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Genomic Approach to Identifying the Putative Target of and Mechanisms of Resistance to Mefloquine in Mycobacteria

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The emergence of mycobacterial resistance to multiple antimicrobials emphasizes the need for new compounds. The antimycobacterial activity of mefloquine has been recently described. *Mycobacterium avium*, *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis* are susceptible to mefloquine in vitro, and activity was evidenced in vivo against *M. avium*. Attempts to obtain resistant mutants by both in vitro and in vivo selection have failed. To identify mycobacterial genes regulated in response to mefloquine, we employed DNA microarray and green fluorescent protein (GFP) promoter library techniques. Following mefloquine treatment, RNA was harvested from *M. tuberculosis* H37Rv, labeled with ^{32}P, and hybridized against a DNA array. Exposure to 4× MIC resulted in a significant stress response, while exposure to a subinhibitory concentration of mefloquine triggered the expression of genes coding for enzymes involved in fatty acid synthesis, the metabolic pathway, and transport across the membrane and other proteins of unknown function. Evaluation of gene expression using an *M. avium* GFP promoter library exposed to subinhibitory concentrations of mefloquine revealed more than threefold upregulation of 24 genes. To complement the microarray results, we constructed an *M. avium* genomic library under the control of a strong sigma-70 (G13) promoter in *M. smegmatis*. Resistant clones were selected in 32 μg/ml of mefloquine (wild-type *M. avium*, *M. tuberculosis*, and *M. smegmatis* are inhibited by 8 μg/ml), and the *M. avium* genes associated with *M. smegmatis* resistant to mefloquine were sequenced. Two groups of genes were identified: one affecting membrane transport and one gene that apparently is involved in regulation of cellular replication.

Mycobacterial diseases remain among the world's leading infection problems. Tuberculosis is a major infectious disease with approximately 8 million new cases worldwide causing an estimated 2 million deaths annually (4). Pathogenic mycobacteria initiate long-term infection in the lungs by entering host macrophages and spreading rapidly. Although effective therapeutic regimens exist, the prevalence of *Mycobacterium tuberculosis* strains resistant to available antimicrobial agents and the emergence of multidrug resistance underscore the need for additional compounds targeted at new pathways. *Mycobacterium avium*, an environmental organism, is a major opportunistic pathogen that causes bacteremia and disseminated disease in patients in the advanced stages of AIDS. In non-AIDS patients, *M. avium* is associated with pulmonary infection (9). Although treatment with new macrolides and rifabutin became available in the last several years, complete killing of the bacteria is usually not achieved. There is an urgent need for new compounds to improve mycobacterial therapy. Based on broad screening of compounds, we found that mefloquine (a derivate of 4-quinoline methanol) is bactericidal against *M. avium* in vivo, and it is active against *M. tuberculosis* in vitro (3). Previously, we have shown that mefloquine is effective against *M. avium* isolates resistant to macrolides, quinolones, and rifamycins (3), which suggests a novel target in mycobacteria. Passages of *M. avium* in the presence of elevated levels of mefloquine, as well as the screening of mycobacterial transposon libraries, did not select for resistant colonies. Attempts to obtain resistant mutants by in vivo selection have also failed, suggesting that the target of mefloquine is either lethal or multiple.

A number of studies with other bacteria have employed DNA microarrays to narrow down the possible target(s) for the drug. Therefore, to examine the effect of mefloquine on the level of gene expression in *M. tuberculosis* and *M. avium*, we applied two different techniques, i.e., the commercially available *M. tuberculosis* DNA microarray and, because the *M. avium* array has not yet been annotated, a green fluorescent protein (GFP) promoter library of *M. avium* genes. Some of the genes identified using both approaches for the two mycobacterial species were then overexpressed in *M. avium*, identifying mechanisms of resistance and a putative mefloquine target.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Mycobacterium avium* 104, a virulent strain isolated from the blood of an AIDS patient; *Mycobacterium smegmatis* strain mc^2155 (provided by William Jacobs, Jr., Albert Einstein College of Medicine, NY); and *Mycobacterium tuberculosis* strain H37Rv (ATCC 25618) were maintained on Middlebrook 7H11 agar supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC; Difco Laboratories, Detroit MI) and 0.1% Tween 80 or grown in Middlebrook 7H9 broth enriched with 0.2% glycerol, 0.1% Tween 80, and OADC. Mycobacteria were grown at 37°C until exponential growth phase. Antibiotics were added at the indicated concentrations when appropriate: mefloquine, 50 μg/ml, and (for *M. smegmatis*) kanamycin, 50 μg/ml

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TABLE 1. Plasmids

Plasmid	Description	Source or reference
pMV261	Template vector used for construction of pEMC1	
pFJS3	Promoterless pMV261; *hps60* promoter was removed with XbaI and PstI restriction endonucleases	Lab stock
pKEN	Plasmid carrying green fluorescent protein gene, *GFPmut2*	5
pEMC1	*GFPmut2* gene cloned into XbaI/PstI sites and its downstream *trpA* transcriptional terminator cloned into EcoRI/HindIII sites in pFJS3; plasmid was used for *M. avium* promoter library construction	This study
pLDG13	PCR product of G13 (*M. marinum* promoter) was cloned into NheI site of pFJS3; plasmid was used for *M. avium* gene library construction	This study
pUC19	Template vector used for construction of transposon plasmid pTNGJC	Commercial source
pTNGJC	Tn*5367*-based transposon was cloned into EcoRI and HindIII sites in pUC19 and *oriM*, mycobacterial origin of replication cloned into HindIII sites	Lab stock

(*M. smegmatis*) or 200 μg/ml (*M. tuberculosis* and *M. avium*). *Escherichia coli* DH5α (Stratagene) was grown on Luria-Bertani broth or agar (Difco Laboratories), and transformants were selected on kanamycin at a concentration of 50 μg/ml.

DNA techniques. *M. avium* strain 104 was used as a source of genomic DNA for library constructions and for specific gene amplification by PCR. Chromosomal DNA was extracted and purified, as previously described (14). Plasmid DNA was isolated from *E. coli* using the QIAprep Miniprep kit (QIAGEN, Valencia, CA). *M. smegmatis* was resuspended in 50 mM glucose, 25 mM Tris (pH 8), 10 mM EDTA (GTE) solution with 10 μg/ml lysozyme and lysed overnight at 37°C in the shaking incubator (250 rpm). The next day, plasmid DNAs were isolated with the QIAprep Miniprep kit, according to the manufacturer's instructions.

GFP promoter library construction. In order to identify *M. avium* gene promoters expressed upon subinhibitory concentrations of mefloquine, the green fluorescent protein reporter system was used. The *E. coli-Mycobacterium* shuttle vector pEMC1 was constructed from the reporter plasmid pMV261 by removing the *hps60* promoter and introducing the promoterless *GFPmut2* gene into XbaI and PstI restriction sites. The *GFPmut2* gene was obtained from Rafael Valdivia and Stanley Falkow, Stanford University (5). The *trpA* transcriptional terminator was replaced upstream of the GFP gene, between the EcoRI and HindIII sites, to ensure that GFP expression was the result of the activation of bacterial promoters. Genomic DNA was isolated from *M. avium* 104 and partially digested with Sau3A enzyme. The digests were gel purified to generate DNA fragments with 300- to 1,000-bp fragments. The inserts were then ligated into the dephosphorylated BamHI site of plasmid pEMC1 (Table 1). *E. coli* DH5α-competent cells were transformed with the genomic library and plated on Luria-Bertani agar containing kanamycin, 50 μg/ml. Plasmid DNA was recovered from a pool of greater than 2×10^4 *E. coli* clones. Screening of 1% of *E. coli* transformants, from each of three separate ligations, by restriction analysis of plasmids, showed that approximately 80% of transformants had inserted DNA. Electrocompetent *M. smegmatis* (mc^2155) was electroporated with an *M. avium* promoter GFP library. For preparation of *M. smegmatis* competent cells, the bacterial pellet was incubated in an ice water bath for 2 h and washed four times with cold washing buffer (10% glycerol, 0.1% Tween 80), and then 100 to 200 μl of competent cells was combined with 1 to 3 μl of library and transferred to a prechilled 0.2-cm cuvette. Electroporation was carried out under the following conditions: capacitance, 25 μF; resistance, 1,000 Ω; voltage, 2.5 kV. Clones were recovered on Middlebrook 7H11 agar plates containing kanamycin, 50 μg/ml. Over 10,000 individual *M. smegmatis* clones were stored in pools of five in 96-well plates containing Middlebrook 7H9 broth with 50% glycerol and stored at −70°C.

Screening of *M. avium* promoter GFP library. The *M. avium* GFP promoter library in *M. smegmatis* has been used with success for the identification of *M. avium* genes upregulated upon macrophage infection. *M. smegmatis* clones from the GFP library were grown (2×10^4 cells/well) in 96-well flat-bottomed tissue culture plates. After 3 days of incubation at 37°C, bacteria were treated with the subinhibitory concentration of mefloquine (4 μg/ml). A baseline level of GFP was recorded before mefloquine treatment, using a Cytofluorometer II (Biosearch, Bedford, MA). The GFP expression level was quantified after 4, 24, and 48 h of incubation at 37°C. Pools from wells that produced at least threefold increase of GFP expression were selected for further evaluation. Selected pools were then diluted and plated on 7H11 agar plates with 50 μg/ml of kanamycin, in order to obtain individual clones. Clones (32 clones were picked from each pool) were then propagated in broth with 50 μg/ml of kanamycin and were again exposed to mefloquine as described above. Clones that resulted in more than a threefold increase in GFP production over baseline were selected for sequencing.

Construction and overexpression of *M. avium* subclones under G13 promoter. The *E. coli-Mycobacterium* shuttle vector pLDG13 was constructed with a *Mycobacterium marinum* G13 promoter (1) inserted into the pFJS3 (Table 1) NheI restriction site. G13-upper (NheI) (5′-TTTGCTAGCGATCGCCACTAGCGC CGCGGT-3′) and G13-lower (NheI) (5′-TTTGCTAGCTCGGTTACCAAGC GTGCATTT-3′) primers were designed to amplify a 457-bp G13 fragment. Amplification was performed in a PCR Express thermal cycler (Hybaid) under the following conditions: heating at 95°C for 5 min and subjection to 30 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min. The PCR product was ligated directly into the pFJS3 plasmid and screened by digestion for the insert; samples were sequenced to determine the correct orientation of the promoter. *Mycobacterium avium* 104 genomic DNA was subjected to partial digestion with Sau3A restriction endonuclease. After electrophoresis on a 1% agarose gel, fragments with an 800- to 3,000-bp size were extracted from the gel and cloned into the BclI site in the pLDG13 plasmid (Table 1). The library was constructed with 70% efficiency, with the number of the clones representing twofold coverage of the *M. avium* genome. The resulting library was expanded in *E. coli* competent cells by electroporation and plated on Luria-Bertani agar containing kanamycin, 50 μg/ml. The pool of approximately 20,000 *E. coli* clones was used as the source of G13 library construction in *M. smegmatis*. Mycobacterial competent cells were prepared, as previously described (14), and electroporated as described above. Transformants were plated on 7H11 agar plates containing kanamycin (50 μg/ml) and mefloquine (16 μg/ml) (2× MIC). Over 8,000 individual *M. smegmatis* clones were stored in pools of 100 and used for screening for mefloquine resistance. *M. smegmatis* mutants with increased MICs were selected and further analyzed by susceptibility testing. Agar dilution testing was performed according to the proportion method as previously described (National Committee for Clinical Laboratory Standards, 1994). Twofold dilutions, ranging from 16 to 128 μg/ml of mefloquine, were used for identification of *M. smegmatis* clones resistant to mefloquine.

Transposon mutagenesis. Temperature-sensitive plasmid pTNGJC (Table 1) was created based on the pUC19 vector, where a temperature-sensitive mycobacterial origin of replication and a Tn*5367*-based transposon were cloned. The transposon plasmid was transformed into *M. smegmatis* mc^2155, *M. avium* 104, and *M. tuberculosis* H37Rv. The presence of the transposon was confirmed by PCR amplification for the kanamycin gene. Single colonies from each mycobacterial transformant were grown at 30°C in the presence of kanamycin. The temperature was then switched to 42°C for 3 to 5 days. Screening of 25 colonies from each mycobacterial species showed that 100% of resulting clones contained the transposon in the chromosome. Library pools from each mycobacterial strain were plated on selective medium containing kanamycin at 50 μg/ml (*M. smegmatis*), kanamycin at 200 μg/ml (*M. tuberculosis* and *M. avium*), and mefloquine at 32 μg/ml for selection of mefloquine-resistant mutants.

Sequence analysis. Samples were sequenced at the Central Service Laboratory, Center for Gene Research and Biotechnology, Oregon State University, Corvallis. Database searches and alignments were performed using the BLAST server from the National Center for Biotechnology Information. The *M. avium* DNA sequence data from the NCBI database and the database at the TIGR Institute (www.tigr.org) were used to confirm and complete the sequences.

RNA preparation and probe synthesis. Total RNA was isolated from *M. tuberculosis* H37Rv in the following manner. Bacterial culture was grown in 7H9 broth at exponential phase and then divided into three parts. Mefloquine was added at a subinhibitory concentration (4 μg/ml) and 4× MIC (32 μg/ml) to two of the cultures, and a control culture of *M. tuberculosis* did not receive antibiotic. The next day, bacterial culture was resuspended in a guanidine thiocyanate-based buffer (Trizol) (GIBCO) and lysed with rapid mechanical agitation in a bead beater. Cells were centrifuged at 13,000 rpm for 5 min at 4°C. RNA was isolated from the supernatant, as previously described (11). RNA samples were treated with DNase I (RNase-free; Clontech) on RNeasy Midi columns for 15 min, according to the manufacturer's instructions (QIAGEN, Valencia, CA). The RNA concentration and quality were determined spectrophotometrically by absorption at an optical density at 260 to 280 nm, as well as verified on a 1% denaturing agarose gel.

cDNA probe synthesis and array hybridization were performed using the Sigma-Genosys Panorama cDNA labeling and hybridization kit according to the manufacturer's protocol. cDNA was synthesized using 1 μg total RNA from *M. tuberculosis* and 4 μl of Sigma-Genosys labeling primers. The cDNA labeling primers were annealed to the RNA template by placing samples in a thermal cycler (Hybaid) at 90°C for 2 min and then cooling to 42°C for an additional 20 min. The following master mix was made for cDNA labeling: 50 U avian myeloblastosis virus reverse transcriptase (Perkin-Elmer), 1× reverse transcriptase buffer, 333 μM dATP, 333 μM dGTP, 333 μM dTTP, 20 Ci [α-^{33}P]dCTP (2,000 to 3,000 Ci/mmol; Amersham Pharmacia Biotech), and RNase-free water. Labeling mix was added to each reaction mixture and incubated at 42°C for 2 h. Unincorporated-radiolabeled nucleotides were removed from labeled cDNA probe by purification over Spandex G-25 gel filtration columns according to the manufacturer's instructions (Sigma).

Hybridization and data analysis. Prior to use of the probe solutions, Panorama *M. tuberculosis* Gene Arrays were rinsed in 50 ml 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) for 5 min and drained. The blots were prehybridized in hybridization solution containing heat-denatured salmon testes DNA at a final concentration of 100 μg/ml. Labeled cDNA was heated at 95°C for 10 min to denature the cDNA, added to the hybridization solution, and hybridized to the arrays at 65°C overnight. The membranes were washed three times with microbial array wash solution (Sigma) containing 0.5× SSPE and 0.2% sodium dodecyl sulfate solution at 65°C for 3 min and agitated. The membranes were wrapped in plastic wrap, exposed to a phosphoimaging screen for 24 h, and then scanned with a phosphoimager (Molecular Dynamics, Amersham Pharmacia Biotech). The data were analyzed by Sigma-Genosys staff using ArrayVision software developed by Imaging Research, Inc. (St. Catharines, Ontario, Canada). Raw fluorescence intensity values were corrected for background by using the Array Vision Software and subsequently normalized by using the Lowess algorithm. The data were filtered for low spot signal intensity by using the threshold of the mean plus 1 standard deviation of the intensity observed from all empty and control spots on the array. Results were analyzed using the Genespring version 6 software, based on the normalized signal intensities. The fold changes were calculated by division of test (mefloquine treatment) spot signal data by the control data. Microarray experiments were repeated two times, and averages of the fold changes are reported in Table 3.

RESULTS

In vitro and in vivo assays for selection of mefloquine-resistant clones. In order to obtain information about the mefloquine target, we attempted to isolate the resistant clones of mycobacteria by sequential plating in the presence of increasing concentrations of the antimicrobial. Passages of mycobacteria in different concentrations of mefloquine did not select for resistant clones, and the in vivo attempt to obtain resistant mutants by treating *M. avium*-infected (intravenously) mice with 40 mg/kg of body weight/day for 4 months failed (data not shown). Based on the findings, we concluded that either the mutation of the target gene is lethal or mycobacteria contained more than one target of mefloquine. We subsequently tested the hypothesis that inactivating the target by the insertion of a transposon would potentially lead to a resistant phenotype. The results, however, showed that transposon libraries of *M. avium*, *M. tuberculosis*, and *M. smegmatis* exposed to 32 μg/ml of mefloquine (4× MIC) did not allow for the identification of resistant clones.

Identification of *M. avium*-expressed promoters after mefloquine treatment using the GFP promoter library. To determine the bacterial genes expressed upon exposure to mefloquine, *M. smegmatis* containing an *M. avium* GFP promoter library was incubated with subinhibitory concentrations of mefloquine, and the increase of GFP expression was monitored. Screening of 2,200 clones of the *M. avium* promoter library in *M. smegmatis* for significantly increased GFP expression identified 26 clones. Promoters were activated at the 24-h time point. No significant changes in GFP expression were observed at 4 h after *M. avium* treatment with mefloquine, compared with the GFP expression at baseline. The promoters upregulated are displayed in Table 2. Twenty-four sequenced clones contained *M. avium* DNA sequences that agree with a definition of a promoter of the predicted open reading frames and were considered natural promoters. The sequences of two clones were apparently located within the gene, indicating the potential for cryptic promoters. These sequences were not further analyzed. Among the identified *M. avium* sequences are promoters for genes associated with lipid metabolism (*accD3*, *fadD19*, and *fadA2*), intermediary metabolism (*guaB2*), information pathways (*rpsT*, *serS*, and *infB*), regulatory proteins (*phoR*), cellular differentiation (Rv3661), and 12 hypothetical integral membrane proteins or transporters from functional classification of cell wall and cell processes.

***M. tuberculosis* gene expression profile in vitro.** The global changes in *M. tuberculosis* gene expression associated with mefloquine treatment were examined using the microarray technique. We determined the level of *M. tuberculosis* gene expression from over 3,800 unique open reading frames. Total RNA was isolated from both untreated *M. tuberculosis* H37Rv and bacteria treated with 4 μg/ml and 32 μg/ml mefloquine for 24 h. DNA probes for hybridization were prepared by reverse transcription using [^{32}P]dCTP and primers specific for each gene, as described above. Genes with expression more than twofold higher than that of untreated control were considered differentially expressed.

A total of 133 genes were identified as responsive after treatment with subinhibitory concentrations of mefloquine, including the majority of genes from functional classification of cell wall and cell processes, intermediary respiration and metabolism pathways, PE/PPE family proteins, and proteins with unknown function. One hundred eight genes showed more than a twofold increase in expression upon mefloquine treatment, while expression of 25 genes was downregulated. The microarray data obtained from *M. tuberculosis* treatment with 4× MIC of mefloquine revealed changes in many genes compared with untreated control. Exposure to high concentrations of mefloquine resulted in significant stress response and expression of genes encoding heat shock proteins. Therefore, comparing the experiments examining subinhibitory and high concentrations of the antimicrobial revealed that much of the response upon exposure to high concentrations of mefloquine was nonspecific. Genes identified by *M. tuberculosis* DNA array in response to different concentrations of mefloquine after 24 h of treatment are shown in Table 3.

Overexpression of *M. avium* proteins using G13 promoter library. Based on the suggestion that the target gene(s) could

TABLE 2. *M. avium* promoters identified by GFP promoter library after exposure to mefloquine

Clone	*M. avium* gene homologue to *M. tuberculosis*	Fold change in GFP induction	Product description	% Similarity
1B11	Rv0359	5.7	Probable conserved integral membrane protein. Contains neutral zinc metallopeptidases, zinc-binding region signature	63
2G6	Rv0904c/*accD3*	3	Putative acetyl-coenzyme A carboxylase carboxyl transferase (beta subunit)	63
3A8	Rv2610c/*pimA*	4.4	Alpha-mannosyltransferase PIMA	84
3D6	Rv3411c/*guaB2*	4.8	IMP dehydrogenase	81
4C2	Rv3779	6.8	Probable transmembrane alanine- and leucine-rich protein	66
5A2	Rv2033c	3.6	Hypothetical protein	70
6EI2	Rv2412/*rpsT*	3.9	30S ribosomal protein	83
7H2	Rv2684/*arsA*	8.6	Integral transmembrane protein related to the arsenical pump	64
8F7	Rv1510	3.5	Hypothetical membrane protein	51
9D5	Rv3834c/*serS*	4.4	Seryl-tRNA synthetase. Contains aminoacyl-tRNA synthetases, class II signature I	76
10A7	Rv0236c	3.5	Hypothetical transmembrane protein	65
10F4	Rv0758/*phoR*	6.7	Alkaline phosphatase synthesis sensor protein	65
11G3	Rv3515c/*fadD19*	5.3	Probable acyl coenzyme A synthetase. Highly similar to products of two adjacent open reading frames from *M. avium* encoding secreted 30-kDa antigen precursor and 34-kDa protein. Contains putative AMP-binding domain signature	84
13C6	Rv2839c/*infB*	4.8	Probable translation initiation factor IF-2. Contains ATP/GTP-binding site motif A	89
14D12	Rv2718c	5	Hypothetical protein	84
14H5	Rv0314	3.9	Hypothetical membrane protein	40
15B10	Rv3661	5.8	Hypothetical protein. Possibly plays a regulatory role in cellular differentiation	77
16A1	Rv0713	3.6	Hypothetical transmembrane protein	56
16G11	Rv2209	3.8	Probable conserved integral membrane protein	
17E7	Rv1431c	4.1	Hypothetical membrane protein. Possibly transporters	70
18A5	Rv3036c/*TB22.2*	3	Conserved hypothetical secreted protein	67
20E3	Rv0243/*fadA2*	3.3	Probable acyl coenzyme A acyltransferase	79
20B12	Rv3197	4.3	Probable conserved ATP binding ABC transporter protein	87
21C11	Rv0936	3.9	Phosphate transport integral membrane protein	29

be essential, we then decided to overexpress the mycobacterial proteins under strong sigma-70 (G13) promoter and select for the mefloquine resistance phenotype. To test if overexpression of genomic library caused increased resistance to mefloquine, pools of 100 colonies were plated on 7H11 plates containing kanamycin (50 μg/ml) and mefloquine (16 μg/ml). Selected colonies were sequenced. Sixteen isolated clones contained *M. avium* genomic fragments with complete open reading frames. To determine if the overexpression of these gene products resulted in increased resistance to mefloquine, the clones were assayed for the MIC of mefloquine (3). Genes identified using *M. smegmatis* expressing the *M. avium* G13 library are shown in Table 4. Although the MIC for wild-type *M. smegmatis* is 8 μg/ml for all overexpressed isolates studied, decreased susceptibilities to mefloquine were detected with increases in the MICs from two- to eightfold dilutions. Susceptibility of *M. smegmatis* clones containing overexpressed *M. avium* genes is summarized in Table 4.

DISCUSSION

Mycobacterial infection is associated with great social burdens around the world. Tuberculosis and infection caused by *M. avium* complex affect many individuals for whom the currently available therapy against the diseases has failed (4, 7). Recently, the screening of drugs for efficacy against *M. avium* identified mefloquine as an active compound (2, 3). Mefloquine is a 4-quinoline methanol, used for prophylaxis and treatment of malaria, including chloroquine-resistant *Plasmodium falciparum* malaria (8). In mice, mefloquine is bactericidal against *M. avium*, and the combination of mefloquine with moxifloxacin or ethambutol has resulted in activity comparable to that achieved by regimens containing the new generation of macrolides (2). In addition, a number of anecdotal reports and one recent publication have attested to the antimycobacterial activity of mefloquine in humans (12).

Identification of the target for mefloquine might facilitate identification of other compounds that would have a novel mechanism of action against mycobacteria. The target for mefloquine in *Plasmodium* is still unknown, although export pumps have been identified as mechanisms of resistance (10). *M. avium* and *M. tuberculosis* are quite different pathogens. However, the MIC of mefloquine for both bacteria is the same, which may imply that the targets and mechanisms of resistance are probably similar. Previous studies have already determined that *M. avium* strains resistant to quinolones, macrolides, rifamycins, and ethambutol were still fully susceptible to mefloquine (3). Our initial attempt to clone the resistant phenotype by identifying a resistant strain was unsuccessful. The fact that we could not isolate a resistant clone using in vitro and in vivo selection suggested that either the mutation of the target was lethal or mefloquine had more than a single target.

Using a couple of strategies, GFP expression and DNA

TABLE 3. *M. tuberculosis* genes identified by DNA microarray[a]

Experimental group, type, and Rv no.	Gene	Function	Fold change
Group I: exposure to subinhibitory concn of mefloquine			
Virulence, detoxification, adaptation			
Rv0587	*yrbE2A*	Hypothetical unknown integral membrane protein	2.5
Rv1901	*cinA*	Probable CinA-like protein, strong similarity to competence damage protein CinA of *Bacillus subtilis*	−2
Rv3100c	*smpB*	Small protein b related to several bacterial small proteins b	−2
Rv3661		Conserved hypothetical protein	2.9
Lipid metabolism			
Rv1350	*fabG2*	3-Oxoacyl-[ACP]reductase	2
Rv1663	*pks17*	Polyketide synthase	−2.1
Rv2604c	*tesb2*	Acyl-CoA thioesterase II	−2
Rv3139	*fadE24*	Acyl-CoA dehydrogenase	2.1
Information pathways			
Rv0071		Possible maturase	2.3
Rv0445c	*sigK*	Alternative RNA polymerase sigma factor	2
Rv0640	*rplK*	50S ribosomal protein L11	2.2
Rv0710	*rpsQ*	30S ribosomal protein S17	2
Rv2710	*sigB*	RNA polymerase sigma factor	2.2
Rv2783c	*gpsI*	Polyribonucleotide nucleotidyltransferase	2
Rv2902c	*rnhB*	RNase HII	2.1
Cell wall and cell process			
Rv0175		Mce-associated membrane protein	2
Rv0341		Isoniazid-inducible gene	−2.4
Rv0394c		Possible secreted protein	2.4
Rv0451c	*mmpS4*	Conserved membrane protein	−2
Rv0541c		Conserved integral membrane protein	−2.4
Rv0842		Conserved integral membrane protein	−2
Rv1567c		Probable membrane protein	2
Rv2077c		Conserved transmembrane protein	3.6
Rv2254c		Probable integral membrane protein	2
Rv2560		Transmembrane protein, Pro-, Gly-rich protein	2.3
Rv2685	*arsB*	Arsenic transport integral membrane protein	2.4
Rv2729c		Conserved integral membrane Ala-, Val-, Leu-rich protein	−2.3
Rv2806		Possible membrane protein	2.3
Rv2856	*nicT*	Nickel transport integral membrane protein	2
Rv2920c	*amt*	Ammonium transport integral membrane protein (Ala-, Gly-, Leu-, Val-rich protein)	−2
Rv3162c		Integral membrane protein	−2.1
Rv3331	*sugI*	Sugar transport integral membrane protein	2.6
Rv3632		Conserved membrane protein	−2.3
Rv3795	*embB*	Integral membrane protein	2.3
Rv3851		Possible membrane protein	2
Rv3885c		Conserved membrane protein	−2
Rv3910		Probable conserved transmembrane protein	2.5
Insertion sequences and phages			
Rv0397		Part of 13E12 repeat family of conserved *Mycobacterium tuberculosis* proteins	−2
Rv1575		Probable phiRVI phage protein	2
Rv1576c		Probable phiRV1 phage protein (capsid subunit)	2.3
Rv1587c		Partial REP13E12 repeat protein	−3.4
Rv1588c		Partial REP13E12 repeat protein	−3.3
Rv1702c		Conserved hypothetical ORF in REP13E12 degenerate repeat	−2.4
Rv2666		Probable transposase	−2.2
Rv3466		Conserved hypothetical ORF in REP13E12 repeat, but extending 5′ of repeat	−2.2
Rv3467		Conserved hypothetical ORF in REP13E12 degenerate repeat	−2.4
Intermediary metabolism and respiration			
Rv0334	*rmlA*	α-D-Glucose-1-phosphate thymidylyl transferase	2.2
Rv0389	*purT*	Phosphoribosylglycinamide formyltransferase 2	2
Rv0777	*purB*	Adenylosuccinate lyase	2
Rv0858c		Probable aminotransferase	2.1
Rv1090	*celA2b*	Second part of cellulase (endoglucanase)	2.7
Rv1092	*coaA*	Pantothenate kinase	−2
Rv1178		Probable aminotransferase	2.5

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TABLE 3—*Continued*

Experimental group, type, and Rv no.	Gene	Function	Fold change
Rv1470	*trxA*	Thioredoxin	−2.2
Rv1620c	*cydC*	Transmembrane ATP-binding protein ABC transporter involved in transport of component linked with the assembly of cytochrome	2
Rv1777		Probable cyp144, cytochrome p450	3.2
Rv1834		Probable hydrolase	2
Rv2217	*lipB*	Lipid biosynthesis protein B	2
Rv2427c	*proA*	Gamma-glutamyl phosphate reductase protein	2
Rv2445c	*ndkA*	Nucleoside diphosphate kinase	2.6
Rv2682c	*dxs1*	I-Deoxy-D-xylulose 5-phosphate synthase	−2.1
Rv2958c		Possible glycosyl transferase	2.1
Rv3094c		Possible transferase	2.1
Rv3109	*moaA*	Molybdenum cofactor biosynthesis protein	2.3
Rv3110	*moaB*	Pterin-4-alpha-carbinolamine dehydratase	2
Rv3283	*sseA*	Thiosulfate sulfurtransferase	2
Rv3299c	*atsB*	Arylsulfatase	−2.3
Rv3549c		Short-chain dehydrogenase/reductase	2
Rv3712		Possible ligase	2.8
Regulatory proteins			
Rv0491	*regX3*	Response regulator protein (sensory transduction protein)	2.1
Rv0600c		Two-component sensor kinase (second part)	−2
Rv0602c	*tcrA*	Two-component DNA-binding response regulator	2
Rv0890c		Probable transcriptional regulatory protein, LuxR family	2.2
Rv1460		Probable transcriptional regulatory protein	3
Rv2989		Transcriptional regulator (Ala-rich protein)	2.4
Rv3219	*whiB1*	WhiB-like regulatory protein	2.4
PE/PPE family proteins	26 genes		−3.1–+3.4
Conserved hypotheticals			
Rv0100, Rv0106, Rv0299, Rv0360c, Rv0637, Rv0874c, Rv0965c, Rv0997, Rv1061, Rv1066, Rv1157c, Rv1619, Rv1710, Rv1975, Rv1991c, Rv2074, Rv2159c, Rv2230c, Rv2255c, Rv2261c, Rv2298, Rv2304c, Rv2369c, Rv2493, Rv2821c, Rv2891, Rv2998, Rv3131, Rv3412, Rv3612c, Rv3819, Rv3899c, Rv3909			−7.7–+3.5
Group II: exposure to 4× MIC of mefloquine			
Virulence, detoxification, adaptation			
Rv0251c	*hsp*	Probable heat shock protein	4.4
Rv0350	*dnaK*	70-kDa heat shock protein	3.2
Rv0352	*dnaJ1*	Chaperone protein	6.1
Rv0384c	*clpB*	Endopeptidase ATP-binding protein (chain B), heat shock protein	2.4
Rv0440	*groEL2*	60-kDa chaperonin 2	1.7
Rv3269		Probable heat shock protein	3.3
Rv3418c	*groES*	10-kDa chaperone	3.0
Rv3846	*sodA*	Superoxide dismutase	1.9
Lipid metabolism			
Rv0649	*fabD2*	Malonyl CoA-[ACP]transacylase	2.0
Rv0824c	*vdesA1*	Acyl-[ACP]desaturase	2.3
Rv1094	*desA2*	Acyl-[ACP]desaturase	2.1
Rv2243	*fabD*	Malonyl CoA-[ACP]transacylase	2.3
Rv2524c	*fas*	Fatty acid synthase	4.2
Rv3139	*fadE24*	Acyl-CoA dehydrogenase	2.9
Rv3140	*fadE23*	Acyl-CoA dehydrogenase	1.8
Rv3505	*fadE27*	Acyl-CoA dehydrogenase	1.8
Information pathways			
Rv1221	*sigE*	ECF subfamily sigma subunit	1.9
Rv2710	*sigB*	RNA polymerase sigma factor	1.2
Cell wall and cell process			
Rv0676c	*mmpL5*	Conserved large membrane protein	2.3
Rv0677c	*mmpS5*	Conserved small membrane protein	3.3
Rv3270	*ctpC*	Cation transport ATPase	2.0
Rv3810	*pirG*	Cell surface protein precursor	2.1

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TABLE 3—*Continued*

Experimental group, type, and Rv no.	Gene	Function	Fold change
Intermediary metabolism and respiration			
Rv0815c	*cysA2*	Thiosulfate sulfurtransferase	2.9
Rv1594	*nadA*	Quinolinate synthase	2.9
Rv2241	*aceA*	Isocitrate lyase	1.9
Rv3117	*cysA3*	Thiosulfate sulfurtransferase	2.0
Rv3283	*sseA*	Thiosulfate sulfurtransferase	1.7
Regulatory proteins			
Rv3160c		Transcriptional regulator	2.0
Rv3583c		Transcriptional regulator	2.1
PE/PPE family proteins	11 genes		2.0–4.2
Conserved hypotheticals			
Rv0236c, Rv0637, Rv0740, Rv0968, Rv1157c, Rv1993c, Rv2022c, Rv2209, Rv2267, Rv2369c, Rv3131, Rv3127, Rv3408, Rv3819			2.0–3.7

[a] Abbreviations: CoA, coenzyme A; ORF, open reading frame; ACP, acylcarrier protein.

microarray, we determined that, upon exposure to subinhibitory concentrations of mefloquine, both *M. avium* and *M. tuberculosis* upregulate a number of genes, the majority of which belong to metabolic pathways and cell wall synthesis. Some of the upregulated genes were membrane proteins of unknown function. It is assumed that the reason that genes are upregulated upon bacterial exposure to antimicrobials is that the target gets inhibited, and upstream genes in the pathway undergo compensatory regulation. Accordingly, the target should not be upregulated but inhibited. If this hypothesis is correct, it is possible that we cannot identify the target of a number of compounds by using the DNA microarray technique as the only tool. Another interesting observation of this study was that both the DNA microarray and the screening of a GFP library identified similar genes, although the results with the GFP library were more limited. The clones of several of the *M. avium* genes identified using the GFP library, under the control of the constitutive promoter, resulted in a twofold-increased MIC (data not shown), which indicates that they are unlikely to be the drug target.

Recently, Wilson and colleagues (15) performed a DNA microarray with RNA from *M. tuberculosis* exposed to isoniazid. Their findings showed that exposure to isoniazid induced several genes that encode proteins physiologically relevant to the drug's mode of action, including several genes encoding type II fatty acid synthase enzymes and *tbpC* (trehalose dimycolyl transferase).

Our results of DNA microarray demonstrated that, when *M. tuberculosis* is exposed to subinhibitory concentrations of mefloquine, a few genes involved in fatty acid metabolism and many in the intermediary metabolism were upregulated. The response to the exposure to 4× MIC resembles a stress response. One of the responses of the bacterium was to upregulate the expression of genes coding for membrane proteins,

TABLE 4. Mycobacterial genes associated with resistance to mefloquine when overexpressed

Gene	Function	MIC of mefloquine (μg/ml)
Rv0545c/*pita*	Low-affinity inorganic phosphate transporter integral membrane protein	16
Rv1304/*atpB*	Key component of the protein channel; it may play a direct role in the translocation of protons (H^+) across the membrane	16
Rv1620c/*cydC*	Probable CydC, transmembrane ATP-binding protein ABC transporter involved in transport of component linked with the assembly of cytochrome	16
Rv1819c	Probable drug transport transmembrane ATP-binding protein ABC transporter	32
Rv2267	Hypothetical protein	16
Rv2643/*arsC*	Probable arsenical resistance transport integral membrane protein	32
Rv2920c/*amt*	Probable ammonium transport integral membrane protein transport binding protein	16
Rv3118/*sseC1*	Hypothetical protein. Thought to be involved in sulfur metabolism 1	16
Rv3195	Hypothetical protein	16
Rv3529c	Conserved hypothetical protein	32
Rv3661	Conserved hypothetical protein. Possibly plays a regulatory role in cellular replication	64
Rv3823c/*mmpL8*	Conserved integral membrane transport protein	16
Rv0359	Probable conserved integral membrane protein	16
Rv0904c/*accD3*	Putative AccD3, acetyl-CoA[a] carboxylase carboxyl transferase, beta subunit	16
Rv3779	Conserved transmembrane Ala-, Leu-rich protein	16
Rv2684/*arsA*	Arsenic transport integral membrane protein	32

[a] CoA, coenzyme A.

many of them involved in transport mechanisms. These results can suggest that either the target for mefloquine is on the cell wall or membrane or many of these proteins are associated with the export of the compound.

The screening for resistant clones using the overexpression library was the employed strategy with more relevant results. All the genes identified were pumps or transport proteins except for the Rv3661 gene, a gene involved in cell replication in *Streptomyces*. The finding that the overexpression of several pumps or transport-related genes can be associated with increased MIC is obvious. However, the fact that many were identified suggests that it is a nonspecific mechanism. In addition, the overexpression of the majority of the transport proteins was associated with a twofold increase in MIC, which cannot be considered significant.

The upregulation of *arsA* and *arsC* and Rv1819c, however, was associated with a fourfold increase in MIC, which is relevant. That all three proteins have a role in mefloquine export is a plausible possibility. The reason that they were not overexpressed in the DNA array and GFP library screening is unknown, but there is a similar study for *Candida albicans* in which export pump genes were not identified in DNA array upon exposure to itraconazole, although they represent the main mechanism of resistance (6).

Rv3661 was the protein that, when overexpressed, resulted in the greater increase in the MIC. Rv3661 was also expressed (5.8-fold) in the GFP promoter screening, suggesting that targets might undergo compensatory overexpression when the pathogen is exposed to subinhibitory concentrations of antimicrobials.

In *Streptomyces azureus*, the gene is associated with mycelium formation, and clones that do not contain a functional gene are aerial mycelium negative. There is some suggestion that the Rv3661 gene encoding a hypothetical protein has homology to the MinD protein gene of *Bacillus subtilis*, the gene for a septum site-determining protein (13). How the inhibition of a gene associated with cell differentiation can be lethal is unknown. The fact that the function of the protein encoded by Rv3661 is not known in mycobacteria, except for some suggestive similarity, might be the reason. The identification of a novel drug target in mycobacteria may have an important impact on the treatment of mycobacterial infections.

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