

Antimicrobial Agent Resistance in Mycobacteria: Molecular Genetic Insights

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“The knowledge acquired about drug resistance, then, will long remain of great practical value, and advances in the field will undoubtedly benefit the fight against tuberculosis considered on a worldwide scale.”

Georges Canetti
J. Burns Amberson Lecture, 1965

INTRODUCTION

Mycobacteria are responsible for considerable human morbidity and mortality worldwide (15, 146, 148). In the United States, bacteria of this genus have significantly increased in importance in the past decade, largely as a consequence of resurgent infections caused by *Mycobacterium tuberculosis* and the emergence of *M. avium* complex (MAC) disease in patients

with AIDS. Tuberculosis has affected humans for at least several millennia (3, 29, 135, 165). It is believed that about one-third of the world's population is infected by *M. tuberculosis* and therefore is at risk of contracting disease (15, 108). An estimated 3 million people globally die of tuberculosis each year, and another 8 million new individuals become infected (15, 86). Although once thought possible to be virtually eradicated by the end of the century (22), tuberculosis in the United States has resurged sharply since the mid-1980s, and an estimated 64,000 excess cases have occurred through 1993 (22–25).

Members of the MAC are also important causes of disseminated disease and death in AIDS patients (10, 66, 71, 82, 114, 163). For example, in the United States, 25 to 50% of adults and approximately 10% of children with AIDS are infected with MAC organisms (71, 114). Adult AIDS patients infected with these bacteria have significantly decreased survival rates compared with individuals without MAC infection (82, 114).

A steady increase in the frequency of *M. tuberculosis* strains resistant to one or more agents commonly used in treatment also has been reported (14, 147). About 13% of new cases are

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now resistant to at least one first-line or primary antituberculosis drug (isoniazid [INH], rifampin [RIF], streptomycin [STR], ethambutol [EMB], and pyrazinamide [PZA]), and 3.2% are resistant to both INH and RIF (14). Several outbreaks of multidrug-resistant *M. tuberculosis* with devastating consequences for individuals infected with human immunodeficiency virus have occurred in communities such as New York City (23, 39, 43, 46, 137, 138). It has been well documented that drug-resistant tuberculosis cases are costly to society (94).

Largely as a consequence of the recent bloom of multidrug-resistant tuberculosis in several communities, and the relatively restricted number of efficacious therapeutic agents available to treat patients infected with MAC organisms, there has been renewed effort to define the molecular genetic basis (that is, at the nucleotide or gene level) of antimicrobial resistance in mycobacteria. One of the assumptions underlying much of this research is that elucidation of the genetic basis of resistance will lead to formulation of rapid and unambiguous strategies for detection of resistant strains, which is currently a slow process (67, 176). It is also thought that knowledge gained from a molecular genetic understanding of resistance mechanisms can be exploited in efforts directed toward rational design of new antimicrobial agents. Progress has been made, largely with *M. tuberculosis*. Inasmuch as most of the significant advances have been made from studies of *M. tuberculosis* and *M. leprae*, rather than MAC organisms, this review will be heavily weighted toward the first two species. In addition, molecular genetic techniques designed to shorten the time required to generate initial antimicrobial susceptibility data will be summarized. This review is specifically designed to cover recent molecular genetic information. Readers interested in other topics in mycobacterial antimicrobial agent research are referred to excellent recent reviews (70–72).

RIFAMPIN

Background

RIF was introduced for use in antitubercular therapy in the early 1970s and is a very important component of current treatment regimens. On the basis of detailed structure-function studies conducted with *Escherichia coli*, the molecular mechanism of RIF activity involves inhibition of DNA-dependent RNA polymerase (48, 173). In *E. coli*, this enzyme is a complex oligomer composed of four different subunits (α , β , β' , and σ encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively), which can occur either as a core enzyme ($\alpha_2\beta\beta'$) or a holoenzyme ($\alpha_2\beta\beta'$ plus σ). RIF binds to the β subunit of *E. coli* RNA polymerase and results in transcription inhibition (97). Recent evidence (91) indicates that the mechanism of action in *M. smegmatis* is similar to that in *E. coli*.

Genetic studies conducted in the early 1980s recorded that *E. coli* strains resistant to RIF had missense mutations and short deletions in the *rpoB* gene (92, 120, 121). In addition, Jin and Gross (74) later described 17 mutational alterations affecting 14 amino acid residues in the *E. coli* RNA polymerase β subunit that mediate RIF resistance in this organism. These *E. coli* studies formed the basis for molecular genetic studies (63) examining the role of mycobacterial *rpoB* mutations in strains found to be RIF resistant by in vitro testing.

M. tuberculosis: Mutations in the Gene (*rpoB*) Encoding the RNA Polymerase β Subunit

Because resistance to RIF in many *E. coli* strains was known to arise as a result of missense and other mutations occurring

in a discrete region of the *rpoB* gene (74, 120, 121), Telenti et al. (158) cloned and sequenced the cognate region of the *M. tuberculosis* gene by using sequence information available from the *M. leprae rpoB* gene (62). The data were used to formulate oligonucleotide primers for amplification and sequencing of a 411-bp fragment of *rpoB* from 66 RIF-resistant and 56 RIF-susceptible strains recovered from patients from several continents. A total of 15 distinct mutations involving eight conserved amino acids clustered in a 23-amino-acid region (69 bp) were identified in 64 of 66 RIF-resistant strains but in none of 56 susceptible organisms. Virtually all of the mutations were missense mutations, and amino acid substitutions at one of two positions (residues 526 and 531) were found in 80% of the resistant strains. Interestingly, no synonymous (silent) nucleotide substitutions were identified in the 122 isolates sequenced for the 411-bp region. For resistant strains bearing many of these *rpoB* mutations, MICs in excess of 8.0 μg of RIF per ml have recently been reported (16).

Although organisms from nine countries were studied by Telenti et al. (158), only 12 U.S. isolates were examined. As a consequence, Kapur et al. (78) used automated DNA sequencing to characterize a collection of 128 isolates from this country, including 121 RIF-resistant organisms, for polymorphism in a 350-bp region of *rpoB* containing the 69-bp stretch with mutations associated with RIF resistance. Twenty-three distinct *rpoB* alleles were identified. The data confirmed that greater than 90% of RIF-resistant strains have sequence alterations in the 69-bp region and showed that the great majority of the changes are missense mutations. The analysis also identified several mutant *rpoB* alleles not previously associated with resistant organisms and one short region of *rpoB* that had an unusually high frequency of insertions and deletions. Twelve of the mutant *rpoB* alleles were created by point mutations affecting seven amino acids, two were due to insertions of 3 or 6 bp, three were made by deletion of 6 bp (two alleles) or 9 bp (one allele), and six were characterized by combinations of two point mutations.

One unexpected and important finding of this study was a difference in the relative abundance of certain mutations compared with that reported by Telenti et al. (158). For example, strains with a CAC \rightarrow TAC codon 526 change (His \rightarrow Tyr) accounted for about 30% of the U.S. collection examined by Kapur et al. (78), whereas organisms with this mutation represented only 12% of the sample studied by Telenti et al. (158). Similarly, only about 25% of strains in the former study had a TCG \rightarrow TTG (Ser \rightarrow Leu) mutation in codon 531, whereas organisms with this change accounted for 47% of the strains in the latter study sample. In the absence of sampling bias, the data are most readily explained by geographic variation in the frequency of occurrence of particular *rpoB* mutations.

A subsequent study of 77 additional strains drawn largely from U.S. patients by Kapur et al. (77) extended knowledge about the spectrum of *rpoB* mutations associated with RIF resistance. Most resistant organisms had one of the previously described *rpoB* mutations, and five new mutations were identified. Interestingly, two of the previously undescribed mutations occurred outside the 69-bp core region containing nucleotide changes in virtually all RIF-resistant *M. tuberculosis* isolates, therefore expanding the size of the core region to 27 amino acids (81 bp) (Fig. 1).

Heym et al. (58) studied 13 RIF-resistant *M. tuberculosis* strains by single-strand conformation polymorphism (SSCP) analysis (118, 119) and DNA sequencing and found *rpoB* mutations in 12 isolates but none in susceptible organisms. Virtually all changes occurred in the 81-bp core region containing most mutations associated with RIF resistance. Four of the

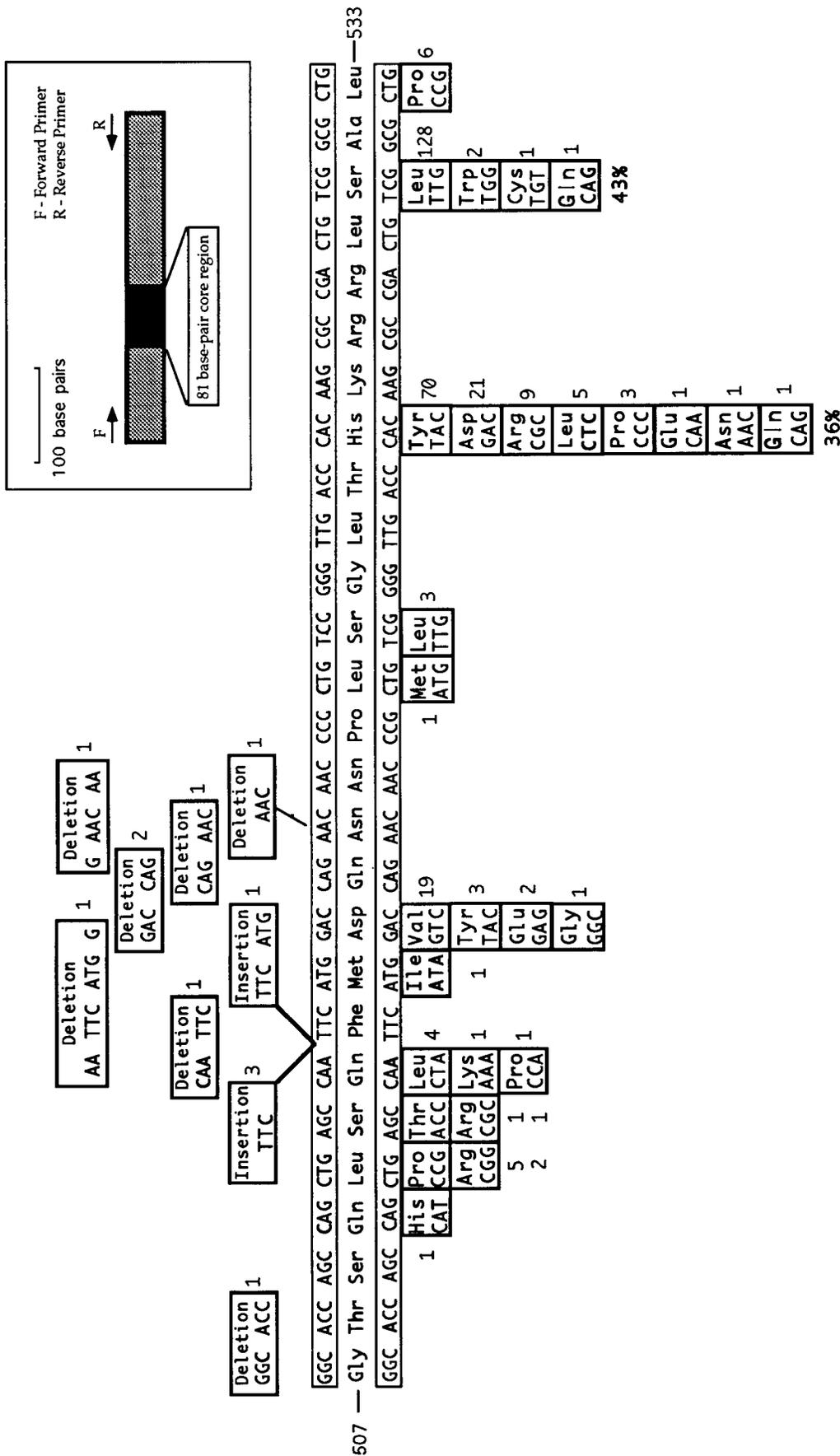


FIG. 1. Mutations occurring in codons 507 through 533 of the *M. tuberculosis rpoB* gene in 307 RIF-resistant isolates. The data are a compilation of mutations described in references 62, 77, 78, 106, 158, and 170 and include only changes occurring in strains with missense mutations at a single nucleotide or organisms with single insertion-deletion events. One RIF-resistant strain described in reference 62 had deletion of three nucleotides (CAT) that make up part of codons 514 and 515, resulting in Leu-514 replacing Phe-514-Met-515. Mutations occurring in *M. tuberculosis* isolates with more than one point mutation or other combinations of mutations are listed in Table 1. The positions of insertions and deletions are illustrated above the wild-type sequence, and missense mutations are recorded below. The codon numbering system initially described by Telenti et al. (158) was used. The codon numbers are designated on the basis of alignment of the translated *E. coli rpoB* sequence with a portion of the translated *M. tuberculosis* sequence and are not the positions of the actual *M. tuberculosis rpoB* codons (103).

TABLE 1. Complex alleles of *rpoB* found in RIF-resistant *M. tuberculosis* isolates

Codon	Variant nucleotides	Amino acid changes
511 ^a 516	CTG→CCG GAC→GAG	Leu→Pro Asp→Glu
511 ^a 516	CTG→CCG GAC→TAC	Leu→Pro Asp→Tyr
526 ^a 531	CAC→CAA TCG→TGT	His→Gln Ser→Cys
528 ^a 531	CGA→CGT TCG→TTG	Arg→Arg Ser→Leu
514 ^a 531	TTC→TTT TCG→TTG	Phe→Phe Ser→Leu
511 ^a 512	CTG→CCG AGC→ACC	Leu→Pro Ser→Thr
510 ^b 526	CAG→CAT CAC→CGC	Gln→His His→Arg
509 ^c 526	AGC→ACC CAC→GAC	Ser→Thr His→Asp
526 ^c 527	CAC→CCC AAG→CAG	His→Pro Lys→Gln
505 ^d 511 531	AGT→CGT CTG→CCG TCG→TGT	Phe→Leu Leu→Pro Ser→Cys
526 ^d 531	CAC→TAC TCG→TTG	His→Tyr Ser→Leu

^a Kapur et al. (78).^b Kapur et al. (77).^c Williams et al. (170).^d Heym et al. (58).

detected mutations had not been previously described (77, 78, 158). No mutations were found in 27 RIF-susceptible strains examined.

RIF-resistant strains of *M. tuberculosis* recovered from patients living throughout the world have also been analyzed by Williams et al. (170). Resistance to this antimicrobial agent was determined by the proportion plate method (1 µg of RIF per ml) (166), and isolates with greater than 1% growth on RIF-containing media compared with growth on control media (no antimicrobial agent) were considered resistant. These investigators examined the DNA sequences obtained from a 305-bp fragment of *rpoB* from 110 RIF-resistant and 10 RIF-susceptible organisms and found missense mutations in the 81-bp target region coding for amino acids 507 through 533 in 100 (91%) of the strains. In addition, one strain each had an insertion and deletion of one amino acid in this region. As described by other investigators (58, 77, 78, 158), most amino acid substitutions affected His-526 or Ser-531. A summary of *rpoB* mutations associated with RIF resistance is presented in Fig. 1 and Table 1. In total, 315 of 329 (96%) RIF-resistant strains have been found to harbor *rpoB* mutations, and 35 distinct allelic variants have been described. The mechanism of resistance in the 14 (4%) strains lacking mutations in the 81-bp *rpoB* core region remains unknown. In this regard, it is noteworthy that sequence analysis of *rpoB* from eight RIF-resistant

strains without *rpoB* core region mutations failed to identify mutations in other regions of the gene (157), including those with amino acid changes associated with RIF-resistant *E. coli* (92). This result implies that at least one additional molecular mechanism mediates RIF resistance in *M. tuberculosis*. Potential mechanisms include alterations in RIF permeability and mutations in other RNA polymerase subunits.

Recent analysis of 29 RIF-resistant organisms recovered from patients in South Korea, Brazil, and Mexico identified missense mutations in *rpoB* in 28 of the strains (106). The bacteria were judged to be resistant on the basis of either the standard proportion or MIC method (164). As described by other investigators, resistance in most strains was associated with amino acid substitutions located at position 516, 526, or 531 (106).

In an effort to directly determine the role of *rpoB* mutations in conferring RIF resistance in mycobacteria, Miller et al. (103) cloned and sequenced the entire *M. tuberculosis rpoB* gene and then used sophisticated genetic techniques to conclusively show that one common mutant *M. tuberculosis rpoB* allele conferred RIF resistance to *M. smegmatis* (103). The results of this experiment contribute to evidence that the *rpoB* mutations identified by Telenti et al. (158), Kapur et al. (77, 78), and others (106, 170) directly confer RIF resistance to *M. tuberculosis*. It remains unknown if RIF-resistant *M. tuberculosis* isolates exhibit pleiotropic phenotypes (including, for example, alterations in growth characteristics or virulence), as described for *E. coli rpoB* mutants (75).

Other Mycobacteria: *rpoB* Mutations

RIF also is a critical component of *M. leprae* chemotherapy and has been used previously in combination with other drugs to treat MAC organisms (82). A related antimicrobial agent, rifabutin, has been widely used in AIDS patients. The in vitro RIF susceptibility varies significantly among MAC serovars. Most MAC organisms are resistant in vitro, and RIF MICs for them exceed 100 µg/ml (70–72).

Resistant mutants are known to arise in leprosy patients receiving RIF monotherapy. The elucidation of the sequence of the *M. leprae rpoB* gene (62) permitted identification of mutations associated with RIF resistance in strains taken from nine patients with lepromatous leprosy (63). Missense mutations affecting Ser-425 (analogous to Ser-531 in *M. tuberculosis*) were identified in eight of the samples, and in the ninth sample, a 6-bp insertion was found in the same area of *rpoB* that contains an unusually high frequency of insertions and deletions in *M. tuberculosis*. Interestingly, no changes were found in the codon corresponding to position 526 (His) that is commonly mutated in RIF-resistant *M. tuberculosis*.

Strains of RIF-resistant *M. leprae* ($n = 4$), *M. africanum* ($n = 2$), and *M. avium* ($n = 4$) have recently been characterized by Williams et al. (170) for mutations in the 81-bp region of *rpoB* containing sequence changes in most RIF-resistant *M. tuberculosis* organisms. For these studies, the *M. leprae* strains were tested for RIF susceptibility by a mouse footpad assay (139), *M. africanum* susceptibility testing was conducted by the proportion plate method (1 µg of RIF per ml) (164), and the susceptibility of *M. avium* strains to RIF was assayed by the proportion method with 40 µg of RIF per ml incorporated into Lowenstein-Jensen slants (170). The four *M. leprae* strains each had a Ser→Leu substitution at amino acid residue 425. One strain of *M. africanum* had the same change, but the second isolate had a wild-type sequence in the 305-bp *rpoB* region studied. For the *M. avium* organisms, one strain had a Ser→Trp alteration in the homolog of amino acid 531, one had

a Ser→Leu change at this position, and the other two had wild-type sequences in the *rpoB* region characterized. It is a curious circumstance that no substitutions occurred at His-526, or its homolog, among non-*M. tuberculosis* (strict sense) isolates, an observation that warrants explanation at the molecular level.

A total of 31 MAC isolates with variable levels of RIF susceptibility were examined for sequence variation in the region of *rpoB* with RIF resistance-associated mutations in *M. tuberculosis* and *M. leprae* (50). Only one of the 31 organisms contained a missense mutation in the target region, a result that was interpreted to mean that alternative mechanisms are responsible for the intrinsic RIF resistance in MAC organisms. Although it is a formal possibility that missense or other mutations elsewhere in *rpoB* mediate resistance, the description (68) of a significant permeability barrier to RIF in one *M. intracellulare* strain suggests that restricted drug access to the target molecule is the main process mediating intrinsic RIF resistance. Additional potential mechanisms, including modifications of drug uptake or efflux and the occurrence of a RIF-inactivating enzyme, have not yet been probed in detail.

RIFABUTIN

M. tuberculosis rpoB Mutations

Rifabutin is a spiro-piperidyl derivative of rifamycin S that has potent antimycobacterial activity (35, 116, 136). In recent years, rifabutin has been commonly used in the prophylaxis against MAC infections in AIDS patients (114, 163).

The observations that some RIF-resistant strains of *M. tuberculosis* remain susceptible to rifabutin (32, 33) and patients with RIF-resistant pulmonary tuberculosis may improve when treated with this drug (49, 61, 126) suggested that *rpoB* mutation position and type of amino acid change influence rifabutin susceptibility. This idea was recently tested by Bodmer et al. (16), who performed quantitative susceptibility testing with RIF, rifabutin, and rifapentine on 36 RIF-resistant strains with 14 distinct *rpoB* mutations. MICs were determined by the BACTEC 460 radiometric method (142), and the following MICs were obtained for wild-type organisms: RIF, 0.25 to 0.5 µg/ml; and rifabutin and rifapentine, <0.015 to 0.125 µg/ml.

A clear correlation between the level of resistance to these drugs and specific mutations in *rpoB* was discovered. All organisms with 9 of the 14 mutant *rpoB* alleles (Gln-513→Leu; His-526→Arg, Asp, Pro, Tyr, or Gln; and Ser-531→Leu, Trp, or Tyr) had high-level cross-resistance to rifabutin and rifapentine (MICs, >4.0 µg/ml). In striking contrast, for strains with Leu-511→Pro, Asp-516→Tyr, Asp-516→Val, or Ser-522→Leu changes, rifabutin MICs were ≤0.5 µg/ml, and therefore the strains were categorized as moderately susceptible or low-level resistant (52). For rifapentine, all organisms were high-level cross-resistant to RIF except one with an Asp-516→Tyr amino acid substitution (MIC = 0.5 µg/ml). Although there is only limited clinical experience in treating RIF-resistant *M. tuberculosis* with rifabutin (49, 61, 126), these data suggest that patients with strains containing certain *rpoB* mutations may be amenable to rifabutin therapy. There remains a need for molecular genetic studies to directly probe the exact relationship between *rpoB* mutations and resistance to rifabutin, rifapentine, and other new rifamycin derivatives (177, 178).

STREPTOMYCIN

Background

STR is an aminocyclitol glycoside antibiotic that is widely used in tuberculosis therapy. The exact mechanism of action of STR has not been extensively investigated in mycobacteria, but in *E. coli* the antibiotic binds to 16S rRNA, interferes with a proofreading step in translation, and inhibits translational initiation, thereby perturbing protein synthesis (48, 105, 115). Expression of aminoglycoside-modifying enzymes is the most common mechanism of aminoglycoside-aminocyclitol resistance in bacteria, and these enzymes are generally encoded by resistance plasmids. However, clinically significant aminoglycoside-modifying enzymes have not yet been described in mycobacteria.

Although most eubacteria have multiple copies of rRNA operons, slowly growing mycobacteria such as *M. tuberculosis* and *M. leprae* have only one copy, and *M. smegmatis*, a rapidly growing species, has only two copies (11, 152). The practical implication of the observation of one 16S rRNA gene copy in the slowly growing mycobacteria is that single nucleotide changes can result in antibiotic resistance (dominant behavior), whereas in *E. coli*, a bacterium with seven 16S rRNA gene copies, or *M. smegmatis*, with two copies, nucleotide changes in a single rRNA gene are not expected to confer resistance (recessive behavior).

M. tuberculosis: Mutations in Genes Encoding 16S rRNA (*rrs*) and Ribosomal Protein S12 (*rpsL*)

Mutations associated with STR resistance in *M. tuberculosis* have been identified in two targets, the 16S rRNA gene (*rrs*) (Fig. 2) and the gene (*rpsL*) encoding ribosomal protein S12 (Fig. 3 and Table 2). In *E. coli*, the ribosomal protein S12 closely interacts with and stabilizes a highly conserved pseudoknot structure formed by 16S rRNA (105, 115). Amino acid replacements in protein S12 affect the higher-order structure of 16S rRNA (1) and confer STR resistance to *E. coli* (47). In addition, alterations in 16S rRNA disrupt interactions between 16S rRNA and paromomycin, a compound related to STR (34). The function of the mycobacterial S12 protein is presumed to be cognate with that identified in *E. coli*, but this has not been formally demonstrated.

Douglass and Steyn achieved the first insight into the molecular basis of STR resistance in *M. tuberculosis* (36). These investigators used PCR, DNA sequencing, and the amplification refractory mutation system method (113) to identify an A→G mutation at position 866 in the 16S rRNA gene of 2 of 11 STR-resistant patient isolates from South Africa. (To maximize clarity, note that position 866 is identical to position 904 described in work summarized below.) Neither the method of susceptibility testing used nor the level of resistance to STR was stated. Position 866 in *M. tuberculosis* 16S rRNA is thought to be functionally analogous to position 913 in *E. coli* 16S rRNA. Studies with the latter organism had provided evidence that mutations in the position 915 region of *E. coli* 16S rRNA reduced binding of STR to the ribosome and thereby protected against antibiotic inhibition of ribosome function in vitro (89, 105). The occurrence of the A→G substitution in STR-resistant organisms, its absence in published mycobacterial 16S rRNA sequences from susceptible organisms, and the data from *E. coli* studies together were interpreted as evidence of a role for the mutation in mediating resistance.

Finken et al. (41) used PCR to amplify the genes encoding 16S rRNA and ribosomal protein S12 from *M. tuberculosis*. A total of 38 STR-resistant and 35 STR-susceptible strains re-

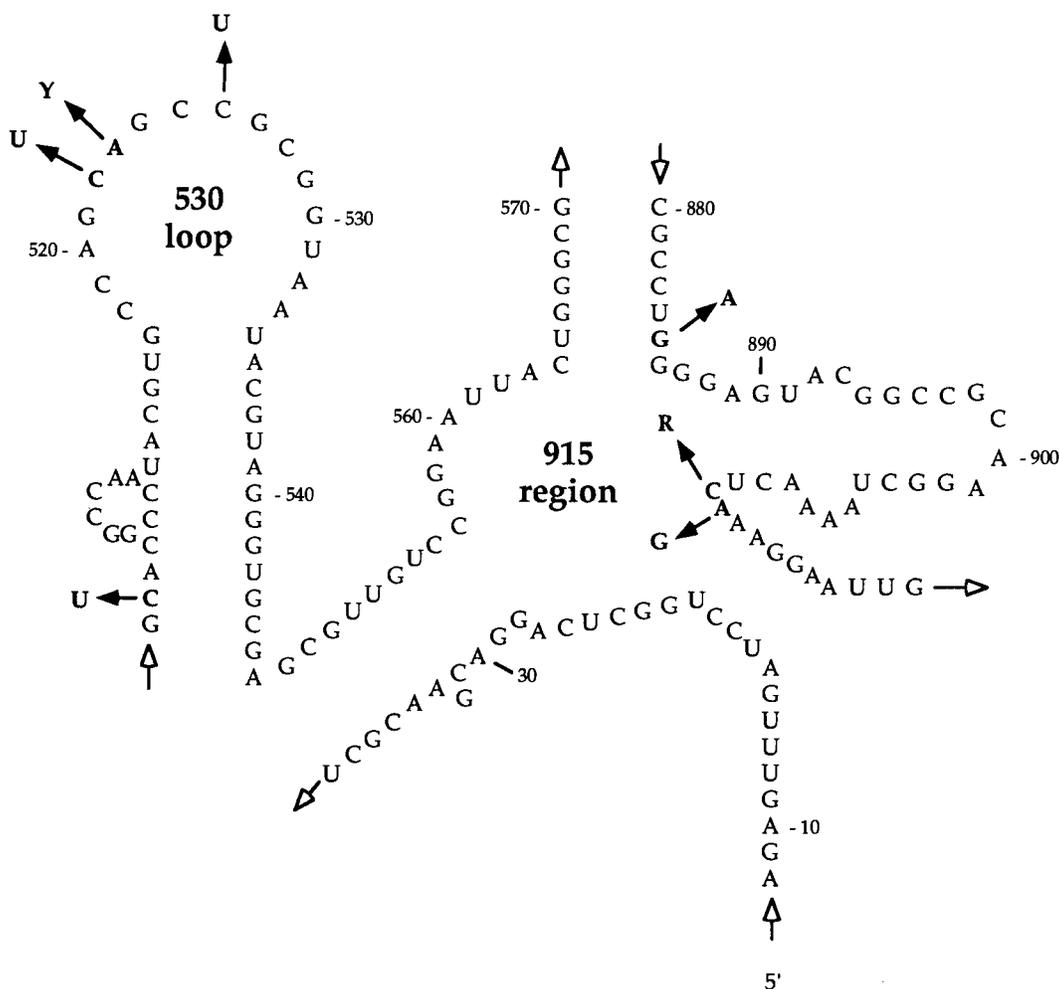


FIG. 2. Mutations located in 16S rRNA associated with STR resistance in *M. tuberculosis*. The drawing is based on a model structure of *E. coli* 16S rRNA (105). The nucleotide numbering system used is based on the publication of Finken et al. (41), and this system is used to maintain continuity with previously reported mutation data. Note that the position designations do not represent the actual *M. tuberculosis* positions. For the actual *M. tuberculosis* positions, subtract 10 from the 530 loop region numbers and subtract 8 from the 915 region numbers. The mutations associated with STR resistance are indicated by the solid arrows and are described in the references cited: position 491 (98), position 512 (98), positions 513 and 516 (41, 110), position 903 (63), and position 904 (98). Y = U or C; R = A or G.

covered from patients were then examined for sequence polymorphism in *rrs* and *rpsL*. Susceptibility testing was conducted by the proportion method (72, 131), and resistance was defined as growth in ≥ 6.0 μg of STR per ml. All 35 STR-susceptible organisms had identical wild-type sequences for *rrs* and *rpsL*. In contrast, 29 of 38 STR-resistant strains had mutations in *rrs* ($n = 9$) or *rpsL* ($n = 20$), and all 29 of these isolates had only a single variant nucleotide in these two genes. Among organisms with alterations in 16S rRNA, five were characterized by an A \rightarrow C transversion at position 513, one had an A \rightarrow T transversion at position 513, and three had C \rightarrow T transitions at position 516 (Fig. 2). The 20 strains with *rpsL* gene mutations had variant codons for amino acids 43 ($n = 14$) and 88 ($n = 6$). All mutations observed in the *rpsL* gene resulted in either Lys \rightarrow Arg or Lys \rightarrow Thr substitutions.

The molecular basis of STR resistance in *M. tuberculosis* was also examined by Nair et al. (112), who independently identified mutations in *rpsL* associated with resistant organisms. The method used to conduct susceptibility testing was not defined, nor were the MICs of STR given. These investigators studied four pairs of resistant *M. tuberculosis* strains recovered sequentially from patients treated with STR and found that all organ-

isms had undergone a transition mutation at bp 134 that results in an Arg \rightarrow Lys (AAG \rightarrow AGG) substitution at amino acid 43, as noted above. The same mutation was identified in a multidrug-resistant organism and a multidrug-resistant derivative of H37 generated by in vitro passage.

More recently, Meier et al. (98) studied the molecular basis of STR resistance in three *M. tuberculosis* patient strains. These organisms were resistant to ≥ 6.0 μg of STR per ml on the basis of testing by the proportion method and radiometric proportion technique (72). One organism had a point mutation (C \rightarrow T) located at position 491 of the 16S rRNA gene, and the second strain had a C \rightarrow T mutation at position 512 of this gene. Interestingly, a third strain that was resistant to >60 μg of STR per ml had a mutation at 16S rRNA gene position 904 (A \rightarrow G) and also a missense mutation in codon 88 of *rpsL* (AAG \rightarrow CAG, Lys \rightarrow Gln).

Honore and Cole (64) analyzed six STR-resistant and three STR-susceptible patient isolates of *M. tuberculosis* for the presence of mutations in the *rpsL* gene. Drug susceptibility testing was performed by the agar dilution method, and organisms capable of growth at ≥ 4 $\mu\text{g}/\text{ml}$ were defined as resistant. Two of the six resistant organisms contained a Lys \rightarrow Arg (AAG \rightarrow

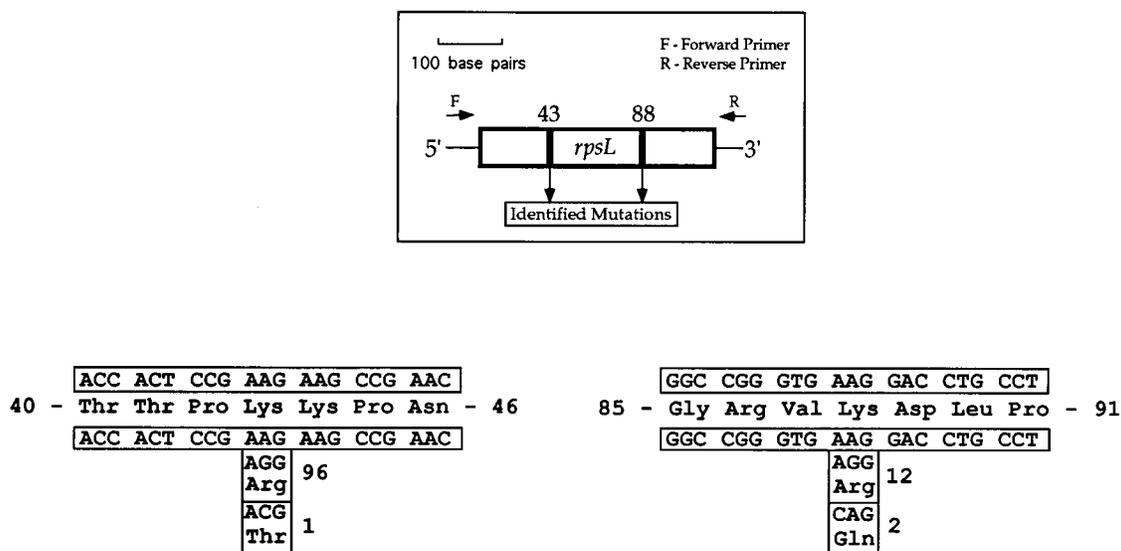


FIG. 3. Schematic representation of mutations located in the gene (*rpsL*) encoding ribosomal protein S12 associated with STR resistance in *M. tuberculosis*. Strains resistant to STR have missense mutations in codons 43 and 88 of the 372-bp *rpsL* gene that are not found in STR-susceptible organisms. Shown at the top is a schematic representation of *rpsL* and the positions of oligonucleotide primer sites used for amplification of the gene for automated sequencing, as described in reference 77. At the bottom of are shown the missense mutations in codons 43 and 88 identified by several investigators (41, 58, 64, 77, 106, 109, 112). The numbers located at the right of variant codons are a compilation of the occurrence of each mutation, as described in references 41, 58, 64, 77, 106, and 109. Not displayed are a CGC→CAC (Arg→His) change located in codon 9 and a GTG→ATG (Val→Met) alteration in codon 93, each found in one STR-resistant *M. tuberculosis* strain (109).

AGG) substitution in amino acid 43, and none of the three susceptible bacteria had mutations. In addition, two other STR-resistant organisms had mutations at position 903 of *rrs*. One of these strains had a C→G change and the other organism had a C→A mutation.

Twenty-five STR-resistant *M. tuberculosis* strains recovered from patients in several countries were analyzed for *rpsL* mutations by SSCP and DNA sequencing by Heym et al. (58). Susceptibility testing was performed by the agar dilution method, and organisms capable of growth at ≥ 4 $\mu\text{g/ml}$ were defined as resistant. Thirteen of 25 strains (52%) had the same amino acid substitution (Lys→Arg) at position 43 (incorrectly designated as position 42 in reference 58), but none of 16

STR-susceptible organisms had this alteration. Similarly, no other *rpsL* changes were detected in this sample of strains by SSCP analysis. Among the 12 STR-resistant strains without *rpsL* mutations, two organisms had nucleotide substitutions in the 16S rRNA gene, but the other 10 strains had no detectable changes by SSCP analysis. Both of the 16S rRNA gene mutations were located at position 903 (designated position 904 in reference 58); one was a C→G change and the other was a C→A substitution. These two organisms are presumably the same strains described in the work cited above (64). Hence, among 25 STR-resistant *M. tuberculosis* strains, 15 (60%) had identifiable mutations in two genes associated with antimicrobial resistance.

TABLE 2. Mycobacterial genes with mutations associated with antimicrobial resistance

Antimicrobial agent	Species	Gene ^a	Size (bp)	Product	Representative reference(s)
RIF	<i>M. tuberculosis</i> , <i>M. africanum</i> , <i>M. leprae</i> , <i>M. avium</i>	<i>rpoB</i>	3,534	β subunit of RNA polymerase	77, 103, 158, 170
INH	<i>M. tuberculosis</i>	<i>katG</i>	2,205	Catalase-peroxidase	57, 106, 111, 180
INH-ETH	<i>M. tuberculosis</i>	<i>inhA</i> locus	810 (<i>inhA</i>) 744 (<i>orf1</i>)	<i>envM</i> analog ^b 3-Ketoacyl-acyl carrier protein reductase analog ^c	5 77, 106
STR	<i>M. tuberculosis</i> , <i>M. smegmatis</i>	<i>rpsL</i>	372	Ribosomal protein S12	41, 83, 112
STR	<i>M. tuberculosis</i>	<i>rrs</i>	1,464	16S rRNA	36, 41
FQ	<i>M. tuberculosis</i> , <i>M. smegmatis</i>	<i>gyrA</i>	2,517	DNA gyrase A subunit	77, 150, 156
Clarithromycin	<i>M. intracellulare</i>	23S rRNA	Unknown	23S rRNA	99

^a GenBank numbers: *rpoB* (LO5910, L27989); *inhA* (UO2492); *gyrA* (L27512); *rpsL* (X70995); *rrs* (X52917); *katG* (X68081). Note that for the *rpoB* gene, accession number LO5910 contains a fragment of the gene described in reference 158, whereas the entire gene is deposited under accession number L27989 (103).

^b The inferred protein encoded by *inhA* shares 40% amino acid identity over 203 amino acids with the EnvM proteins (12, 162) of *Salmonella typhimurium* and *E. coli* (5).

^c Orf1 has significant sequence similarity to several proteins participating in fatty acid biosynthesis (5).

Kapur et al. (77) characterized five STR-resistant *M. tuberculosis* strains by automated DNA sequencing of the region of *rpsL* that contains the mutations of interest. Two of the five strains had missense mutations in codon 43 (AAG→AGG; Lys→Arg). More recent analysis of a sample of greater than 125 STR-resistant strains recovered from patients from global sources has found missense mutations in codon 43 or 88 in approximately 30% of the organisms (109, 110).

In summary, on the order of two-thirds of the STR-resistant strains of *M. tuberculosis* have detectable mutations associated with resistance to this antimicrobial agent. This observation implies that there is at least one additional mechanism conferring STR resistance, and future molecular genetic studies should be targeted to identify the gene(s) involved. In addition, there is a need for unambiguous molecular genetic demonstration that the mutations recorded in STR-resistant *M. tuberculosis* actually mediate resistance to this antimicrobial agent.

M. smegmatis: Mutations in *rpsL*

Mutations conferring STR resistance to *M. smegmatis* in the laboratory have been examined by Kenney and Churchward (83). (*M. smegmatis* is frequently used in genetic studies because it grows rapidly, is nonpathogenic, and like several other mycobacterial species, is transformable in the laboratory by electroporation.) Twenty-eight independent spontaneous mutants resistant to STR (≥ 20 $\mu\text{g/ml}$) were characterized for mutations in *rpsL* and *rpsG*, encoding ribosomal proteins S12 and S7, respectively. Twenty-five of the 28 strains were resistant to >1 mg of STR per ml. Complementation analysis was used to prove that the observed mutations were responsible for STR resistance. To determine the identity of the mutations conferring resistance, chromosomal DNA was isolated from the 28 strains and the *rpsL* gene was amplified by PCR and sequenced. All mutations were missense point mutations and occurred in codon 43 or a short region containing codons 86, 88, and 91. Twenty-four of 28 (86%) of the mutations affected Lys-43 ($n = 16$ isolates) or Lys-88 ($n = 8$ isolates), and 13 of the combined 24 identified mutations resulted in substitution of Arg for Lys. Most of the amino acid changes in the *Mycobacterium* S12 protein have been recorded in *E. coli* mutants resistant to STR, and many have been identified in naturally occurring STR-resistant strains of *M. tuberculosis* (41, 98). Interestingly, all of the STR-resistant *M. smegmatis* strains had mutations in *rpsL*, and none had changes in the gene encoding the 16S rRNA subunit, unlike the situation for resistant *M. tuberculosis*. This observation was attributed to the presence of two copies of the 16S rRNA gene in *M. smegmatis* but to only a single copy in the slow-growing mycobacteria.

ISONIAZID

Background

Although INH (isonicotinic acid hydrazide) was first synthesized in the early part of this century, it was not introduced as an antituberculosis medication until the 1950s. INH and RIF together form the backbone of *M. tuberculosis* chemotherapy globally. The INH MIC for susceptible *M. tuberculosis* strains is usually less than 0.02 to 0.05 $\mu\text{g/ml}$, and other members of the *M. tuberculosis* complex are also highly susceptible to this agent. In contrast, INH is inactive in vitro against most MAC isolates (70) and has a restricted role in therapy of infections caused by other mycobacterial species.

INH has been used widely in *M. tuberculosis* treatment for over 40 years, but neither the bacterial target nor the mode of

action is well understood. Potential insight into the mechanism of INH resistance occurred in 1954 when Middlebrook and colleagues discovered that INH-resistant organisms had decreased catalase activity (28, 100, 102). This observation was extended by Hedgecock and Faucher (51), who studied INH-resistant organisms and noted an inverse correlation between INH MIC and catalase-peroxidase activity. Although the exact metabolic pathway by which INH exerts its effect is unknown, one working hypothesis postulates that INH or an INH metabolite blocks the synthesis of mycolic acids in drug-susceptible organisms (155, 173, 174). Evidence that the toxicity of INH against *M. tuberculosis* is potentiated by the peroxidase activity of the mycobacterial catalase-peroxidase has also been presented (140, 141).

M. tuberculosis: Mutations in *katG* Encoding Catalase-Peroxidase

Primary resistance of *M. tuberculosis* to INH arises in the laboratory at an estimated frequency of 10^{-5} to 10^{-7} (31), which is several orders of magnitude greater than that reported for RIF (181). In an effort to elucidate the molecular mechanism responsible for the observed decreased catalase activity associated with INH-resistant organisms, Zhang et al. (180) cloned and Heym et al. (59) characterized the gene (*katG*) coding for the *M. tuberculosis* catalase-peroxidase. The *M. tuberculosis* enzyme is an 80,000-Da protein with substantial homology to hydroperoxidase I from *E. coli*, catalase-peroxidase from *M. intracellulare* (107), and other bacterial catalase-peroxidases. Transformation of *M. smegmatis* and *M. tuberculosis* with wild-type *katG* restored INH susceptibility to resistant isolates (179), a result confirming that the protein product of *katG* (KatG) participated in INH action. In addition, it was observed that in two of three high-level (MIC, >50 $\mu\text{g/ml}$) resistant patient isolates *katG* was deleted from the chromosome (180). These investigators concluded that resistance in a subset of *M. tuberculosis* strains is due to loss of the complete *katG* gene. However, they also observed by Southern analysis that most INH-resistant strains had an apparently intact *katG* gene, although the levels of catalase-peroxidase activity recorded for these organisms were greatly decreased. In addition, Stoeckle et al. (149) studied by PCR 80 randomly selected isolates recovered from patients in New York City and found that 35 (90%) of 39 INH-susceptible and 31 (76%) of 41 INH-resistant strains contained apparently intact *katG* sequences and therefore did not have a gross deletion of this gene. Moreover, there was no simple relationship between absence of *katG* and occurrence of high-level (>1 $\mu\text{g/ml}$) INH resistance. These observations suggested that for most strains either simple base-pair changes resulting in (for example) missense or stop mutations or small deletions were associated with resistance.

The observation that most INH-resistant *M. tuberculosis* strains did not have gross *katG* deletions suggested the need to more precisely analyze the structure of *katG* present in resistant organisms. Several groups have now reported that many INH-resistant strains contain missense and other types of mutations. For example, Altamirano et al. (2) studied nine INH-resistant isolates and one INH-susceptible patient isolate and found that a 237-bp fragment of *katG* could be amplified from eight of nine INH-resistant isolates. These eight INH-resistant organisms had missense mutations, deletions of 1 base, or insertions of up to 3 bases in the region studied (nucleotides 3 through 239 located at the 5' end of the gene).

Heym et al. (58) recently reported the results of characterization of the *katG* gene in 20 INH-resistant organisms and

16 strains that were resistant to both INH and ethionamide (ETH). For these organisms, the MICs of INH ranged from 0.2 to >10 µg/ml, and the ETH MICs were equal to or greater than 10 µg/ml. These investigators found that 23 of 36 (64%) organisms had mutations in *katG*, as defined by SSCP analysis. In addition, 5 of the 23 INH-resistant organisms also had SSCP-associated variation in the *inhA* locus (see next section), and five strains had only *inhA* locus alterations.

Characterization of *katG* sequences in 18 INH-resistant organisms with altered SSCP patterns found several types of mutations located predominantly in the region encoding the amino-terminal one-half of the protein (57). The most common mutation ($n = 7$ strains) was a CGG→CTG change resulting in an Arg→Leu substitution at amino acid position 463. Four additional missense mutations were identified, including GTG→GCG (f-Met→Ala, position 1) in one strain, GAC→GCC (Thr-275Pro) in one strain, AGC→ACC (Ser-315Thr) in five strains, and CTG→ATG (Leu-587Met) in one strain. Two strains that had a deletion of 12 bp resulting in absence of amino acids 120 to 123 were identified, and one strain had an insertion of 3 bp (CAT, Ile) between codons 125 and 126.

The availability of crystal structures for related enzymes, including yeast cytochrome *c* peroxidase (42), permitted several inferences to be made about structure-function activities of the *katG* gene product. The deletion of residues 120 to 123 and the insertion of Ile between amino acids 125 and 126 were predicted to result in diminution of catalase activity, and it was shown that organisms bearing these mutations had only approximately 10% residual catalase-specific activity relative to strains with wild-type *katG* alleles. The amino acid replacements at positions 275 (Thr→Pro) and 315 (Ser→Thr) were also predicted to result in decreased catalase activity because amino acid residues located close to the equivalent positions in yeast cytochrome *c* peroxidase and *E. coli* hydroperoxidase are functionally important (93, 124, 144). These predictions were borne out by enzymatic studies demonstrating that *M. tuberculosis* strains with the position 275 or 315 substitutions have virtually no detectable catalase activity (58). In contrast to the diminished catalase activity observed among organisms with the mutations described above, strains with the Arg-463→Leu substitution had essentially wild-type levels of enzyme activity.

Cockerill et al. (27) have also characterized the *katG* gene in 15 strains of *M. tuberculosis* for which INH MICs ranged from <0.12 to >32 µg/ml, including nine resistant and six susceptible strains. Sequencing of *katG* from all organisms found that six of nine INH-resistant strains had one or more missense mutations, one strain had a nonsense mutation, one had an 8-bp deletion, and one had no mutations in the coding sequence. In striking contrast to the data presented by Heym et al. (58), in which virtually all strains had single amino acid substitutions, nine of the strains had multiple missense mutations, including one organism reported to have nine amino acid substitutions. Five of six INH-resistant strains with missense mutations had, in addition to other changes, a G→T transversion in codon 463, resulting in the substitution of Leu for Arg. The six INH-susceptible strains had none to 11 amino acid substitutions compared with a consensus sequence formulated from the data available for all 15 organisms; however, none of the mutations affected Arg-463. The G→T change in codon 463 results in loss of a restriction enzyme site recognized by *NciI* and *MspI* and thereby generates a convenient strategy to rapidly screen large collections of organisms for this polymorphism. Analysis of 32 INH-susceptible and 43 INH-resistant strains demonstrated that 19 of 43 (44%) of resistant strains, but only 1 of 32 (3%) of INH-susceptible strains, had lost this

restriction site and were presumed to contain the G→T mutation resulting in an Arg→Leu substitution.

On the basis of these data, two divergent views regarding the level of *katG* allelic variation in natural isolates of susceptible and resistant *M. tuberculosis* and the characteristics of *katG* variants associated with INH resistance emerged. To provide additional data bearing on the issue of *katG* allelic variation in INH-susceptible and -resistant organisms, Musser et al. (111) sequenced *katG* and the *inhA* locus in entirety from 34 resistant and 12 susceptible strains from global sources. Virtually all resistant organisms had amino acid changes in KatG or nucleotide substitutions in a presumed upstream regulatory region of *inhA*. Moreover, sequence analysis of a region of *katG* encoding residues Ser-315 and Arg-463, and the *inhA* locus, in 10 susceptible and 51 INH-resistant organisms from The Netherlands found that 84% of resistant bacteria had mutations in *katG* or the *inhA* locus. Interestingly, all 16 strains of *M. bovis* and *M. microti* had Leu-463 rather than Arg-463 in *katG*, a result consistent with the hypothesis that Leu-463 is the ancestral condition in *M. tuberculosis*. Taken together, the data are consistent with results showing that very little *katG* allelic variation occurs in INH-susceptible strains, and a restricted subset of missense changes are repeatedly associated with INH resistance.

To summarize, missense and other mutations in *katG* that are associated with resistance to INH have been identified (Fig. 4). Although there is evidence to suggest that one or more of the variant *katG* alleles confers INH resistance, there is clearly a need for additional molecular genetic data addressing the exact role of some of the nonsynonymous substitutions described by several investigators.

M. tuberculosis: *inhA* Locus Polymorphisms

The observations that only a relatively modest percentage of INH-resistant *M. tuberculosis* strains are catalase negative and have gross alterations (149) or missense or other mutations involving *katG* (27, 57) indicated that other molecular mechanisms mediating INH resistance remained to be discovered. As a consequence, there has been a concerted effort by several groups to isolate additional genes involved in INH resistance. Banerjee et al. (5) identified a locus containing two contiguous open reading frames (designated *orf1* and *inhA*) coding for products that may participate in resistance to both INH and ETH (Fig. 5). ETH is a structural analog of INH that is sometimes used as a second-line antituberculosis drug. ETH, like INH, is thought to inhibit mycolic acid biosynthesis in *M. tuberculosis* and other mycobacteria (173, 175). In addition, studies (21, 60, 90) have demonstrated that for certain strains low-level INH resistance correlated with coacquisition of ETH resistance rather than loss of catalase activity. Taken together, these observations suggested that the two antimicrobial agents may share a common target and led to formulation of an elegant molecular genetic strategy to identify the structural gene encoding a putative target. Single-step spontaneous INH-ETH-resistant mutants of *M. smegmatis* and *M. bovis* NZ were independently used as sources of chromosomal DNA to construct cosmids that conferred INH-ETH resistance to *M. smegmatis*. Subsequent genetic manipulations resulted in identification of the locus containing two contiguous open reading frames, designated *orf1* and *inhA*, encoding 25.7- and 28.5-kDa proteins, respectively. DNA sequence analysis found that the resistant organisms had a single base change (T→G in nucleotide 280) resulting in a Ser-94→Ala-94 amino acid substitution in *inhA*. Subcloning experiments demonstrated that the presence of *inhA* alone was sufficient to confer INH resistance to *M. smegmatis* in the laboratory. In *M. tuberculosis* and *M.*

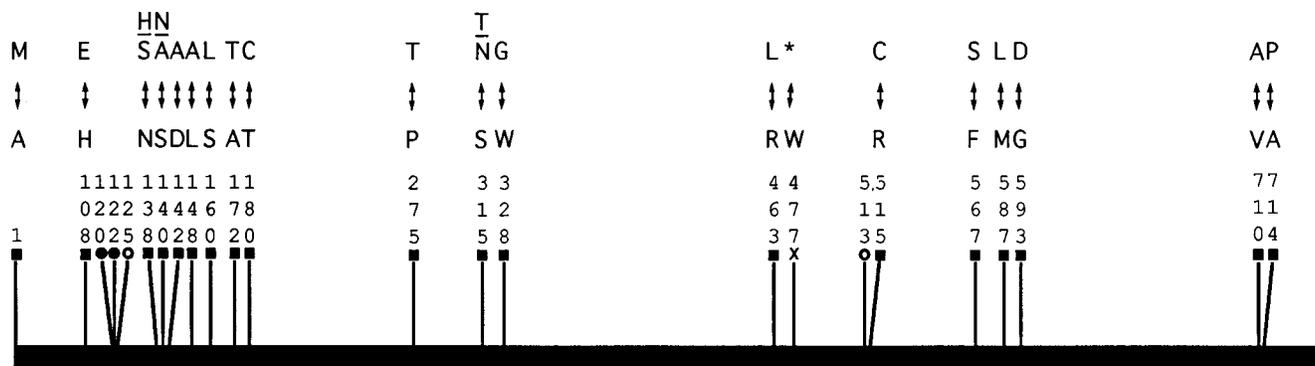


FIG. 4. Schematic representation of polymorphism in the translated KatG protein. The data were compiled from mutations identified in references 57, 106, and 111. The locations of variant amino acids are numbered in vertical format. The single-letter amino acid abbreviations immediately above the codon numbers refer to residues found in the protein inferred from the DNA sequence deposited in GenBank under accession number X68081 (59). A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; S, serine; T, threonine; W, tryptophan, V, valine. *, ×, termination mutation; ■, simple missense mutation; ●, deletion; ○, insertion.

bovis, the two genes are separated by a short noncoding region of 21 bp that lacks a readily identifiable promoter. Hence, *orf1* and *inhA* together may constitute a two-gene operon that is transcribed from a promoter located in front of *orf1* (Fig. 5).

The inferred proteins made by *inhA* of *M. tuberculosis* (and *M. bovis* and *M. smegmatis*) have greater than 40% sequence identity over a stretch of 203 amino acids with the EnvM proteins of *E. coli* and *Salmonella typhimurium*. EnvM is thought to participate in fatty acid biosynthesis (12, 162) and has been shown to catalyze the reduction of crotonyl-acyl carrier protein (13). In this regard, it is noteworthy that a Gly-93→Ser-93 substitution in EnvM in *E. coli* resulted in resis-

tance to diazaborine, an inhibitor of fatty acid, phospholipid, and lipopolysaccharide biosynthesis (162). The predicted protein made by *orf1* has greatest identity with 3-ketoacyl-acyl carrier protein reductase coded for by *fabG* of *E. coli*, an enzyme thought to be involved in fatty acid biosynthesis (5). On the basis of the hypothesis that INH and ETH act by inhibiting synthesis of mycolic acid, it is noteworthy that analogs of the predicted proteins made by *orf1* and *inhA* have roles in *E. coli* fatty acid synthesis. Moreover, cell extracts prepared from the resistant mutant strain, or from resistant merodiploids harboring multiple *inhA* copies, had significant resistance to INH-mediated inhibition of mycolic acid biosynthesis.

The identification of the missense mutation in *inhA* conferring mycobacterial resistance to INH and ETH in the laboratory led to the plausible hypothesis that missense mutations in *inhA* would constitute a very abundant cause of resistance among strains of *M. tuberculosis* recovered from patients. Surprisingly, this is not the case. The entire *inhA* gene, including the upstream putative regulatory region, was sequenced by Kapur et al. (77) in a sample of 37 INH-resistant organisms (including some resistant to INH plus ETH) collected from diverse localities in the United States. With only one exception, all strains had the identical wild-type *inhA* allele, and none contained the Ser-94→Ala-94 substitution. The one exceptional strain had a missense mutation (ATC→ACC, Ile-16→Thr-16) located at nucleotide 47. Variation was then examined in the 744-bp *orf1* gene in 24 resistant patient isolates, and surprisingly, polymorphisms were identified at two nucleotides flanking a presumed ribosomal binding site in four of these isolates (77). Although one strain had a synonymous substitution at nucleotide position 102, no other *orf1* mutations were found in resistant organisms or in upstream or coding regions of seven susceptible strains (77). On the basis of these studies, it is possible that the base-pair substitutions flanking the presumed *orf1* ribosomal binding site result in altered regulation of Orf1 and/or InhA in some *M. tuberculosis* strains (Fig. 5).

Related data have also been generated recently by Morris et al. (106), who examined the *inhA* locus for sequence polymorphisms by a combination of SSCP and DNA sequencing in a sample of 42 INH-resistant isolates. No alterations were identified in the *inhA* coding region, but five isolates had mutations in the putative regulatory region containing a possible ribosome binding site. Two strains had a T→G transversion, two strains had a C→T transition, and one organism had an A→G

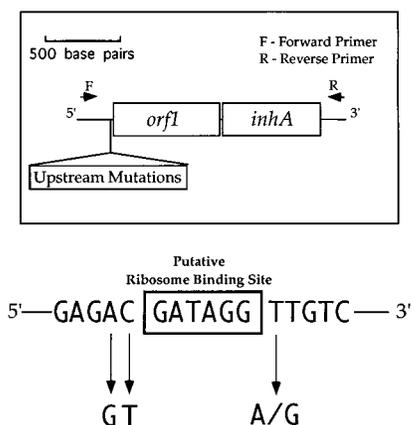


FIG. 5. Schematic representation of mutations identified in a putative ribosome binding site area at the *inhA* locus associated with isoniazid resistance in *M. tuberculosis*. The *inhA* locus is composed of two contiguous open reading frames (designated *orf1* and *inhA*) coding for protein products that may participate in resistance to INH and perhaps ETH (5). The 28.5-kDa protein product encoded by the 810-bp *inhA* gene has significant homology with the EnvM protein of *E. coli*, a molecule thought to participate in fatty acid biosynthesis (11, 162). The predicted 25.7-kDa protein made by *orf1* (744 bp) has significant similarity to 3-ketoacyl-acyl carrier protein reductase encoded by *fabG* of *E. coli*, and this enzyme is also thought to be involved in fatty acid biosynthesis (5). In *M. tuberculosis* and *M. bovis*, *orf1* and *inhA* are separated by a 21-bp noncoding region that lacks a readily identifiable promoter (5), and the genes are presumed to constitute a two-gene operon that is transcribed from a promoter located upstream of *orf1*. Variation has been identified by automated DNA sequencing and other strategies in a putative ribosome binding site area located immediately upstream of *orf1* in some *M. tuberculosis* strains resistant to INH alone or to both INH and ETH (77, 106). No sequence variation has been identified in this region, or in other nucleotides of the *inhA* locus, in susceptible organisms (77, 105).

change in nucleotides located in the region upstream from the *orf1* structural gene (106) (Fig. 5). These observations are consistent with the identification of putative *inhA* regulatory region mutations in 10 of 36 INH-resistant organisms studied by Heym et al. (58). It is noteworthy that in the studies by Kapur et al. (77), Morris et al. (106), and Heym et al. (58), mutations in the putative *inhA* locus regulatory region occur in a disproportionately higher percentage of strains resistant to both INH and ETH than in organisms resistant to INH but susceptible to ETH. This observation suggests a causal relationship between the putative regulatory region nucleotide substitutions and resistance to the two antimicrobial agents. The lack of Ser-94→Ala-94 substitutions among the patient isolates clearly provides a sobering reminder of the importance of correlating laboratory observations with molecular study of large samples of patient strains.

Recently, additional data about structure and function relationships of the InhA protein have become available (33). Wild-type InhA catalyzed the reduction of 2-*trans*-octenoyl-acyl carrier protein, a result demonstrating that the *inhA* gene product is an enoyl-acyl carrier protein reductase. This result is consistent with the hypothesis that InhA participates in mycolic acid biosynthesis. Comparison of enzyme kinetic parameters of wild-type InhA with those of the Ser-94→Ala-94 InhA variant found no difference in the K_m and V_{max} values. However, the Michaelis constant for NADH was five times higher in the Ser-94→Ala-94 mutant, a result suggesting that the mechanism of drug resistance is related to specific enzyme-cofactor interactions in the NADH binding site. Interestingly, the side chain of Ile-16 (an amino acid replaced by Thr-16 in one INH-resistant clinical isolate [78]) is also located in the NADH-binding cleft and is positioned close to the adenine ribose portion of NADH. Hence, it is very likely that substitution of the hydroxyl side chain of Thr-16 for the alkyl group of Ile-16 perturbs the hydrogen-bonding pattern of the NADH-binding site and leads to significant differences in cofactor interaction.

Despite the availability of these detailed molecular interaction data, many issues about the role of *inhA* locus mutations in INH resistance remain unresolved. First, thus far, neither INH nor ETH has been shown to bind to InhA, which suggests that activation of these antimicrobial agents may be necessary. Second, it remains unknown if InhA and KatG interact, and if so, how it occurs, and if the mutations identified at the *inhA* locus and *katG* result in altered interactions. In this regard, Dessen et al. (33) have hypothesized that the Ser-94→Ala-94 and Ile-16→Thr-16 substitutions result in a decreased binding affinity of InhA for NADH. The change in kinetics of enzyme-cofactor interactions then alters subsequent binding of a putative activated form of INH, ultimately resulting in INH resistance. Although no detailed hypothesis has been advanced in light of the new InhA structure-function data to explain the mechanism by which *orf1* regulatory region mutations might produce resistance to INH, the upstream mutations may result in increased InhA protein expression.

In summary, the molecular basis of resistance to INH for some *M. tuberculosis* strains appears to involve mutations in *katG* and perhaps putative *inhA* locus regulatory sequences. However, mutations in these genes clearly do not account for all INH-resistant strains. The observation (132, 133) that mutations in the *E. coli* *oxyR* regulon influence the susceptibility of this organism to INH suggests possible additional mechanisms involved in INH resistance. Moreover, there has been essentially no work done to examine the potential role of INH permeability changes in mediating resistance. Clearly, much additional genetic and biochemical work is needed to elucidate

the complex mechanism of resistance to this antimicrobial agent.

FLUOROQUINOLONES

Background

The increasing frequency of recovery of multidrug-resistant *M. tuberculosis* strains resistant to most first- and second-line antituberculosis agents has resulted in substantial use of other antimicrobial agents in patients infected with these problem strains (150). Fluoroquinolones (FQ), mainly ciprofloxacin (CIP), have been employed, but resistance to these agents develops relatively rapidly (61, 150). FQ antimicrobial agents are variably active in vitro against MAC organisms and other mycobacterial species. CIP has been employed in combination-therapy regimens to treat MAC infections (82).

Study of quinolone activity in many bacterial species has found that the primary target of these antimicrobial agents is DNA gyrase, a type II DNA topoisomerase that is composed of two A and two B subunits, encoded by *gyrA* and *gyrB*, respectively (65). Missense mutations in the putative FQ binding region of the A subunit have been found to confer high-level resistance in several bacterial species (65). This region is highly conserved in *E. coli*, *Staphylococcus aureus*, and *Campylobacter jejuni* and is referred to as the quinolone resistance-determining region. Other mutations conferring FQ resistance have been found in the gene (*gyrB*) for the B subunit, although these mutations tend to confer lower-level resistance (65).

Ciprofloxacin-Resistant *M. tuberculosis*: Mutations in *gyrA* Encoding the A Subunit of DNA Gyrase

As a first step toward understanding the molecular mechanism of FQ resistance in *M. tuberculosis*, Takiff et al. (156) cloned and sequenced the wild-type *gyrA* and *gyrB* genes encoding the A and B subunits of DNA gyrase, respectively. The *gyrA* gene (2,517 bp) is located 36 bp downstream of *gyrB* (2,060 bp), and the inferred *M. tuberculosis* GyrA and GyrB proteins have 69 and 63% similarity to the *E. coli* gyrase subunits, respectively. Four CIP-susceptible reference organisms, six CIP-resistant laboratory mutants of the *M. tuberculosis* complex, 54 patient isolates of *M. tuberculosis* (39 CIP-susceptible and 15 CIP-resistant organisms), and one CIP-susceptible patient isolate of *M. africanum* were then screened by PCR-SSCP for *gyrA* and/or *gyrB* polymorphisms. With a single exception, MICs for all resistant patient strains were greater than or equal to 4 $\mu\text{g/ml}$. A 320-bp PCR product of *gyrA* and a 428-bp PCR-generated fragment of *gyrB* corresponding to regions in genes from *E. coli* and other bacterial species with CIP-conferring mutations were studied. The 320-bp *gyrA* fragment was sequenced from all strains in the sample. Two *gyrA* SSCP patterns were identified among CIP-susceptible organisms and eight *gyrA* SSCP patterns were found among resistant bacteria; all strains had a single *gyrB* SSCP pattern. A total of five polymorphic codons (positions 88, 90, 91, 94, and 95) were identified in *gyrA* by DNA sequencing. Codon 95 contained a naturally occurring polymorphism (AGC, Ser; or ACC, Thr) that may be unrelated to CIP resistance because it occurred in both FQ-susceptible and FQ-resistant organisms. In contrast, the other four polymorphic codons contained changes unique to the CIP-resistant strains, with position 94 being the most highly variable. All patient isolates for which MICs was greater than or equal to 4 $\mu\text{g/ml}$ had variant codons not represented among susceptible strains. No simple relationship between dis-

tinct variant codons and CIP MICs was observed, although all eight strains for which MICs were $>8 \mu\text{g/ml}$ had mutations in codon 94. Hence, *gyrA* mutations were identified in all strains for which the CIP MIC was $>2 \mu\text{g/ml}$, a value that has been proposed as a cutoff for clinical CIP resistance (54).

On the basis of the observation that CIP-resistant *M. tuberculosis* strains for which MICs were $>4.0 \mu\text{g/ml}$ contain missense mutations in *gyrA*, Kapur et al. (77) sequenced a 320-bp region of *gyrA* in 17 isolates, including a sample of 12 isolates that were resistant to CIP. Virtually all (16 of 17) strains, including both resistant and susceptible organisms, had an AGC \rightarrow ACC (Ser \rightarrow Thr) mutation in codon 95 of *gyrA* that is apparently unrelated to CIP resistance. Among the resistant strains, three organisms had missense mutations in codon 94, and two resistant strains had missense mutations in codon 90. No other mutations were identified in the region of *gyrA* studied. Taken together, these data show that there may not be a simple one-to-one correlation of missense mutations and CIP resistance in *M. tuberculosis* and imply that the molecular basis of resistance to this drug is complex and remains to be fully elucidated. Additional well-characterized strain samples will need to be studied in order to fully understand the relationship of missense mutations to antibiotic resistance. Moreover, there is a need for additional molecular genetic studies probing the role of variant *gyrA* alleles in mediating CIP resistance.

Ofloxacin: *gyrA* Mutations in Resistant *M. tuberculosis* and *M. smegmatis*

Ofloxacin (OFX) has also been used to treat patients with pulmonary tuberculosis (161). Cambau et al. (20) characterized one strain of OFX-resistant *M. tuberculosis* recovered from a patient with a long history of multidrug-resistant tuberculosis. This organism was resistant to $32 \mu\text{g}$ of OFX per ml on the basis of results determined by the proportion method (70). DNA sequence analysis of part of the *gyrA* gene in the initial susceptible organism and the OFX-resistant derivative strain identified a missense mutation (GAC \rightarrow CAC, Asp \rightarrow His) in codon 89 (numbering system used in reference 156).

Revel et al. (129) studied polymorphism occurring in five mutant strains of *M. smegmatis* selected in the laboratory for which OFX MICs were greater than or equal to $2 \mu\text{g/ml}$. All organisms had missense mutations in codon 90 (GCG \rightarrow GTG, Ala \rightarrow Val) or codon 94 (GAC \rightarrow GGC, Asp \rightarrow Gly) of *gyrA*.

OTHER ANTIMICROBIAL AGENTS USED IN MYCOBACTERIAL THERAPY

Ethionamide

As noted above, ETH (2-ethylpyridine-4-carbonic acid thioamide) is a derivative of isonicotinic acid with potent activity against *M. tuberculosis* and other mycobacteria (38, 60, 90, 130). Most *M. tuberculosis* strains are inhibited in vitro by ETH concentrations in the range of 10 to $20 \mu\text{g/ml}$. On the basis of the observation that cells treated with ETH lose the property of acid fastness, and some experimental data (5, 175), it is thought that the mechanism of action involves inhibition of mycolic acid biosynthesis. The exact mechanism of ETH resistance is unknown, but some strains resistant to both INH and ETH harbor mutations in a putative regulatory region located upstream of the *orfI* gene in the *inhA* locus (Fig. 5) (5, 58, 77).

Ethambutol

EMB is a very specific and effective drug that is used in combination with INH to treat *M. tuberculosis* infection (76, 80, 160, 171). The molecular mechanism of EMB action is unknown, but experimental data have been generated and several hypotheses have been advanced to explain its action (9, 26, 44, 45, 73, 84, 88, 123, 125, 128, 143, 153, 154). Most of the data have been generated from study of the effects of EMB on mycobacterial species other than *M. tuberculosis*. Among the effects attributed to EMB are inhibition of RNA metabolism (44, 45), phospholipid synthesis (26, 84), transfer of mycolic acids to cell wall-linked arabinogalactan (153), spermidine synthesis (123, 125), and an early step of glucose conversion into the monosaccharides used for the cell wall polysaccharides arabinogalactan, arabinomannan, and peptidoglycan (143). Thus far, no genes known to encode products participating in EMB resistance have been described.

Pyrazinamide

PZA is a synthetic derivative of nicotinamide that has been used in some short-course antituberculosis treatment regimens. Neither the biochemical mechanism of action nor the molecular basis of resistance is fully known. Several investigators have reported that PZA-susceptible *M. tuberculosis* strains have a pyrazinamidase that metabolizes PZA to pyrazinoic acid and that PZA-resistant organisms have lost pyrazinamidase activity (19, 87, 96). Although the exact antimycobacterial moiety is unknown, Heifets et al. (53) have demonstrated that pyrazinoic acid is likely to participate. On the basis of these observations, one can speculate that any molecular mechanism resulting in loss of or decreased PZA activity (e.g., loss of pyrazinamidase structural gene or missense mutations resulting in an altered allele) may lead to PZA resistance. However, that fact that highly PZA-resistant strains do not always lack pyrazinamidase activity (19) suggests that more than a single resistance mechanism may operate.

Capreomycin, Viomycin, and Kanamycin

Capreomycin and viomycin are polypeptide antimicrobial agents that are sometimes used in combination therapy for treatment of drug-resistant *M. tuberculosis* strains (56). Kanamycin is an aminoglycoside that is also used to treat resistant organisms. Although strains that acquire resistance to capreomycin generally remain susceptible to other antituberculosis medications, cross-resistance with kanamycin and viomycin can occur (95, 151). For example, McClatchy et al. (95) reported complete cross-resistance between viomycin and capreomycin among laboratory-generated resistant mutants of *M. tuberculosis*. However, cross-resistance between kanamycin and capreomycin and between kanamycin and viomycin was variable. In addition, review of the medical histories of 27 patients with kanamycin-resistant *M. tuberculosis* isolates indicated that cross-resistance with capreomycin and viomycin occurs but, like the in vitro situation, is unpredictable. Currently, no progress has been made in the understanding of the molecular basis of resistance of *Mycobacterium* spp. to these agents.

Clarithromycin

Clarithromycin is a macrolide antibiotic with substantial in vitro activity against nontuberculosis mycobacteria. Although a clinical trial of clarithromycin monotherapy in AIDS patients demonstrated that within 6 weeks a dramatic decrease occurred in the number of *M. avium-M. intracellulare* complex

bacteria culturable from blood (30), it has also been observed that drug resistance emerges during monotherapy (134). Macrolides are bacteriostatic antibiotics that inhibit the peptidyltransferase region of the 50S ribosomal subunit (reviewed in reference 48). Studies of several organisms have found that a peptidyltransferase center located in a small region of 23S rRNA is implicated in macrolide resistance. In *E. coli*, methylation of an adenine residue in a generally conserved loop of domain V confers resistance to macrolides and several other antibiotics (145).

Because there is increasing interest in using clarithromycin and the related macrolides azithromycin and roxithromycin as therapy for nontuberculous mycobacteria (17, 18, 55), the mechanism responsible for clarithromycin resistance in *M. intracellulare* was investigated in six pairs of susceptible and resistant organisms cultured from patients with chronic pulmonary infections undergoing monotherapy with clarithromycin. For the resistant isolates, MICs were >32 $\mu\text{g/ml}$ as assayed by broth microdilution (17, 18). Three of the six independent clarithromycin-resistant isolates, but none of the susceptible strains, had a single point mutation in domain V of 23S rRNA. One organism each had an A \rightarrow C, A \rightarrow G, or A \rightarrow T change. The position mutated corresponds to *E. coli* position 2058, which has been implicated in resistance to erythromycin and macrolide-lincomide-streptogramin B antibiotics (99). The other three resistant organisms had wild-type sequences in the stretch of approximately 400 nucleotides studied. The authors also observed that two *M. avium* patient isolates with acquired resistance to azithromycin both showed a single point mutation in 23S rRNA (A-2058 \rightarrow T and A-2059 \rightarrow C). Finally, in a note added in proof, it was stated that two *M. avium*-*M. intracellulare* complex strains had an A \rightarrow C transition at position 2059. Hence, of the total of 10 clarithromycin-resistant *M. avium*-*M. intracellulare* isolates studied, 7 (70%) had a mutation of 23S rRNA at position 2058 or 2059.

Tetracycline

Tetracycline and related compounds are sometimes used to treat infections caused by certain rapidly growing nontuberculous mycobacteria. Pang et al. (122) examined a total of seven isolates of tetracycline-resistant *M. fortuitum*, *M. fortuitum* third biovariant complex, and *M. peregrinum* for the presence of sequences homologous to genes encoding resistance to this antimicrobial agent in gram-positive organisms. One isolate each of the *M. fortuitum* third biovariant complex and *M. peregrinum* had sequences homologous with genes conferring tetracycline resistance. PCR-based sequence analysis of one of the genes (*tetK*) found in a strain of the *M. fortuitum* third biovariant complex revealed 98% identity in the 300-bp region studied. In addition, the sequence had a G+C content of only 30%, a value that differs significantly from the 70% G+C typifying mycobacterial genes. Together, the data suggest that these *Mycobacterium* spp. acquired *tetK* from a heterologous source, perhaps by conjugation.

MOLECULAR STRATEGIES FOR DETECTION OF MUTANT ALLELES

Several molecular techniques have been formulated to rapidly detect mutations in target genes of interest (6, 40, 113, 118, 119, 127, 167, 168), including those associated with antimicrobial resistance in *Mycobacterium* spp. (Table 3). These strategies have been used for two main purposes: definition of the frequency distribution of distinct mutations occurring in resistant strains, and rapid indirect identification of resistant organ-

isms. The utility of these strategies is enhanced in *M. tuberculosis* because with the exception of mutations associated with resistance, there is a striking paucity of nucleotide sequence diversity in many structural genes among isolates recovered from global sources (79). *M. leprae* has also been reported to have restricted genetic diversity based on restriction fragment length polymorphism analysis (169).

The most widely used technique has been DNA sequencing of PCR-amplified targets generated from DNA obtained from mature cultures. Both manual and automated DNA sequencing approaches have been employed. Representative data obtained by automated DNA sequencing of a segment of *rpoB* in RIF-resistant organisms are shown in Fig. 6. Other methods applied to identify mutations associated with antimicrobial resistance include simple PCR, PCR-SSCP analysis, the amplification refractory mutation system, PCR-heteroduplex analysis, heminested PCR (167), and restriction enzyme analysis of PCR products to identify loss or gain of a restriction site (Table 3). Several of these strategies, including automated DNA sequencing, PCR-SSCP analysis, and PCR-heteroduplex formation, have been employed to a limited extent to analyze early positive BACTEC 460 cultures and primary patient specimens (77). Thus far, there have been no published reports of application of a microtiter format point mutation assay that has been successfully applied to detect drug resistance-conferring mutations in human immunodeficiency virus type 1 from patients treated with zidovudine (81).

One of the promising new strategies for ultrafast, unambiguous, low-cost DNA sequence analysis currently in the developmental stage is termed sequencing by hybridization (4, 7, 8, 37, 85). This approach exploits the natural base-pairing specificity of multiple DNA probes to "decode" the nucleotide sequence of a target DNA molecule. Sequencing by hybridization is also sometimes referred to as matrix hybridization DNA sequencing or simply matrix sequencing. This approach was independently proposed by several groups as a new sequencing strategy about 5 years ago. In sequencing by hybridization, DNA samples are interrogated by hybridization to a complete set of all nucleotide probes of a given length to determine the oligonucleotide content of the DNA sample. As initially described, interrogation would employ a complete set of all oligonucleotide probes of a given length, for example, all 65,536 (4⁸) octamers. Then, the complete sequence is generated by computer with an algorithm that automatically identifies successive sequence overlaps between oligonucleotide "words."

In one of the sequencing by hybridization formats being developed for rapid identification of *M. tuberculosis* mutations associated with antimicrobial resistance, numerous probes are attached to a surface such as a glass microscope slide. A DNA sample such as a PCR product is then hybridized to the complete set of probes simultaneously. This strategy permits acquisition of the complete sequence information in a single experiment. Both microelectronic and optical detection schemes are being developed to detect hybridization within the oligonucleotide array (7).

VIRULENCE AND DRUG RESISTANCE

One of the very important questions relating to the study of resistant *M. tuberculosis* is the relative virulence of drug-resistant organisms. This has been a controversial issue for several decades, since it was reported that INH-resistant strains that lack catalase activity have significantly reduced virulence for guinea pigs (101), and Mitchison et al. (104) reported that a significant number of INH-resistant *M. tuberculosis* strains isolated from patients in India tended to be of low virulence in a

TABLE 3. Molecular strategies used to detect mutations in genes associated with antimicrobial resistance in mycobacteria

Technique and study	Species	Antimicrobial agent
DNA sequencing		
Douglass and Steyn (36)	<i>M. tuberculosis</i>	STR
Finken et al. (41)	<i>M. tuberculosis</i>	STR
Nair et al. (112)	<i>M. tuberculosis</i>	STR
Meier et al. (98)	<i>M. tuberculosis</i>	STR
Telenti et al. (158)	<i>M. tuberculosis</i>	RIF
Honore and Cole (64)	<i>M. tuberculosis</i>	RIF
Kapur et al. (78)	<i>M. tuberculosis</i>	RIF
Williams et al. (170)	<i>M. tuberculosis</i> , <i>M. africanum</i> , <i>M. avium</i> , <i>M. leprae</i>	RIF
Hunt et al. (69)	Several	RIF
Altamirano et al. (2)	<i>M. tuberculosis</i>	INH
Cockerill et al. (27)	<i>M. tuberculosis</i>	INH
Musser et al. (111)	<i>M. tuberculosis</i>	INH
Banerjee et al. (5)	<i>M. tuberculosis</i>	INH, ETH
Heym et al. (58)	<i>M. tuberculosis</i>	RIF, STR
Kapur et al. (77)	<i>M. tuberculosis</i>	RIF, STR, INH, ETH, CIP
Morris et al. (106)	<i>M. tuberculosis</i>	RIF, STR, INH, ETH
Cambau et al. (20)	<i>M. tuberculosis</i>	OFX
Takiff et al. (156)	<i>M. tuberculosis</i>	FQ
Sullivan et al. (150)	<i>M. tuberculosis</i>	FQ
Honore and Cole (63)	<i>M. leprae</i>	RIF
Guerrero et al. (50)	<i>M. avium</i> , <i>M. intracellulare</i>	RIF
Meier et al. (99)	<i>M. intracellulare</i>	Clarithromycin
Kenney and Churchward (83)	<i>M. smegmatis</i>	STR
Revel et al. (129)	<i>M. smegmatis</i>	FQ
Restriction enzyme polymorphism		
Nair et al. (112)	<i>M. tuberculosis</i>	STR
Cockerill et al. (27)	<i>M. tuberculosis</i>	INH
Amplification refractory mutation system		
Douglass and Steyn (36)	<i>M. tuberculosis</i>	STR
PCR-SSCP analysis		
Telenti et al. (159)	<i>M. tuberculosis</i>	RIF
Honore and Cole (64)	<i>M. tuberculosis</i>	RIF
Takiff et al. (156)	<i>M. tuberculosis</i>	FQ
Heym et al. (58)	<i>M. tuberculosis</i>	RIF, STR, INH, ETH
Morris et al. (106)	<i>M. tuberculosis</i>	INH, STR
PCR-heteroduplex formation		
Williams et al. (170)	<i>M. tuberculosis</i>	RIF
Other		
Zhang et al. (180)	<i>M. tuberculosis</i>	INH
Altamirano et al. (2)	<i>M. tuberculosis</i>	INH
Stoeckle et al. (149)	<i>M. tuberculosis</i>	INH
Felmlee et al. (40)	<i>M. tuberculosis</i>	RIF
Whelen et al. (167)	<i>M. tuberculosis</i>	RIF

guinea pig model of infection. Recently, Ordway et al. (117) studied a panel of 15 clinical isolates of *M. tuberculosis*, including several resistant to one or more antimycobacterial agents, and found that drug-resistant strains had a range of virulence for mice. In apparent contrast to Mitchson's data, no simple relationship was identified between degree of drug resistance and relative virulence, as defined by growth in lungs following aerogenic exposure to a low-dose inoculum. Although these data are important, unfortunately the exact molecular basis of drug resistance in these organisms was not defined. An elegant study recently conducted by Wilson et al. (172) provided strong evidence that *katG* participates in virulence in a guinea model of *M. bovis* disease. Isogenic strains were used to demonstrate that *katG* was essential for virulence, whereas the presence or absence of the *inhA* locus had no apparent role in virulence. Critically, the investigators restored virulence to an avirulent,

catalase-negative, INH-resistant *M. bovis* strain by integration of a functional *katG* gene into the genome. Clearly, additional studies of this type, employing defined mutant *katG* alleles, should be conducted once strategies are developed to generate isogenic *M. tuberculosis* strains.

CONCLUSIONS

Considerable progress has been made in recent years toward understanding the molecular basis of antimicrobial resistance in mycobacteria. Most work has been conducted with *M. tuberculosis*, and the primary theme emerging from these studies is that resistance is usually due to simple nucleotide substitutions rather than to acquisition of new genetic elements encoding antibiotic-altering enzymes. Multidrug-resistant isolates of *M. tuberculosis* arise as a consequence of sequential accu-

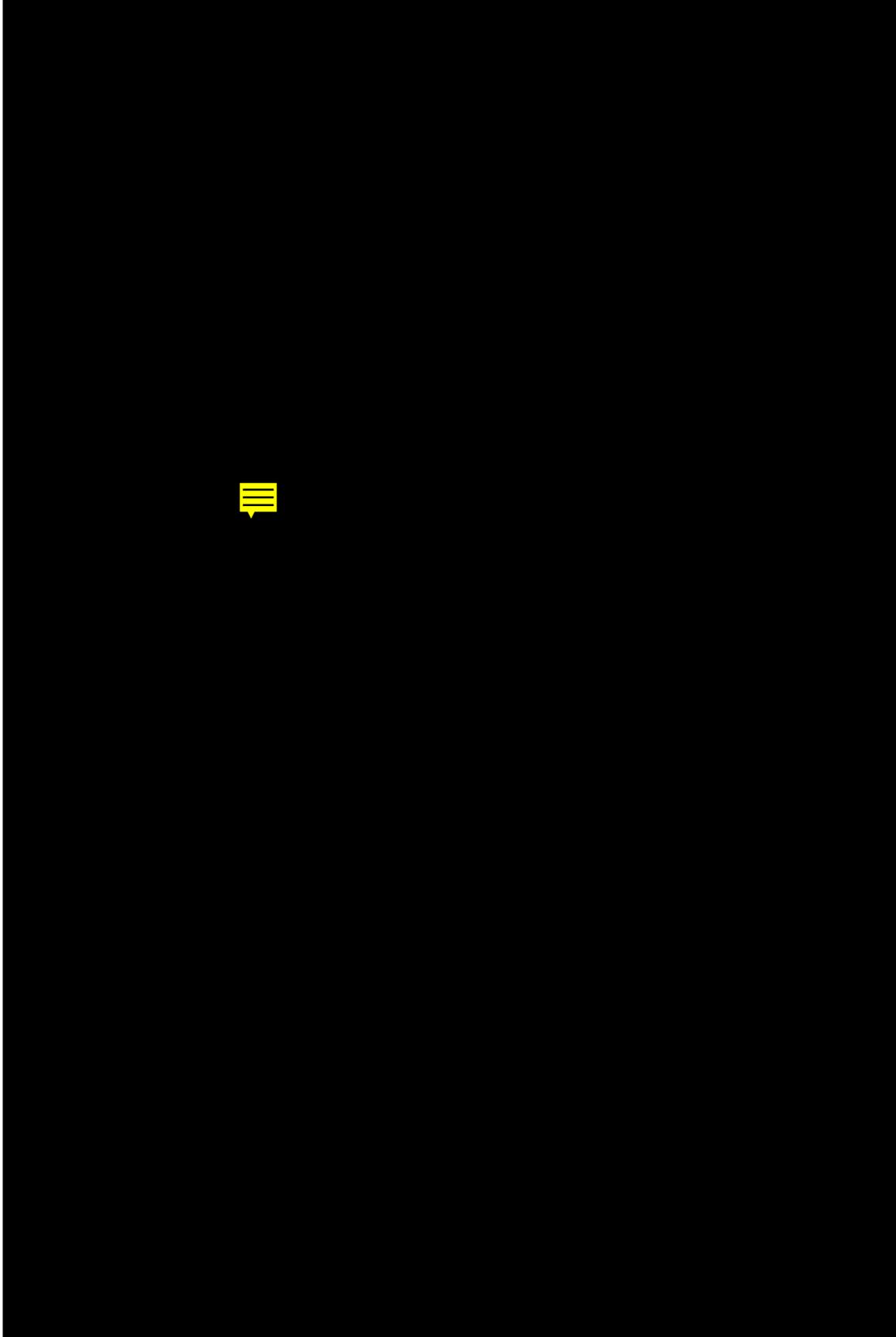


FIG. 6. Chromatogram illustrating mutations associated with RIF-resistant mycobacteria in a region of the gene (*proB*) encoding the β subunit of RNA polymerase. Shown are representative data generated by an Applied Biosystems model 373A automated DNA sequencer. An area of approximately 70 bp encoding amino acids 511 through 533 is shown. The wild-type sequence is presented at the top, and three variant alleles characterized by deletions of 6 or 9 bp are shown below the wild-type allele. The deleted nucleotides are shown above each of the three mutant alleles. The GAC CAG deletion results in deletion of amino acids 516 (Asp) and 517 (Gln), and deletion of CAG and AAC results in deletion of amino acids 517 and 518 (Asn). The AAT TCA TGG deletion involves codons 513 through 517 (CAA TTC ATG GAC) and results in conversion of the wild-type Ser-Gln-Phe-Met-Asp-Gln sequence into a Ser-His-Gln primary sequence. See Fig. 1 for additional clarification.

mulation of mutations conferring resistance to single therapeutic agents. Despite substantial progress, there is clearly much work to be done to fully elucidate the molecular basis of antimicrobial resistance in *M. tuberculosis* and other mycobacteria. With the exception of mutations in *rpoB* conferring RIF resistance, for no other antitubercular medication can the basis of resistance be explained in a substantial percentage (>90%) of strains. Several strategies show considerable promise for rapid detection of mutations associated with antimicrobial resistance, although none of these approaches at present is amenable to utilization in a general clinical microbiology laboratory. The important question of the consequences of resistance acquisition to relative virulence in its broadest definition (transmissibility, colonization, invasion, etc.) remains an area for which insight is needed. As Canetti (21) noted 30 years ago, "... drug resistance in tuberculosis is a field of admirable diversity. This field has something to offer to almost everybody: the clinician, the pathologist, the pharmacologist, the biochemist, the epidemiologist, the geneticist." To this group must surely be added the clinical microbiologist.

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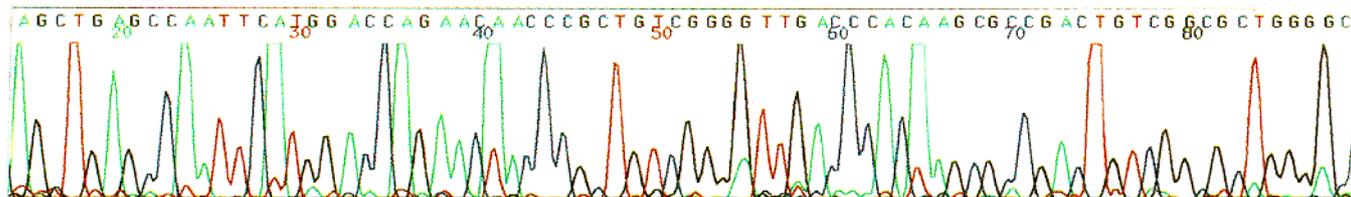
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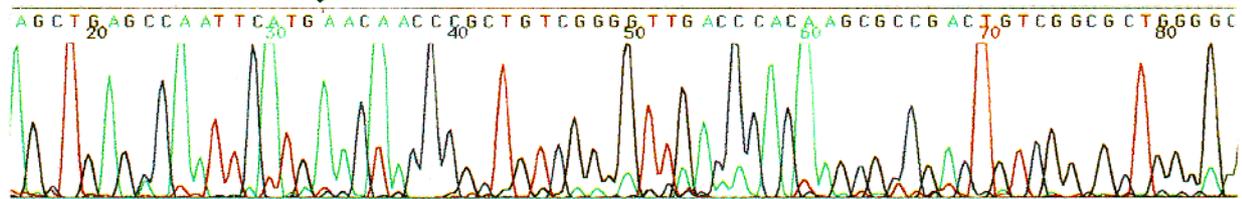
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Wild Type



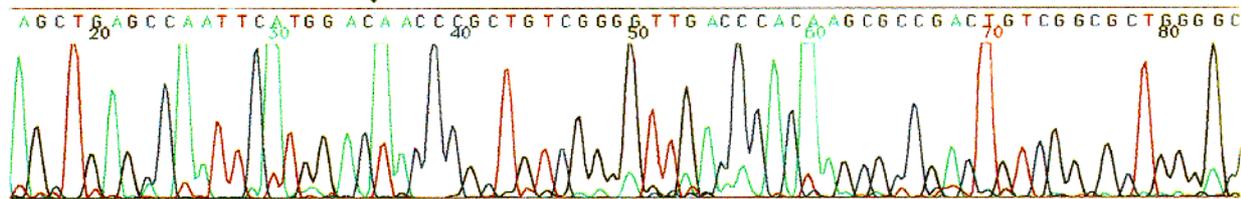
GAC CAG

516/517
Deletion: GAC CAG



CAG AAC

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Deletion: CAG AAC



AAT TCA TGG

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Deletion: AAT TCA TGG

