ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2004, p. 3412–3418 Vol. 48, No. 9
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.9.3412–3418.2004

Genes Required for Intrinsic Multidrug Resistance in *Mycobacterium avium*

Julie S. Philalay, Christine O. Palermo, Kirsten A. Hauge, Tige R. Rustad, and Gerard A. Cangelosi*

Seattle Biomedical Research Institute, Seattle, Washington

Received 12 February 2004/Returned for modification 28 April 2004/Accepted 8 May 2004

Genes required for intrinsic multidrug resistance by *Mycobacterium avium* were identified by screening a library of transposon insertion mutants for the inability to grow in the presence of ciprofloxacin, clarithromycin, and penicillin at subinhibitory concentrations. Two genes, *pks12* and Maa2520, were disrupted in multiple drug-susceptible mutants. The *pks12* gene (Maa1979), which may be cotranscribed with a downstream gene (Maa1980), is widely conserved in the actinomycetes. Its ortholog in *Mycobacterium tuberculosis* is a polyketide synthase required for the synthesis of dimycocerosyl phthiocerol, a major cell wall lipid. Mutants of *M. avium* with insertions into *pks12* exhibited altered colony morphology and were drug susceptible, but they grew as well as the wild type did in vitro and intracellularly within THP-1 cells. A *pks12* mutant of *M. tuberculosis* was moderately more susceptible to clarithromycin than was its parent strain; however, susceptibility to ciprofloxacin and penicillin was not altered. *M. avium* complex (MAC) and *M. tuberculosis* appear to have different genetic mechanisms for resisting the effects of these antibiotics, with *pks12* playing a relatively more significant role in MAC. The second genetic locus identified in this study, Maa2520, is a conserved hypothetical gene with orthologs in *M. tuberculosis* and *Mycobacterium leprae*. It is immediately upstream of Maa2521, which may code for an exported protein. Mutants with insertions at this locus were susceptible to multiple antibiotics and slow growing in vitro and were unable to survive intracellularly within THP-1 cells. Like *pks12* mutants, they exhibited increased Congo red binding, an indirect indication of cell wall modifications. Maa2520 and *pks12* are the first genes to be linked by mutation to intrinsic drug resistance in MAC.

The environmental pathogen *Mycobacterium avium* complex (MAC) opportunistically infects susceptible humans, especially AIDS patients with low $CD4^+$ cell counts (9, 10, 13, 22). MAC infections are difficult to treat due to the intrinsic multidrug resistance of the organism. Drugs such as clarithromycin, azithromycin, rifabutin, ethambutol, amikacin, clofazamine, and fluoroquinolones, which are effective against primary isolates, frequently lose effectiveness unless administered in combination.

The multidrug resistance of MAC is usually ascribed to intrinsic properties of the organism's lipid-rich cell wall, although additional factors may contribute (2, 19, 23, 27, 36). A role for the cell wall has been inferred from indirect observations. Exposure to detergents, drugs, and other agents that compromise cell wall integrity can result in increased susceptibility to multiple drugs (19, 27, 29). Aminoglycosides are more active on ribosomes in cell extracts than on intact MAC cells (24). Finally, there is a correlation between drug susceptibility and colony type of MAC. Transparent colony variants, which predominate in patient samples, are significantly more resistant to multiple antibiotics than are their opaque counterparts (18, 28, 35).

An additional morphotypic switch, termed red-white, also affects multidrug resistance in MAC. Red and white colony types are visible when the bacteria are grown on media containing the lipoprotein stain Congo red (CR) (5–7, 20, 25). The red-white switch operates independently of the opaque-transparent switch, such that red opaque (RO), red transparent (RT), white opaque (WO), and white transparent morphotypes can be distinguished by CR staining. White variants are more common than their red counterparts in patient samples, and they grow better in disease models. However, the RT colony type can also be recovered from patient samples (25). The red-to-white switch is accompanied by increased resistance to multiple antibiotics, including macrolides, rifamycins, and quinolones. WO variants are more resistant to these drugs than their RO counterparts are, and white transparent variants are more resistant than their RT counterparts are (6).

Due in part to the instability of the transparent morphotype in vitro (35), the multidrug resistance associated with the transparent morphotype remains uncharacterized. The red and white morphotypes are more stable in vitro, which makes them relatively amenable to genetic dissection. Mutational analysis with use of a transposome mutagenesis system identified an apparent acetyltransferase gene, *crs*, that is required for CR binding in red variants. It also identified the major nonribosomal peptide synthetase involved in the synthesis of the serovar-specific glycopeptidolipid (20). For the present study, the mutagenesis procedure was modified to improve the efficiency of mutagenesis of the relatively drug-resistant WO morphotype. This led to the identification of genes required for the multidrug resistance associated with this morphotype.

MATERIALS AND METHODS

Bacterial strains. White and red variants of strain HMC02, a clinical isolate of *Mycobacterium avium* subsp. *avium*, have been described previously (6). A *pks12* mutant of *Mycobacterium tuberculosis*, along with its parent strain, H37Rv, was

* Corresponding author. Mailing address: Seattle Biomedical Research Institute, 4 Nickerson St., Seattle, WA 98109. Phone: (206) 284-8846, ext. 313. Fax: (206) 284-0313. E-mail: gcang@sbri.org.

kindly provided by P. Kolattukudy, University of Central Florida, Orlando. This mutant had been constructed by replacing a 2,193-bp fragment near the center of the *pks12* gene with a hygromycin resistance gene cassette (32). The mutant was originally designated Δ*msl6* after an older name for the *pks12* gene. For clarity, it is designated H37Rv::Δ*pks12* in this paper.

Transposome mutagenesis of WO cells. The WO variant of *M. avium* subsp. *avium* strain HMC02 was mutagenized by using the commercial EZ::TN <KAN-2> system (Epicentre, Madison, Wis.). The electroporation method described previously (20) worked well on red variants, which form dispersed suspensions, but was relatively inefficient at mutagenizing white variants, which are flocculent in broth culture. Therefore, the procedure for preparing electrocompetent cells (15) was modified by growing the cells in the presence of sucrose as described by Lee et al. (21). This resulted in dispersed growth of WO cells and improved transformation efficiency. Under the modified protocol, cells were grown to an optical density at 600 nm of 0.3 to 0.5 in Middlebrook 7H9 broth with albumin-dextrose-catalase (ADC) enrichment and 0.5 M sucrose. One day before harvest, glycine was added to 0.2 M. Cells were pelleted at 5,000 × *g* and resuspended in electroporation solution at 8× their original concentration (15). After two more washes in electroporation solution, cells were resuspended in electroporation solution at 100× their original concentration and stored at −80°C in 100-μl aliquots.

Competent WO cells (100-μl aliquots) were mutagenized with the EZ::TN <KAN-2> transposome, and mutants were selected by growth on Middlebrook 7H10 agar with albumin enrichment, glycerol, 100 μg of CR/ml, and 100 μg of kanamycin/ml (MAG-CR-KAN) as described previously (20). Kanamycin-resistant mutants were transferred to fresh MAG-CR-KAN plates containing 0.2 μg of ciprofloxacin/ml and control MAG-CR-KAN plates without ciprofloxacin. After 3 weeks of incubation under air at 37°C, clones that exhibited altered CR staining characteristics, inability to grow in the presence of 0.2 μg of ciprofloxacin/ml, or both were retested to confirm their phenotypes. Confirmed mutants were subjected to further examination.

Identification of EZ::TN insertion sites. Transposon insertion sites were mapped as described previously (20). Briefly, genomic regions adjoining EZ::TN insertions were amplified by arbitrary primer PCR or inverted PCR. Purified PCR products were submitted for automated sequencing at the Seattle Biomedical Research Institute. The Unfinished Microbial Genomes searching tool at The Institute for Genomic Research website (http://tigrblast.tigr.org/ufmg/) was used to precisely locate insertion sites within the draft *M. avium* subsp. *avium* strain 104 genome sequence. Regions of up to 20 kb surrounding the insertion sites were then analyzed using the Basic Local Alignment Search Tool (BLAST) and the ORF Finder tool at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) to identify sequences in the National Center for Biotechnology Information nonredundant database that are closely homologous to open reading frames (ORFs) in the region of the insertion. Gene designations (e.g., Maa2520) were derived from the recent annotation of the *M. avium* subsp. *avium* 104 genome sequence by M. Semret and M. Behr (31a).

Drug susceptibility in vitro. The drug susceptibilities of wild-type and mutant cells of *M. avium* subsp. *avium* and *M. tuberculosis* were tested by the proportion method (17) with a breakpoint of 1% and critical concentrations of the following antibiotics: clarithromycin (0, 0.05, 0.25, 1.25, 6.25, 16, and 64 μg/ml), penicillin (0, 0.5, 2.5, 12.5, and 62.5 μg/ml), and ciprofloxacin (0, 0.025, 0.05, 0.25, 1.25, and 6.25 μg/ml). Clarithromycin was kindly provided for this study by Abbott Laboratories, North Chicago, Ill. Other drugs were obtained from commercial vendors (ICN Biomedicals, Aurora, Ohio, and Sigma Chemical Co., St. Louis, Mo.). To prevent artifactual results due to degradation of antibiotics, plates were read after no more than 2 weeks of incubation at 37°C. *M. tuberculosis* cells were incubated under 5% CO_2. *M. avium* subsp. *avium* cells were also tested for susceptibility to rifampin by using AB Biodisk E-test strips (Remel, Lenexa, Kans.) as described previously (34). All drug susceptibility tests were conducted in triplicate. In addition to MICs, the presence or absence of rare (<1%) drug-resistant variants was recorded.

Survival during intracellular incubation in THP-1 cells. The myelomonocytic THP-1 cell line (ATCC T1B-202) was grown in RPMI culture medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 50 U of penicillin-streptomycin per ml, at 37°C in the presence of 5% CO_2 (16). Seventy-two hours prior to infection, cells were treated for differentiation and adherence to 12-mm-diameter glass coverslips with use of 100 nM phorbol 12-myristate 13-acetate (11). Bacterial cultures were grown with shaking at 37°C in Middlebrook 7H9 broth with 0.2% glycerol and 10% Middlebrook ADC enrichment to an optical density at 600 nm of 0.3 to 0.5. Bacteria were diluted in RPMI infection medium (10% fetal bovine serum and 2 mM L-glutamine). Immediately prior to infection with bacteria, two coverslips with host cells were removed for the 0-h time point, lysed with double-distilled water, and plated onto MAG-CR plates. Remaining coverslips were washed twice with RPMI to remove nonadherent THP-1 cells and residual antibiotic-containing culture medium. New RPMI infection medium with no antibiotics was added to the coverslips, and cells were then infected at a 10:1 ratio of bacteria to THP-1 cells (11) and incubated at 37°C in 5% CO_2. At time points of 2, 24, and 48 h and 4 and 7 days postinfection, triplicate coverslips were washed three times with RPMI culture medium to remove extracellular bacteria (12). Cells on each coverslip were lysed in double-distilled water, serially diluted, and plated onto MAG-CR plates. After incubation for 2 to 3 weeks at 37°C, results were quantified based on the number of CFU per coverslip. Means and standard deviations of each set of triplicate readings were plotted.

Comparison of predicted macrolide and penicillin resistance genes of *M. tuberculosis* and *M. avium* subsp. *avium*. *M. tuberculosis* genes previously linked to innate resistance to macrolide and penicillin drugs, and genes predicted by homology to play roles in resistance, were identified in the literature, in the *Mycobacterium tuberculosis* Structural Genomics Consortium Database (http://www.doe-mbi.ucla.edu/TB/), and in the TubercuList database (http://genolist.pasteur.fr/TubercuList/). The nucleotide sequence of each *M. tuberculosis* H37Rv gene was compared to the *M. avium* subsp. *avium* strain 104 genome sequence by using the unfinished microbial genomes searching tool at the Institute for Genomic Research website (http://tigrblast.tigr.org/ufmg/). Gene sequences were further analyzed for cross-species similarity by using EMBOSS::needle software publicly available at http://www.ebi.ac.uk/emboss/align/.

RESULTS

Mutagenesis of white variants of *M. avium* subsp. *avium*. Genetic analysis of MAC is complicated by the fact that the most clinically significant morphotypes (white and transparent) are also the most difficult to genetically transform. The modified procedure described in Materials and Methods improved the efficiency of transformation of WO variants of *M. avium* subsp. *avium* strain HMC02, such that we were able to generate an incomplete library of approximately 1,500 kanamycin-resistant transposon mutant clones. Previous observations indicated that the EZ::TN transposon inserted at random sites in this strain (20).

The library was screened for mutants that were unable to grow on 0.2 μg of ciprofloxacin/ml (to which WO variants are resistant), or exhibited elevated CR staining comparable to that of the RO morphotype, or both. Fifteen ciprofloxacin-sensitive mutants were isolated, all of which exhibited elevated CR staining relative to that of the parent strain. Three additional mutants exhibited elevated CR staining but were not more sensitive to ciprofloxacin than the parent strain was.

Transposon insertion sites were mapped. Most insertion sites were disrupted in no more than one mutant, so their roles in the observed phenotypes could not be confirmed. However, two genetic loci, Maa2520 and Maa1979, were mutagenized in multiple ciprofloxacin-sensitive mutants and were studied further.

Bioinformatic characterization of Maa2520 and Maa2521. Two ciprofloxacin-sensitive mutants, 6.389 and 7.1034, had insertions into a 1,242-bp ORF, Maa2520 (Fig. 1A). The two mutants were generated in independent mutagenesis procedures. The putative 414-amino-acid product of Maa2520 was 89% identical to conserved hypothetical protein Rv1697 of *M. tuberculosis*, and 88% identical to MLC1351.11c of *Mycobacterium leprae*, by BLAST analysis (expect value 0.0). Weaker homologs were found in *Corynebacterium*, *Thermoanaerobacter*, *Thermobifida*, and *Bacillus* species (expect values 1E-28 to 5E-84). These proteins belong to a cluster of orthologous uncharacterized membrane-anchored proteins conserved in bacteria (COG4825).

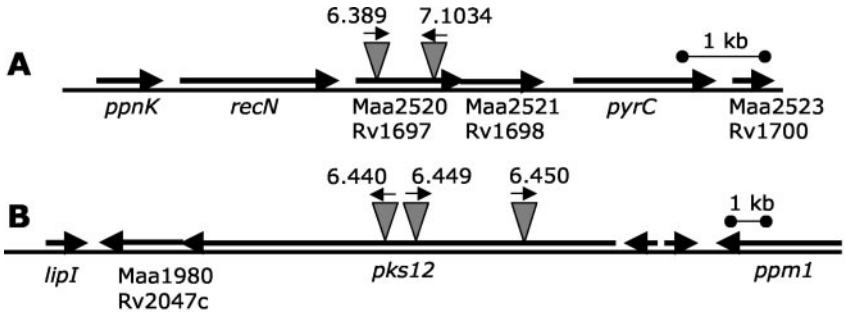

FIG. 1. Genetic maps of the Maa2520-Maa2521 (A) and *pks12* (B) regions of the *M. avium* subsp. *avium* 104 genome. Positions and orientations of transposon insertions are shown. Gene order, orientation, and approximate genetic distances within both regions are identical in *M. avium* subsp. *avium* 104 and *M. tuberculosis* H37Rv. *M. avium* subsp. *avium* genome annotation numbers (MaaNNNN) are shown above corresponding *M. tuberculosis* genome annotation numbers (RvNNNN). Where descriptive gene designations have been assigned to *M. tuberculosis* H37Rv genes (for example, *pks12*), those designations are used for both species. Note that lines A and B are drawn to different scales.

Maa2520 ends 3 bp upstream of the start codon of a 951-bp ORF, Maa2521, suggesting that the two genes may be cotranscribed. The 317-amino-acid Maa2521 gene product is 82% identical to ML1362 of *M. leprae* and 75% identical (86% conserved) to Rv1698 of *M. tuberculosis*. Weaker homologs were found in *Corynebacterium* and *Thermobifida* species. The function of these proteins is not known; however, the *M. leprae* homolog has been described as a possible exported protein (http://www.sanger.ac.uk/Projects/M_leprae/CDS/ML1362.shtml). The genomic region surrounding Maa2520-Maa2521 in *M. avium* subsp. *avium* strain 104 (Fig. 1A) is genetically identical to the orthologous region in *M. tuberculosis* H37Rv.

Phenotypic characterization of Maa2520-Maa2521 mutants. Mutants 6.389 and 7.1034 exhibited identical pleiotropic phenotypes. In addition to ciprofloxacin sensitivity, they were more sensitive to clarithromycin and penicillin than the parent strain was in vitro (Table 1). They were also more sensitive to rifampin in E-tests (Fig. 2A). They did not form drug-resistant colony type variants and had well-defined MIC cutoffs in all drug susceptibility tests. This contrasted with wild-type cells that spontaneously formed drug-resistant colony type variants, including transparent variants, at typical frequencies of 10^{-4} to 10^{-6} (35). For example, growth of the RO variant of strain HMC02 was substantially inhibited by 1.25 μg of ciprofloxacin/ml; however, drug-resistant subpopulations always formed a few isolated colonies at this concentration. In contrast, mutants 6.389 and 7.1034 formed no colonies under these conditions (Fig. 2B).

TABLE 1. Drug susceptibility of *M. avium* subsp. *avium* HMC02 WO and mutants derived from this strain

Strain	MIC (μg/ml)		
	Clarithromycin	Ciprofloxacin	Penicillin
HMC02 WO (parent strain)	6.25	1.25	12.5
6.389 (Maa2520)	0.25[a]	0.25[a]	2.5[a]
7.1034 (Maa2520)	0.25[a]	0.25[a]	2.5[a]
6.440 (*pks12*)	0.25	0.25	2.5
6.449 (*pks12*)	0.25	ND[b]	2.5
6.450 (*pks12*)	ND	0.25	2.5

[a] No drug-resistant subvariants observed. All other strains formed isolated drug-resistant colonies at frequencies of <1%.

[b] ND, not determined in the experiment shown. Separate experiments showed increased drug susceptibility consistent with the other data in this table.

Additional phenotypes observed in vitro were elevated CR staining and slow growth relative to the parent strain. Two-week-old colony diameters were about half that of the parent strain (Fig. 2B)

Drug resistance and virulence tend to correlate with each other when spontaneously occurring colony types of MAC are compared. Therefore, we examined the ability of Maa2520-Maa2521 mutants to survive intracellular incubation within the macrophage-like THP-1 cell line. A complicating factor was that the mutagenesis had been carried out on an opaque variant of strain HMC02, due to the difficulty of genetically transforming transparent variants. Opaque variants of MAC are unable to grow intracellularly and are not virulent (6, 25). We attempted to derive spontaneous transparent variants from parent and mutant strains for comparison with each other; however, mutants 6.389 and 7.1034 did not form transparent variants even under antibiotic selective pressure (Fig. 2B). Therefore, we infected THP-1 cells with opaque variants of each strain and tested their ability to survive (if not grow) intracellularly. The parent strain exhibited an initial decline in viability for a day or two postinfection and then stabilized, possibly by virtue of a subpopulation of virulent variants. In contrast, both Maa2520-Maa2521 mutants declined to undetectable levels by day 7 postinfection, a phenotype that was markedly different from that of the parent strain (Fig. 3A).

Bioinformatic characterization of Maa1979 and Maa1980. Three ciprofloxacin-sensitive mutants, 6.440, 6.449, and 6.550, had insertions into an ORF, Maa1979 (Fig. 1B). At 12,513 bp, this ORF is the second longest in the *M. avium* subsp. *avium* strain 104 genome (M. Semret and M. Behr, personal communication). The putative 4,170-amino-acid product of Maa1979 was 80% identical and 87% homologous by BLAST analysis to the Pks12 protein (Rv2048c) of *M. tuberculosis* (expect value 0.0). Homologs are present throughout the genera *Mycobacterium* and *Streptomyces* and in other bacteria as well (COG3321, polyketide synthase modules). In *M. tuberculosis*, *pks12* encodes a polyketide synthase required for the synthesis of dimycocerosyl phthiocerol (DIM), a major cell wall lipid (32). Maa1979 ends 4 bp upstream of a 2,606-bp ORF, Maa1980, which may be cotranscribed with Maa1979. The 869-amino-acid gene product of Maa1980 is 73% identical and 80% homologous to the conserved hypothetical protein Rv2047c of *M.*

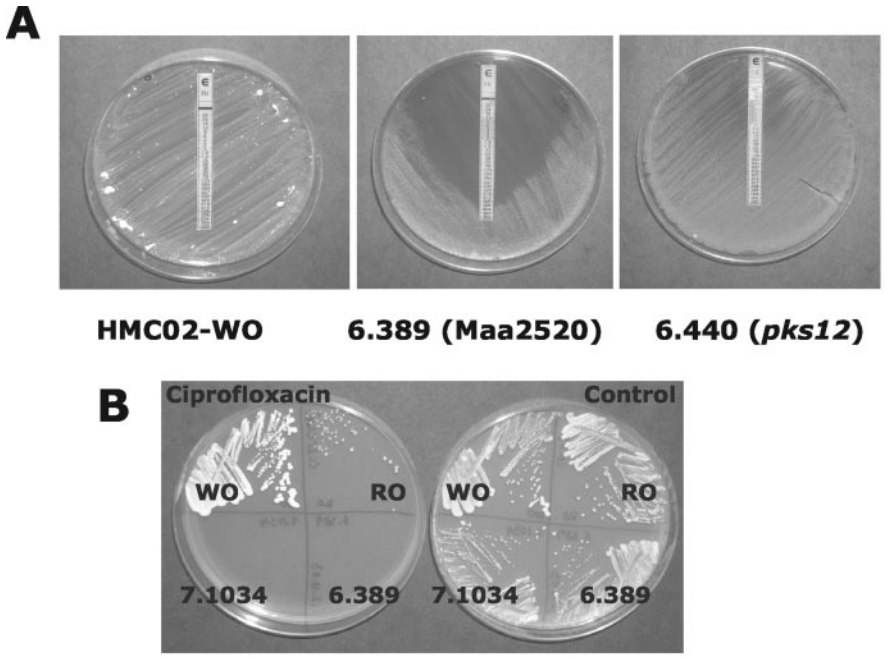

FIG. 2. Drug susceptibility phenotypes of wild-type and mutant clones on agar medium. (A) Rifampin E-tests were conducted on MAG plates as described previously (34), except that plates were incubated for an extended 3-week period to yield visible growth of drug-resistant subclones on mutant 6.440 (*pks12*). (B) Colony formation by wild-type and transposon mutant clones of strain HMC02 streaked on MAG plates with (left) or without (right) 0.5 μg of ciprofloxacin/ml. The formation of isolated ciprofloxacin-resistant colonies by the otherwise drug-susceptible HMC02 RO clone and the relatively small size of colonies formed by mutants 6.389 and 7.1034 are visible.

tuberculosis (expect value 0.0) and only slightly less homologous to MLCB2052.18 of *M. leprae*. As with Maa2520-Maa2521, the apparent operon structure of Maa1979-Maa1980 is seen in *M. tuberculosis* as well as in *M. avium* subsp. *avium*, and the surrounding genomic regions are nearly identical in the two species.

Phenotypic characterization of Maa1979-Maa1980 (*pks12*) mutants. The *pks12* mutants 6.440, 6.449, and 6.550 exhibited identical phenotypes. All three mutants formed rough colonies that stained with CR, in contrast to the smooth WO parent strain, which resisted CR staining. Colony size and apparent growth rates were identical to those of the parent strain. Increased susceptibility was observed against all drugs tested; however, in contrast to the Maa2520-Maa2521 mutants, the *pks12* mutants formed isolated drug-resistant variants at low frequencies (Table 1). This was also evident after extended incubation on rifampin E-test plates, on which *pks12* mutants formed diffuse zones of inhibition (Fig. 2A). Drug-resistant variants formed by the mutants were not transparent and did not otherwise differ in appearance from the main population of colonies.

Because the *pks12* mutants did not form archetypical transparent colonies, they were tested for survival during intracellular growth following the same strategy used for the Maa2520-Maa2521 mutants. The *pks12* mutants 6.440 and 6.450 did not differ from the parent strain in their ability to survive intracel-

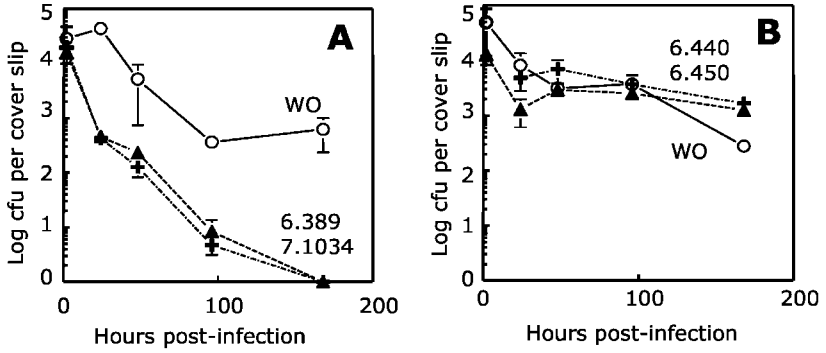

FIG. 3. Survival of wild-type and mutant clones within THP-1 cells. All data points represent the means and standard deviations of triplicate measurements. (A) The parent strain HMC02 WO (open circles) and Maa2520-Maa2521 mutants 6.389 (closed triangles) and 7.1034 (plus signs) were inoculated into THP-1 cells, and their intracellular survival was tracked as described in Materials and Methods. (B) The same experiment was conducted on parent strain HMC02 WO (open circles) and *pks12* mutants 6.440 (closed triangles) and 6.450 (plus signs).

TABLE 2. Drug susceptibility of *M. tuberculosis* H37Rv and H37Rv::Δ*pks12*

Strain	MIC (μg/ml)		
	Clarithromycin	Ciprofloxacin	Penicillin
H37Rv	64.0	0.25	>62.5
H37Rv::Δ*pks12*	16.0	0.25	>62.5

lularly. Upon infection of THP-1 cells, the mutants declined to lower stable numbers, similar to the parent strain (Fig. 3B).

Drug susceptibility of a *pks12* mutant of *M. tuberculosis*. The three Maa1979-Maa1980 insertions were distributed over a 3.5-kb section of the gene, indicating that they were not siblings. However, all three were isolated in a single mutagenesis procedure. Therefore, as an independent test of the role of this locus in intrinsic drug resistance, a *pks12* deletion mutant of *M. tuberculosis* described previously (32) was also examined. As shown in Table 2, the mutant H37Rv::Δ*pks12* was more susceptible to clarithromycin than was its parent strain, consistent with a role for *pks12* in resistance to this macrolide drug. However, the effect was more modest than that seen in *M. avium* subsp. *avium*, and clarithromycin resistance was higher overall in *M. tuberculosis*. Moreover, the *pks12* mutation did not affect resistance of *M. tuberculosis* H37Rv to ciprofloxacin and penicillin.

DISCUSSION

MAC infections of humans are very difficult to treat because of the intrinsic multidrug resistance of the pathogen. A role for the cell wall has been inferred from indirect observations; however, the mechanisms of intrinsic drug resistance remain uncharacterized. The genes described here are the first to be linked by mutational analysis to intrinsic drug resistance by MAC.

Maa2520 and Maa2521 code for conserved hypothetical proteins that exhibit sequence characteristics associated with the cell surface. Strong homologs were found only in the genomes of two other slow-growing mycobacterial pathogens, *M. tuberculosis* and *M. leprae*. Weaker homologs were found in a few additional bacterial species including two thermophiles. These gene products may be required for cell wall stability, and their roles in intrinsic multidrug resistance may be indirect. The pleiotropy of mutants with insertions into this gene cluster (slow growth in vitro and sensitivity to killing by THP-1 cells) is consistent with this hypothesis. Sassetti et al. (30), in their transposon site hybridization (TraSH) analysis of *M. tuberculosis* genes required for growth in vitro, gained evidence that Rv1697, the *M. tuberculosis* ortholog of Maa2520, was required for normal growth of *M. tuberculosis* in vitro. It was not determined whether this was due to an absolute requirement for Rv1697 by *M. tuberculosis* or was simply the result of a lower growth rate.

The *pks12* gene has been well characterized in *M. tuberculosis*. It codes for a polyketide synthase required for the synthesis of DIM, a major cell wall constituent in some pathogenic mycobacteria (32). DIM is thought to contribute to the impermeability of the *M. tuberculosis* cell wall (4). It is not yet known whether Pks12 functions in the synthesis of a DIM-like lipid in MAC. Mutations in the *pks12* gene of MAC resulted in increased multidrug susceptibility, perhaps as a result of increased cell wall permeability. Colonies of *pks12* mutants were rough and stained with CR, consistent with altered cell walls relative to those of the smooth, CR-resistant colonies formed by the parent strain. Polar effects on a downstream gene present in both species, Maa1980, may account for the observed phenotypes. Colonies of the *pks12* mutant of *M. tuber-*

TABLE 3. Comparison of genes associated with macrolide and penicillin resistance in the genomes of *M. tuberculosis* H37Rv and *M. avium* subsp. *avium* 104

M. tuberculosis gene designation	Product	Description[a]	Reference(s)	Homology to *M. avium* subsp. *avium* genes[b]		
				e score	% Amino acid identity	% Protein similarity
Rv1258	Tap	MF efflux pump	1	1.6e-143	74	80
Rv1473		Possible macrolide efflux pump	8	8.6e-180	74	77.2
Rv1667		Possible macrolide efflux pump	8	1.1e-62	74	34.4
Rv1668		Possible macrolide efflux pump	8	3.1e-148	79	49.1
Rv1988	ErmMT/38	23S rRNA methyltransferase	3, 26	—[c]	—	—
Rv2477		Possible macrolide efflux pump	8	3.7e-296	88	98.7
Rv0016	PbpA	Probable PBP	8	3.0e-216	81	92.5
Rv0050	PonA1	Class A1 PBP	8	1.9e-303	81	73.1
Rv0406		Possible β-lactamase	8	6.6e-97	78	76.8
Rv1730		Possible PBP	8	—	—	—
Rv2068c	BlaC	β-Lactamase	8, 14, 31, 33	—	—	—
Rv2163	PbpB	Probable PBP	8	1.8e-278	79	94.4
Rv2864		Possible PB lipoP	8	5.9e-284	83	92.2
Rv2911	DacB2	Probable PBP	8	1.1e-129	82	88.1
Rv3330	DacB1	Probable PBP	8	3.6e-176	82	84.7
Rv3682	PonA2	Class A1 PBP	8	0.0	82	94.6

[a] Annotations are from the *Mycobacterium tuberculosis* Structural Genomics Consortium and TubercuList databases. PBP, penicillin binding protein; lipoP, lipoprotein; MF, major facilitator.

[b] Expect values (e scores) and percent amino acid identity were generated by BLAST analysis. Percent protein similarity values were generated by using EMBOSS software.

[c] —, no homologous ORF (expect value, $<10^{-13}$) found in the *M. avium* subsp. *avium* 104 genome.

culosis did not differ in appearance from those of the parent strain. Mutants of both species grew at normal rates in vitro.

In contrast to Maa2520-Maa2521, which was required for intracellular survival within THP-1 cells, mutants in Maa1979-Maa1980 exhibited no pronounced defects relative to the parent strain under these conditions. This finding contrasts with observations made for the *pks12* mutant of *M. tuberculosis*, which was slightly attenuated for growth within the MH-S mouse alveolar macrophage cell line and more strongly attenuated for growth within intranasally infected C57BL/6J mice (32). However, given that the pathogens as well as the host systems differed in the two studies, the two findings cannot be considered contradictory.

The relative sensitivity of *M. tuberculosis* H37Rv::Δ*pks12* to clarithromycin indicates that *pks12* or its cotranscript plays a role in intrinsic drug resistance in both species. However, the effect was more modest, and its spectrum more narrow, in *M. tuberculosis*. Genomic comparisons indicate that *M. tuberculosis* and *M. avium* subsp. *avium* have different genetic mechanisms of intrinsic resistance to macrolides and penicillins. A 23S rRNA methyltransferase gene, *ermMT*, has been proposed to play a role in the high-level resistance of *M. tuberculosis* to macrolides (3, 26). Resistance of *M. tuberculosis* to penicillins is thought to be mediated by at least one major β-lactamase, *blaC*, and possibly by altered expression of several penicillin binding proteins (14, 31, 34). *M. avium* subsp. *avium* 104 has homologs to penicillin binding proteins and putative macrolide efflux pumps found in *M. tuberculosis*; however, homologs to *ermMT* and *blaC* were not found in its genome (Table 3). The picture that emerges from these comparisons is that *M. tuberculosis* and MAC have separate, but overlapping, mechanisms of resistance to these drugs, with the nonspecific resistance mediated by *pks12* playing a relatively more important role in MAC.

The nonspecific drug resistance of MAC affects a broad spectrum of drugs including fluoroquinolones and rifamycins, to which *M. tuberculosis* strains are intrinsically sensitive. Therefore, it is perhaps unexpected that the MAC genes identified in this study were present in *M. tuberculosis* as well. If they play direct roles in multidrug resistance, they may be expressed to higher levels in MAC relative to *M. tuberculosis* or have minor modifications that increase or alter their activity. Alternatively, they may play indirect roles in drug resistance, for example by stabilizing the activity of separate cell surface components which are unique to MAC.

In summary, transposon mutational analysis identified two genetic loci that are directly or indirectly required for intrinsic multidrug resistance in MAC. Continued mutagenesis of the approximately 4,500 genes in the *M. avium* subsp. *avium* genome will likely identify additional required gene products. Biochemical and physiological characterization of these gene products will yield a clearer understanding of this clinically significant phenotype.

ACKNOWLEDGMENTS

We are grateful to David Sherman and Tanj Bennett for helpful discussions and to Makeda Semret and Marcel Behr for providing prepublication information on annotation of the *M. avium* subsp. *avium* 104 genome sequence. We are also grateful to Pappachan Kolattukudy for providing the H37Rv::Δ*pks12* mutant and its parent *M. tuberculosis* strain.

This work was supported by grant AI25767 from the National Institutes of Health. Analysis of *M. tuberculosis* drug resistance genetics was supported by a generous grant from the Akibene Foundation. Preliminary *M. avium* genome sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of the *M. avium* genome is being carried out by TIGR with support from the National Institutes of Health.

REFERENCES

1. **Ainsa, J. A., M. C. Blokpoel, I. Otal, D. B. Young, K. A. De Smet, and C. Martin.** 1998. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. J. Bacteriol. **180:**5836–5843.
2. **Barrow, W. W., E. L. Wright, K. S. Goh, and N. Rastogi.** 1993. Activities of fluoroquinolone, macrolide, and aminoglycoside drugs combined with inhibitors of glycosylation and fatty acid and peptide biosynthesis against *Mycobacterium avium*. Antimicrob. Agents Chemother. **37:**652–661.
3. **Buriankova, K., F. Doucet-Populaire, O. Dorson, A. Gondran, J. C. Ghnassia, J. Weiser, and J. L. Pernodet.** 2004. Molecular basis of intrinsic macrolide resistance in the *Mycobacterium tuberculosis* complex. Antimicrob. Agents Chemother. **48:**143–150.
4. **Camacho, L. R., P. Constant, C. Raynaud, M. A. Laneelle, J. A. Triccas, B. Gicquel, M. Daffe, and C. Guilhot.** 2001. Analysis of the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. J. Biol. Chem. **276:**19845–19854.
5. **Cangelosi, G. A., J. E. Clark-Curtiss, M. Behr, T. Bull, and T. Stinear.** 2004. Biology of waterborne pathogenic mycobacteria, p. 39–54. *In* J. Bartram and G. Rees (ed.), Pathogenic mycobacteria in water. World Health Organization-U.S. Environmental Protection Agency, Geneva, Switzerland.
6. **Cangelosi, G. A., C. O. Palermo, and L. E. Bermudez.** 2001. Phenotypic consequences of red-white colony type variation in *Mycobacterium avium*. Microbiology **147:**527–533.
7. **Cangelosi, G. A., C. O. Palermo, J. P. Laurent, A. M. Hamlin, and W. H. Brabant.** 1999. Colony morphotypes on Congo red agar segregate along species and drug susceptibility lines in the *Mycobacterium avium-intracellulare* complex. Microbiology **145:**1317–1324.
8. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
9. **Falkinham, J. O., III.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9:**177–215.
10. **Ford, T., J. Hermon-Taylor, G. Nichols, G. Cangelosi, and J. Bartram.** 2004. Approaches to risk management in priority settings, p. 169–179. *In* J. Bartram and G. Rees (ed.), Pathogenic mycobacteria in water. World Health Organization-U.S. Environmental Protection Agency, Geneva, Switzerland.
11. **Garcia, R. C., E. Banfi, and M. G. Pittis.** 2000. Infection of macrophage-like THP-1 cells with *Mycobacterium avium* results in a decrease in their ability to phosphorylate nucleolin. Infect. Immun. **68:**3121–3128.
12. **Greenwell-Wild, T., N. Vazquez, D. Sim, M. Schito, D. Chatterjee, J. M. Orenstein, and S. M. Wahl.** 2002. *Mycobacterium avium* infection and modulation of human macrophage gene expression. J. Immunol. **169:**6286–6297.
13. **Guthertz, L. S., B. Damsker, E. J. Bottone, E. G. Ford, M. F. Thaddeus, and M. J. Janda.** 1989. *Mycobacterium avium* and *Mycobacterium intracellulare* infection in patients with and without AIDS. J. Infect. Dis. **160:**1037–1041.
14. **Hackbarth, C. J., I. Unsal, and H. F. Chambers.** 1997. Cloning and sequence analysis of a class A β-lactamase from *Mycobacterium tuberculosis* H37Ra. Antimicrob. Agents Chemother. **41:**1182–1185.
15. **Hatfull, G. F., and W. R. Jacobs, Jr.** 2000. Molecular genetics of mycobacteria. ASM Press, Washington, D.C.
16. **Hayashi, T., A. Catanzaro, and S. P. Rao.** 1997. Apoptosis of human monocytes and macrophages by *Mycobacterium avium* sonicate. Infect. Immun. **65:**5262–5271.
17. **Heifets, L., and G. A. Cangelosi.** 2002. Antibiotic susceptibility testing of *Mycobacterium tuberculosis*—a neglected problem at the turn of the century. Int. J. Tuberc. Lung Dis. **3:**564–581.
18. **Inderlied, C. B., C. A. Kemper, and L. E. M. Bermudez.** 1993. The *Mycobacterium avium* complex. Clin. Microbiol. Rev. **6:**266–310.
19. **Jarlier, V., and H. Nikaido.** 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol. Lett. **123:**11–18.
20. **Laurent, J. P., K. A. Hauge, K. Burnside, and G. A. Cangelosi.** 2003. Mutational analysis of cell wall biosynthesis in *Mycobacterium avium*. J. Bacteriol. **185:**5003–5006.
21. **Lee, S.-H., M. Cheung, V. Irani, J. D. Carroll, J. M. Inamine, W. R. Howe, and J. N. Maslow.** 2002. Optimization of electroporation conditions for *Mycobacterium avium*. Tuberculosis **82:**167–174.
22. **Marras, T. K., and C. L. Daley.** 2002. Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin. Chest Med. **23:**553–567.

23. **Mdluli, K., J. Swanson, E. Fischer, R. E. Lee, and C. E. Barry.** 1998. Mechanisms involved in the intrinsic isoniazid resistance of *Mycobacterium avium*. Mol. Microbiol. **27:**1223–1233.
24. **Mizuguchi, Y., T. Uodo, and T. Yamada.** 1983. Mechanism of antibiotic resistance in *Mycobacterium intracellulare*. Microbiol. Immunol. **27:**425–431.
25. **Mukherjee, S., M. Petrofsky, K. Yaraei, L. E. Bermudez, and G. A. Cangelosi.** 2001. The white morphotype of *Mycobacterium avium-intracellulare* is common in infected humans and virulent in infection models. J. Infect. Dis. **184:**1480–1484.
26. **Nash, K. A.** 2003. Intrinsic macrolide resistance in *Mycobacterium smegmatis* is conferred by a novel *erm* gene, *erm*(38). Antimicrob. Agents Chemother. **47:**3053–3060.
27. **Nikaido, H., and V. Jarlier.** 1991. Permeability of the mycobacterial cell wall. Res. Microbiol. **142:**437–443.
28. **Prinzis, S., B. Rivoire, and P. J. Brennan.** 1994. Search for the molecular basis of morphological variation in *Mycobacterium avium*. Infect. Immun. **62:**1946–1951.
29. **Rastogi, N., K. S. Goh, and S. Clavel-Seres.** 1997. Stazyme, a mycobacteriolytic preparation from a *Staphylococcus* strain, is able to break the permeability barrier in multiple drug resistant *Mycobacterium avium*. FEMS Immunol. Med. Microbiol. **19:**297–305.
30. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol. Microbiol. **48:**77–84.
31. **Segura, C., M. Salvado, I. Collado, J. Chaves, and A. Coira.** 1998. Contribution of β-lactamases to β-lactam susceptibilities of susceptible and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrob. Agents Chemother. **42:**1524–1526.
31a. **Semret, M. G. Zhai, S. Mostowy, C. Cleto, D. C. Alexander, G. A. Cangelosi, D. Cousins, D. M. Collins, D. van Soolingen, and M. A. Behr.** Extensive genomic polymorphism within *Mycobacterium avium*. J. Bacteriol., in press.
32. **Sirakova, T. D., V. S. Dubey, H. J. Kim, M. H. Cynamon, and P. E. Kolattukudy.** 2003. The largest open reading frame (*pks12*) in the *Mycobacterium tuberculosis* genome is involved in pathogenesis and dimycocerosyl phthiocerol synthesis. Infect. Immun. **71:**3794–3801.
33. **Voladri, R. K., D. L. Lakey, S. H. Hennigan, B. E. Menzies, K. M. Edwards, and D. S. Kernodle.** 1998. Recombinant expression and characterization of the major β-lactamase of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **42:**1375–1381.
34. **Wanger, A., and K. Mills.** 1994. E-test for susceptibility testing of *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare*. Diagn. Microbiol. Infect. Dis. **19:**179–181.
35. **Woodley, C. L., and H. L. David.** 1976. Effect of temperature on the rate of transparent to opaque colony type transition in *Mycobacterium avium*. Antimicrob. Agents Chemother. **9:**113–119.
36. **Young, L. S., and L. E. Bermudez.** 1996. Animal models in anti-*Mycobacterium avium*-complex drug development, p. 141–161. *In* J. A. Korvik and C. A. Benson (ed.), *Mycobacterium avium*-complex infection: progress in research and treatment. Marcel Dekker, Inc., New York, N.Y.