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Efflux Pump-Mediated Intrinsic Drug Resistance in *Mycobacterium smegmatis*

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The *Mycobacterium smegmatis* genome contains many genes encoding putative drug efflux pumps. Yet with the exception of *lfrA*, it is not clear whether these genes contribute to the intrinsic drug resistance of this organism. We showed first by reverse transcription (RT)-PCR that several of these genes, including *lfrA* as well as the homologues of *Mycobacterium tuberculosis* Rv1145, Rv1146, Rv1877, Rv2846c (*efpA*), and Rv3065 (*mmr* and *emrE*), were expressed at detectable levels in the strain mc^{2}155. Null mutants each carrying an in-frame deletion of these genes were then constructed in *M. smegmatis*. The deletions of the *lfrA* gene or *mmr* homologue rendered the mutant more susceptible to multiple drugs such as fluoroquinolones, ethidium bromide, and acriflavine (two- to eightfold decrease in MICs). The deletion of the *efpA* homologue also produced increased susceptibility to these agents but unexpectedly also resulted in decreased susceptibility to rifamycins, isoniazid, and chloramphenicol (two- to fourfold increase in MICs). Deletion of the Rv1877 homologue produced some increased susceptibility to ethidium bromide, acriflavine, and erythromycin. The upstream region of *lfrA* contained a gene encoding a putative TetR family transcriptional repressor, dubbed LfrR. The deletion of *lfrR* elevated the expression of *lfrA* and produced higher resistance to multiple drugs. Multidrug-resistant single-step mutants, independent of LfrA and attributed to a yet-unidentified drug efflux pump (here called LfrX), were selected in vitro and showed decreased accumulation of norfloxacin, ethidium bromide, and acriflavine in intact cells. Finally, use of isogenic β-lactamase-deficient strains showed the contribution of LfrA and LfrX to resistance to certain β-lactams in *M. smegmatis*.

Mycobacteria, among which are important human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*, are gram-positive bacteria that display marked intrinsic resistance to a variety of antimicrobial agents, and this property is caused by their unique cell wall structure, which is rich in long-chain fatty acids such as C_{60} to C_{90} mycolic acids (5). Mycolic acids are covalently linked to the peptidoglycan-associated polysaccharide arabinogalactan. Moreover, mycobacterial porins, the water-filled channel proteins which form the hydrophilic diffusion pathways, are sparse (34). A major porin of *Mycobacterium smegmatis*, MspA, forms a tetrameric complex with a single central pore, but the density of this protein is 50-fold lower than that of porins of gram-negative bacteria (15). Thus, the mycobacterial cell wall functions as an even more efficient protective barrier than the outer membrane of gram-negative bacteria and limits the access of drug molecules to their cellular targets (5).

The cell wall barrier alone, however, is not sufficient to explain the intrinsic drug resistance of these bacteria. Drug efflux, another drug resistance mechanism, is now known to contribute to intrinsic or acquired resistance in a wide variety of bacteria (22). Drug efflux transporters in bacteria fall into several major families, such as the major facilitator superfamily (MFS) and the multidrug and toxic extrusion, resistance-nodulation-cell division, small multidrug resistance (SMR), and ATP-banding cassette (ABC) families (22, 30). All of these classes of drug efflux transporters can be identified in the genome sequences of several mycobacteria including *M. tuberculosis* (http://www.sanger.ac.uk/Projects/M_tuberculosis). Indeed, drug efflux pumps have been described in several mycobacteria to date. For example, *M. smegmatis* LfrA, an MFS transporter homologous to the QacA multidrug pump of *Staphylococcus aureus*, was the first multidrug efflux pump reported for mycobacteria (33). When expressed on a plasmid, LfrA mediates low-level resistance to fluoroquinolones and other toxic compounds such as ethidium bromide (24, 33). EfpA, Tap, and P55 are three other MFS pumps reported for several mycobacterial species, and of these pumps, Tap and P55 are known to produce low-level resistance to aminoglycosides and tetracyclines when introduced on multicopy plasmids (2, 14, 32). In addition to the MFS pumps, Mmr (an SMR pump) and DrrAB (an ABC exporter) were reported in *M. tuberculosis* (8, 13). When expressed from plasmids, these exporters mediated low-level resistance to certain antimicrobial agents (8, 13).

Nevertheless, the role of these drug exporters in intrinsic drug resistance of mycobacteria remains largely unknown, except for the study of *lfrA* gene disruption strain in *M. smegmatis* (31). In this study, we carried out studies to characterize efflux pump-mediated multidrug resistance in mycobacteria by using *M. smegmatis* as the model organism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids are listed in Table 1. The mycobacteria were cultivated at 37°C (except that 30 or

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TABLE 1. *M. smegmatis* strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
M. smegmatis		
mc^{2}155	Wild type, transformationally competent	Laboratory collection
XZL1317	mc^{2}155 ΔRv1145/Rv1146	This study
XZL1507	mc^{2}155 ΔRv2846c (*efpA*)	This study
XZL1587	mc^{2}155 ΔRv1257	This study
XZL1594	mc^{2}155 ΔRv1877	This study
XZL1675	mc^{2}155 Δ*lfrA*	This study
XZL1676	mc^{2}155 ΔRv3065 (*mmr, emrE*)	This study
XLZ1705	MDR derivative of XZL1675, single-step selected by exposure to ethidium bromide (1 μg/ml)	This study
XLZ1706	MDR derivative of XZL1675, single-step selected by exposure to ethidium bromide (4 μg/ml)	This study
XLZ1716	XZL1675 Δ*blaA*	This study
XLZ1717	mc^{2}155 Δ*blaA*	This study
XZL1718	XZL1705 Δ*blaA*	This study
XZL1720	mc^{2}155 Δ*lfrR*	This study
XZL1721	XZL1675 Δ*lfrR*	This study
Plasmids		
pBluscript II SK(+)	Cloning vector, 2.96 kb, ampicillin resistant	Stratagene
pSUM36	*E. coli*-mycobacterium shuttle vector, 5.2 kb, kanamycin resistant	3
pPR23-1	*E. coli*-mycobacterium shuttle vector, 8 kb, *oriM* temperature sensitive, *sacB* counterselection, gentamicin resistant	27

42°C was used for the construction of deletion mutants) in Difco 7H9 broth supplemented with 10% (vol/vol) Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Fisher Scientific) and 0.05% (wt/vol) Tween 80 or on Difco 7H11 agar plates supplemented with 10% (vol/vol) OADC. *Escherichia coli* cells were grown at 37°C in Luria-Bertani broth. Plasmids were maintained in *E. coli* with appropriate antibiotics for selection (100 μg of ampicillin, 50 μg of kanamycin, or 40 μg of gentamicin per ml).

Antimicrobial agents. Most antimicrobial agents, including acriflavine and ethidium bromide, were purchased from Sigma (St. Louis, Mo.). Piperazine-[U-^{14}C]norfloxacin (specific radioactivity, 14.8 mCi/mmol) was a generous gift from Merck, Sharp, & Dohme Research Laboratory (Rahway, N.J.). Nitrocefin was purchased from Becton Dickinson (Cockeysville, Md.).

Antimicrobial susceptibility. The MICs of several antimicrobial agents for *M. smegmatis* were determined with the twofold serial dilution method in 7H9 medium supplemented with Tween 80 and OADC. Visible growth was scored after 2 to 3 days of incubation at 37°C. Because the alteration in susceptibility was often small, MIC determination was repeated at least three times, and when single values are listed they are those where identical values were obtained in all repetitions. For the same reason, in some cases, drug susceptibilities were measured by a second method with drug gradient plates. Linear concentration gradients of drugs were prepared in square 7H11 agar plates (6). Log-phase cultures were diluted to ca. 10^7 cells/ml and then were streaked as a linear inoculum across the plate, parallel with the drug gradient. Bacterial growth across the plates from low to high drug concentrations was recorded in millimeters after 3 to 5 days at 37°C.

RT-PCR. Total bacterial RNA was isolated from 7H9-grown, overnight mid-exponential-phase cultures at an optical density at 600 nm (OD_{600}) of 0.8 to 1.0 (50 ml) of *M. smegmatis* by the Trizol method (20). Following a further treatment of the RNA samples with RNase-free DNase (4 U of enzyme/μg of RNA for 60 min at 37°C; Promega), the DNase was inactivated at 65°C for 20 min. These RNA samples (0.001 to 0.5 μg) were used as the template for reverse transcription (RT)-PCR with the OneStep RT-PCR system according to the protocol supplied by the manufacturer (QIAGEN, Inc., Valencia, Calif.). The gene-specific primers (50 pmol) (the primer sequences are available upon request) were used per reaction (final volume of 50 μl), which involved a 30-min incubation at 50°C, followed by 15 min at 95°C and 30 to 35 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 68°C, before finishing with 10 min at 68°C. RT-PCR products were analyzed by agarose (1.7% [wt/vol]) gel electrophoresis for the expected RT-PCR products. To control for DNA contamination of RNA samples, non-RT reactions (i.e., standard PCRs) were carried out. In no instance was a product obtained in the absence of the RT reaction mixture.

Generation of in-frame gene deletion mutants. Using the known or putative drug efflux genes of *M. tuberculosis* H_{37}Rv as probes (http://www.sanger.ac.uk) (10), genes homologous to them were identified in the nearly complete genome sequence of *M. smegmatis* mc^{2}155 (http://www.tigr.org) by using the program tblastn (Table 2). To investigate any involvement of these putative drug efflux genes in intrinsic drug resistance of *M. smegmatis*, we constructed in-frame gene deletions by using a homologous genetic recombination approach as follows. PCR was performed to amplify upstream and downstream sequences (ca. 1 to 1.5 kb each), respectively, of each target gene with genomic DNA of *M. smegmatis* mc^{2}155 as the template (the primer sequences are available upon request). The reaction mixture contained 0.1 μg of genomic DNA, 40 pmol of each primer, 200 μM concentrations of each deoxynucleoside triphosphate, 2 mM $MgSO_4$, 10% (vol/vol) dimethyl sulfoxide and 2 U of Vent DNA polymerase (New England Biolabs) in 1× Thermo reaction buffer and was heated for 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1.5 min at 72°C. The PCR products were purified, digested with the appropriate restriction enzymes (BamHI and HindIII or HindIII and XbaI), and cloned into the BamHI-XbaI-digested pBluescript II SK(+) via a three-piece ligation. The ligation mixtures were used to transform *E. coli* DH5α. Following verification of the cloned sequences by DNA sequencing, the fragments were subsequently cloned into the temperature-sensitive suicide vector pPR23-1 (27, 28). The resultant plasmids were electroporated into *M. smegmatis* mc^{2}155, and the plasmids were allowed to replicate in the cells by cultivation overnight at 30°C. Cells in which the plasmids became integrated into the chromosome were selected then by plating the culture on gentamicin (20 μg/ml) plates and incubating the plates for 3 to 5 days at 42°C. The mutants in which the plasmid sequence was eliminated via homologous recombination were finally selected on 7H11 agar plates containing 5% (wt/vol) sucrose, since expression of the levansucrase-encoding *sacB* gene, present on the vector sequence, was lethal to mycobacteria (28). The sucrose-resistant, gentamicin-sensitive colonies were examined for the presence of the intended gene deletions by PCR amplification of the genomic DNA.

Construction of *blaA* deletion mutants. *blaA* deletion mutants were constructed in a manner similar to that of deletion mutants of putative efflux proteins, described above, by using the *M. smegmatis blaA* sequence obtained from the genome sequence at The Institute for Genomic Research (www.tigr.org). The deleted sequence corresponds to amino acid residues 50 to 257 of this protein.

Assay of ethidium bromide and acriflavine accumulation in intact cells. Mycobacterial cells were cultivated at 37°C for 2 to 3 days in 7H9 medium and subsequently diluted 50-fold in the same medium. Following an overnight growth to mid-exponential phase (OD_{600}, ca. 0.8 to 1.0), the cells were harvested at 5,000 × *g* for 10 min at room temperature, washed once with 50 mM sodium phosphate buffer (pH 7.2), and resuspended in the same buffer at an OD_{600} of 0.4 to 0.7. Drug entry into these cells was monitored at room temperature with an RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia,

TABLE 2. Classification and amino acid identities of some putative drug efflux proteins of *M. tuberculosis* H37Rv and *M. smegmatis* mc^{2}155 and the mRNA expression of the *M. smegmatis* efflux genes measured by RT-PCR

Gene(s) of *M. tuberculosis*	Gene(s) of *M. smegmatis*	Family[a]	Amino acid residue identity (%)[b] (*M. tuberculosis* vs *M. smegmatis*)	mRNA expression in *M. smegmatis*[c]
Rv1145-Rv1146[d]	4208498–6201[e]	RND	62	+
Rv1250	4074929–3495	MFS	63	+
Rv1258c (*tap*)	4057327–7998	MFS	67	+
Rv1410c (P55)	2073408–1882	MFS	69	++
Rv1634	2814796–3420	MFS	64	+
Rv1819c	3395875–7779	ABC	63	++
Rv1877	2555505–2748	MFS	58	+
Rv2136c	3203468–2646	ABC	73	++
Rv2836c	1641611–2909	MATE	74	+
Rv2846c (*efpA*)	1629942–31402	MFS	81	±
Rv2994	1393778–2456	MFS	54	+
Rv3065 (*mmr, emrE*)	2666199–373	SMR	40	+
Absent	*lfrA*[f]	MFS	NA	±

[a] The classification is based on the published literature (2, 8, 12, 13, 14, 33) and information online at http://www.membranetransport.org and http://www.sanger.ac.uk/Projects/M_tuberculosis/. RND, resistance-nodulation-cell division family; MATE, multidrug and toxic extrusion family.
[b] The percent identity figures are based on BLAST alignment without the use of a filter.
[c] The mRNA expression levels assessed by RT-PCR were semiquantitatively determined to be strong (++), medium (+), and weak (±).
[d] The homologues of these two *M. tuberculosis* genes (i.e., Rv1145 and Rv1146) belong to a single gene in *M. smegmatis*.
[e] Nucleotide residue numbers in the *M. smegmatis* genome database at The Institute for Genomic Research.
[f] Homologues of the *M. smegmatis lfrA* gene were not identified in the *M. tuberculosis* genome. NA, not applicable.

Md.) at room temperature. The final concentrations of ethidium bromide and acriflavine used were both 5 μM. In ethidium bromide accumulation, the excitation and emission wavelengths used were 520 nm and 590 nm, respectively. In acriflavine accumulation, the excitation and emission wavelengths were 485 nm and 501 nm, respectively. In some cases, cells were pretreated with a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (at 100 to 400 μM), for 15 min at room temperature before the initiation of the assays.

Assay of [^{14}C]norfloxacin accumulation in intact cells. Cells in the mid-exponential phase (OD_{600} of 0.8 to 1.0) were diluted to an OD_{600} of 0.5 with the 7H9 broth. The cell suspension was maintained at 37°C with aeration by shaking (200 rpm), and the assay was started by the addition of the radiolabeled drug at a final concentration of 20 μM. At various time points, 0.1 ml of the suspension (in triplicate) was removed and filtered through a Gelman Metricel GN-6 membrane filter (0.45-μm pore size). The filter was washed with 5 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 100 mM LiCl, and the radioactivity retained on the filter was quantitated with an LS6500 scintillation spectrometer (Beckman Coulter Inc., Fullerton, Calif.).

Selection of MDR mutants from LfrA-deficient strain. Cells of *M. smegmatis* Δ*lfrA* strain XZL1675 were plated on 7H11 agar plates containing 0.25, 1, or 4 μg of ethidium bromide/ml (i.e., 2, 8, and 32 times the MIC), and the plates were incubated at 37°C for 3 to 5 days. Colonies from the drug plates were restreaked on drug-free 7H11 agar plates and further assessed for susceptibility to ethidium bromide and other structurally unrelated antimicrobial agents. Of the 24 randomly selected colonies, 14 colonies displayed a multidrug resistance phenotype showing elevated resistance not only to ethidium bromide but also to acriflavine and fluoroquinolones. Two of these multidrug-resistant (MDR) mutants, XZL1705 and XZL1706, were characterized in detail.

β-Lactamase activity assays. Cells at the mid-exponential growth phase were harvested, and cell pellets were washed once with 50 mM sodium phosphate buffer (pH 7.2). Following disruption of the cells (8 cycles of 30 s each) on ice with a Soniprep 150 sonicator (Gallenkamp, Belton Park, United Kingdom), the cell lysates were used as sources of β-lactamases. Hydrolysis of a chromogenic β-lactam compound, nitrocefin (100 μM), was assessed at 482 nm at room temperature as a measure of β-lactamase activity.

Cell wall permeability. To assess the cell wall permeability of *M. smegmatis*, the hydrolysis of nitrocefin was carried out in intact cells. Briefly, cells at the mid-exponential phase were harvested, washed, and resuspended at an OD_{600} of 0.2 in the sodium phosphate buffer, pH 7.2. The hydrolysis of nitrocefin (100 μM) by intact cells was measured at room temperature at the OD_{482} with a Uvicon 850 spectrophotometer (Kontron, Zürich, Switzerland). The maximal cellular β-lactamase activity was also determined with ultrasonicated broken cells.

RESULTS

Drug efflux pumps in *M. smegmatis*. Drug-specific or multidrug efflux pumps are known to be distributed widely in bacteria. Analysis of the *M. tuberculosis* H37Rv genome supports this conclusion (10), indicating the presence of at least two dozen putative drug exporters, which fall into all five classes of drug exporters mentioned in the introduction (http://www.sanger.ac.uk; http://www.membranetransport.org). The majority of these transporters belong to either MFS or ABC, the two largest transport families (4, 12). Based on the bioinformatic data of the *M. tuberculosis* genome, we carried out the BLAST (tblastn) search of the soon-to-be-completed genome of *M. smegmatis* (http://www.tigr.org) and found the presence of a number of putative drug efflux proteins. The comparison of these proteins of *M. smegmatis* and *M. tuberculosis* showed that they were homologous proteins with up to 81% residue identity (Table 2). The homologues of two adjacent genes, Rv1145 and Rv1146 of *M. tuberculosis*, were found to form a single open reading frame in *M. smegmatis*. Interestingly, the homologue of LfrA of *M. smegmatis* was not present in *M. tuberculosis*. (In this paper, *M. smegmatis* homologues of *M. tuberculosis* genes are designated with the "Rv" nomenclature of the *M. tuberculosis* genes for the sake of brevity.)

To select the genes to be tested for their drug efflux functions, we first assessed the expression of these putative efflux protein genes in the wild-type strain of *M. smegmatis* at the exponential phase of growth by RT-PCR with gene-specific primers. Given the fact that the gene-specific primers were used, precise comparison of expression levels was not possible. Nevertheless, semiquantitative results are summarized in Table 2. Genes *lfrA* and *efpA* appeared to be expressed weakly, as larger amounts of RNA templates (ca. 0.5 μg) were needed to produce a visible amplification.

TABLE 3. Antimicrobial susceptibility of LfrA and 4 putative efflux pump-deficient mutants of *M. smegmatis* mc^{2}155[a]

Strain	Relevant characteristic	MIC (μg/ml)[b]											
		EB	ACR	CIP	NOR	RIF	INH	CHL	ERY	GEN	KAN	NOV	TET
mc^{2}155	Wild type	1	4	0.25	2	1	8	8	32	8	4	4	0.25
XZL1675	Δ*lfrA*	**0.13**	**0.5**	**0.13**	**1**	1	**4**	8	32	8	4	4	**0.13**
XZL1705	Δ*lfrA* MDR (LfrX)	**1–2**	**8**	**0.25**	**16**	1	**8**	8	32	8	4	4	**0.5**
XZL1676	Δ*mmr* (Rv3065)	**0.5**	**0.5**	**0.13**	**1**	1	8	8	32	8	4	**2**	0.25
XZL1507	Δ*efpA* (Rv2846c)	**0.5**	**0.5**	**0.13**	**1**	**4**	**16**	**32**	**64**	**4**	4	4	0.25
XZL1594	ΔRv1877	**0.5**	**0.5**	0.25	2	1	8	8	**16**	8	**2**	**2**	**0.13**

[a] XZL1317 (ΔRv1145/Rv1146) and XZL1587 (ΔRv1257) showed MICs identical to mc^{2}155; thus, their MICs are not shown.

[b] MICs were determined by twofold serial dilutions. Because the mutants often had minimal changes in MIC, the MIC determinations were repeated several times, and the difference between the wild type and the mutant was confirmed in each case. Abbreviations: EB, ethidium bromide; ACR, acriflvine; CIP, ciprofloxacin; NOR, norfloxacin; RIF, rifampin; INH, isoniazid; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; NOV, novobiocin; TET, tetracycline. MICs of pyrazinamide (measured at pH 5.5) and triclosan were 16,000 and 16 μg/ml, respectively, for all strains. Values in boldface type represent changes from the MIC for the parent strains (mc^{2}155, or XZL1675, in the case of the MDR strain XZL1705). There was also a twofold increase in the MIC for strain XZL1705, which became more resistant to nalidixic acid (MIC, 128 μg/ml), enoxacin (MIC, 2 μg/ml), ampicillin (MIC, 128 μg/ml), and amoxicillin (MIC, 256 μg/ml) than its parent strain XZL1675.

Role of *lfrA* and four putative efflux genes in intrinsic drug resistance. Although many putative drug efflux genes are present in the *M. smegmatis* genome (Table 2), any involvement of these putative pumps in intrinsic drug resistance remains unknown, with the exception of *lfrA* (31). To assess the contribution of these putative pumps, several deletion mutants were constructed, and the impact of the gene deletion on drug susceptibility was measured in broth medium or on gradient drug plates as described in Materials and Methods. Deletion of *lfrA* (strain XZL1675) rendered the strains more susceptible to several antimicrobials, such as ethidium bromide, acriflavine, and fluoroquinolones (up to an eightfold decrease in MICs) (Table 3), largely confirming the results of Sander et al. (31). A similar change was observed for the deletion of *mmr* (*emrE*) (strain XZL1676) (Table 3). Consistent with these observations, the accumulation of ethidium bromide, acriflavine, and norfloxacin by intact cells of the LfrA-deficient mutant (XZL1675) was higher than that of wild-type cells (Fig. 1 and 2). It should be mentioned that ethidium fluorescence increases as it enters the cell and interacts with DNA molecules. On the contrary, acriflavine fluorescence is quenched as it gets into the cell. The *mmr* deletion mutant (strain XZL1676) was also found to accumulate about twice as much ethidium bromide as its parental wild-type strain (data not shown). The deletion of Rv1877 (XZL1594) produced increased susceptibility to cationic dyes, erythromycin, novobiocin, tetracycline, and kanamycin (Table 3).

Intriguingly, the deletion of *efpA* (strain XZL1507) produced an unexpected phenotype where the mutant had an increased susceptibility to several drugs (i.e., ethidium bromide, acriflavine, and fluoroquinolone, with a twofold decrease in MICs) and at the same time showed a decreased susceptibility to some other drugs, such as rifamycins (rifampin, rifamycin SV, and rifabutin), isoniazid, chloramphenicol, and erythromycin (two- to eightfold increases in MICs) (Table 3). Unlike all other deletion mutants constructed in this study, the *efpA* mutant had a slower growth rate than its parental wild-type strain, with a growth rate about 60% of that of the wild-type strain. Thus, the increased susceptibility of the *efpA* mutant could be a result of the impaired growth behavior of the mutant.

In spite of their in vivo expression (see RT-PCR results above), neither the deletion of the Rv1145-Rv1146 homologue nor deletion of the nonefflux Rv1257 homologue (a gene of unknown function located upstream of the putative drug efflux gene Rv1258c [*tap*], included as negative control) produced any notable alterations in drug susceptibility. Attempts for the construction of deletion mutants of Rv1410c, Rv1819c, Rv2136c, and Rv2994 (Table 2) were unsuccessful for unknown reasons.

Identification and characterization of a TetR family repressor gene, *lfrR*, located upstream of the *lfrA* gene. Inspection of the upstream region of the *lfrA* gene identified an open reading frame (570 bp) that encoded a putative protein of 189 residues, which showed homology to several TetR family transcriptional proteins, including a putative transcriptional repressor of *Streptomyces coelicolor* (GenBank accession number CAB76088) and the NfxB repressor of the *Pseudomonas aeruginosa* MexCD-OprJ multidrug efflux system (GenBank accession number X65646). Given its location close to *lfrA*, we thought that the gene may regulate *lfrA*, and hence, we call tentatively it *lfrR*. The *lfrR* and *lfrA* genes are transcribed in the same orientation, and the intergenic sequence between the two genes contains only 71 bp. The deduced LfrR protein was predicted by the Jpred software (http://www.compbio.dundee.ac.uk) to possess a helix-turn-helix motif.

An examination of the upstream sequence of the *lfrR* gene by the neural prediction method (http://www.fruitfly.org/seq_tools/promoter.html) identified a strong candidate promoter sequence starting from 30 bp upstream of the *lfrR* start codon. However, this sequence seemed too close to the putative translational start codon. Translation of LfrR may start from an alternative start AUG codon at position 67. In any case, RT-PCR with *lfrR*-specific primers readily yielded an expected product of 441 bp in uninduced wild-type *M. smegmatis* (data not shown), demonstrating that *lfrR* is indeed expressed in the wild-type strain.

To determine whether LfrR had any impact on LfrA expression, a 390-bp in-frame deletion in the *lfrR* gene was constructed from the wild type and its *lfrA* deletion strains. Deletion of *lfrR* in the wild-type background rendered the strain XZL1720 markedly more resistant to ciprofloxacin, norfloxacin, ethidium bromide, and acriflavine (2- to 16-fold increases in the MICs) (Table 4), all of them typical substrates for the

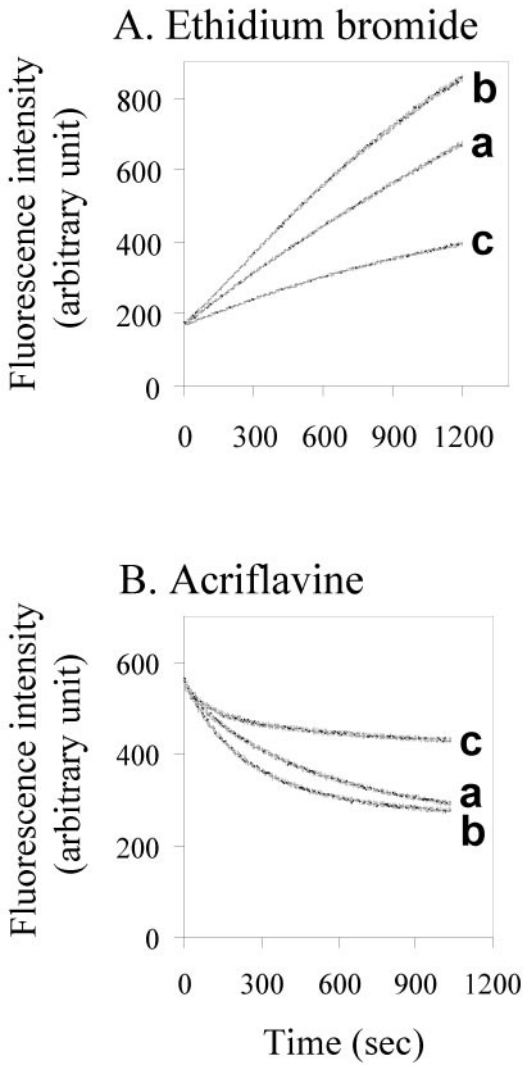

FIG. 1. Accumulation of ethidium bromide (A) and acriflavine (B) by intact cells of *M. smegmatis* monitored in a time course with a spectrofluorometer. The three isogenic strains compared are mc^{2}155 (wild type) (a), XZL1675 (Δ*lfrA*) (b), and XZL1705 (MDR Δ*lfrA* mutant) (c). Cells were grown in 7H9 medium, harvested, washed, and resuspended in a sodium phosphate buffer as described in Materials and Methods. Ethidium bromide and acriflavine were used at a final concentration of 5 μM.

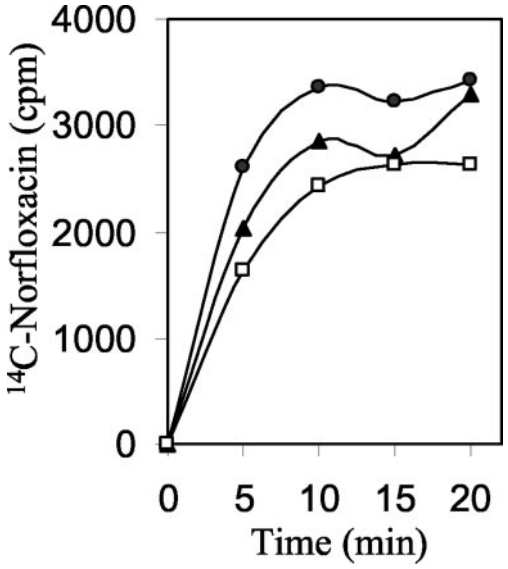

FIG. 2. Accumulation of norfloxacin by intact cells of *M. smegmatis*. The three isogenic strains compared are mc^{2}155 (wild type) (▲), XZL1675 (Δ*lfrA*) (●), and XZL1705 (MDR Δ*lfrA* mutant) (□). Cells were grown in 7H9 medium, harvested, washed, and resuspended in a sodium phosphate buffer as described in Materials and Methods. Radiolabeled norfloxacin was used at a final concentration of 20 μM. Data shown represent the means of the results from two separate experiments in which triplicate samples were taken at each time point.

LfrA efflux pump. In contrast, *lfrR* deletion had less effect on the drug susceptibility of the LfrA-deficient strain XZL1721, confirming that LfrR affects drug resistance mainly (but not completely, see below) via altered expression of LfrA pump. Comparison of *lfrA* expression in wild-type and *lfrR*-deficient strains by RT-PCR also demonstrated that *lfrA* expression was strongly increased as a result of the *lfrR* deletion (Fig. 3). Together, these results clearly indicate that LfrR negatively regulates LfrA expression. LfrR may also regulate pump(s) other than LfrA; thus, even though LfrA was absent, the deletion of *lfrR* gene in XZL1721 made the mutant more resistant to several agents, especially acriflavine, in comparison with XZL1675. Of interest, cells with the *lfrR* deletion grown in 7H9 medium appeared somewhat sick, perhaps due to the overexpressed LfrA that may pump out physiological substrates or metabolites.

Multidrug resistance independent of the LfrA multidrug efflux pump. Although we have identified in this study several drug efflux transporters in *M. smegmatis*, their contribution to the innate drug resistance appears to be limited. One possible explanation is that additional mutations may be required to derepress these or other efflux genes. To uncover such mutations, derivatives of an LfrA-deficient strain were selected in the presence of 8 times the MIC of ethidium bromide, a representative substrate of a number of bacterial drug exporters. Resistant colonies developed after 4 to 5 days of incubation with a frequency of 10^{-8} to 10^{-9}. These colonies were screened for their susceptibility to ethidium bromide, ciprofloxacin, and norfloxacin. Two mutants, XZL1705 and XZL1706, which showed decreased susceptibility to multiple drugs (Table 3), were selected for further characterization. The resistant mutant XZL1705 accumulated less drug (ethidium bromide, acriflavine, and norfloxacin) than its parental strain XZL1765 (Fig. 1 and 2). (Because XZL1706 showed a drug

TABLE 4. Effect of *lfrR* deletion on drug susceptibility of wild-type and LfrA-deficient strains of *M. smegmatis*

Strain	Relevant characteristic(s)	MIC (μg/ml)[a]					
		CIP	NOR	ACR	EB	INH	RIP
mc^{2}155	Wild-type	0.25	2	4	1	8	1
XZL1720	Δ*lfrR*	4	8	16	4	16	1
XZL1675	Δ*lfrA*	0.13	1	0.5	0.13	4	1
XZL1721	Δ*lfrA* Δ*lfrR*	0.25	1	4	0.13	8	1

[a] Abbreviations: CIP, ciprofloxacin; NOR, norfloxacin; ACR, acriflavine; EB, ethidium bromide; INH, isoniazid; RIF, rifampin.

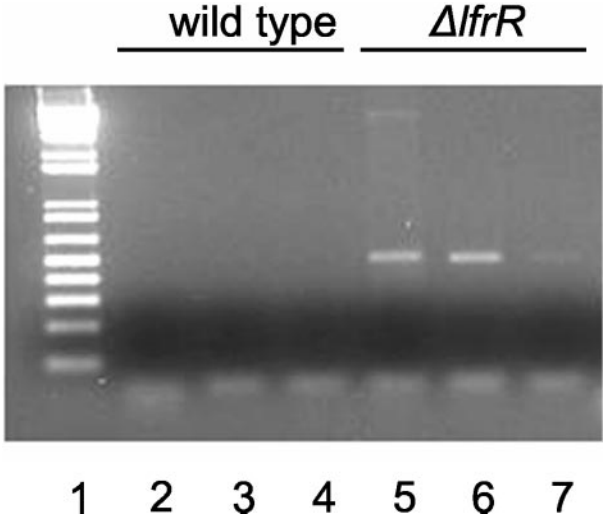

FIG. 3. Influence of *lfrR* deletion on expression of *lfrA* in *M. smegmatis* (lanes 2 to 7) measured by RT-PCR amplification of RNA with primers specific for *lfrA* (expected 494-bp product). The RNA templates were isolated from strains mc^{2}155 (wild type) (lanes 2 to 4) and XZL1720 (Δ*lfrR*) (lanes 5 to 7), and different amounts of RNA were used in the amplification (lanes 2 and 5, 0.2 μg; lanes 3 and 6, 0.02 μg; lanes 4 and 7, 0.002 μg). DNA mass markers (0.1 to 12 kb) (1 kb plus DNA ladder; Invitrogen) are shown in lane 1. For each sample, an RT-PCR with primers specific for the *blaA* gene was run as a control (data not shown).

resistance pattern very similar to that of XZL1705 and accumulated less drug in comparison to the parent strain, only the results for XZL1705 are shown.) Treatment of the mutant cells with carbonyl cyanide *m*-chlorophenylhydrazone resulted in a marked increase of ethidium bromide accumulation (data not shown). These data suggest that an efflux mechanism, attributable to a yet-unidentified drug efflux pump (tentatively dubbed as LfrX), is likely responsible for the elevated multidrug resistance observed in strain XZL1705. The identity of the putative LfrX pump is currently under investigation with a transposon mutagenesis approach.

Cell wall permeability. Nitrocefin hydrolysis by intact cells was measured as an indicator of cell wall permeability. Compared with that of the wild-type strain, the hydrolysis rates of nitrocefin by either the efflux gene deletion mutants (Δ*lfrA*, Δ*emrE*, and Δ*efpA*) or the MDR XZL1705 were not altered (data not shown).

Contributions of LfrA and LfrX efflux pumps to β-lactam resistance. It has been established that multidrug efflux pumps contribute to β-lactam resistance in gram-negative bacteria (21, 25). β-Lactamase production often plays, however, a predominant role in β-lactam resistance. A chromosomal β-lactamase, encoded by Rv2068c (also called *blaC* [GenBank accession number Z73966] or *blaA* [GenBank accession number U67924]), has been reported in *M. tuberculosis* (10, 17). Using this *M. tuberculosis* β-lactamase as a probe, a BLAST search of the *M. smegmatis* genome at The Institute for Genomic Research website (www.tigr.org) revealed a homologue, which we call *blaA*. The *M. smegmatis* BlaA enzyme belongs to an Ambler class A β-lactamase.

A deletion of the *M. smegmatis blaA* gene was constructed in strains mc^{2}155 (wild-type), XZL1675 (Δ*lfrA*), and XZL1705 (Δ*lfrA* MDR), and was confirmed by both PCR amplification and β-lactamase activity assay. Thus, the *blaA* gene deletion in these three strains decreased the β-lactamase activity more than 130-fold (Table 5), suggesting that this enzyme is likely the major, if not the sole, determinant for β-lactamase activity in this organism. (Very recently, another group also showed that *blaA* [called *blaS* by this group] contributes overwhelmingly to the β-lactamase activity in *M. smegmatis*, although they showed the presence of another gene, *blaE*, apparently coding for a class C enzyme.) (A. R. Flores and M. S. Pavelka, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 674, 2003). With the availability of these virtually β-lactamase-deficient strains, the contribution of the multidrug efflux pumps LfrA and LfrX was assessed. In the absence of BlaA, deletion of LfrA rendered the strain more susceptible to ampicillin, benzylpenicillin, and amoxicillin (two- to fourfold decrease in MICs) (compare XZL1716 with XZL1717 in Table 5), and the LfrA-independent MDR mutant XZL1705 showed elevated resistance to these penicillins (two- to fourfold increase in MICs) (data not shown). These β-lactam susceptibility alterations were also confirmed on β-lactam gradient plates (data not shown). These data suggest that LfrA and the yet-unidentified LfrX efflux pump also contribute, in a limited way, to resistance to certain β-lactam antibiotics.

Of interest, the absence of the BlaA enzyme appeared not to produce significant enhanced susceptibility to some β-lactams. For example, MICs of several β-lactams such as cloxacillin, nafcillin, cephamandole, ceftriaxone, and cefpirome for the BlaA-deficient strains still remained >512 μg/ml. This could be due, at least partly, to the poor affinity of the target, penicillin-binding proteins, to some of these compounds. Indeed, one of the penicillin-binding proteins of *M. tuberculosis*, with an apparent size of 52 kDa, bound nafcillin very poorly, although it bound ceftriaxone with a high affinity (7).

TABLE 5. β-Lactam susceptibility of *blaA* mutants derived from wild-type and LfrA-deficient strains of *M. smegmatis*

Strain	Relevant characteristic(s)	β-Lactamase activity (U)[a]	MIC of (μg/ml)[b]:					
			AMP	AMX	PEN	IMI	MEM	LOR
mc^{2}155	Wild type	6,500	128	64	>512	4	8	256
XZL1717	Δ*blaA*	20	32	16–32	128	2–4	4–8	128
XZL1675	Δ*lfrA*	8,000	128	128	>512	8	8	512
XZL1716	Δ*lfrA* Δ*blaA*	60	8	8	64	4–8	4–8	128

[a] β-Lactamase activity was determined by hydrolysis of nitrocefin (units are changes in OD_{482} per minute per milliliter.
[b] Abbreviations: AMP, ampicillin; AMX, amoxicillin; PEN, penicillin G; IMI, imipenem; MEM, meropenem; LOR, cephaloridine.

DISCUSSION

Contribution of drug efflux pumps to drug resistance in *M. smegmatis*. Earlier studies of drug accumulation in intact cells have suggested that drug-resistant mycobacteria accumulated less drug than the parental strains, and these results were often attributed to the lower cell envelope permeability in resistant cells (18, 23). However, some of these drug accumulation data will probably need to be reinterpreted as the consequence of increased drug efflux on the basis of current understanding of drug influx and efflux processes across the bacterial cell envelope. Indeed, a drug accumulation study carried out more recently in intact cells in the presence and absence of an efflux pump inhibitor revealed small but reproducible activity of efflux systems towards rifampin in *M. tuberculosis*, *Mycobacterium aurum*, and *M. smegmatis* (29).

The first mycobacterial multidrug efflux pump, LfrA, was identified in 1996 in *M. smegmatis* (33), and since then, several other mycobacterial drug efflux pumps have been reported as described in the introduction. Despite the presence of a large number of putative drug efflux genes in the genomes of *M. tuberculosis*, *Mycobacterium bovis*, and *M. smegmatis*, the involvement of the efflux pumps in the intrinsic drug resistance in mycobacteria remains largely unknown. In this study, we made a comparison of the putative drug efflux genes and pumps between *M. tuberculosis* and *M. smegmatis* and found that many homologous pumps are present in both organisms. We therefore believe that studies on drug efflux pumps that use *M. smegmatis* as a model organism would provide us with data to understand efflux-mediated drug resistance mechanisms in other mycobacteria, including *M. tuberculosis*.

We first showed that many putative efflux genes were expressed in the wild-type strain at detectable levels. We then examined deletions of five of these genes.

(i) *lfrA*. Although *lfrA* is weakly expressed in wild-type cells, its contribution to drug resistance appears to be the highest among the genes analyzed. Consistent with the results of Sander et al. (31), inactivation of the chromosomal *lfrA* gene demonstrated the involvement of LfrA in the intrinsic resistance to cationic dyes, fluoroquinolones, and tetracycline. These results are also strengthened by our identification of the repressor of LfrA expression, LfrR, and the finding that the deletion of this repressor gene makes the mutant strain highly resistant to these drugs (Table 4). Among mycobacterium-specific drugs, *lfrA* deletion seems to produce marginally increased susceptibility to isoniazid (Table 3) and this result is strengthened somewhat by the slight increase in resistance in the *lfrR* mutant. Possibly, LfrA contributes to the efflux of isoniazid observed earlier (9; Y. Tokue and H. Nikaido, unpublished data).

(ii) *emrE*. *M. tuberculosis mmr* (or *emrE*) produces increased resistance to cationic dyes and erythromycin when expressed in *M. smegmatis* from multicopy plasmids (13). The deletion of the *emrE* homologue produced increased susceptibility similar to the deletion of *lfrA* (Table 3) (except tetracycline), thus showing that this pump plays an important role in the intrinsic resistance of *M. smegmatis* to dyes and, importantly, fluoroquinolones but apparently not to erythromycin.

(iii) *efpA*. In *M. tuberculosis*, a gene encoding an MFS efflux pump of the QacA family, EfpA, was reported (14). Interestingly, genome-wide microarray analysis of the *M. tuberculosis* genes revealed that *efpA* expression was increased in the presence of isoniazid (36). However, there have not been attempts to express EfpA even from plasmids, and its role in drug resistance and its substrate specificity are not known to this day. In this study, we showed that deletion of the *efpA* gene in *M. smegmatis* resulted in increased susceptibility to cationic dyes and fluoroquinolones (Table 3), a result indicating its contribution to the intrinsic resistance of the wild-type organism. Surprisingly, the deletion mutant also showed increased resistance to several drugs, particularly to rifamycins. Since this result was unexpected, the construction of the *efpA* deletion mutant was repeated several times; an identical phenotype was observed with all newly generated *efpA* deletion mutants. These independent reconstructions of the *efpA* mutants ruled out the possibility that drug-resistant mutants were inadvertently selected during the mutant construction. At present, we cannot offer a concrete explanation of this phenotype, although it seems possible that the decreased efflux of an internal signaling molecule, due to the absence of EfpA, may cause the induction of other efflux pump(s).

Doran et al. (14) reported, by Southern hybridization, that the presence of *efpA* homologues are limited to slow-growing, pathogenic mycobacterial species and that a homologue was absent in *M. smegmatis*. A BLAST search of the unpublished *M. smegmatis* genome at The Institute for Genomic Research, using the sequence of the probe used by Doran et al. (14), shows that the correct homologue is identified with 76% identity at the nucleotide level. Thus, this seems to be another example showing the limitation of the Southern hybridization approach for the detection of homologues.

(iv) Rv1877. The *M. smegmatis* homologue of Rv1877 appears to code for a protein that belongs to the QacA 14-TMS family of efflux transporters of the MFS superfamily (see reference 26). The protein, however, is somewhat unusual in its large size (686 residues) in comparison to the well-known members of this family (475 to 548 residues). A BLAST search shows that it is most closely related to the *M. tuberculosis* Rv1877 (58% identity), and those proteins with high similarity and known (or reasonably predictable) functions include the putative antibiotic exporters (identity, 38 to 40%) in antibiotic-producing actinomycetes (1, 16, 35). Deletion of the Rv1877 homologue made the strain more susceptible to cationic dyes, erythromycin, tetracycline, and kanamycin but not to fluoroquinolones.

(v) Rv1145-Rv1146. As mentioned in Results, the closest homologues of Rv1145-Rv1146 form a single fused gene in *M. smegmatis*. The disruption of this gene did not alter the drug susceptibility (Table 3). This is perhaps not surprising, as the protein encoded by this gene shows homology with MmpL proteins of *M. tuberculosis*, which to our knowledge have not been shown to be involved in drug efflux, although one member, MmpL7, was shown to be involved in the transport of a complex lipid, phthiocerol dimycocerosate, to the cell envelope (11).

Other multidrug efflux pumps may obviously contribute to the intrinsic drug resistance of *M. smegmatis*. This is clearly shown by the generation of LfrA-independent MDR mutants in a single step, described in this study. Furthermore,

we could not construct deletion mutants of several putative efflux genes (see Results); they may be performing essential functions for the cell. Obviously more study is needed to understand the contribution of drug efflux in the resistance of this organism.

In this study, we showed that LfrA expression is regulated by the repressor gene LfrR. The deletion mutant of *lfrR* showed a striking increase in resistance to many drugs (for example, a 16-fold increase in the MIC of ciprofloxacin). This finding suggests that regulatory mutations may arise in pathogenic mycobacteria in the future and may produce clinical problems. It also underscores the need to study the regulation of other efflux pump genes.

β-Lactam susceptibility of *M. smegmatis*. So far, only the cell wall permeation barrier and β-lactamase-mediated hydrolysis have been considered as factors affecting the β-lactam resistance of mycobacteria (7, 19). By eliminating most of the β-lactamase activity through the inactivation of the *blaA* gene of *M. smegmatis*, we could show, for the first time, that some efflux transporters, including LfrA and the yet-unidentified LfrX, have detectable effects on β-lactam susceptibility of this organism. Since LfrA activity, for example, could be increased strongly by mutations in LfrR, this efflux-mediated mechanism of resistance appears worthy of further study, especially because β-lactams may be considered as an alternative means of treatment for MDR strains of *M. tuberculosis* (7).

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