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Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*

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ABSTRACT **Due to the resurgence of tuberculosis and the emergence of multidrug-resistant strains, fluoroquinolones (FQ) are being used in selected tuberculosis patients, but FQ-resistant strains of *Mycobacterium tuberculosis* have rapidly begun to appear. The mechanisms involved in FQ resistance need to be elucidated if the effectiveness of this class of antibiotics is to be improved and prolonged. By using the rapid-growing *Mycobacterium smegmatis* as a model genetic system, a gene was selected that confers low-level FQ resistance when present on a multicopy plasmid. This gene, *lfrA*, encodes a putative membrane efflux pump of the major facilitator family, which appears to recognize the hydrophilic FQ, ethidium bromide, acridine, and some quaternary ammonium compounds. It is homologous to *qacA* from *Staphylococcus aureus*, *tcmA*, of *Streptomyces glaucescens*, and *actII* and *mmr*, both from *Streptomyces coelicoler*. Increased expression of *lfrA* augments the appearance of subsequent mutations to higher-level FQ resistance.**

The worldwide reemergence of tuberculosis as a major public health problem has been accompanied by an ominous increase in multidrug resistant strains (1). This increase has stimulated an intense search for new antimycobacterial agents, but at present only one additional class of drugs, the fluoroquinolones (FQ), has been added to the traditional anti-tuberculosis armamentarium (2). Introduced into clinical practice in the 1980s, the FQ were initially active against many pathogens (3), but their use has been limited by the rapid appearance of resistance in a large percentage of clinical isolates, especially in *Staphylococcus aureus* and *Pseudomonas aeruginosa* (41, 42). The therapeutic use of the FQ in tuberculosis began only within the past 3–4 years, and they are generally reserved for infections resistant to other agents. However, most FQ are only moderately active against the mycobacteria, and unfortunately, FQ-resistant (FQ^r) clinical isolates of *Mycobacterium tuberculosis* have already appeared (4, 5). If more can be learned about what determines the effectiveness of a particular FQ against the mycobacteria and the mechanisms by which resistance develops, new agents or strategies may be designed that can prevent or circumvent this resistance.

The principal targets of the FQ are bacterial type II topoisomerases, including both the bacterial DNA gyrase, an essential type II topoisomerase that introduces supercoils into the DNA chromosome (6), and the highly homologous topoisomerase IV, which deconcatenates the chromosome after DNA replication (7, 8). Mutations in a particular region of *gyrA*, which encodes the gyrase A subunit, have been associated with moderate-to-high level [>5× minimal inhibitory concentration (MIC)] FQ resistance in many species of bacteria, including *M. tuberculosis* (5), and similar mutations have been found in the homologous region of topoisomerase IV (9, 10). Mutations conferring low-level resistance have also been found in *gyrB* (11) encoding the B subunit of the DNA gyrase, and the most highly resistant strains often contain mutations in several sites (12), as well as double *gyrA* mutations (13). The principal candidates for nontarget (non-type II topoisomerase) mutations are genes that control the intracellular concentration of the FQ (14). These include the genes for membrane porins, such as *ompF* (15, 16), presumably mediating entry of the drug into the bacteria, and genes for membrane efflux pumps, such as *norA* (17, 18), that extrude drug out of the bacteria in exchange for protons (19). Many of the described mutations do not alter the amino acid sequence of these membrane proteins but rather affect their expression, by either gene amplification (20), promoter-up mutations (21), or mutations in regulatory loci such as *mar* (22), *sox* (23), or *pqr* (24).

In this report, using the rapid growing *Mycobacterium smegmatis* as a model genetic system (25) for *M. tuberculosis*, we describe a membrane efflux pump whose presence on a plasmid confers low-level FQ resistance and increases the frequency of mutations to higher-level resistance.§

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *M. smegmatis* high-frequency transformation strain mc^2155 was grown as described (26). For cloning and preparation of sequencing templates, *Escherichia coli* strains XL1Blue and DH5α were grown in LB broth and agar medium at 37°C. Antibiotics were used at the following concentrations: carbenicillin at 50 μg/ml, kanamycin (Kan) at 10 μg/ml for *M. smegmatis* and at 25 μg/ml for *E. coli*; tetracycline (Tet) at 2.5 μg/ml for *M. smegmatis*, and at 12.5 μg/ml for *E. coli*.

Drugs and Resistance Testing. Testing for resistance to drugs and toxic agents was performed in 96-well microtiter plates. After overnight growth in 7H9-OADC-Tween, 10^5 colony-forming units of mc^2155 were added to wells containing 7H9-OADC without Tween and various concentrations of the drugs. After 48 hr of growth at 37°C, wells were scored for cell pellets, and aliquots were plated for colony counts.

Ciprofloxacin (Cip) was obtained from Miles, sparfloxacin was given by Parke–Davis, and ofloxacin, levofloxacin, and DU-6859-a were provided by Daiichi Pharmaceutical (Tokyo). Acriflavine, benzalkonium chloride, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), chloramphenicol, cetyldimethylethylammonium bromide (CTAB), ethidium bromide, and nalidixic acid were obtained from Sigma.

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Abbreviations: MIC, minimal inhibitory concentration; WT, wild type; FQ, fluoroquinolones; FQ^r, fluoroquinolone-resistant; Cip, ciprofloxacin; Cip^r, Cip-resistant; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CTAB, cetyldimethylethylammonium bromide; Kan, kanamycin; Kan^r, Kan resistant; Tet, tetracyclin; Tet^r, Tet-resistant.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U40487).

Construction of Genomic Libraries and Isolation of lfrA. Genomic DNA was isolated from Cip-resistant (Cip^r) strain mc^{2}552 as described (25). After a partial digestion with *Msp* I, 3- to 6-kb fragments were ligated into the *Cla* I site of vector pYUB53 (25), transformed into *E. coli* DH5α, and plated on LB Tet. Colonies were pooled, and plasmid DNA was isolated (27) and electroporated into mc^{2}155 (25), which was then plated on 7H11 plates with Cip at 1 μg/ml and Tet at 2.5 μg/ml. Plasmid pMN1 was isolated from Tet-resistant (Tet^r) Cip^r *M. smegmatis* colonies and transformed into *E. coli*. Plasmid DNA for analysis, cloning, and sequencing was isolated from *E. coli* (27).

Subcloning and Sequencing of lfrA. Clone pMN1 was cut with various enzymes to generate a restriction map, and fragments were subcloned into vectors pMV203 (26) (pSB51-pSB53) or pMD31 (28) (Fig. 1). Miniprep DNA of the subclones was electroporated into mc^{2}155, and kanamycin-resistant (Kan^r) colonies were tested for Cip resistance. The insert from pSB51, which conferred Cip resistance, was subcloned into vectors pUC118 and pUC119, and serial deletions were created by using Promega Erase-a-Base. Helper phage M13K07 was used to obtain single-stranded DNA for sequencing (27), and double-stranded DNA was prepared (27) by alkaline lysis or with Magic Minipreps (Promega). The deletion plasmids were sequenced with United States Biochemical Sequenase 2.0 using M13 universal and reverse primers.

Sequence assembly and analysis were done by using the ASSEMBLYLIGN and MACVECTOR programs (IBI). Protein comparisons were done with the BESTFIT program of the Wisconsin Genetics Computer Group package. BLASTX (29) was also used for homology searches.

Selection of Wild-Type (WT) Copy of *lfr*. A colony blot of an mc^{2}6 *M. smegmatis* cosmid library (25) was hybridized to a 1.1-kb *Hin*cII fragment from pSB51. Restriction fragments from a hybridizing cosmid were subcloned into vector pMD31 and electroporated into mc^{2}155 to test for Cip resistance.

Plasmids (Fig. 1) were constructed using restriction enzymes and DNA ligase purchased from New England Biolabs, Promega, or Amersham.

Partial Sequencing of *gyrA*. A cosmid containing the *M. smegmatis gyrA* was obtained by hybridization to the mc^{2}6 cosmid library as described (5). A shotgun library of a *gyrA* hybridizing subclone was constructed in vector pUC119 and hybridized to a 0.9-kb *Bam*HI fragment containing the segment of *gyrA* from *M. tuberculosis* where FQ^r mutations have been found (5). On the basis of the *M. smegmatis gyrA* sequence obtained from the hybridizing clones, primers were constructed and used as described (5) to amplify and sequence this region from strain mc^{2}552.

RESULTS

Selection of a Cip^r Mutant of *M. smegmatis*. Overnight cultures of *M. smegmatis* mc^{2}155 were spread on plates with increasing concentrations of Cip. Isolated colonies appeared on plates with Cip at 0.5, 1.0, and 2.0 μg/ml, at frequencies of 2 $\times$ 10^{-5}, 4 $\times$ 10^{-5} and 1 $\times$ 10^{-7}, respectively. A colony that arose on 2 μg/ml was passed successively to plates with Cip at 1, 8, and 16 μg/ml. The strain mc^{2}552 was taken from a large colony on a plate with Cip at 16 μg/ml.

Isolation and Characterization of lfrA. A genomic library of mc^{2}552, constructed in shuttle vector pYUB53, was transformed into *E. coli*. Tet^r colonies were pooled, and plasmid DNA was isolated and electroporated into WT Cip-sensitive mc^{2}155, which was then plated on medium with Cip at 1 μg/ml and Tet at 2.5 μg/ml. Analysis of plasmid DNA from six of the many Tet^r Cip^r colonies showed that all contained the same clone, designated pMN1.

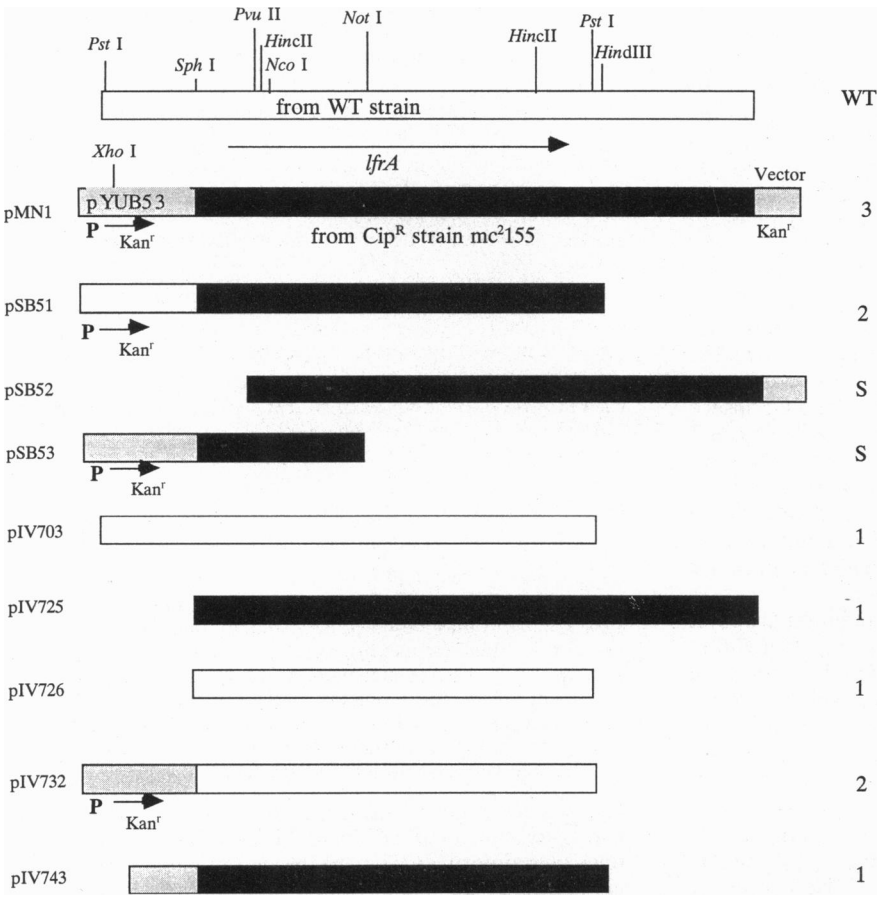

FIG. 1. Subclones of plasmid pMN1 and pIV703. Filled bars represent DNA from Cip^r strain mc^{2}552, open bars are DNA from Cip-sensitive strain mc^{2}6, and shaded bars show DNA from plasmid vector pYUB53. Letters on the right show whether the plasmid conferred Cip resistance in mc^{2}155: **S**, sensitive; or resistant to **1**, **2**, or **3** μg/ml; **P** is the promoter of the Kan gene on pYUB53. All restriction enzyme sites shown were used to construct the plasmids, except for *Hin*cII sites, which were used to isolate a hybridization probe.

To obtain the minimum fragment of DNA conferring Cip resistance, pMN1 was digested with several restriction enzymes and subcloned into vector pMV203 (Fig. 1). The smallest fragment conferring Cip^r colonies was found to be the 2.3-kb insert in pSB51, although the level of resistance (Cip at 2 μg/ml) was slightly less than that conferred by pMN1 (Cip at 3 μg/ml). It is notable that all plasmid constructions conferred much lower-level resistance to Cip than seen in the original mutant strain mc^{2}552, which grew well on plates with 16 μg/ml.

Sequence of *lfrA*. Sequence analysis showed that the insert of pSB51 contained an open reading frame of 1674 bp with no obvious consensus promoter sequence (Fig. 1). Similarity searches with BLASTX revealed homology to the membrane efflux pumps of the major facilitator family. On the basis of this homology and the location of a putative ribosome-binding site, the methionine shown in Fig. 1 was designated as the translational start of a protein composed of 504 amino acids. The encoded protein is most homologous to *qacA* (30) (35/60% identity/% similarity) of *S. aureus*, but also similar to *actII* (31), *tcmA* (32), and *mmr* (33), (34/58, 32/57, and 27/52% identity/% similarity, respectively) all from streptomyces (Fig. 2). Like these, the predicted *M. smegmatis* protein appears to have 14 hydrophobic, membrane-spanning regions. The gene was named *lfrA*, for low-level FQ resistance.

LfrA on pMN1 Is Expressed from the Kan Promoter. Initially, it was expected that *lfrA* from strain mc^{2}552 would contain a mutation changing its specificity. However, the membrane efflux pumps norA (21) and bmr (20) confer FQ resistance when their expression is increased, and emrB (35) was found on the basis of a multicopy effect, so it was possible that *lfrA* also conferred resistance solely because of its presence on a multicopy plasmid. To test this hypothesis, a WT *lfrA* gene was selected by hybridization to a cosmid library of Cip-sensitive *M. smegmatis* and subcloned into pMD31 to create pIV703 (Fig. 1). The presence of the WT *lfrA* gene on pIV703 in mc^{2}155 confers resistance to Cip at 1 μg/ml, suggesting a multicopy effect, but the level of resistance was less than that seen with pMN1 (3 μg/ml).

To define the basis for the higher resistance conferred by pMN1, a series of plasmids were constructed to compare the *lfrA* genes on pMN1 and pIV703 (Fig. 1). When the *lfrA* protein-coding region from pIV703 was ligated to the upstream region from plasmid pMN1, the resulting plasmid, pIV732, conferred resistance to Cip at 2 μg/ml. This result suggested that *lfrA* expression on pMN1 was driven by the strong Kan promoter on parent vector pYUB53; this is possible because pMN1 was constructed by cloning into a *Cla* I site in vector pYUB53, which lies within the kanamycin resistance (*aph*) gene. Examination of the ligated sequence shows the Aph protein to be in the same reading frame as LfrA, with one stop codon, UGA, at the beginning of the cloned insert. Expression from the Kan promoter seems likely because resistance is reduced to Cip at 1 μg/ml when the entire vector segment (pIV725), or merely the promoter of the *aph* gene, is removed (pIV743). Therefore the increased level of Cip resistance conferred by pMN1 is not due to a mutation in either the *lfrA* protein coding or upstream segments but rather to increased expression from the *aph* promoter.

mc^{2}551 Contains a Mutation in GyrA. As mentioned above, the original mutant strain mc^{2}552 grew on plates with Cip at 16 μg/ml, but mc^{2}155-containing pMN1 will grow only on plates containing no more than 2–3 μg/ml. It was therefore not surprising that mc^{2}552 was found to have a *gyrA* point mutation, changing aspartate to asparagine in codon 94 (data not shown), an amino acid often mutated in FQ^r *M. tuberculosis* clinical isolates and laboratory mutants (5).

Specificity of LfrA. To define the range of substrates for LfrA, it was first tested against several other FQ. Table 1 shows that compared with vector pYUB53 alone, pMN1 confers at least a 2- to 4-fold increase in resistance to the hydrophilic FQs Cip, ofloxacin, levofloxacin, and DU-6859-a. However, pMN1 does not confer resistance to the hydrophobic FQs sparfloxacin and nalidixic acid (Tables 1 and 2).

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QacA   LISFFTKT-TDM--M.SK----KR.T.-.V...VSLFVVTM.M.I.IM..
LfrA   MSTCIEGTPSTT--RTPT----RAWVA-LAVLALPVLLIAIDNTVLAFAL
Mmr    .T.-VRTGGAQ.AEVPAGGRRDVPSGVKITA..TGFVMATL.V..VNV.G
ActII  VTA-NP.R.GGPADQGHP----.R.AI-.G..V.SLVG.IL.....NVT.
TcmA   ...ETHDE..GVAHTPASGLRG.P.PT-.LAV.VG.MMV.L.S.IV.I.N
                                                  * *

QacA   .ELV---RELE..G.Q......I......GFIIPLSAFA.KW..KKA..T
LfrA   PLIA---EDFRPSATTQLWIVDVYSLVLAALLVAMGSLGDRLGRRRVLLI
Mmr    AT.---Q.SLDTTL.QLT....G.V.TF.S..MLA.G.AN.I.AKT.Y.W
ActII  .TLTDPEQGLGA.HSQVE.VLSA.T.AF..T.FTW.V...........L
TcmA   PA.---QQ.LHA.LADVQ.ITNG.L.A..VS.ITA.K....F.H.QTF.V
                        *    * *  *            *     *

QacA   .F.L.GL..-..I.F.E.A.FVIAI.F...IA..LI..T...M..V..EN
LfrA   GGAGFAVVSALAAF-APSTELLVGARALLGVFGAMLMPSTLSLIRNIFTD
Mmr    .MGV.FL-AS..CAL..T..TLIA. LVQ.AGA.LF...S...LVFS.PE
ActII  .LGL.GL-SS..GAY.G.P.QLIA...CM..S..AVL....AT.AAV.P-
TcmA   .V.....-TSA.IGLSG.VAAI.VF.V.Q.L...LMQ..A.G.LRVT.P-
       *   *       *              *   *   *   *  *      *

QacA   PKE.-ATAL.V.SIASSI.AVF...I......Q.S.HSA..IN..FAIIA
LfrA   ASAR-RLAIAIWASCFTAGSALGPIVGGALLEHFHWGAVFLVAVPILLPL
Mmr    KRQ.T.M-LGL.SAIVATS.G...T...LMVSA.G.ESI..LNL..GAIG
ActII  LRE.-PK.LG...ASVGFALGI..VT..I..A..W..S.L..N..LMAGC
TcmA   -PGKLNM..G..SGVVG.ST.A...I..L.VQ.VG.E...FIN..VG.AA
              *          **  **      *       *

QacA   V.A.LF.L...KLSKEK-SHSW.IP.TI..IAG.IGL..SI.EFSK-E..
LfrA   LVLGPRLVPESR--DPN-PGPFDPVSIVLSFTTMLPIVWAVKTAAH-DGL
Mmr    MAMTYRYIAATESRATR----LAVPGHL.WIVALAAVSF.LIEGPQL-.W
ActII  ...VVLV...T.GTAGR-RV--.AAGLL..IAGVV.L.Y.IIE.GRSG.V
TcmA   ....L--.ILTDARAERA.KS..VSG...LSGAMFCL..GLIK.PAW-.W
                                 *                   *

QacA   ADIIPWVVIV-LA.TMIVI..K.NLS.SD....VR...KRS.SAGTI.-A
LfrA   SAAAA-AAFA-VGIVSGALFVRRQNRSATPMLDIGLFKVMPFTSSILA-N
Mmr    T.GPVLT.Y-A.AVTAA..LAL.EH.VTN.VMPWQ..RGPG..GAN.-VG
ActII  TRP.V...GL-A.LGLLLV.LWHER.TPE.SLEL.F.RMKA.STAVA.VG
TcmA   GDLRTL-GFLAAAVLAFAG.TLRES.ATE.LMPLAM.RSV.LSAGTV-LM
                              *     *

QacA   .MTMFAMASVLLLA..W..V.EE...FK...YL..M.IGD.V--F.PIAP
LfrA   FLSIIGLIGFIFFISQHLQLVLGLSPLTAGLVTLPGAVVSMIAGLAVVKA
Mmr    ..FNFA.F.ST.MLGLYF.HAR.AT.FQ...EL..MTIF-FPVA-NI.Y.
ActII  .V.-FAMM..L..SAFY..S.R.YT..Q..GC.VAL..ANVVC.--PLST
TcmA   V.MAFSF..GL..VTFY..N.H.M..VES.VHL.PLTGM-..V.-.P.SG
                        *     *   *

QacA   GLAA..G.KIVLPS.IGTA-.I.MFIMYF.G.P.-----SYSTMALALIL
LfrA   --AKRFAPDTLMVTGLVFV-AVGFLMILLFRHNL-----TVAAIIASFVV
Mmr    RISA..SNG..LTAF.LLAG.ASL-SMVTITAS-----TPYWVVAVAVG.
ActII  VLVRSIG.RNVCAA.MLA.-TASLCGVTFVTQHA-----P.WL.LVL.AA
TcmA   IVIS..G.GGPL.V.MLLT-.ASLWGMSTLEADSGMGITSLWFVLLGLGL

QacA   VGA.MA-.LA.ASAL.MLET.TS.A.N.A..E.SM.D..N.F.V.V..SL
LfrA   LELGVGVSQTVSNDTIVASVPAAKSGAASAVSETAYELGAVVGTATLGTI
Mmr    ANI.A.IISPGMTAAL.DAAGPENANV.GS.LNANRQI.SL..I.AM.VV
ActII  .GA..ACVMPTAAVS.MNAI.REKA.V...MNN.VRQ..GAL.V.V..SL
TcmA   APV---MV--GTT.V..SNA..ELA.V.GGLQQS.MQV.GSL...V..VL
                              *           *   * * *

QacA   S----SML..VFL.ISSFSSK.IVGDLAHV.E..VV..VE..K---.TGI
LfrA   F----TAFYRSNVDVPA----GLTPEQTGAAAESIGGAAAVAADLPAATA
Mmr    LHS-TSD-----W.HG----------AAI-------------------
ActII  M----G.A..RGIE-DELAV--.P.SARHQ.G.SLDATLLA.TR.GESG-
TcmA   MASRVGDVFPDKWAEANLPRV.-PR.AAAIEDAAEV..VPP.GT..GRH.

QacA   K..ANE.VTS.NDAFVA..LVGGIIMIIISI..YLLIPKSL--------D
LfrA   TQLLDSARAAFDSGIAPTAVIAAMLVLAAAAVV----------------G
Mmr    ---------S.LA-VGLAYLLGG---.--S--AWRLIAR---PERRSA--
ActII  --.VGP.RQ..LDAMHLA.GA..AVA.VG.LA.LRWLPSSVTTPTPPAGA
TcmA   GT.SEVVHSS.I..MGLAFTV.GAVA.V...--AVALFTRKAEPDERAPEE
                 *

QacA   ITKQK----------
LfrA   VAFRR----------
Mmr    --VTAAT--------
ActII  .PG.EHSDHLKVQGS
TcmA   FPVPASTA---GRG-
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FIG. 2. Clustal V alignment of efflux pumps most homologous to Lfra: QacA is a plasmid-based efflux pump found in *S. aureus* strains resistant to disinfectants (30). Mmr (33), ActII (31), and TcmA (32) are export pumps associated with the streptomyces biosynthetic gene clusters producing the antibiotics methylenomycin, actinorhodin, and tetracenomycin, respectively. Amino acid identity with LfrA is indicated with a period (.), residues identical among all five proteins are underscored with an asterisk, and gaps are shown with hyphens.

Table 1. LfrA-mediated resistance to various FQ

FQ	Inhibitory concentration,* mg/ml	
	pYUB53	pMN1
Cip	0.5	3.0
Ofloxacin	0.5	1.0
Levofloxacin	<0.125	0.5
DU-6859-a	<0.03	0.25
Sparfloxacin†	0.25	0.25

mc^2155 containing either vector pYUB53 alone or *lfrA* on plasmid pMN1 was grown overnight and spread on 7H10 plates containing a range of concentrations for the antibiotics shown.
*Parent resistant strain mc^2552 grew well on all FQ concentrations tested. On the drug concentrations indicated, colony growth was inhibited by at least 50% after 72 hr at 37° C.
†Both strains showed diffuse, weak growth on sparfloxacin at 0.25 μg/ml.

Plasmids pMN1 and pSB51 were then tested against drugs to which other efflux pumps confer resistance (20, 35) (Table 2). They were found to increase the MIC 2- to 4-fold to ethidium bromide, acriflavine, and CTAB. *LfrA* on pIV703 also increased resistance to ethidium bromide 4-fold (data not shown). These studies were then repeated in the presence of CCCP, an energy uncoupler that has been shown to inhibit the action of other efflux pumps (19, 20). CCCP reduced the MIC for all drugs tested, suggesting that the MICs of these compounds are at least partially determined by Lfr and other proton antiporter efflux pumps. At 10 μg/ml, CCCP does not appear to completely inhibit the highly expressed LfrA on pMN1, as there remains a 4-fold increase in resistance to Cip, compared with WT mc^2155 without CCCP. Notably, CCCP also reduced Cip resistance in strain mc^2552, suggesting that Lfr or another efflux pump plays a role in its high-level Cip resistance.

Increased Frequency of Secondary Mutations. Although Cip-resistant colonies of *M. smegmatis, M. tuberculosis*, or *Mycobacterium bovis* bacillus Calmette–Guérin do not appear in a primary selection on plates with a Cip concentration of 3 μg/ml or more, by restreaking colonies isolated on 0.5, 1, or 2 μg/ml, one can easily isolate colonies resistant to Cip at 3, 4, or even 8 μg/ml (unpublished results). We therefore studied whether strains carrying *lfrA* on plasmid pMN1 would show the same pattern. When spread on plates with Cip at 3, 4, or 8 μg/ml, mc^2155 (pMN1) showed diffuse weak growth, but when a broad swath of this growth was restreaked, colonies grew on plates with 4 or 8 μg/ml. By comparison, mc^2155 with no plasmid or containing the vector alone, showed no growth on any plate with at least Cip at 0.5 μg/ml. Thus, the presence of *lfrA* on a plasmid appears to increase the frequency of mutations to high-level resistance.

DISCUSSION

LfrA belongs to the growing family of membrane efflux pumps termed the major facilitators (19), members of which have been found to confer drug resistance in various species of bacteria, and recently, in *Saccharomyces cerevisiae* (34). These translocases have 12–14 membrane-spanning regions and function by transporting drug out of the bacteria in exchange for protons. The range of substrates that can be pumped out varies from protein to protein. Some of these translocases, such as QacA, eliminate a wide range of toxic compounds and antibiotics and have been termed multidrug resistant pumps, although they bear no homology to the eucaryotic P-glycoprotein MDR (19) and show no evidence of ATPase activity. These translocases are generally inhibited by the energy-uncoupler CCCP, which presumably destroys the proton gradient driving force. Some efflux pumps, such as Mmr, Tcm, and ActII, apparently function to pump out the product of the streptomyces polyketide synthetase operon with which they are associated.

The determinants of the specificity of the pumps are poorly understood, but, like the plasmid-based *qacA*, to which *lfrA* is most homologous, the presence of *lfrA* on a plasmid confers a 2- to 4-fold increase in resistance to ethidium bromide, acriflavine, and CTAB, as well as to the hydrophilic FQ. The natural substrate of LfrA is unknown, but like Mmr, Tcm, and ActII, it could be associated with a polyketide synthetase operon and function to export the operon's product. The segment of DNA downstream of *lfrA* on pMN1 increases the level of resistance slightly compared with pSB51.

Increased FQ resistance seen with *bmr, emrB*, and *norA* is due to increased expression (20, 21, 35) rather than structural mutations in these pumps. Similarly, plasmid pMN1 was apparently selected in our screen for clones that confer Cip resistance because it contains the *lfrA* gene expressed from the strong Kan resistance promoter of vector pYUB53. The endogenous *lfrA* promoter remains undefined, as there is no obvious promoter consensus sequence within the 170 bases sequenced upstream of the apparent translational start. However, the absence of a consensus promoter is not unusual for the genus *Actinomyces* (36), and, as plasmid pIV703 confers a 4-fold increase in Cip resistance, a functional promoter must be present.

High-level resistance to the FQs appears to evolve in a multistep process of additive mutations (12, 13). Cip^r *M. smegmatis* strain mc^2552 was obtained by three successive

Table 2. Influence of CCCP (15 μg/ml) on the MICs of *M. smegmatis* strain mc^2155 alone, mc^2155 with various plasmids, and Cip^r parent strain mc^2552

Drug	Relative resistances							
	mc^2155* WT	pSB51 lfr	pMN1 lfr	mc^2552 Cip^r	mc^2155† WT CCCP	pSB51 lfr CCCP	pMN1 lfr CCCP	mc^2552 Cip^r CCCP
CCCP	1 (30)*	1	1	1				
Ethidium bromide	1 (8)	2	4	4	0.5	0.5	1	1
CTAB	1 (8)	2	2	1	0.125	0.25	1	0.125
Benzalkonium chloride	1 (32)	1	1	1	0.125	NT	0.125	0.125
Acriflavine	1 (6.25)	4	4	4	<0.5	<0.5	>0.5	>0.5
Tet	1 (2)	0.5	4	1	0.25	0.25	2	<0.065
Chloramphenicol	1 (16)	2	2	2	0.125	0.25	0.25	0.125
Nalidixic acid	1 (128)	1	1	1	0.125	0.25	0.25	0.062
Cip	1 (0.25)	16	32	64	0.5	4	8	8

Relative resistance is the ratio of the MIC of each strain tested to the MIC of WT strain mc^2155 without a plasmid. NT, not tested.
*MICs for mc^2155 without a plasmid are shown in parentheses (μg/ml).
†mc^2155 with vector pYUB53, the backbone for pMN1, was the same as mc^2155 without a plasmid except that the vector carries a Tet^r gene.

selections on increasing Cip concentrations. The *gyrA* mutation apparently occurred in one of these steps, but the mutations responsible for the other steps and the contribution of the *gyrA* mutation alone are unclear. Although we initially expected that one of these steps was a mutation to increase the expression of *lfrA*, our chimeric plasmid studies suggest there is no mutation in the region of *lfrA* contained on pMN1. It is possible, however, that mc^{2}552 has a mutation in another effux pump, distinct from LfrA, or in a regulatory locus such as *mar* (22) *sox* (*23*), or *pqr* (*24*), that would increase the expression of either LfrA or the relevant efflux pump.

Increased expression of *lfrA* seems to raise the frequency of subsequent mutations to higher levels of resistance; this could provide a model of how strains become progressively more resistant. Increased expression of the efflux pump may permit the bacteria to survive at low Cip concentrations and subsequently allow selection for a second mutation to an increased level of resistance, at a frequency that is substantially higher than that of selecting both mutations in a single step (37). If the mutation frequency is augmented by the mutagenic effect described for the FQ (38), one would also expect other, unrelated mutations to be more frequent, but this hypothesis has not been studied.

While the factors that determine a bacteria's intrinsic MIC for a particular FQ are poorly defined, they may include its permeation into the bacteria and its affinity as a substrate for the efflux pumps present in the organism (14, 39). The mycobacteria are likely to have other efflux pumps besides Lfr, including perhaps one more homologous to EmrB, which could extrude the hydrophobic FQ. It is possible, however, that the greater effectiveness of Lfr against hydrophilic compounds is not due to specificity of the pump but rather reflects the greater permeability of hydrophobic compounds through the lipid-rich mycobacterial cell wall (43). As the steady-state intracellular level of a drug is determined by the balance between influx and efflux, an efflux mechanism would be less effective against a drug that can penetrate the cell wall with a reasonably (40) high influx rate.

Finally, one would like to know whether an *lfrA*-mediated FQ^r mechanism could be relevant to the pathogenic mycobacteria. Hybridizations to genomic DNA suggest that a gene homologous to *lfrA* exists in *M. tuberculosis* and *Mycobacterium avium* (unpublished results). Efflux pumps such as LfrA could influence the MIC and, with increased expression, confer resistance to antibiotics. If multidrug resistance continues to emerge as a problem in the control of tuberculosis, it will be important to elucidate and characterize the multidrug resistance pumps of *M. tuberculosis* that affect MIC and therapeutic efficacy of the FQ and other chemotherapeutic agents.

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