ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2006, p. 4044–4052 Vol. 50, No. 12
0066-4804/06/$08.00+0 doi:10.1128/AAC.00656-06

LfrR Is a Repressor That Regulates Expression of the Efflux Pump LfrA in *Mycobacterium smegmatis*▽

Silvia Buroni, Giulia Manina, Paola Guglierame, Maria Rosalia Pasca, Giovanna Riccardi, and Edda De Rossi*

Department of Genetics and Microbiology, University of Pavia, Pavia, Italy

Received 30 May 2006/Returned for modification 30 July 2006/Accepted 30 September 2006

The *lfrA* gene of *Mycobacterium smegmatis* encodes an efflux pump which mediates resistance to different fluoroquinolones, cationic dyes, and anthracyclines. The deletion of the *lfrR* gene, coding for a putative repressor and localized upstream of *lfrA*, increased the *lfrA* expression. In this study, reverse transcription-PCR experiments showed that the two genes are organized as an operon, and *lacZ* reporter fusions were used to identify the *lfrRA* promoter region. The *lfrRA* promoter assignment was verified by mapping the transcription start site by primer extension. Furthermore, we found that some substrates of the multidrug transporter LfrA, e.g., acriflavine, ethidium bromide, and rhodamine 123, enhance *lfrA* expression at a detectable level of transcription. LfrR protein was purified from *Escherichia coli* as a fusion protein with a hexahistidine tag and found to bind specifically to a fragment 143 bp upstream of *lfrR* by gel shift analysis. Furthermore, acriflavine was able to cause the dissociation of the LfrR from the promoter, thus suggesting that this molecule interacts directly with LfrR, inducing *lfrA* expression. These results suggest that the LfrR repressor is able to bind to different compounds, which allows induction of LfrA multidrug efflux pump expression in response to these ones. Together, all data suggest that the LfrA pump is tightly regulated and that the repression and induction can be switched about a critical substrate concentration which is toxic for the cell.

Multidrug resistance has emerged as a major clinical problem and can arise through a number of mechanisms, including the action of efflux transporters that pump out a wide variety of structurally and chemically dissimilar drugs, dyes, and other compounds (49). Multidrug efflux transporters are membrane proteins found in both prokaryotes and eukaryotes and are classified into five families. Two of these are large and ancient superfamilies known as the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS). The other three are smaller families: the resistance-nodulation-cell division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (25). The mechanism used to bind and export a broad range of substrates remains poorly understood, largely due to the difficulties posed by the structural analysis of integral membrane proteins. In the case of bacteria, an alternative approach has been the study of proteins that regulate the expression of specific multidrug transporters. The data available today show that multidrug transporters are often expressed under precise and elaborate control at the level of transcription (13). Examples of both repressors (12, 27, 28) and activators (1, 24) of transcription whose genes are adjacent to that for the transporter have been described. Many of these regulators are local repressors that directly interact with the promoter regions of multidrug resistance (MDR) efflux genes or operons. For example, repressors QacR (*Staphylococcus aureus*), MtrR (*Neisseria gonorrhoeae*), AcrR (*Escherichia coli*), and MexR (*Pseudomonas aeruginosa*) bind specifically to the promoter sequences of *qacA*, *mtrCDE*, *acrAB*, and *mexAB* efflux pump-encoding genes, respectively, thereby inhibiting transcription of these genes (9, 12, 15, 29).

Overexpression of multidrug resistance pumps, resulting in increased bacterial resistance, is usually due to mutations in these regulatory genes (9, 12, 16, 36). For these reasons, the study of the regulation of MDR efflux gene expression is an important issue in the field of antibiotic resistance. Furthermore, an increasing number of efflux pump genes has also been found to be controlled by global transcriptional activator proteins (13) or by two-component regulatory systems (25).

Mycobacterium tuberculosis is the infectious agent responsible for tuberculosis. Tuberculosis is often difficult to treat, because *M. tuberculosis* is intrinsically resistant to most common antibiotics, apparently because of its extremely low cell wall fluidity and permeability (3, 20, 46). The situation is made worse by the dramatic increase in multidrug-resistant strains. About 50 million people are presently infected with MDR *M. tuberculosis* strains, defined as resistant to both isoniazid and rifampin, the two first-line drugs used to treat tuberculosis (8). Along with cell wall permeability, active efflux systems also provide resistance by extruding the drugs that enter the cell. Therefore, it seems reasonable to characterize efflux pump-mediated multidrug resistance in mycobacteria by using *Mycobacterium smegmatis* as the model organism. Despite several mycobacterial efflux pumps having been characterized (5, 26, 48), their involvement in intrinsic and acquired drug resistance in mycobacteria remains unresolved (5), except for the study of the *lfrA* gene in *M. smegmatis* (40). LfrA is an MFS transporter that confers resistance to ethidium bromide, acriflavine, and some fluoroquinolones when overexpressed from a multicopy plasmid (43). Disruption of the *lfrA* gene rendered the mutant more susceptible to ethidium bromide, acriflavine, ciprofloxa-

* Corresponding author. Mailing address: Department of Genetics and Microbiology, Via Ferrata 1, 27100 Pavia, Italy. Phone: 39-0382-985561. Fax: 39-0382-528496. E-mail: derossi@ipvgen.unipv.it.

▽ Published ahead of print on 16 October 2006.

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Comment(s)
RG389	TGCTGGTCCTGGCCGATCAC	Sense primer, amplifying a 672-bp fragment of the *lfrRA* operon
prexA	GCGATGAGCAGCACCGGAAGT	Antisense primer, amplifying a 672-bp fragment of the *lfrRA* operon
RG400	TTGGATCCATCGGCGTCACTC (BamHI)	Sense primer for *lfrR-lfrA* intergenic region-*lacZ* fusion, amplifying a 75-bp DNA fragment
RG401	TTGGATCCACTCGCGGCCTAC (BamHI)	Sense primer for *lfrR-lfrA* intergenic region-*lacZ* fusion, amplifying a 297-bp DNA fragment
RG402	TAGGTACCTCATGAGAAGAGCT (KpnI)	Antisense primer for *lfrR-lfrA* intergenic region-*lacZ* fusion, used with RG400 and RG401
RG404	TAGGATCCATCCTCGGCGAG (BamHI)	Sense primer for upstream *lfrR-lacZ* fusion, amplifying a 351-bp DNA fragment of the *lfrR* upstream region
RG405	TAGGATCCCGCCTCCTCCGC (BamHI)	Sense primer for upstream *lfrR-lacZ* fusion, amplifying a 227-bp DNA fragment of the *lfrR* upstream region
RG406	TAGGATCCTGCCCGCCTATTC (BamHI)	Sense primer for upstream *lfrR-lacZ* fusion, amplifying a 143-bp DNA fragment of the *lfrR* upstream region
RG407	TAGGATCCAGGTGAGTGGGA (BamHI)	Sense primer for upstream *lfrR-lacZ* fusion, amplifying a 62-bp or a 125-bp DNA fragment of the *lfrR* upstream region with primers RG409 and RG410, respectively
RG409	TAGGTACCTGGTCATGTATCAA (KpnI)	Antisense primer for upstream *lfrR-lacZ* fusion, used with RG404, RG405, RG406 and RG407, respectively
RG408	TAGGATCCACCAGCCCGAGCA (BamHI)	Sense primer for upstream *lfrR-lacZ* fusion, amplifying a 67-bp DNA fragment of the *lfrR* gene
RG410	TAGGTACCGCATGGCGGCATCAA (KpnI)	Antisense primer for upstream *lfrR-lacZ* fusion, amplifying a 67-bp DNA fragment of the *lfrR* gene with RG408
prexR	GAGTGCGGCGGTGGGGTGAT	Primer for primer extension
RG391	CGCCCCGAGCACCGAGTT	Sense primer, amplifying a 547-bp DNA fragment of the *lfrA* gene
RG392	GATGATCGACAGGAAGTTC	Antisense primer, amplifying a 547-bp DNA fragment of the *lfrA* gene
RG168	TTGGATCCGATGACCAGCCCGAGCAT (BamHI)	Sense primer for *lfrR* expression into pET-15b, amplifying a 570-bp fragment of the *lfrR* gene
RG169	TTGGATCCTCAGGTGCGCGGCAGG (BamHI)	Antisense primer for *lfrR* expression into pET-15b, amplifying a 570-bp fragment of the *lfrR* gene
A19	GCGGCCTTTATCTATGTCAC	Sense primer, amplifying a 369-bp DNA fragment of the Rv0191 gene
A20	CAGACTGGTTCCGATGTAGA	Antisense primer, amplifying a 369-bp DNA fragment of the Rv0191 gene
RG106	CAGCTACATCGACTACGCC	Sense primer, amplifying a 317-bp DNA fragment of the *gyrA* gene
RG107	GCGCTTCGGTGTAACGCAT	Antisense primer, amplifying a 317-bp DNA fragment of the *gyrA* gene

[a] Restriction sites are underlined in the sequence, and the corresponding enzymes are listed in parentheses following the sequences.

cin, doxorubicin, and rhodamine 123 (two- to eightfold decrease in MICs) (40). These results were also confirmed by Li et al. (26). The upstream region of *lfrA* contains a gene coding for a putative TetR family transcriptional repressor, named LfrR, hypothesized to be responsible for regulation of *lfrA* gene expression (6, 26). The deletion of *lfrR* increased the expression of *lfrA* and resulted in higher resistance to several drugs (26).

In this study, we demonstrate that *lfrR* and *lfrA* genes are cotranscribed by a common promoter. LfrR represses the transcription of the *lfrRA* operon by directly binding to the promoter region of *lfrR-lfrA*. We expressed and purified the LfrR protein from *Escherichia coli* cells, and we demonstrated that it binds to a 143-bp region upstream of the *lfrR* gene. Furthermore, we identified both the *lfrRA* promoter region and the transcriptional start site. To investigate the regulation of the *lfrA* gene, we analyzed its expression by reverse transcription-PCR (RT-PCR) in the presence of compounds hypothesized to be transported by the LfrA pump. Our results indicate that the LfrA pump is tightly regulated.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All cloning steps were performed in *Escherichia coli* DH5α grown in Luria-Bertani (LB) broth or on LB agar (38). *M. smegmatis* mc^2155 wild-type and $mc^2$11 mutant strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% (vol/vol) oleic acid/albumin/dextrose/catalase (OADC enrichment) and 0.05% Tween 80 or on Middlebrook 7H11 medium (Difco) supplemented with 10% (vol/vol) OADC. When necessary, antibiotics (Sigma) were added at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 34 μg/ml; carbenicillin, 50 μg/ml; and kanamycin, 25 μg/ml (for mycobacteria) and 50 μg/ml (for *E. coli*). All strains were grown aerobically at 37°C with shaking at 200 rpm.

DNA techniques. DNA manipulations were performed by standard procedures as described by Sambrook and Russell (38). DNA restriction and modifying enzymes were used as recommended by the manufacturer (Amersham Biosciences). DNA fragments and PCR products were purified from agarose gels with the Qiaex kit (QIAGEN), unless otherwise specified, according to the manufacturer's instructions. Isolation of plasmid DNA was performed using the Plasmid Mini kit (QIAGEN) according to the manufacturer's instructions. Plasmid DNA was sequenced with the SP6 promoter primer by using an automatic DNA sequencer (ABI-PRISM 3100) (Applied Biosystems). Electroporation of *E. coli* cells was done as described previously (38). Electrocompetent mycobacterial cells were prepared and electroporated by using a Bio-Rad Gene Pulser, as described by Parish and Stoker (35). Following electroporation, *M. smegmatis* was plated onto Middlebrook 7H11 medium supplemented with 10% (vol/vol) OADC and 25 μg/ml of kanamycin.

PCR amplification. All primers used for PCR are listed in Table 1. PCR amplifications were performed in a volume of 40 μl containing 200 μM of each deoxynucleoside triphosphate, 500 nM of each primer, 2.5 mM $MgCl_2$, 2% dimethyl sulfoxide, 100 ng of mycobacterial DNA, and 5 U of *Taq* DNA polymerase (Sigma). The protocol used for amplification was as follows: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at a temperature dependent on the primer pair used, and elongation at 72°C for a time dependent on the expected sizes of the products, with a final elongation at 72°C for 10 min.

Construction of *lfrR*::*lacZ* and *lfrA*::*lacZ* fusions. Various portions of the upstream *lfrR* region and of the *lfrR-lfrA* intergenic region were PCR amplified

from *M. smegmatis* mc^{2}155 genomic template by using different pairs of primers as follows: RG407-RG409, RG406-RG409, RG405-RG409, RG404-RG409, RG408-RG410, RG407-RG410, RG400-RG402, and RG401-RG402. The PCR fragments were cloned into pGEM-T Easy (Promega) to yield pECR1, pECR2, pECR3, pECR4, pECD1, pECD2, pECA1, and pECA2, respectively. All plasmids were sequenced to make sure that no mutations were introduced in the corresponding regions. Next, the various fragments were fused to *lacZ* in the 9.3-kb promoter probe vector pJEM12 (45). To do this, the pEC plasmid series was digested with BamHI and KpnI; the DNA fragments were purified from an agarose gel and then ligated to pJEM12 digested with the same enzymes to produce p*lfrR1*, p*lfrR2*, p*lfrR3*, p*lfrR4*, p*lfrD1*, p*lfrD2*, p*lfrA1*, and p*lfrA2*, respectively. The fusion plasmids were then transformed into *M. smegmatis* mc^{2}155 by electroporation as described above for DNA techniques.

β-Galactosidase assay. β-Galactosidase activity was measured as described by Miller (32). Activities were determined in *M. smegmatis* mc^{2}155 strains containing the plasmid pJEM12 and all promoter fusion constructs. The assay was carried out at 28°C using *ortho*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate, and the enzyme activity, expressed in terms of Miller units, was detected by measuring the optical density at 420 nm (OD_{420}). Briefly, transformed mycobacterial cells were grown in 7H9 medium supplemented with 10% (vol/vol) OADC, 0.05% Tween 80, and kanamycin (25 μg/ml) to an optical density at 600 nm of 0.5. No antibiotic or sublethal concentrations (0.4× MIC) of ethidium bromide (MIC, 8 μg/ml) or acriflavin (MIC, 12.5 μg/ml) were added, and the cultures were reincubated for 2 h. The cells were then harvested by centrifugation (4,000 × *g*, 15 min, 4°C), and the pellets were washed and resuspended in 1 ml of phosphate-buffered saline (20 mM phosphate buffer, 150 mM NaCl, pH 7.4). After adding 20 μl of chloroform and 10 μl of 10% sodium dodecyl sulfate (SDS), aliquots of the lysates were incubated with ONPG at 28°C. The enzymatic reaction was followed spectrophotometrically at A_{420}. The β-galactosidase activity was determined as follows: $A = (OD_{420}\ \text{min}^{-1})/(OD_{600} \times \text{milliliters of culture})$. Triplicate samples were measured for each bacterial clone.

RNA extraction and RT-PCR. *M. smegmatis* was grown to an OD_{600} of 0.6 in 7H9 medium supplemented with 10% (vol/vol) OADC and 0.05% Tween 80. Ten milliliters of culture was harvested at 5,000 × *g* for 15 min at 4°C; the cells were resuspended in 200 μl of Tris-EDTA buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0) containing 4 mg/ml of lysozyme and sonicated twice for 10 s at 40 W. Total RNA was then extracted using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol and treated with DNase I-RNase free (10 U/μg of RNA) (Roche) for 30 min at room temperature; the DNase was inactivated at 70°C for 10 min. The RT reactions were carried out by using 2 μg of RNA template in the presence of M-MLV Reverse Transcriptase (Promega). Reverse transcription was carried out as follows. Two micrograms of RNA and 100 pmol of gene-specific primers (prexA and RG392, respectively; see Table 1) were incubated at 70°C for 5 min and then cooled on ice. Five microliters of 5× reaction buffer, 5 μl of 10 mM deoxynucleoside triphosphates, and 200 U of M-MLV Reverse Transcriptase enzyme were added, and the reaction was carried out at 37°C for 1 h. The enzyme was inactivated at 95°C for 5 min, and the reaction was ethanol precipitated. cDNA was dissolved in 20 μl of deionized water, and 4 μl was used for subsequent PCRs. cDNAs were amplified using primer pairs prexA-RG389 and RG391-RG392 (Table 1), as described above, with an annealing temperature of 64°C and 54°C, respectively, and an elongation time of 1 min. Samples were analyzed by electrophoresis on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualized under UV light. The same reactions were carried out for each sample without M-MLV Reverse Transcriptase to ensure that amplification was a result of cDNA and not of contaminating DNA molecules.

Primer extension analysis. The primer prexR (Table 1), which is complementary to the beginning of the coding sequence of the *lfrR* gene, was used to map the 5′ transcriptional start site. It was end labeled with 3,000 Ci mmol^{-1} [γ-^{32}P]ATP (Amersham Biosciences) by using T4 polynucleotide kinase, as described in the primer extension kit (Promega). Briefly, the labeled primer (6 pmol) was incubated with 60 μg of RNA and 5 μl of 2× avian myeloblastosis virus primer extension buffer at 70°C for 10 min to melt secondary structures within the template and chilled on ice. The annealing was performed at 68°C for 20 min and at room temperature for 10 min. Extension was carried out with avian myeloblastosis virus reverse transcriptase (Promega), according to the manufacturer's instructions, at 42°C for 30 min; the reaction was stopped by the addition of 20 μl of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). The reaction products were separated on a 6% polyacrylamide–8 M urea sequencing gel and were run alongside the sequencing products obtained with the prexR primer. Sequencing reactions were carried out with the Sequenase version 2.0 kit (USB) according to the manufacturer's instructions. The gels were dried and then exposed to X-ray film for 24 h at −80°C.

Induction experiments. In order to detect the expression of the *lfrA* gene by RT-PCR, *M. smegmatis* mc^{2}155 cells were grown in 7H9 medium supplemented with 10% (vol/vol) OADC and 0.05% Tween 80 to an OD_{600} of 0.5. The culture was then split, sublethal concentrations (0.05× to 0.4× MIC) of ethidium bromide (MIC, 8 μg/ml), acriflavine (MIC, 12.5 μg/ml), ciprofloxacin (MIC, 0.16 μg/ml), doxorubicin (MIC, 4 μg/ml), and rhodamine 123 (MIC, 5 μg/ml) were added, and the cultures were reincubated for 1, 2, and 4 h, respectively. Cells were harvested at 4,000 × *g* for 15 min and stored at −20°C until RNA extraction and RT-PCR experiments.

Overproduction and purification of LfrR protein. To produce hexahistidine-tagged LfrR (His_6-LfrR), the *lfrR* gene was amplified from *M. smegmatis* mc^{2}155 genomic DNA by PCR with primers RG168 and RG169, which incorporate a BamHI restriction site (Table 1). PCR conditions were as described above, except that the annealing temperature was 54°C. The PCR fragment was gel purified and cloned into pGEM-T Easy cloning vector (Promega) to generate pGEM/*lfrR*. The 570-bp BamHI fragment from pGEM/*lfrR* was then cloned into BamHI-restricted pET15-b (Novagen) to produce pET/*lfrR*. A fresh colony of *E. coli* BL21(DE3)pLysS harboring plasmid pET/*lfrR* was grown overnight at 37°C in LB medium containing chloramphenicol and carbenicillin at a final concentration of 34 μg/ml and 50 μg/ml, respectively. The culture was diluted 1:100 into 200 ml of the same medium and incubated at 37°C until it reached an OD at 600 nm of 0.5. Isopropyl-β-thiogalactopyranoside (IPTG) was then added at a final concentration of 0.125 mM, and incubation was continued for 2 h. Bacterial cells were harvested, resuspended in sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole [pH 8.0]), and disrupted by sonication. The lysate was centrifuged at 10,000 × *g* for 45 min at 4°C, and the His_6-LfrR protein was recovered from the supernatant by Ni-nitrilotriacetic acid chromatography (QIAGEN) as recommended by the manufacturer. The LfrR protein was eluted with the sonication buffer containing 200 mM imidazole. The homogeneity of the eluted protein was estimated to be 90% by Coomassie blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. His_6-LfrR protein concentration was determined by using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) (Sigma) as the protein standard. Total yield was about 20 mg of purified His_6-LfrR from 200 ml of culture.

One milliliter of protein was dialyzed overnight at 4°C against 1 liter of dialysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol) to remove imidazole and used for gel mobility shift assay.

Electrophoretic mobility shift assay. The DNA probes for gel shift experiments were obtained by BamHI-KpnI digestion of pECR1, pECR2, and pECA2 plasmids and isolated from agarose gel by using a Ultrafree-DA kit (Millipore) according to the manufacturer's instructions. The fragments were end labeled with [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) (Amersham Biosciences) by using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions. Approximately 3.5 nM of the labeled DNA fragments was incubated with 2 μM LfrR in reaction buffer (10 mM Tris HCl [pH 7.5], 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$, 50 ng of salmon sperm DNA, 1 mg/ml of BSA, 4% glycerol) at room temperature for 20 min. The samples were immediately loaded onto a 6% native polyacrylamide gel containing 0.5× TBE (0.05 M Tris base, 0.05 M boric acid, 1 mM EDTA-$Na_2 \cdot 2H_2O$) and subjected to electrophoresis. The gels were then vacuum dried and exposed overnight to Biomax radiography film (Kodak). Electrophoretic mobility shift assay was also performed with acriflavine and ciprofloxacin (0.05× and 2× MIC) to test the LfrR modulatory effect upon binding to target DNA. Electrophoretic mobility shift assay competitions were performed with specific and nonspecific competitor DNA. The nonspecific DNA was amplified by PCR with primers A19 and A20 (Table 1) and 100 ng of *M. tuberculosis* DNA. The 369-bp PCR product, consisting of an internal portion of the Rv0191 gene coding for a probable conserved integral membrane protein (http://genolist.pasteur.fr/tuberculist), was isolated from agarose gel as described above.

Unlabeled specific and nonspecific competitor DNA (50- to 100-fold molar excess) were incubated with His_6-LfrR for 10 min at room temperature, followed by the addition of the labeled probe and incubation for 20 min at room temperature. The resulting DNA-protein complexes were then subjected to electrophoresis and autoradiographed as described above.

RESULTS

Transcriptional analysis of the *lfrRA* operon using RT-PCR. Previously, it has been reported that the upstream region of *lfrA* contained a gene coding for a putative TetR family transcriptional repressor, named LfrR, and that the *lfrR* deletion

increased the *lfrA* expression (26). These data suggest that LfrR negatively regulates the production of LfrA. Moreover, *lfrA* disruption in *M. smegmatis* increased susceptibility to ethidium bromide (40). To demonstrate that *lfrR* and *lfrA* genes are part of the same transcriptional unit, RT-PCR experiments were performed. To this aim, total RNA from *M. smegmatis* strain mc^{2}155, grown in the absence or in the presence of ethidium bromide at a final concentration of 3.2 μg/ml, was isolated and retrotranscribed with primer prexA that annealed to the *lfrA* gene (Table 1). The cDNA was amplified by PCR using primers internal to *lfrR* and *lfrA* sequences (RG389 and prexA, respectively), allowing the detection of cotranscription of the *lfrR* and *lfrA* genes. An amplification product of 672 bp according to the size predicted from the DNA sequence was obtained in the case of RNA isolated from cells grown in the presence of ethidium bromide (data not shown). The sequence analysis of the amplified product confirmed that this fragment corresponds to the region defined by RG389 and prexA primers. This result demonstrates clearly and for the first time the presumed operon structure of the *lfrR* and *lfrA* genes. In our conditions, no amplification was observed when the RNA was isolated from cells grown in the absence of ethidium bromide (data not shown).

Mapping of the *lfrRA* promoter. Transcriptional *lacZ* gene fusions carrying various portions of the *lfrR* upstream regions as well as of the *lfrR-lfrA* intergenic region were constructed and used to map the *lfrRA* promoter region. The sizes of the fragments contained in the *lacZ* fusion plasmids are shown in Fig. 1A. The fusion plasmids were electroporated into *M. smegmatis* strain mc^{2}155, and β-galactosidase activities were measured. Cells containing vector pJEM12 did not show any detectable β-galactosidase activity, indicating that the endogenous level of expression of the promoterless *lacZ* was low and negligible (Fig. 1B). Also, the cells harboring the p*lfrD1* plasmid, which contains the *lfrR* region from nucleotide positions +2 to +69 devoid of obvious promoter sequences, did not show any detectable activity (Fig. 1B). In contrast, plasmid p*lfrR3* (−220 to +7) showed the highest activity; however, expression of this activity was not higher in the case of p*lfrR4* (−351 to +7), which showed an activity comparable to that of p*lfrR3* plasmid, suggesting that the entire promoter region is located in the R3 fragment (Fig. 1B). This hypothesis was consistent with the fact that the plasmid p*lfrR2* (−136 to +7) directed expression of a reduced level of β-galactosidase activity (Fig. 1B), followed by plasmid p*lfrR1* (−55 to +7), which exhibited a low level of β-galactosidase activity.

The β-galactosidase activity of the different constructs has also been determined with cultures grown in the presence of ethidium bromide and acriflavine (data not shown). The promoter activity of each construct was similar to that observed in the case of cultures grown without inducers, indicating that these compounds did not affect the promoter activity. The high expression level of the reporter without inducer could be explained by the amplification effect of this region due to the cloning into pJEM12 vector.

In summary, these experiments localized the *lfrRA* promoter in a region extending 220 nucleotides upstream of *lfrR*.

Mycobacterial cells containing p*lfrA1*, which carries the entire *lfrR-lfrA* intergenic region of 71 bp, showed a minimal activity, as did cells containing p*lfrA2* (−294 from *lfrA* to +3),

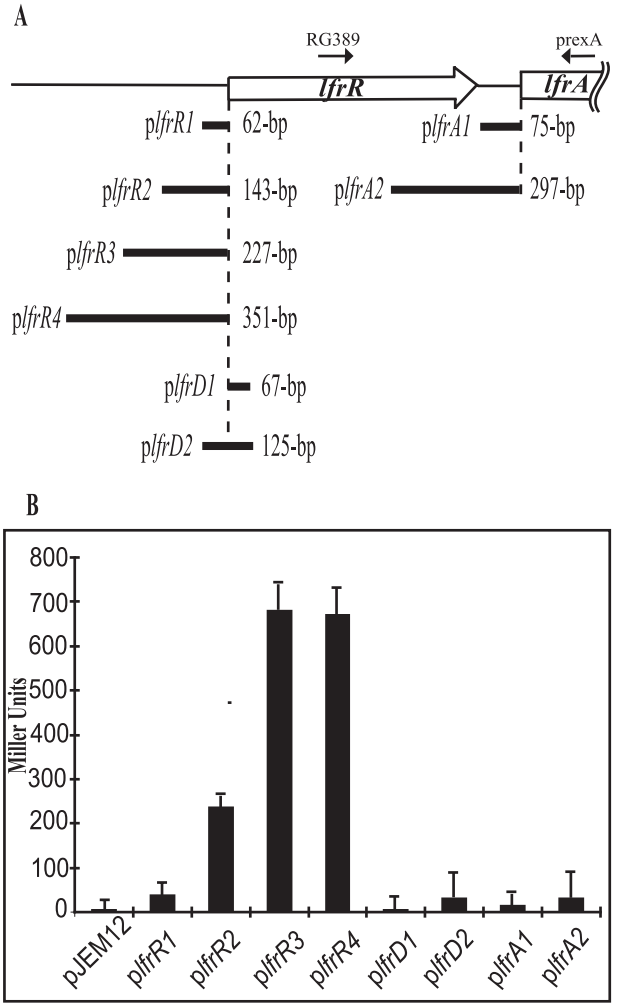

FIG. 1. Localization of the *lfrRA* promoter region using *lacZ* transcriptional fusions. A. Diagram showing the sizes and the positions of the various fragments (shown as black boxes) contained in the *lacZ* fusion plasmids. The white arrows indicate the *lfrR* and *lfrA* genes. B. β-Galactosidase activity of *M. smegmatis* cells containing the different constructs. Error bars indicate standard deviations from triplicate determinations of β-galactosidase activity.

indicating that no promoter activity was present in the intergenic region, thus confirming the RT-PCR data. The β-galactosidase activity of a plasmid containing the *lfrR-lfrA* intergenic region and the entire *lfrR* coding sequence was not evaluated.

Identification of the transcriptional start point of the *lfrRA* operon. The transcription initiation point of the *lfrRA* operon was mapped in cells growing in the absence or presence of ethidium bromide. When total RNA, prepared from *M. smegmatis* mc^{2}155 cells grown in the absence of ethidium bromide, was subjected to primer extension analysis, no signal could be identified, even using different oligonucleotides (data not shown). On the contrary, when total RNA was extracted from an *M. smegmatis* mc^{2}155 culture grown in the presence of ethidium bromide (3.2 μg/ml) for 2 h, a single product was identified (Fig. 2A). The 5′ end of *lfrRA* is the A residue that is the first base of the translational initiation codon (Fig. 2A). No other signal could be identified further upstream with this

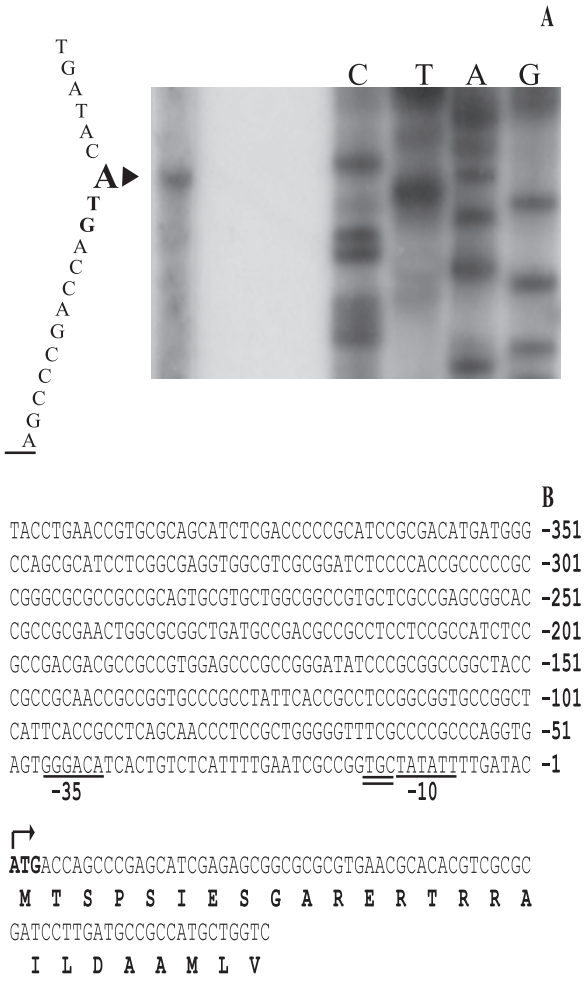

FIG. 2. A. Mapping of the transcriptional start point for the *lfrRA* operon. The position of the transcription start site was determined by primer extension with the oligonucleotide prexR (Table 1). Sequencing reactions, performed with the same oligonucleotide on a plasmid containing the entire *lfrRA* operon and the regions upstream and downstream of the genes, are reported in the last four lanes of the panel. The coordinates of the 5′ end are reported on the left of the panel. B. Nucleotide sequence of the *lfrRA* promoter region. The nucleotide sequences around the promoter region of the *lfrRA* genes are presented. The first 25 amino acids and the corresponding nucleotide sequence of the LfrR protein are also reported. The transcription start site is marked by an arrow. The putative −10 promoter element and the unusual −35 region at a distance of 28 bp are underlined. The "extended −10 promoter" is double underlined (see Discussion).

oligonucleotide or with an oligonucleotide internal to the *lfrA* coding sequence (oligonucleotide prexA) (Table 1 and data not shown). It is noteworthy that the prexA oligonucleotide gave a product of the right size, in agreement with the prexR primer. The position of the 5′ end is consistent with the RT-PCR data, which indicated that the two genes are organized as an operon. It is likely that the primer extension experiment identified a bona fide transcriptional start site, because computer analysis of this region of the mRNA did not reveal any significant features, such as putative hairpin structures, known to prematurely arrest reverse transcription. When the sequence upstream of the *lfrRA* transcriptional start site was compared with sequences of other mycobacterial promoter structures, a putative −10 box (TATATT) could be identified (Fig. 2B). No homologous −35 regions with a canonical distance of 16 to 18 bp have been found; a −35 box (GGGACA) similar to the consensus sequence of *E. coli* was identified, but it was at a 28-bp distance from the −10 box (Fig. 2B).

Some substrates of the LfrA transporter induce transcription of the *lfrA* gene. It was shown previously that the resistance of *M. smegmatis* to multiple structurally unrelated compounds, such as ethidium bromide, acriflavine, ciprofloxacin, doxorubicin, and rhodamine 123, can occur when LfrA is overexpressed by cloning the *lfrA* gene into a multicopy vector (40, 43). On the other hand, the disruption of the *lfrA* gene decreased resistance to ethidium bromide and acriflavine but resulted only in a twofold decrease in MICs for the other compounds (40). Since many of the known bacterial mechanisms of drug resistance are inducible by the corresponding drugs (23), it was interesting to determine if the expression of LfrA in *M. smegmatis* is inducible by LfrA substrates. To screen for factors influencing transcription of the *lfrA* gene, RT-PCR experiments were performed with primers RG391 and RG392 specific for *lfrA* (with an expected 547-bp product) (Table 1). The expression of the *lfrA* efflux pump gene was studied by analyzing the relative amount of *lfrA* mRNA expressed in *M. smegmatis* mc^{2}155 grown in the absence and in the presence of ethidium bromide, acriflavine, ciprofloxacin, doxorubicin, and rhodamine 123, previously reported to be substrates of the LfrA efflux pump (40, 43). No detectable levels of the *lfrA* mRNA was observed when *M. smegmatis* was grown in the absence of any compounds (Fig. 3A, lanes 2 and 7). On the contrary, a detectable level of the *lfrA* mRNA was observed in response to different amounts of ethidium bromide (Fig. 3A, lanes 3 to 6 and 8 to 11). The lower concentration of ethidium bromide and the shorter time of the treatment were sufficient to induce the *lfrA* expression (Fig. 3A, lane 3). The same result was achieved after treatment of the *M. smegmatis* culture with 0.05× and 0.4× MIC of acriflavine (Fig. 3B, lanes 2 and 3) and with the highest concentration (0.4× MIC) of rhodamine 123 after 2 h of induction (Fig. 3B, lane 7). No *lfrA* mRNA was detected in the case of the RT-PCR amplification of RNA isolated from cultures treated either with doxorubicin or ciprofloxacin at concentrations corresponding to 0.05× and 0.4× MIC (Fig. 3B, lanes 4 and 5 as well as 10 and 11, respectively). In all experiments, the expression of the *gyrA* gene coding for the A subunit of the DNA gyrase was determined as an internal control to ensure that the differences observed were not due to variability in the RNA isolation and/or in the RT-PCR technique. The *M. smegmatis gyrA* expression is unaltered in the mid-logarithmic phase under different growth conditions (Fig. 3A and B), demonstrating that the differences detected in the amount of *lfrA* mRNA under different growth conditions are genuine.

Taken together, these results are in favor of the hypothesis that the LfrR protein could act as a repressor of the *lfrA* transcription. Previously in our laboratory we isolated the *M. smegmatis* mutant mc^{2}11 resistant to different fluoroquinolones, cationic dyes, and anthracyclines (6, 40). We demonstrated that the LfrA efflux pump was involved in the resistance profile (6, 40).

Further characterization pointed out that this mutant has an

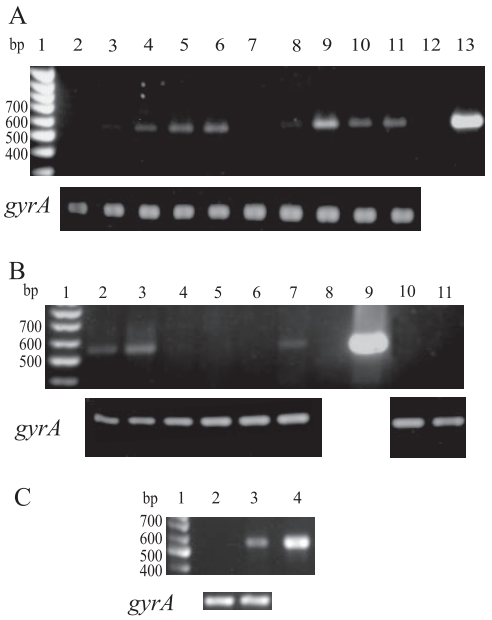

FIG. 3. Effects of different LfrA substrates on *lfrA* expression. The expression of *lfrA* was analyzed by RT-PCR with primers RG391 and RG392 on RNA isolated from *M. smegmatis* mc^{2}155 cultures. A. Expression of *lfrA* in the presence of increasing concentrations of ethidium bromide after 1 (lanes 3 to 6) and 4 h (lanes 8 to 11) of treatment. Lane 1, molecular size marker (Fermentas); also, the sizes of some bands in base pairs are provided on the left; lanes 2 and 7, no ethidium bromide; lanes 3 and 8, 0.4 μg/ml (0.05× MIC); lanes 4 and 9, 0.8 μg/ml (0.1× MIC); lanes 5 and 10, 1.6 μg/ml (0.2× MIC); lanes 6 and 11, 3.2 μg/ml (0.4× MIC); lane 12, negative control; lane 13, positive control, which is DNA amplification with primers RG391 and RG392 (expected size, 547 bp). The expression of the *gyrA* gene (317 bp) of *M. smegmatis* was determined as an internal control of all the RT-PCRs and is shown at the bottom of the panel. B. Expression of *lfrA* in the presence of acriflavine (lanes 2 and 3, 0.625 and 5 μg/ml [0.05× and 0.4× MIC]), doxorubicin (lanes 4 and 5, 0.2 and 1.6 μg/ml [0.05× and 0.4× MIC]), rhodamine 123 (lanes 6 and 7, 0.25 and 2 μg/ml [0.05× and 0.4× MIC]), and ciprofloxacin (lanes 10 and 11, 0.008 and 0.064 μg/ml [0.05× and 0.4× MIC]) after 2 h of treatment. Lanes 1, 8, and 9 are the same as lanes 1, 12, and 13 in panel A. The expression of the *gyrA* gene (317 bp) of *M. smegmatis* was determined as an internal control of all the RT-PCRs and is shown at the bottom of the panel. C. Expression of *lfrA* in *M. smegmatis* mc^{2}155 wild-type (lane 2) and *M. smegmatis* mc^{2}11 mutant (lane 3) strains grown in the absence of inducers. Lanes 1 and 4 are the same as lanes 1 and 13 in panel A. The expression of the *gyrA* gene (317 bp) of *M. smegmatis* was determined as an internal control of all the RT-PCRs and is shown at the bottom of the panel.

insertion of 18 bp within the *lfrR* coding region (E. De Rossi, personal communication). Consequently, we hypothesized that the mutated LfrR protein does not repress the *lfrA* transcription. To verify this hypothesis, RT-PCR experiments with primers RG391 and RG392 were performed using total RNA isolated from *M. smegmatis* mc^{2}11 cells grown in the absence of proper inducer. As shown in Fig. 3C (lane 3), a good level of *lfrA* expression was observed, thus confirming that LfrR acts as a repressor. On the contrary, no *lfrA* transcription was observed when RT-PCR experiments were performed with total RNA extracted from *M. smegmatis* mc^{2}155 wild-type cells (Fig. 3C, lane 2). The *M. smegmatis gyrA* expression is unaltered (Fig. 3C), demonstrating that the differences detected in the amount of *lfrA* mRNA are genuine.

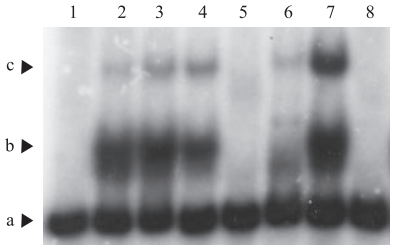

FIG. 4. LfrR binds specifically to the DNA sequence upstream of the *lfrRA* operon. Lane 1, free labeled 143-bp probe; lanes 2 and 3, probe with 20 and 40 ng of LfrR, respectively; lane 4, probe with 20 ng of LfrR and a 100-fold molar excess of nonspecific Rv0191 fragment (noncompetitive probe); lanes 5 and 6, probe with 20 ng of LfrR and 100- and 50-fold molar excesses of 143-bp fragment (competitive probe), respectively; lane 7, probe with 20 ng of LfrR and 0.32 μg/ml of ciprofloxacin (2× MIC); lane 8, probe with 20 ng of LfrR and 25 μg/ml of acriflavine (2× MIC). The letter a indicates the free labeled 143-bp probe, while letters b and c indicate two different retarded complexes (see Results).

Expression and purification of the LfrR protein. The *M. smegmatis lfrR* gene was amplified by PCR, cloned, overexpressed, and purified as a hexahistidine-tagged protein under control of the T7 promoter of the plasmid pET-15b (Novagen), creating a His-tagged protein. The His_6-LfrR fusion protein was expressed in *E. coli* BL21(DE3)pLysS cultures, after induction with IPTG at a final concentration of 0.125 mM, and purified to near homogeneity by Ni^{2+} affinity chromatography. Only one band was visualized on SDS-polyacrylamide gel electrophoresis, which represents the purified protein; this band has an apparent molecular mass of about 21 kDa (data not shown), which is close to value calculated for the 189-amino-acid fusion protein monomer. The purified LfrR protein was used for all subsequent band-shift analyses.

Binding of LfrR to the region upstream of *lfrR*. To determine if LfrR regulates the *lfrRA* operon via direct interaction with the promoter upstream of *lfrR*, gel mobility shift assays were performed. The purified His_6-LfrR protein, when incubated with the end-labeled 143-bp *lfrR* promoter, isolated by BamHI-KpnI digestion of plasmid p*lfrR2* (Fig. 1A), showed a clear shift in the DNA-binding pattern, although two different LfrR-DNA complexes could be detected (Fig. 4, lanes 2 and 3); these retarded complexes are shown in Fig. 4 by the letters b and c, while the letter a indicates the free labeled 143-bp probe. This would suggest the labile formation of higher-molecular-weight complexes because of multimerization of LfrR on the DNA probe. Alternatively, this would indicate the presence of two potential binding sites in the upstream sequence of *lfrR* and that LfrR binds with different affinities to these two sites within the *lfrR* upstream sequence. The observed shift was not complete, as demonstrated by the presence of free labeled probe, probably due to incomplete labeling of the promoter fragment leaving unlabeled "competitor" DNA. However, similar results were achieved when the 227-bp fragment from p*lfrR3* was used. His_6-LfrR binding to the 143-bp fragment was specific, since the band shift was completely and partially inhibited in the presence of a 100- and 50-fold molar excess of unlabeled fragment, respectively (Fig. 4, lanes 5 and 6). The

specificity of binding was further investigated in a competition assay with a 100-fold molar excess of an unrelated labeled fragment (a 369-bp fragment amplified from the Rv0191 gene of *M. tuberculosis*) (Fig. 4, lane 4); the specific binding activity of LfrR to the 143-bp fragment is retained, indicating that LfrR binding to the *lfrRA* promoter region is sequence specific. Gel shift assays were also performed with His_6-LfrR and the end-labeled 62-bp or 297-bp fragment (Fig. 1A), isolated by BamHI-KpnI digestion of plasmids p*lfrR1* and p*lfrA2*, respectively. These two fragments did not cause any band shift when incubated with the His_6-LfrR protein (data not shown).

To confirm the hypothesis that LfrR acts as a repressor of the *lfrRA* operon, dissociating from the upstream region upon drug binding, the effects of ciprofloxacin and acriflavine were also tested for their ability to dissociate the LfrR-DNA complex. Addition of an excess amount of acriflavine (25 μg/ml; 2× MIC) abolished the LfrR binding to the 143-bp labeled fragment (Fig. 4, lane 8), suggesting that this drug acts as an inducer that antagonizes the interaction between LfrR and DNA. These data are consistent with RT-PCR experiments, which showed a detectable level of *lfrA* mRNA only in the presence of acriflavine as well as ethidium bromide, both substrates of the LfrA efflux pump. This indicates that the presence of this compound causes the dissociation of the protein from the DNA, thus allowing the transcription to proceed. On the contrary, the addition of ciprofloxacin (0.32 μg/ml; 2× MIC) did not have any effect on LfrR binding to the 143-bp labeled fragment (Fig. 4, lane 7), suggesting that this drug does not dissociate the LfrR-DNA complex. These data are broadly consistent with the substrate specificity of the LfrA pump and strongly confirm RT-PCR results.

DISCUSSION

The first efflux pump described in mycobacteria, the protein LfrA, was identified in *M. smegmatis*. LfrA confers resistance to ciprofloxacin, other fluoroquinolones, ethidium bromide, and acriflavine (43), but also to anthracyclines and rhodamine 123 when cloned into a multicopy plasmid (40). More recently, a gene coding for a putative TetR family transcriptional repressor, named LfrR, was identified upstream of the *lfrA* gene (26).

In this work we show, by RT-PCR experiments, that *lfrR* and *lfrA* genes are organized as an operon. The use of *lacZ* fusions narrowed the *lfrRA* promoter to a region located within 220 nucleotides upstream of *lfrR*. Primer extension analysis confirmed that the transcriptional start site of *lfrA* is indeed upstream of the *lfrR* gene, and it was found that the A residue is the first base of the translational initiation codon of the *lfrR* gene. The leaderless mRNA is consistent with the lack of a Shine-Dalgarno sequence upstream of the *lfrR* gene (11, 19). Alignment of the region centered around position −10 (TATATT) consensus sequences from the transcriptional start site of the *lfrRA* genes showed significant sequence similarities to *E. coli* consensus promoters according to the study of Bashyam et al. (2). Sequence similarities were found also in respect to other mycobacterial promoters, in particular to the acetamidase gene (100% identity) (30) and to the S16 promoter (83% identity) (2) of *M. smegmatis*. One putative −35 region (GGGACA) was found positioned at an unusual spacing, 28 bp from the −10 region, showing 66% identity with the *E. coli* consensus promoter and with the −35 region (TT GACA) of the *M. smegmatis rrnAP3* and *rrnAPCL1* promoters (10). No other homologous −35 regions with a canonical 16- to 18-bp distance have been found; it is noteworthy that in mycobacteria there is a greater heterogeneity at the −35 region, reflecting a higher GC content, and in many cases there is no identifiable −35 element (42, 47). This could also explain why p*lfrR1* does not express LacZ: maybe the p*lfrR1* construct does not contain the *lfr* promoter. Moreover, the upstream region is also important for regulation and promoter activity, as demonstrated with p*lfrR3* and p*lfrR4* constructs. However, promoters that lack a canonical −35 sequence but are still functional have been reported in mycobacteria (2). For mycobacterial promoters, where apparent conservation in −35 region is absent, many of them possess TGN nucleotides immediately upstream of the −10 region, and thus they are termed "extended −10 promoters" (42). This "extended −10 promoter" is also present as TGC upstream of the −10 region of the *lfrR* gene (Fig. 2B).

It was not surprising that *lfrA* expression was inducible by ethidium bromide and acriflavine and, in part, by rhodamine 123, as this was reported for other drug efflux pumps of different bacteria, such as Bmr of *Bacillus subtilis* (1) and TtgABC (44) and SrpABC (24) of *Pseudomonas putida*. It is interesting to note that the two compounds for which the MICs strongly decrease in an *M. smegmatis lfrA*-deleted strain (40) are the ones that can induce the expression of the pump itself. In this way we demonstrate what was postulated in the previous work (40): ethidium bromide is a better inducer of the expression of *lfrA* than ciprofloxacin. It is noteworthy that the *lfrA* gene is highly expressed in the *M. smegmatis* $mc^2$11 mutant characterized by a mutated LfrR protein.

The critical role of LfrR on the regulation of LfrA in *M. smegmatis* and its binding site were defined by electrophoretic mobility shift assay: LfrR represses the transcription of *lfrA*. This feature resembles the control of TetA by TetR, in which the basal level of expression of *tetA* is minimal in the absence of tetracycline (13). This is probably due to the fact that both LfrA and the TetA pumps are specific for specific substrates and constitutive expression of *lfrA*, and *tetA* is not required in the absence of these compounds. On the other hand, the overproduction of efflux pumps in the absence of selection pressure or substrates has been demonstrated to be deleterious to some organisms (7, 33, 39). Therefore, there is a need for regulatory systems to modulate the expression of MDR efflux pumps in bacteria. In this respect, LfrR acts as a moderator to maintain balanced production of LfrA to meet the physiological needs and facilitate the adaptation of *M. smegmatis* to environmental changes, including antibiotic treatments.

It was previously reported that the expression of MDR efflux pumps can be conditionally induced by structurally diverse substrates of these pumps (1, 4, 12, 21, 22, 29, 31, 37). This induction is due to the direct interaction of the substrates with repressor molecules, which interferes with the binding of repressors to operator DNA and which results in increased levels of expression of MDR pump genes. Here we show that the inducible expression of LfrA following treatment with ethidium bromide, acriflavine, or rhodamine 123 is mediated by LfrR. Indeed, acriflavine is able to abolish the binding of

LfrR to the 143-bp promoter region (Fig. 4), thus allowing the transcription to proceed. This finding is consistent with the in vivo induction of *lfrA* expression by acriflavine and strongly indicates that acriflavine-mediated inhibition of LfrR binding to *lfrRA* promoter is responsible for the enhanced transcription of *lfrA*.

Transcriptional regulators of the TetR family are characterized by a conserved helix-turn-helix-containing DNA-binding domain at the N-terminal region and a divergent C-terminal sequence that is involved in binding to various inducing ligands (14, 17, 18). Binding by an inducing compound to the C-terminal region triggers conformational changes in the N-terminal DNA-binding domain, reducing the affinity of a regulator to its target promoter DNA (13). Such structural changes in a repressor induced by the binding of a ligand have been confirmed for several regulator proteins in the TetR family, such as TetR and QacR (34, 41). Similar to other TetR family regulators, LfrR has a typical N-terminal DNA-binding helix-turn-helix motif and a potential ligand-binding region in the C-terminal portion (26). Therefore, it is likely that acriflavine interacts with the C-terminal region of LfrR and induces conformational changes in the repressor, resulting in a great reduction in its DNA-binding affinity.

The absence of an interaction among ciprofloxacin and LfrR was surprising given earlier observations of reproducible ninefold increases in the MIC of this compound when *lfrA* was overexpressed, establishing that it is a LfrA substrate (40). The explanation for this apparent paradox simply may be that not all LfrA substrates must be inducers, and LfrA may be more promiscuous than LfrR. A relevant example of such a behavior is given by the QacA efflux pump, which extrudes compounds that do not induce its expression or bind to the QacR repressor (12).

The ability of LfrR to interact functionally with dissimilar molecules can seemingly have two explanations. The first hypothesis suggests that the function of the LfrR-LfrA system is to protect bacteria from diverse environmental toxins. The alternative hypothesis suggests that LfrA has evolved to efflux-specific compounds while the ability of this protein to bind and efflux diverse drugs is merely a fortuitous side effect of its normal function.

There is no known homolog of the *lfrA* gene in the *M. tuberculosis* genome. This indicates that *M. smegmatis* (for which a genome size of 7.5 Mb has been estimated, versus the 4.4-Mb size of *M. tuberculosis*) will contain many other MFS transporters different from those identified in *M. tuberculosis*.

In conclusion, we demonstrate definitively that LfrR is the *lfrA* transcriptional repressor. Furthermore, the expression of the LfrA efflux pump is regulated by some of its substrates, and this regulation is mediated by its repressor LfrR.

ACKNOWLEDGMENTS

We thank J. Timm for supplying plasmid pJEM12, required for this study.

This work was supported by Fondo d'Ateneo per la Ricerca 2004 and 2005 (E.D.R.), Istituto Superiore Sanità, Ricerca Finalizzata 2005 (G.R.), and Integrated Research Actions Italy-Spain (E.D.R.).

REFERENCES

1. **Ahmed, M., M. Borsch, S. S. Taylor, N. Vazquez-Laslop, and A. A. Neyfakh.** 1994. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. J. Biol. Chem. **269:**28506–28513.
2. **Bashyam, M. D., D. Kaushal, S. K. Das Gupta, and A. K. Tyagi.** 1996. A study of mycobacterial transcriptional apparatus: identification of novel features in promoter elements. J. Bacteriol. **178:**4847–4853.
3. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
4. **Brown, M. H., T. Paulsen, and R. A. Skurray.** 1999. The multidrug efflux protein NorM is a prototype of a new family of transporters. Mol. Microbiol. **31:**394–395.
5. **De Rossi, E., J. A. Ainsa, and G. Riccardi.** 2006. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol. Rev. **30:**36–52.
6. **De Rossi, E., M. C. Blokpoel, R. Cantoni, M. Branzoni, G. Riccardi, D. B. Young, K. A. De Smet, and O. Ciferri.** 1998. Molecular cloning and functional analysis of a novel tetracycline resistance determinant, *tet*(V), from *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **42:**1931–1937.
7. **Eckert, B., and C. F. Beck.** 1989. Overproduction of transposon Tn*10*-encoded tetracycline resistance protein results in cell death and loss of membrane potential. J. Bacteriol. **171:**3557–3559.
8. **Espinal, M. A., A. Laszlo, L. Simonsen, F. Boulahbal, S. J. Kim, A. Reniero, S. Hoffner, H. L. Rieder, N. Bikin, C. Dye, R. Williams, M. C. Raviglione, et al.** 2001. Global trends in resistance to antituberculosis drugs. N. Engl. J. Med. **344:**1294–1303.
9. **Evans, K., L. Adewoye, and K. Poole.** 2001. MexR repressor of the *mexA BoprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. **183:**807–812.
10. **Gonzalez-y-Merchand, J. A., M. J. Colston, and R. A. Cox.** 1996. The rRNA operons of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*: comparison of promoter elements and of neighbouring upstream genes. Microbiology **142:**667–674.
11. **Grill, S., C. O. Gualerzi, P. Londei, and U. Blasi.** 2000. Selective stimulation of translation of leaderless mRNA by initiation factor 2: evolutionary implications for translation. EMBO J. **19:**4101–4110.
12. **Grkovic, S., M. H. Brown, N. J. Roberts, I. T. Paulsen, and R. A. Skurray.** 1998. QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. J. Biol. Chem. **273:**18665–18673.
13. **Grkovic, S., M. H. Brown, and R. A. Skurray.** 2002. Regulation of bacterial drug export systems. Microbiol. Mol. Biol. Rev. **66:**671–701.
14. **Grkovic, S., K. M. Hardie, M. H. Brown, and R. A. Skurray.** 2003. Interactions of the QacR multidrug-binding protein with structurally diverse ligands: implications for the evolution of the binding pocket. Biochemistry **42:**15226–15236.
15. **Hagman, K. E., and W. M. Shafer.** 1995. Transcriptional control of the *mtr* efflux system of *Neisseria gonorrhoeae*. J. Bacteriol. **177:**4162–4165.
16. **Hagman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd, and W. M. Shafer.** 1995. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. Microbiology **141:**611–622.
17. **Hillen, W., and C. Berens.** 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. **48:**345–369.
18. **Hinrichs, W., C. Kisker, M. Duvel, A. Muller, K. Tovar, W. Hillen, and W. Saenger.** 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. Science **264:**418–420.
19. **Janssen, G. R.** 1993. Eubacterial, archaebacterial, and eukaryotic genes that encode leaderless mRNA, p. 59–67. *In* R. H. Baltz, G. D. Hegeman, and P. L. Skatrud (ed.), Industrial microorganisms: basic and applied molecular genetics. ASM Press, Washington, D.C.
20. **Jarlier, V., and H. Nikaido.** 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol. Lett. **123:**11–18.
21. **Kaatz, G. W., and S. M. Seo.** 1995. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **39:**2650–2655.
22. **Kato, S., J. Nishimura, Y. Yufu, H. Ideguchi, T. Umemura, and H. Nawata.** 1992. Modulation of expression of multidrug resistance gene (mdr-1) by adriamycin. FEBS Lett. **308:**175–178.
23. **Kieboom, J., J. J. Dennis, G. J. Zylstra, and J. A. M. De Bont.** 1998. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J. Bacteriol. **180:**6769–6772.
24. **Köhler, T., S. F. Epp, L. K. Curty, and J.-C. Pechère.** 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **181:**6300–6305.
25. **Li, X. Z., and H. Nikaido.** 2004. Efflux-mediated drug resistance in bacteria. Drugs **64:**159–204.
26. **Li, X. Z., L. Zhang, and H. Nikaido.** 2004. Efflux pump-mediated drug resistance in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **48:**2415–2423.
27. **Lomovskaya, O., K. Lewis, and A. Matin.** 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J. Bacteriol. **177:**2328–2334.
28. **Lucas, C. E., J. T. Balthazar, K. E. Hagman, and W. M. Shafer.** 1997. The MtrR repressor binds the DNA sequence between the *mtrR* and *mtrC* genes of *Neisseria gonorrhoeae*. J. Bacteriol. **179:**4123–4128.

29. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
30. **Mahenthiralingam, E., P. Draper, E. O. Davis, and M. J. Colston.** 1993. Cloning and sequencing of the gene which encodes the highly inducible acetamidase of *Mycobacterium smegmatis*. J. Gen. Microbiol. **139:**575–583.
31. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino.** 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:** 2242–2246.
32. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Nguyen, T. N., Q. G. Phan, L. P. Duong, K. P. Bertrand, and R. E. Lenski.** 1989. Effects of carriage and expression of the Tn*10* tetracycline-resistance operon on the fitness of *Escherichia coli* K12. Mol. Biol. Evol. **6:**213–225.
34. **Orth, P., D. Schnappinger, W. Hillen, W. Saenger, and W. Hinrichs.** 2000. Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. Nat. Struct. Biol. **7:**215–219.
35. **Parish, T., and N. G. Stoker.** 1998. Electroporation of mycobacteria, p. 129–144. *In* T. Parish, and N. G. Stoker (ed.), Methods in molecular biology, vol. 101: mycobacteria protocols. Humana Press, Totowa, N.J.
36. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
37. **Rosenberg, E. Y., D. Bertenthal, M. L. Nilles, K. P. Bertrand, and H. Nikaido.** 2003. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. Mol. Microbiol. **48:**1609–1619.
38. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sanchez, P., J. F. Linares, B. Ruiz-Diez, E. Campanario, A. Navas, F. Baquero, and J. L. Martinez.** 2002. Fitness of *in vitro* selected *Pseudomonas aeruginosa nalB* and *nfxB* multidrug resistant mutants. J. Antimicrob. Chemother. **50:**657–664.
40. **Sander, P., E. De Rossi, B. Boddinghaus, R. Cantoni, M. Branzoni, E. C. Bottger, H. Takiff, R. Rodriquez, G. Lopez, and G. Riccardi.** 2000. Contribution of the multidrug efflux pump LfrA to innate mycobacterial drug resistance. FEMS Microbiol. Lett. **193:**19–23.
41. **Schumacher, M. A., M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, and R. G. Brennan.** 2001. Structural mechanisms of QacR induction and multidrug recognition. Science **294:**2158–2163.
42. **Smith, I., W. R. Bishai, and V. Nagaraja.** 2005. Control of mycobacterial transcription, p. 219–231. *In* S. T. Cole, K. D. Eisenach, D. N. McMurray, and W. R. Jacobs, Jr. (ed.), Tuberculosis and the tubercle bacillus. ASM Press, Washington, D.C.
43. **Takiff, H. E., M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. Jacobs.** 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. USA **93:**362–366.
44. **Teràn, W., A. Felipe, A. Segura, A. Rojas, J. L. Ramos, and M. T. Gallegos.** 2003. Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. Antimicrob. Agents Chemother. **47:**3067–3072.
45. **Timm, J., E. M. Lim, and B. Gicquel.** 1994. *Escherichia coli*-mycobacteria shuttle vectors for operon and gene fusions to *lacZ*: the pJEM series. J. Bacteriol. **176:**6749–6753.
46. **Trias, J., and R. Benz.** 1994. Permeability of the cell wall of *Mycobacterium smegmatis*. Mol. Microbiol. **14:**283–290.
47. **Unniraman S., M. Chatterji, and V. Nagaraja.** 2002. DNA gyrase genes in *Mycobacterium tuberculosis*: a single operon driven by multiple promoters. J. Bacteriol. **184:**5449–5456.
48. **Viveiros, M., C. Leandro, and L. Amaral.** 2003. Mycobacterial efflux pumps and chemotherapeutic implications. Int. J. Antimicrob. Agents. **22:**274–278.
49. **Walsh, C.** 2003. Antibiotics: actions, origins, resistance, p. 89–155. ASM Press, Washington, D.C.