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Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update

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Summary Knowledge of the molecular genetic basis of resistance to antituberculous agents has advanced rapidly since we reviewed this topic 3 years ago. Virtually all isolates resistant to rifampin and related rifamycins have a mutation that alters the sequence of a 27-amino-acid region of the beta subunit of ribonucleic acid (RNA) polymerase. Resistance to isoniazid (INH) is more complex. Many resistant organisms have mutations in the *katG* gene encoding catalase-peroxidase that result in altered enzyme structure. These structural changes apparently result in decreased conversion of INH to a biologically active form. Some INH-resistant organisms also have mutations in the *inhA* locus or a recently characterized gene (*kasA*) encoding a β-ketoacyl-acyl carrier protein synthase. Streptomycin resistance is due mainly to mutations in the 16S rRNA gene or the *rpsL* gene encoding ribosomal protein S12. Resistance to pyrazinamide in the great majority of organisms is caused by mutations in the gene (*pncA*) encoding pyrazinamidase that result in diminished enzyme activity. Ethambutol resistance in approximately 60% of organisms is due to amino acid replacements at position 306 of an arabinosyltransferase encoded by the *embB* gene. Amino acid changes in the A subunit of deoxyribonucleic acid gyrase cause fluoroquinolone resistance in most organisms. Kanamycin resistance is due to nucleotide substitutions in the *rrs* gene encoding 16S rRNA. Multidrug resistant strains arise by sequential accumulation of resistance mutations for individual drugs. Limited evidence exists indicating that some drug resistant strains with mutations that severely alter catalase-peroxidase activity are less virulent in animal models. A diverse array of strategies is available to assist in rapid detection of drug resistance-associated gene mutations. Although remarkable advances have been made, much remains to be learned about the molecular genetic basis of drug resistance in *Mycobacterium tuberculosis.* It is reasonable to believe that development of new therapeutics based on knowledge obtained from the study of the molecular mechanisms of resistance will occur.

BURDEN OF DRUG-RESISTANT TUBERCULOSIS

The acid-fast bacillus *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). This infection is the leading cause of mortality in adults due to an infectious agent and accounts for 26% of all preventable adult deaths globally.[1,2] Strains of *M. tuberculosis* resistant to antimycobacterial agents are recovered from immunocompetent and immunocompromised patients worldwide.[3] Clusters of disease caused by drug-susceptible and -resistant strains have been reported globally.[3–10] TB will undoubtedly increase in prevalence in most countries due to the human immunodeficiency virus (HIV) pandemic. In response to these alarming statistics and trends, the World Health Organization declared TB to be a global public health emergency.[2]

The US Centers for Disease Control and Prevention national surveillance system recorded 93 449 TB cases from 1993 through 1996, including 1457 cases of multidrug resistant TB (MDR-TB).[11] Recent US data show that about 13% of all new cases are resistant (primary resistance) to at least one first-line drug, and 1.6% are resistant to both isoniazid (INH) and rifampin (RIF).[11] Cohn et al[3] reviewed the results of 63 surveys of resistance to antituberculous drugs performed globally between 1985 and

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1994. The median rates of primary resistance to first-line drugs were INH (4.1%), streptomycin (STR) (3.5%), RIF (0.2%), and ethambutol (EMB) (0.1%). The median rates of acquired (secondary) resistance were much higher than those of primary resistance. Given the enhanced difficulty of treating drug-resistant strains,[12] the emergence and widespread dissemination of *M. tuberculosis* clones resistant to antimicrobial agents has adverse implications for TB control in the 21st century.

Substantial progress has been made in our understanding of the molecular basis of *M. tuberculosis* drug resistance in the last 3 years.[13] The goal of this review is to update the reader on recent progress in this critical area. Individuals interested in other topics pertaining to antimycobacterial agent research are referred elsewhere.[14–18]

CHEMOTHERAPEUTICS AND DRUG RESISTANCE

The current short course *M. tuberculosis* drug treatment regimen uses an initial 2-month phase of daily therapy with INH, RIF, and pyrazinamide (PZA), plus either STR or EMB.[19,20] This regimen is followed by daily therapy with INH and one other primary drug for the next 4 months. Drugs such as ethionamide (ETH), cycloserine, p-aminosalicylic acid, thioacetazone, kanamycin (KAN), capreomycin (CAP), viomycin (VIO), amikacin (AMI) and fluoroquinolones (FQs) are used as secondary or alternative agents to treat infections caused by strains resistant to commonly used medicines. Most expert panels and professional organizations, including the Centers for Disease Control and Prevention, the American Thoracic Society, and the World Health Organization recommend the use of directly observed therapy for patients with active TB.[21,22] Successful chemotherapy results in conversion of sputum to a culture-negative state in 2 months and clearing of infiltrates on the chest radiograph between 4 and 6 months.[23,24] Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate.[6,25,26] Several factors influence the degree of success of treatment programs including duration and complexity of therapy, ease of health care access, treatment cost, patient adherence, and drug side effects. In addition, some data suggest that certain HIV-positive patients may be at elevated risk of developing RIF-resistant TB, perhaps due to drug absorption problems.[27–31]

One of the assumptions associated with much of the research investigating drug resistance is that knowledge of the molecular genetic basis of this phenomenon will assist development of new chemotherapeutics by rational drug design strategies. It is also reasonable to think that rapid, sensitive, and unambiguous strategies for the detection of drug resistant strains may result. Both areas of research will benefit from the availability of a complete genome sequence from one *M. tuberculosis* strain.[32]

RIFAMPIN AND RELATED RIFAMYCINS

RIF is a semi-synthetic derivative of rifamycin that is used as a first-line TB medication. The highly effective bactericidal action of this drug against *M. tuberculosis* has made it a key component of therapy.[21,22] The mechanism of RIF activity and resistance have been extensively studied in *Escherichia coli*.[33,34] RIF binds to the β-subunit of ribonucleic acid (RNA) polymerase resulting in inhibition of transcription initiation.[33,34] A similar mechanism of action has been demonstrated in *M. smegmatis*.[35]

It was known for many years that missense mutations and short deletions in the central region of the RNA polymerase beta subunit gene (*rpoB*) result in RIF^R *E. coli*.[36–39] This insight led to characterization of the *M. tuberculosis rpoB* gene and to identification of a wide variety of mutations conferring RIF resistance.[13,40,41] Compilation of data available from many studies indicated that 315 of 329 (96%) of epidemiologically unrelated RIF^R patient isolates of *M. tuberculosis* had 35 distinct point mutations, or short insertions and deletions located in an 81-bp core region (rifampin resistance determining region [RRDR] of *rpoB* codons 507–533 encoding 27 amino acids).[13,41–47] Importantly, 43% of the strains had missense mutations in codon 531 (Ser) and 36% of organisms had codon 526 (His) alterations that would result in amino acid replacements. The most common amino acid substitutions were Ser531Leu (42%) and His526Tyr (23%).[13] The mechanism of resistance was not identified in 4% of RIF^R clinical isolates because no mutations were detected in the 81-bp core region of *rpoB* nor elsewhere in the *rpoB* gene.

Several studies published from late 1995 to early 1998 have confirmed and extended the basic principles delineated by these findings.[48–55] Caugant et al[48] analyzed the RRDR of *rpoB* in 66 *M. tuberculosis* patient isolates from Mozambique, including 41 RIF^R isolates identified by the BACTEC radiorespiratory method. Most (39 of 41) RIF^R strains had point mutations conferring amino acid replacements, and the frequency distribution of the affected codons was closely similar to that reported in earlier studies. Rinder et al.[49] determined the distribution of *rpoB* mutations in 49 RIF^R isolates recovered in Germany and Sierra Leone. Resistance to RIF was determined by the proportion method. Similar to all other studies, mutations in codons 526 and 531 were the most common. Interestingly, the investigators reported differences in the frequency distribution of mutations in codon 516 and 526 in the isolates from the two geographic areas. However, inasmuch as the strains were not analyzed by IS*6110* typing to determine epidemiologic independence, and there is no apparent reason to account for the reported geographic differences, it is possible that redundant sampling of clonally related isolates occurred.

Recently, Escalante et al.[50] conducted the first molecular genetic analysis of RIF^R strains cultured from patients in Latin American countries. The investigators analyzed 19 epidemiologically-independent resistant strains from Peru and identified RRDR mutations in 18 organisms. Most strains had amino acid replacements at position 526 or 531.

PCR-SSCP and deoxyribonucleic acid (DNA) sequencing were used to analyze the core region of *rpoB* in 32 RIF^R and 26 RIF^S *M. tuberculosis* strains from Korea.[52] Susceptibility testing was done by the absolute concentration method. Most mutations occurred in codons 526 (37.8%) and 531 (24.4%). Two previously undescribed mutations in codons 507 and 518 were identified. In contrast to other studies, a high frequency of double mutations occurring in two separate codons (15.6%) and in a single codon (6.3% in codon 526; CAC->TGC, His-> Cys) was identified.

Several groups have conducted studies with patient isolates designed to gain understanding of the relationship between RIF MICs and RpoB structural changes.[53–56] This work is largely an extension of the investigations originally reported by Bodmer et al.,[53] who found strong correlation of MICs for RIF and related rifamycins with certain RpoB variants. In particular, amino acid substitutions located at position 526 and 531 conferred high-level resistance to rifampin, rifabutin, and rifapentine. Ohno et al[54] analyzed the RRDR of RpoB in 40 clinical isolates of *M. tuberculosis* recovered in Japan. RIF susceptibility testing was done by the broth microdilution method and strains with MIC of ≥ 2 μg/ml were considered to be RIF^R. All 13 strains with high level RIF resistance (MIC ≥ 64 μg/ml) had missense mutations in codons 516, 526, or 531. In contrast, all 21 susceptible organisms lacked changes at these positions. Of the six strains with MICs between 2 and 32 μg/ml, three strains had a missense mutation in either codon 516 or 526, and three strains lacked mutations in the RRDR.

The relationship between the degree of RIF resistance and RpoB missense changes was also analyzed by Taniguchi et al.[55] in 21 isolates of *M. tuberculosis* recovered from patients in Japan. RIF susceptibility testing was done by the proportion method or by broth dilution method. Twenty strains had amino acid alterations in the RRDR, and one strain had an Ala381Val substitution in the N-terminal region of RpoB. Isolates with mutations in codons 513 (5%), 526 (33%), and 531 (43%) had high level drug resistance (MIC ≥ 50 μg/ml). In contrast, amino acid substitutions located at position 514, 521, or 533 resulted in low-level resistance (MIC 12.5 μg/ml).

Similar findings were reported by Moghazeh et al.,[56] who tested 24 RIF^R clinical isolates of *M. tuberculosis* with known *rpoB* mutations against three rifamycin derivatives, RIF, rifapentine, and the related investigational drug KRM-1648[57,58] to correlate levels of resistance with specific mutations. Susceptibility testing was done by the agar diffusion method. As recorded in other studies,[53–55] mutations in codons 513, 526, and 531 had high level resistance to RIF (MIC >32 μg/ml), with the exception of strains with His526Leu or His526Asn substitutions, which had MICs of 8 and 16 μg/ml, respectively. In contrast to results reported by other investigators,[53–55] a Leu533Pro substitution was associated with an MIC >32 μg/ml for RIF, rather than a low level MIC. The molecular explanation for this discrepancy is unknown. Importantly, all strains resistant to RIF were also cross resistant to rifapentine, and the same MIC values were identified. In contrast, KRM-1648 was able to overcome rifampin resistance in strains with four different genetic alterations. This finding suggested the theoretical possibility that KRM-1648 could be used to treat patients with certain types of *rpoB* mutations.

A recent analysis of *M. tuberculosis* strains constructed by molecular genetic strategies to contain defined *rpoB* mutant alleles on a plasmid has confirmed that mutations in the RRDR are responsible for resistance to this antimicrobial agent.[59] In addition, the study confirmed that KRM-1648 can overcome resistance to certain other rifamycins in vitro.

In summary, analysis of approximately 500 RIF^R strains from global sources has found that 96% of RIF^R clinical isolates of *M. tuberculosis* have mutations in the 81-bp core region of *rpoB* that alter the primary structure of RpoB (Fig. 1). These mutations are absent in susceptible organisms. Although minor discrepancies have been reported, in general there is a strong correlation of specific amino acid substitutions and MIC. Missense mutations in codons 513, 526, or 531 result in high level RIF resistance, whereas amino acid changes at position 514 or 533 usually result in low level RIF resistance. Interestingly, Zhou & Jin[60] have recently shown that in *E. coli,* amino acid changes at positions 526, 531, 532, 533, and 563 destabilize initiation complexes at stringently controlled promoters. In essence, these RNA polymerase mutants behave like 'stringent' RNA polymerases in the absence of the stringent response in vivo. The molecular mechanism of resistance in 4% of the RIF^R tuberculosis isolates that lack RRDR changes is unknown. Although a recent report demonstrated that ribosylative inactivation of rifampin by *M. smegmatis* is a principal contributor to low-level resistance to this antibiotic, there is no evidence that this process participates in *M. tuberculosis* drug resistance.[61] The wealth of data available from study of precisely defined and characterized resistant mutants recovered from humans with TB, and susceptibilities to four related rifamycins, will be of additional use if the crystal structure of the RpoB subunit is solved.

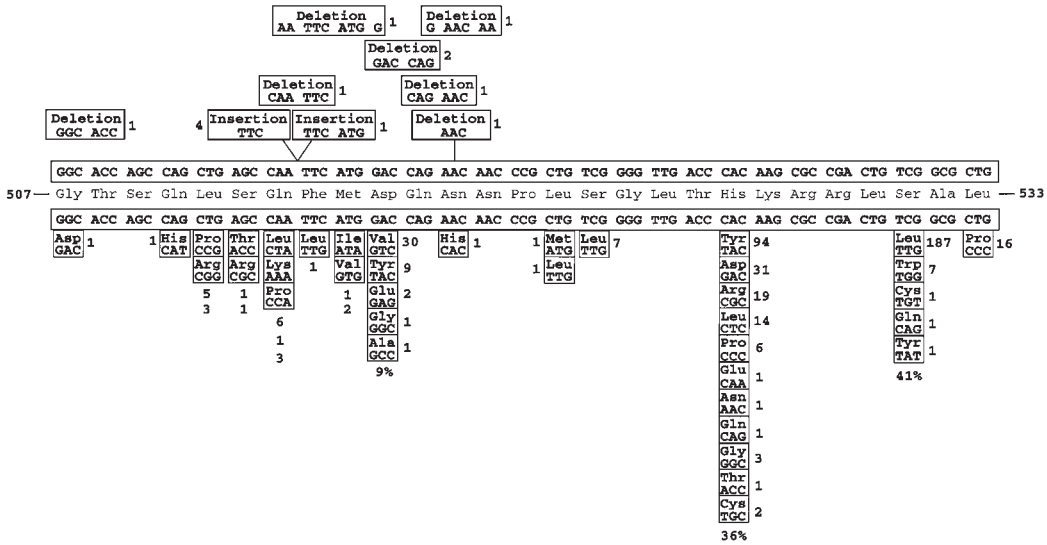

Fig. 1 Mutations located in codons 507 through 533 of the *M. tuberculosis rpoB* gene in 478 RIF^R isolates. The codon numbering system described previously[38] was used. The figure summarizes mutations reported elsewhere.[41–55,129] Only changes occurring in strains with missense mutations at a single codon or organisms with single insertion-deletion events are included. One RIF^R strain described previously[129] had a deletion of three nucleotides (CAT) that make up part of codons 514 and 515, resulting in Leu514 replacing Phe514-Met515. Mutant sequences with more than one point mutation or other combinations of mutations have been reported previously.[13] The codon numbers are designated on the basis of alignment of the translated *E. coli rpoB* sequence with the homologous part of the translated *M. tuberculosis* sequence and are not the positions of the actual *M. tuberculosis rpoB* codons. The figure is modified from one presented previously.[13]

ISONIAZID AND ETHIONAMIDE

INH is a synthetic, bactericidal agent that is used as a first-line drug against TB worldwide. Susceptible strains have an MIC of less than 0.05 μg/ml. Virtually all other mycobacteria and prokaryotes are INH resistant, and hence, this drug is used mainly to treat infections caused by *M. tuberculosis* complex members. Despite its widespread application in TB therapy and prophylaxis, and intensive laboratory investigation, there is much that is not yet understood about the bacterial targets and mode of action of INH.

katG and INH resistance

Decades ago, study of patient isolates of *M. tuberculosis* noted a correlation between INH resistance and loss of catalase-peroxidase activity.[62–65] This observation eventually led to the cloning and sequencing of the structural gene (*katG*) for the enzyme.[66,67] Molecular genetic studies confirmed that *katG* participated in mediating susceptibility to INH.[68] Moreover, *katG* was deleted from the chromosome of two strains with high level INH^R isolates (MIC >50 μg/ml).[66] However, many subsequent studies have shown that the vast majority of INH^R strains contain the *katG* gene, a result indicating that deletion of this gene is very rare among patient isolates.[69–82]

The observation that most INH^R *M. tuberculosis* strains did not have gross deletions in *katG* stimulated a more precise analysis of the structure of *katG* in resistant isolates. Investigators on several continents have reported that many (~50–60%) INH^R patient isolates have missense mutations, or small deletions or insertions that are not represented among INH^S control strains.[13]

Although a diverse array of distinct KatG changes are uniquely represented among INH^R organisms, amino acid substitutions located at position 315Ser are the most abundant (Fig. 2). For example, Musser et al.[74] studied 85 INH^R strains from global sources, and identified changes in amino acid 315 (Ser) in 49 (58%) epidemiologically unassociated isolates. Most organisms had a Ser315Thr amino acid replacement. Similarly, Haas et al.[75] reported that 68% of INH^R strains from Africa had codon 315 missense changes (usually Ser315Thr substitutions), and Dobner et al.[76] found that 26 of 27 INH^R isolates from Germany and Sierra Leone had codon 315 mutations. Marttila et al.[82] recently reported that 22 of 24 INH^R isolates from the St Petersburg area in Russia had Ser315Thr substitutions. Similar results were reported by Escalante et al.[50] on the basis of analysis of isolates from Peru. Statistically, the most common amino acid substitution is AGC (Ser)->ACC (Thr), but ACA (Thr), ATC (Ile), AGA (Arg), CGC (Arg), AAC (Asp), and GGC (Gly) changes also have been identified.[13,72–80]

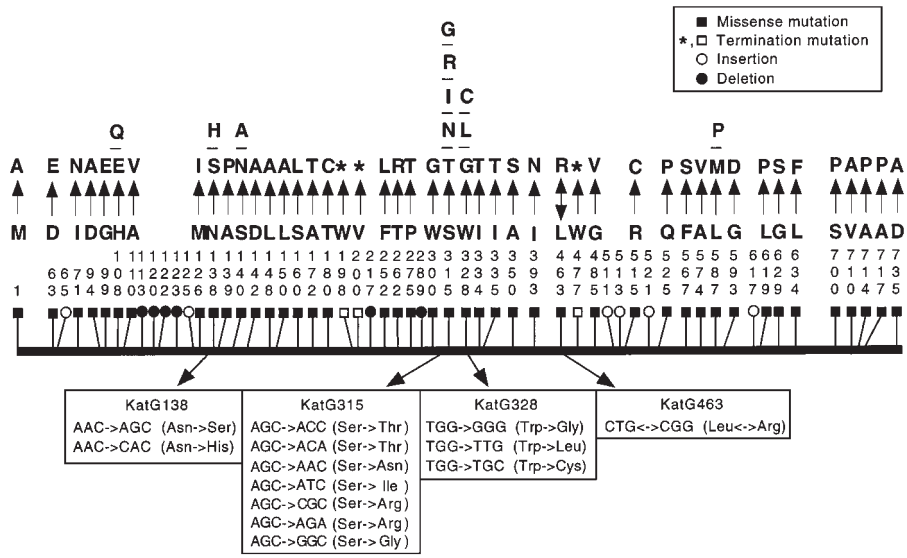

Fig. 2 Polymorphism in the KatG protein identified in INH^R *M. tuberculosis.* The data were compiled from mutations reported previously.[44,66–68,70–80] The variant amino acids are numbered vertically. The single-letter amino acid abbreviations are used. Shown below the schematic are nucleotide and amino acid changes occurring at codons with two or more variant codons. The KatG463 Leu<–>Arg substitution is a commonly-occurring natural polymorphism that is not associated with INH susceptibility levels judged to be clinically significant. A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; W: tryptophan; V: valine.

Three studies failed to identify amino acid substitutions in KatG 315Ser in high frequency. Pretorius et al.[77] did not find these changes in a sample of 39 strains recovered from South Africa, the USA, and Switzerland. However, this report was retracted soon thereafter when it was found that the PCR-SSCP strategy initially used to screen resistant strains failed to detect codon 315 polymorphisms present in 13 of 39 (33%) of the organisms.[78] Marttila et al.[79] examined 54 INH^R isolates recovered from patients in Finland and identified only four organisms with Ser315 substitutions. The reason for the relatively low frequency of occurrence of KatG315 substitutions in the Finnish data set is not clear. Rouse et al.[80] analyzed 26 INH^R organisms from South Korea and the USA and identified only one isolate with a KatG315 amino acid substitution. One possible explanation is that the sample included a large number of strains with low level INH MICs (1–2 μg/ml).

A plausible explanation for the abundance of KatG315 amino acid changes in most INH^R organisms can be inferred from data published recently. Wengenack et al.[83] studied the enzymatic properties of purified wild-type and the Ser315Thr mutant KatG proteins made in *E. coli.* Compared with the activities for wild-type KatG, the catalase activity of the Ser315Thr mutant was reduced 6-fold, whereas the peroxidase activity was only decreased < 2-fold. The mutant and wild-type proteins each had one heme group per subunit, a result indicating that the difference in enzymatic activity of the Ser315Thr mutant protein was not due to incomplete heme cofactor incorporation in the heterologous *E. coli* host. The wild-type KatG was more efficient than the mutant enzyme in ability to convert INH (prodrug) to isonicotinic acid (activated INH). Taken together, the results are consistent with the idea that the Ser315Thr mutant is a competent catalase-peroxidase that has reduced ability to metabolize INH. Hence, amino acid substitutions at position 315 appear to strike a balance between the need to maintain active catalase-peroxidase activity to detoxify host antibacterial radicals, and reduce conversion of prodrug to active INH, a process that would normally kill the bacterium.

The most commonly occuring polymorphism found in the *katG* gene is a CGG<->CTG change resulting in Arg<->Leu variation at amino acid position 463.[13,72,84,85] An initial analysis suggested that the KatG463 Leu substitution was causally involved in INH resistance.[72] However, a subsequent study demonstrated that most isolates with this amino acid had other KatG structural changes, a result suggesting that the KatG463Leu variant was merely a convenient surrogate marker for INH^R organisms.[74] Several studies suggest that this is the case. Strains with Arg463Leu substitutions have essentially wild-type levels of catalase-peroxidase activity.[72,86] Site-

directed mutagenesis demonstrated that the presence of Leu at codon 463 (rather than Arg) did not alter catalase-peroxidase activity.[87] The Arg463Leu substitution is present in many INH^S strains, especially organisms recovered in China, the former Soviet Union, and certain other areas of Asia.[84,85,88] Detailed kinetic and spectroscopic studies failed to identify differences in the properties of KatG463Arg and KatG463Leu proteins, moreover, no significant difference in catalase or peroxidase activity, or other enzymatic parameters between the two purified proteins has been found in vitro.[86] For example, both proteins oxidize INH to isonicotinic acid, and display the same dependence on INH concentration.

However, data derived from study of isogenic strains of bacille Calmette-Guérin (BCG) constructed to express either KatG463Arg or 463Leu were noteworthy because they suggested that subtle differences may exist between the two proteins.[87] Most notably, the MIC of BCG transformed with the 463Leu variant was slightly higher than BCG expressing the 463Arg protein (1.0 μg/ml vs 0.5 μg/ml). This result is consistent with the observation that *Mycobacterium bovis* strains, all of which naturally have KatG463Leu,[74,89] are slightly less susceptible to INH than most *M. tuberculosis* strict sense organisms. In addition, of the 14 low-level INH-resistant catalase-positive organisms reported by Rouse et al.,[87] nine had Leu at KatG codon 463, and none of these organisms had other mutations in *katG* or the *inhA* region.

Rouse et al.[87] used site-directed mutagenesis to alter the wild-type *katG* gene from *M. tuberculosis* at 13 codons previously shown to be mutated in INH^R clinical isolates. The effects on drug susceptibility of the resulting amino acid changes were determined with complementation assays in *katG*-defective, INH^R strains of *M. smegmatis* and *M. bovis* BCG. Importantly, nine of the 13 variant amino acids were shown to confer INH resistance, including Arg104Leu, His108Gln, Asn138Ser, Leu148Arg, His270Gln, Thr275Pro, Ser315Thr, Trp321Gly, and Asp381Gly. These results were consistent with modelling studies suggesting that residues 104 and 108 are located at or near the enzyme catalytic site, and residues 270, 275, and 315 participate in heme binding.[90,91]

inhA locus and INH resistance

ETH is a structural analog of INH that is used as a second-line drug against TB. ETH is thought to inhibit mycolic acid biosynthesis in *M. tuberculosis.*[92,93] Several studies have shown that for certain strains, low level INH resistance is correlated with co-acquisition of ETH resistance, suggesting that INH and ETH share a molecular target.[94–96]

Molecular genetic study of single-step spontaneous INH-ETH resistant mutants of *M. smegmatis* and *M. bovis* identified a two-gene operon with contiguous open reading frames designated *mabA* and *inhA,* coding for products that participated in resistance to both INH and ETH[95] (Fig. 3). The predicted protein product of *mabA* had greatest identity with 3-ketoacyl-acyl carrier protein (ACP) reductase coded for by *fabG* of *E. coli,* an enzyme involved in fatty acid biosynthesis.[96] The mycobacterial *inhA*-encoded enoyl reductase had 40% amino acid sequence identity with *E. coli envM*-encoded enoyl reductase. InhA from *M. tuberculosis* has been purified, crystallized, and shown to be an NADH-dependent enoyl-ACP (acyl carrier protein) reductase with specificity for long-chain enoyl thioester substrates.[96–98] The sum of the evidence suggests that MabA and InhA participate in mycolic acid biosynthesis.

The identification of a Ser94Ala substitution in InhA conferring resistance to INH and ETH in the laboratory led to the hypothesis that missense mutations in *inhA* would occur in resistant *M. tuberculosis* strains recovered from patients. DNA sequence analysis of the *inhA* locus in

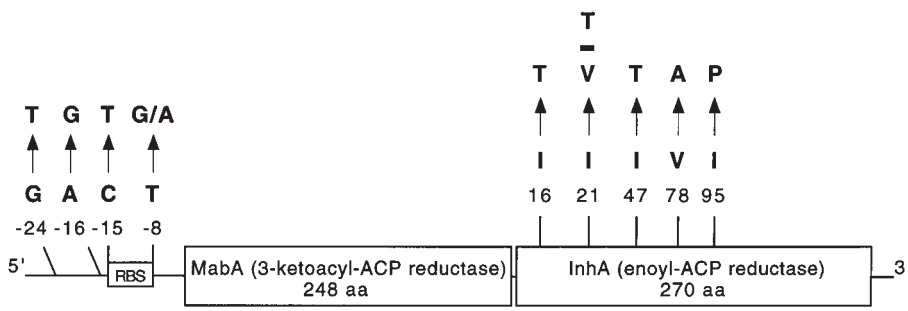

Fig. 3 Schematic representation of mutations identified in the *inhA* locus in INH^R and/or ETH^R isolates of *M. tuberculosis.* The *inhA* locus is composed of two contiguous open reading frames designated *mabA* (encoding 3-ketoacyl-acyl carrier protein reductase) and *inhA* (encoding enoyl-acyl carrier protein reductase). In *M. tuberculosis* complex organisms, *mabA* and *inhA* are separated by a 21-bp noncoding region that lacks a readily identifiable promoter. RBS: ribosome binding site. The single-letter amino acid designations used are A: alanine; I: isoleucine; P: proline; T: threonine; and V: valine.

115 INH^R clinical isolates identified an Ile16Thr substitution in InhA, but no mutations in *mabA*.[46,74] Interestingly, the Ser94Ala amino acid replacement was not found in the patient isolates. However, 15 isolates had mutations in the upstream putative regulatory region of the *inhA* locus. Study of INH^R clinical isolates from the Netherlands showed that 11 of 51 strains had a C–>T substitution flanking the 5′ side of the presumed ribosome binding site upstream of *mabA*.[74] These strains lacked *katG* codon 315 mutations.

On the basis of identification of mutations in the upstream region of *inhA* locus, it was hypothesized that these changes result in increased InhA protein expression, thereby elevating the drug target levels and producing INH resistance via a titration mechanism. To test this hypothesis, a multicopy plasmid containing the *inhA* gene was transformed into *M. smegmatis* and *M. tuberculosis* strains.[99] High-level INH resistance was conferred only to *M. smegmatis*. The promoter region containing mutations at positions thought to be located at –8 and –15 upstream from translational start site of *mabA* were cloned into an integrating, single copy vector and then transformed into *M. smegmatis* and *M. tuberculosis*.[99] The results indicated that the mutations had no measurable effect on INH resistance in *M. smegmatis* but had a modest effect on *M. tuberculosis*. Comparison of the lipid biosynthetic responses to INH treatment revealed that the lipid profiles were different between *M. smegmatis* and *M. tuberculosis*. The *M. smegmatis* profiles were consistent with an inhibition of short-chain fatty acid biosynthesis by INH, presumably due to inhibition of InhA. In contrast, the *M. tuberculosis* lipid profiles suggested that INH directly inhibits desaturation of tetra or hexacosanoic acids. On the basis of these results, it was suggested that InhA is not the major target for activated INH in *M. tuberculosis*. However, studies with *M. smegmatis* and results obtained with *Mycobacterium aurum* support the idea that InhA is a target for KatG activated INH.[100]

Recently, six low-level INH^R *M. tuberculosis* isolates were identified that had missense mutations in the *inhA* structural gene, resulting in Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala, and Ile95Pro replacements.[101] INH^S organisms lacked these variant amino acids. Strains containing the first five amino acid substitutions did not have *katG* mutations that would confer INH resistance; *katG* in the fifth organism was not characterized. Several of the mutant enoyl reductases were expressed and purified, and their steady-state kinetic parameters and NADH binding properties were characterized. The steady state K_m values for fatty acyl-coA substrate were not altered significantly compared to wild-type enzyme. In striking contrast, all amino acid replacements caused a 2–12-fold increase in K_m for NADH (Table 1). These results were similar to the previous observation that the Ser94Ala substitution produced a five-fold increase in K_m for NADH binding.[96] Furthermore, all mutant enzymes had an increased K_d for NADH. On the basis of crystal structure analysis, all amino acid substitutions would be located in the NADH binding site (Fig. 4). Taken together, the data are consistent with the idea that INH resistance in these strains is related to the reduced NADH binding affinity for enoyl reductase.[101]

Several recent papers have described the detailed in vitro chemical interactions between INH and mycobacterial proteins.[102–107] INH activated by catalase-peroxidase binds exclusively to enoyl-reductase-NADH complex in the presence of manganese and oxygen.[104,106] The three dimensional structure for the ternary complex of wild-type enoyl reductase of *M. tuberculosis* with NADH and activated INH has been determined.[107] The acylpyridine fragment of INH is found to be covalently attached to the C4 position of NADH. It is believed that a similar adduct between the electrophilic, activated species of INH produced by KatG and the enzyme-NADH complex would form a ternary complex in vivo, resulting in the inactivation of enoyl reductase followed by inhibition of mycolic acid biosynthesis.

In summary, amino acid replacements in the NADH binding site of InhA apparently result in INH resistance by preventing the inhibition of mycolic acid biosynthesis. No mutations have been identified in *mabA*. Mutations in the upstream region of the *mabA-inhA* operon are thought to increase the target levels, thereby causing resistance by a drug titration mechanism. Mutations in *katG* or *inhA* do not account for all INH^R strains since 15–25% of INH^R clinical isolates have both wild-type *katG* and *inhA* genes. The identification of additional targets for activated INH is an important future endeavour.

Inhibition of a β-ketoacyl ACP synthase (KasA) by INH

The observation that mutations in *katG* and the *inhA*

Table 1 Steady-state kinetic parameters of *M. tuberculosis* wild-type and mutants of InhA

Parameter	WT	I16T	I21V	I47T	I95P
V_{max} (U/mg)	33 ± 1	39 ± 2	25 ± 1	23 ± 1	0.39 ± 0.04
NADH K_m(μM)	56 ± 4	149 ± 10	104 ± 7	243 ± 25	662 ± 11
trans-Δ^2-$C_{12:1}$-CoA K_m (μM)	75 ± 5	85 ± 8	102 ± 13	42 ± 4	141 ± 29

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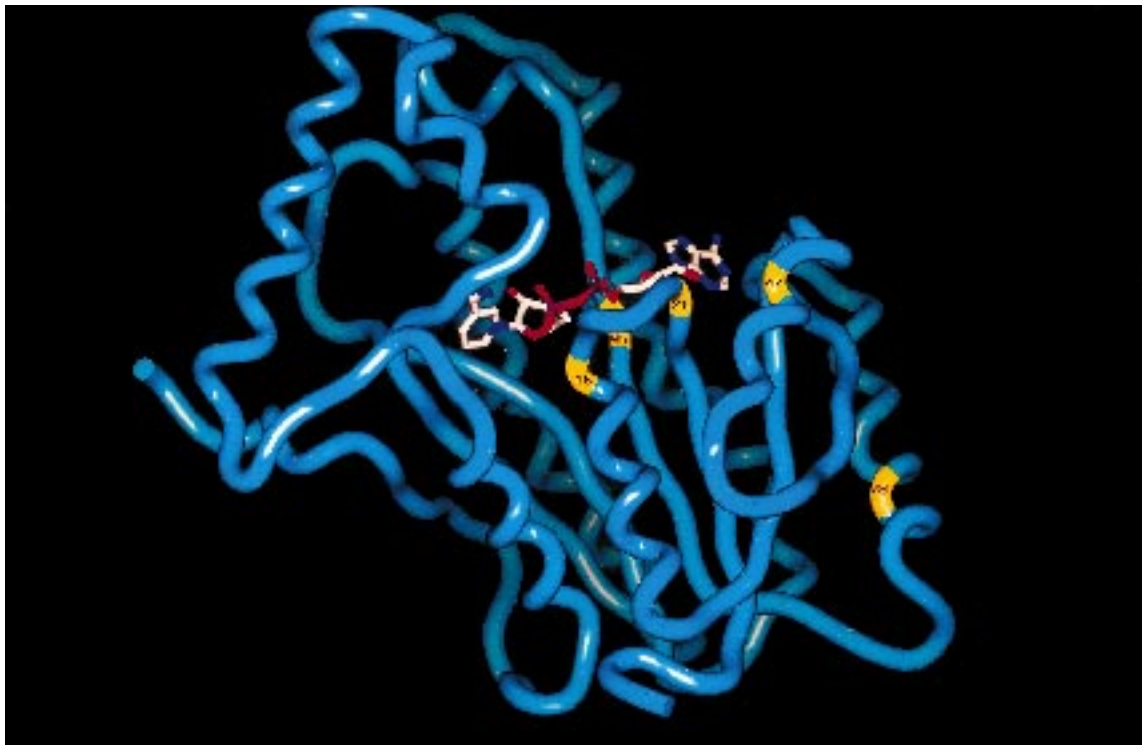

Fig. 4 Schematic drawing of InhA crystal structure showing amino acid replacements found in isoniazid-resistant *M. tuberculosis* patient isolates. The amino acid positions marked have been identified in INH-resistant, but not INH-suspectible organisms. One NADH molecule is shown in the NADH binding fold. Adapted from ref. 97.

locus do not account for INH^R in all patient isolates in part has stimulated additional investigations designed to identify targets of this drug. Recently, Mdluli et al.[108] reported that in response to INH treatment, a saturated hexacosanoic acid (C26:0) accumulated on a 12 kDa acyl carrier protein referred to as AcpM. Interestingly, a protein with the same aminoterminus as AcpM, but with an apparent molecular mass of 80 kDa, was upregulated upon INH treatment and labeled with [^{14}C]-INH. Characterization of the 80 kDa molecule from INH-treated *M. tuberculosis* showed that it consisted of a covalent complex of INH, AcpM, and a β-ketoacyl ACP synthase termed KasA. Automated DNA sequence analysis discovered that four amino acid-altering mutations were present in *kasA* in recent INH-resistant patient isolates (Fig. 5). Susceptible strains lacked mutations in this gene. Importantly, two of the resistant isolates lacked mutations in *katG* and the *inhA* locus. The crystal structure of the *E. coli* homologue of KasA has recently been reported.[109] Three of the four *M. tuberculosis* KasA amino acid changes mapped to the active site of the *E. coli* molecule. The fourth amino acid substitution was located at the carboxyterminus of KasA and may alter protein-protein interactions. Taken together, the data suggest that KasA is a new INH target

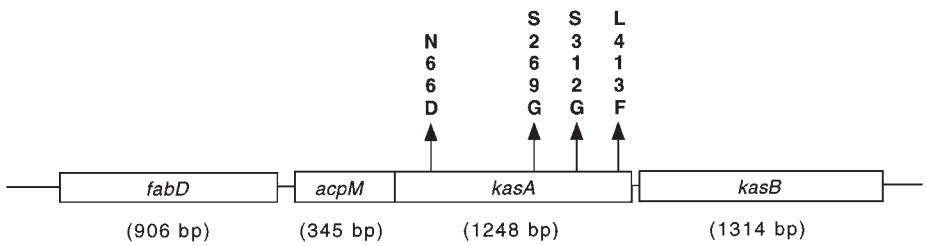

Fig. 5 Kas genomic locus from *M. tuberculosis. fabD* encodes a presumed malonyl CoA-acyl carrier protein (ACP) transacylase; *acpM* encodes AcpM, an acyl carrier protein; *kasA* encodes a presumed β-ketoacyl-ACP synthase; *kasB* also encodes a presumed β-ketoacyl-ACP synthase. See ref. 108 for additional details.

useful for the development of new therapeutics and the identification of drug-resistant strains.

oxyR, *ahpC*, and INH resistance

In *E. coli* and *Salmonella typhimurium, katG* is part of an oxidative stress regulon induced by *oxyR* in response to the detrimental effects of H_2O_2.[110–112] The *oxyR* gene controls expression of *katG* and several other genes including *ahpC,* encoding the small subunit of alkylhydroperoxide reductase.[113,114] Mutations in either *oxyR* or *ahpC* in *E. coli* confer INH susceptibility to this otherwise INH-insensitive bacterium.[115]

To determine if mutations in *oxyR* or *ahpC* participate in INH resistance in *M. tuberculosis,* these genes were recovered from a genomic library made from strain H37Rv.[116,117] *oxyR* and *ahpC* were discovered to be tightly linked and divergently transcribed,[116–119] attributes typical of the LysR family of transcriptional regulators in enteric organisms.[120] Surprisingly, the *oxyR* gene was naturally inactivated by accumulation of multiple genetic lesions including several frameshift mutations, point mutations, and deletions.[116,117] These alterations were also present in other members of the *M. tuberculosis* complex; however, an intact *oxyR* gene was located 109 bp upstream of *ahpC* in *Mycobacterium leprae.* Transformation of the *oxyR-ahpC* region of *M. leprae* into *M. tuberculosis* resulted in strain H37Rv acquiring resistance to 5 μg/ml of INH. These results were interpreted to mean that the natural *oxyR* lesions found in *M. tuberculosis* are at least partially responsible for the exquisite sensitivity of *M. tuberculosis* to INH.[116,117,121–123]

A corollary of the hypothesis that susceptibility of *M. tuberculosis* to INH is in part due to loss of OxyR function is that mutations that result in enhanced expression of other genes whose products are involved in the INH pathway might compensate for the loss of *oxyR.* Consistent with this idea, mutations in the *ahpC* promoter sequences were identified in a small number of INH^R organisms deficient in KatG activity, but were not present in INH^S bacteria.[124] Reporter gene constructs using *lacZ* combined with western immunoblot analysis showed that several of the *ahpC* mutant alleles had enhanced transcriptional activity. Transformation of a multicopy plasmid containing the wild-type *ahpC* gene into *M. tuberculosis* H37Rv resulted in increased resistance to cumene hydroperoxide, an observation suggesting that increased AhpC activity could compensate for the loss of KatG activity in the detoxification of organic peroxides[124] (Fig. *6*). Hence, the weight of the genetic and biochemical evidence indicated that the *ahpC* mutations represented compensatory alterations occuring as a consequence of loss of catalase-peroxidase activity. These observations raised the possibility that upregulation of AhpC expression may participate in INH resistance in *M. tuberculosis* strains lacking mutations in *katG* or *inhA* locus.

Because all studies cited above used laboratory generated INH^R strains or very few clinical isolates, it was important to determine the frequency with which *oxyR-ahpC* mutations were present in a large group of epidemiologically independent human TB isolates. Sequence analysis of 229 *M. tuberculosis* isolates from intercontinental sources characterized previously for polymorphisms in *katG* and the *inhA* locus was conducted.[125] The entire 1221-bp region was studied in 118 isolates, and 111 other isolates were sequenced for *oxyR, ahpC,* or the 105-bp *oxyR-ahpC* intergenic region. Only nine mutations were identified in the 105 bp *oxyR-ahpC* intergenic region of INH^R strains, indicating that these mutations were relatively rare among a broad spectrum of INH^R strains. Importantly, most INH^R strains with *katG* codon 315 sub-

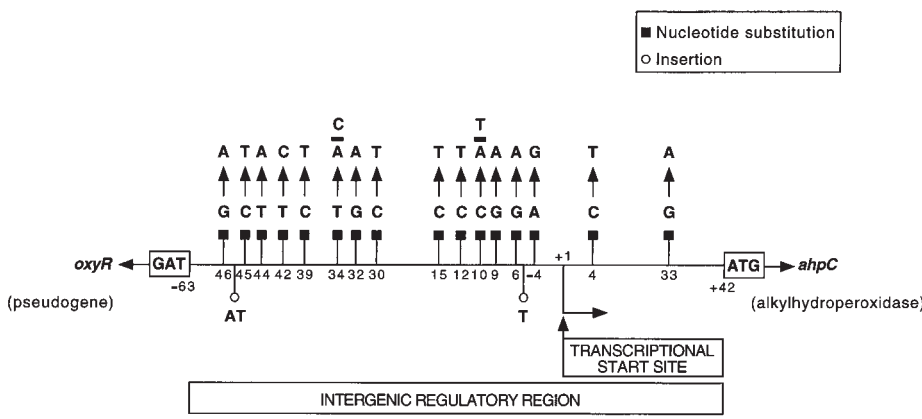

Fig. 6 Schematic of the *M. tuberculosis oxyR-ahpC* intergenic region showing nucleotide changes identified in INH^R and INH^S isolates. The data are a compilation of mutations described previously.[121,124–126,128,240] Numbering is based on nucleotide position relative to the mRNA start site.[119]

stitutions that significantly decrease catalase activity and confer high-level MICs for INH, lacked alterations in *ahpC* or *oxyR-ahpC* intergenic region. The relative paucity of *ahpC* compensatory mutations was attributed to rare occurrence of a *KatG* null phenotype among epidemiologically independent INH^R strains. Taken together, the data are consistent with the hypothesis that some polymorphisms located in the *oxyR-ahpC* intergenic region are selected due to reduction in catalase or peroxidase activity attributable to *katG* changes arising with INH therapy. The analysis also found two mutations at codons 73 (GAC->CAC, Asp->His) and 191 (CTC->CGC, Leu->Arg) in INH^R isolates, although the exact relationship of these amino acid changes to INH resistance is unknown. There was no preferential association of polymorphisms in *oxyR* with INH^R organisms, the expected result given that *oxyR* is a pseudogene.[116,117] An interesting offshoot of the analysis of *ahpC-oxyR* polymorphism was the identification of a point mutation in *oxyR* that specifically differentiates *M. bovis* from other members of the *M. tuberculosis* complex.[126]

Essentially identical results were recently reported on the basis of analysis of 57 INH-resistant clinical strains of *M. tuberculosis* from patients in Korea, Brazil, Mexico, China, and the USA.[127] All strains had a wild-type *inhA* gene as assessed by PCR-SSCP. No *ahpC* promoter or structural mutations were identified in 25 catalase-positive, isoniazid-resistant isolates, or 25 INH^S organisms. Eight catalase-negative strains highly resistant to INH (MICs ranging from 10 to 125 μg/ml) had alterations in both *katG* and *ahpC* regulatory sequences. Three strains had *katG* deletions, one strain had a nonsense mutation, one strain had a single base pair insertion, and three strains had missense mutations. The promoter mutations were located at -6, -9, -10, -12, -30, and -42 relative to the transcriptional start site; with the exception of the -42 change (T->C), AhpC overproduction was documented by immunoblot analysis. The commonly occurring KatG Ser315Thr substitution was not associated with *ahpC* upstream mutations, a result that confirmed previous findings.[125]

In an attempt to determine the frequency distribution of mutations in targeted areas of *katG, inhA,* and *ahpC* among INH^R strains, Telenti et al.[128] analyzed 95 *M. tuberculosis* isolates from Spain by PCR-SSCP. Susceptibility testing was done by the proportional method. The mutation frequencies were as follows for INH^R strains – *katG* (36.8%), *inhA* (31.6%), *katG-inhA* (2.6%), *ahpC* (13.2%) and *katG-ahpC* (2.6%). All five distinct mutations detected in the *ahpC* promoter region had been previously reported by other investigators. Interestingly, the frequency of *inhA* regulatory region mutations was considerably higher than reported by other investigators. Although the exact reason is unclear, we note that strains were classified as a distinct genotype on the basis of a rep-PCR method that, unlike the IS*6110* profiling strategy, has not yet been validated in large studies. Hence, there is a formal possibility that some redundant sampling of epidemiologically related strains was a factor.

Notwithstanding a report[125] that the *M. tuberculosis ahpC* gene can confer low-level INH resistance on *M. smegmatis,* most investigators do not currently favor the idea that AhpC has a direct role in mediating INH resistance in human isolates of *M. tuberculosis.* Rather, the bulk of the evidence suggests that *ahpC* changes are selected as a consequence of acquisition of a null phenotype for catalase-peroxidase activity.

FurA

Song et al.[130] have discovered two transcriptional start sites for the *katG* gene in *M. tuberculosis.* One of the transcriptional start sites is located 471 bp upstream of the *katG* initiation codon. Analysis of two *M. tuberculosis* strains revealed that this upstream region contains an open reading frame (*furA*) encoding a homolog of Fur, a ferric uptake regulator found in enteric bacteria such as *E. coli* and *S. typhimurium.* Although the exact function of *furA* is not known, the hypothesis was put forth that FurA is a regulatory factor, possibly responding to fluctuations in the availability of transition metals such as iron. Recent comparative sequence analysis of *furA* has identified mutations in INH^R *M. tuberculosis* organisms that are lacking in INH^S strains. The relationship between these mutations and INH resistance is being examined.

In summary, mutations in a relatively large number of genes have been associated with isoniazid resistance. Evidence implicates mutations in *katG, inhA,* and *kasA* as causally involved in mediating resistance to this critical drug. *oxyR* in *M. tuberculosis* is a pseudogene. Mutations in the promoter region of *ahpC* occur in many or most catalase-negative, INH^R isolates. These mutations appear to be compensatory and are presumably selected after reduction in catalase-peroxidase activity attributable to *katG* changes arising with INH therapy. However, this sequence of events has not yet been documented to occur in humans. AhpC itself does not appear to directly mediate INH resistance. The mechanism of INH resistance in some strains remains to be determined.

STREPTOMYCIN

STR is an aminocyclitol glycoside antibiotic that is one of the first line drugs used to treat TB. In *E. coli* STR binds to 16S rRNA, inhibits translational initiation, and detrimentally affects translation fidelity.[131,132] Mutations associated with STR resistance in *M. tuberculosis* have been identified in the 16S rRNA gene (*rrs*) and *rpsL* gene encoding

ribosomal protein S12.[133–140] In contrast to other bacteria which have multiple copies of rRNA genes, *M. tuberculosis* complex members have only one copy.[141,142] Hence, single nucleotide changes can potentially produce antibiotic resistance. Point mutations in *rrs* are clustered in two regions around nucleotides 530 and 915. The 530 loop of 16S rRNA is highly conserved and is located adjacent to the 915 region in secondary structure models. Several nucleotides in both regions interact with the ribosomal S12 protein.[143] In the 530 loop, C->T transitions are observed at positions 491, 512, and 516, and transversions (A -> C or T) have been reported at position 513. In the 915 region, C->A or G alterations at position 903 and A->G changes at position 904 are common. These observations are consistent with footprinting experiments done in *E. coli* that show STR interacts directly with the nucleotide sites in the 530 loop and 915 regions of 16S rRNA and that mutations at these sites result in STR^R *E. coli*.[131,132]

The majority of point mutations producing STR resistance occur in *rpsL*. The most common mutation is an AAG->AGG change in codon 43 resulting in a Lys->Arg substitution; less frequently, an AAG->ACG (Lys->Thr) substitution is observed.[13,134–140] Mutations also occur in codon 88, and these result in Lys->Arg (AAG->AGG) or Lys->Gln (AAG->CAG) amino acid replacements. The analogous mutations have been recorded in STR^R strains in several bacterial species, presumably because the S12 protein is highly conserved.

Sreevatsan et al.[138] determined the frequency distribution of *rpsL* and *rrs* mutations in 78 STR^R and 61 STR^S isolates recovered from patients worldwide. *rrs* mutations were found in the 915 or 530 loop regions in 8% of the resistant isolates. Three previously undescribed mutations were identified in the 915 region of *rrs*, an area encoding a STR binding site in 16S rRNA. The nucleotide positions were 798 (C->T), 877 (G->A), and 906 (A->C). In addition, 54% of the STR^R isolates had missense mutations in codons 43 or 88 of *rpsL* gene. Two different isolates each had two unique combinations of mutations. One isolate from Vietnam had Lys88Arg and Val93Met substitutions and an isolate from Hong Kong had Arg9His and Lys43Arg changes. No mutations were detected in *rpsL* or the 530 loop or 915 regions of *rrs* in 25–35% of STR^R isolates, indicating the existence of additional mechanisms mediating STR resistance.

To gain insight into the relationship of RpsL and *rrs* changes and STR MIC levels, Cooksey et al.[139] analyzed 45 STR^R isolates for phenotypic resistance and mutations. Antimicrobial susceptibility testing was performed by the agar proportion method. Twenty-four isolates had high level resistance (MIC >500 μg/ml) and 20 isolates had low level resistance (MIC, 10 μg/ml). All 24 high level resistance mutants had a Lys43Arg change in RpsL. Of the low level STR^R mutants, only one isolate had a C->G change at position 903 in the 915 region of *rrs* gene. No mutations were detected in the 530 loop of the *rrs* gene, indicating the existence of alternative mechanisms for 19 of 45 (42%) low level STR^R isolates. The investigators suggested that cell permeability changes, production of aminoglycoside-modifying enzymes, or alterations in other ribosomal molecules may contribute to low level drug resistance in *M. tuberculosis* strains lacking *rpsL* and *rrs* changes.

Meier et al.[140] also studied the association of particular RpsL amino acid replacements and *rrs* mutations with STR MIC level. MIC was determined by agar diffusion method and resistance was defined as MIC of >2 μg/ml. Of the 19 STR^R isolates, four had Lys43Arg substitutions and another four isolates had Lys88Arg changes in RpsL. Six isolates had changes in the 530 loop region of 16S rRNA and the remaining five (26%) isolates had wild-type *rpsL* and *rrs* genes. Amino acid changes in RpsL correlated with high level resistance (MIC > 500 μg/ml) and mutations in *rrs* correlated with intermediate level of resistance (MIC < 250 μg/ml). Low level resistance (MIC < 50 μg/ml) was also identified in strains that lacked mutations in the regions of *rpsL* and *rrs* studied. Interestingly, the susceptibilities of STR^R and STR^S isolates were investigated in the presence or absence of Tween, a membrane-active agent capable of reducing membrane barriers. The MICs for STR^S and mutants with high and intermediate levels of resistance were not affected when Tween was included. However, the MICs of low level STR^R mutants decreased significantly by approximately 10 fold suggesting a permeability barrier may contribute to resistance.

In summary, about 65–75% of STR^R isolates have resistance-associated changes in RpsL or *rrs*. Failure to identify resistance-associated variation in these genes in 25–35% of organisms indicates that other molecular mechanisms of STR resistance exist. The hypothesis that low level resistance is associated with permeability barrier requires further evaluation. Although resistance to aminoglycoside antibiotics in some bacteria is due to drug inactivation via acetylation,[144] this mechanism has not been described in *M. tuberculosis*.

PYRAZINAMIDE

PZA is a structural analog of nicotinamide that is used as a first-line TB drug.[145,146] PZA kills semi-dormant tubercle bacilli under acidic conditions.[147,148] It is hypothesized that in the acidic environment of phagolysosomes, the tubercle bacilli produce pyrazinamidase, an enzyme which converts PZA to pyrazinoic acid, the active derivative.[149] Consistent with this hypothesis, PZA^R *M. tuberculosis* isolates lose pyrazinamidase activity.[150,151]

To define the molecular mechanism of PZA resistance, Scorpio et al.[152] cloned and characterized the *M. tuber-*

culosis gene (*pncA*) encoding pyrazinamidase. Sequence analysis revealed that *pncA* encodes a protein with 186 amino acids that is 35.5% identical to *E. coli* nicotinamidase (pyrazinamidase).[153] DNA sequencing of four PZA^R clinical isolates identified missense mutations in codons 63, 138, and 141 in three of the organisms, and deletion of nucleotide 162 in the fourth isolate, a change resulting in the production of a truncated polypeptide.[152] All four strains lacked pyrazinamidase activity and had MICs >500 μg/ml. In contrast, susceptible organisms had the identical wild type sequence. Transformation of the wild type *pncA* gene into a PZA^R mutant derived from H37Rv restored pyrazinamidase activity and PZA susceptibility. The results provided strong molecular genetic and biochemical evidence that *pncA* mutations conferred PZA resistance.

To gain additional insight into the role of *pncA* mutations in PZA resistance, Sreevatsan et al.[154] sequenced this gene in 67 PZA^R and 51 PZA^S *M. tuberculosis* clinical isolates from diverse geographical localities, and with a wide diversity of IS*6110* subtype. PZA susceptibility testing was done by BACTEC or the proportion method. All PZA^S isolates had the identical *pncA* allele, whereas 72% of PZA^R isolates had *pncA* mutations. A total of 17 previously undescribed mutations, including missense changes, upstream presumed regulatory mutations, nucleotide insertions and deletions, and termination mutations were found (Fig. 7). Interestingly, approximately 40% of the distinct amino acid substitutions involved replacement with a proline residue. Although the significance of this observation is not clear, we note that if these changes occurred in alpha-helical regions, protein structure might be altered, thereby detrimentally affecting pyrazinamidase activity. Lack of *pncA* mutations in 19 of 67 (28%) PZA^R isolates suggested the existence of at least one additional gene participating in PZA resistance.

Similar results were reported by Scorpio et al.[155] who sequenced *pncA* in 34 PZA^R clinical isolates and found that 33 of the organisms had 26 distinct mutations, most of which were previously undescribed. Nucleotide substitutions, insertions, and deletions were identified, and these sequence alterations were dispersed throughout the *pncA* gene. In addition, in vitro generated resistant mutants lost pyrazinamidase activity, and had high level drug resistance (MIC >900 μg/ml). Sequence analysis of 14 laboratory generated resistant strains identified nucleotide substitutions, insertions, and deletions in eight organisms, results indicating that many of these mutants had the same mechanism of resistance as PZA^R clinical isolates. Recently, Hirano et al.[156] reported that 32 of 33 PZA^R strains cultured from patients in nine countries had *pncA* mutations, whereas PZA^S strains lacked changes in this gene.

Hence, a remarkably wide array of *pncA* mutations resulting in structural changes in PncA have been identified in greater than 70% of drug resistant clinical isolates.

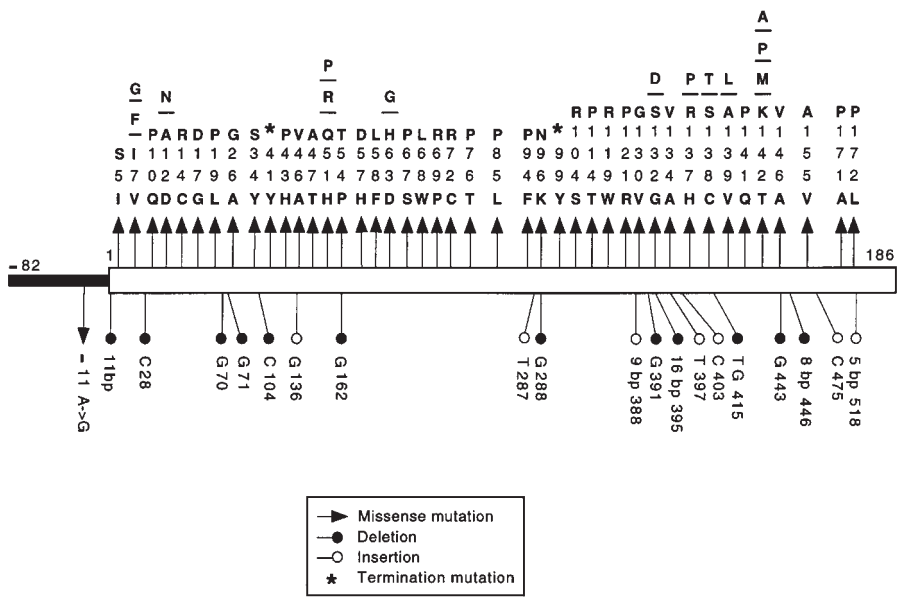

Fig. 7 Schematic representation of polymorphism in *pncA* in PZA^R *M. tuberculosis.* Mutations reported previously[152,154–156] are included. Variant amino acids are numbered vertically. The single-letter amino acid abbreviations are used. A: alanine; C: cysteine; D: aspartic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine, W: tryptophan; Y: tyrosine.

It is presumed that these structural changes detrimentally change enzyme function, thereby altering conversion of PZA to its bioactive form. One hypothesis to account for the extreme diversity in resistance-associated mutations is that because the enzyme consists of only 186 amino acids, any amino acid substitution may be likely to detrimentally alter function. Susceptible organisms lack *pncA* changes. The details of the molecular mechanism of PZA-pyrazinamidase interaction, and pyrazinoic acid formation are unknown. Studies designed to delineate pyrazinamidase structure-function relationships are clearly needed to assist understanding of the mechanism of action of PZA, and resistance to this drug.

ETHAMBUTOL

EMB ([S,S']-2,2'-[ethylenediimino])di-1-butanol is a bactericidal first-line drug used to treat TB. Early studies reported that EMB inhibited incorporation of mycolic acids into the cell wall.[157,158] Takayama & Kilburn[158] showed that EMB inhibited the transfer of arabinogalactan into the cell wall of *M. smegmatis,* a process that led to the accumulation of mycolic acids and their cognate trehalose esters. The identification of β-D-arabinofuranosyl-1-monophosphodecaprenol as the major intermediate in the biosynthesis of arabinogalactan, and the rapid accumulation of this intermediate following EMB administration suggested that the EMB target was an arabinosyl transferase.[159] Only the dextro isomer of EMB is biologically active, an observation consistent with the idea that the drug binds to a specific cellular target.[160] Inasmuch as EMB has been proposed to be an arabinose analog, the specific target is likely to be an arabinosyl transferase, presumably a functionally-important site.

To gain insight into the molecular genetics of arabinan biosynthesis, Belanger et al.[161] identified a two gene locus (*embAB*) in *Mycobacterium avium* that encodes arabinosyl transferases mediating polymerization of arabinose into arabinogalactan. Working in parallel, Telenti et al.[162] cloned and sequenced genes encoding the putative EMB target in *M. smegmatis.* Three genes organized as an operon were identified and designated *embCAB.* The corresponding *M. tuberculosis embCAB* genes were then identified from an ordered cosmid library. Sequence analysis and genetic studies revealed that the three genes were organized as a 10 kbp operon. A collection of 72 strains of *M. tuberculosis* including 28 EMB^R isolates was examined for mutations in the *embCAB* operon by automated DNA sequencing and SSCP analysis. Among epidemiologically unrelated EMB^R isolates, 13 of 28 (47%) had mutations in codon 306 of *embB* that would produce Met->Ile or Met->Val substitutions (Fig. 8). These mutations were not present in 44 EMB^S strains. In addition, analysis of *M. tuberculosis* isolates cultured from a single patient before and after development of EMB resistance identified a Met306Val substitution in the resistant, but not in the susceptible organism. Mutations located at a site corresponding to codon 306 were also found in EMB^R *M. smegmatis* strains. Furthermore, gene transfer experiments in *M. smegmatis* showed that mutations in *embB* conferred EMB resistance. Secondary structure analysis suggested that EmbCAB are integral membrane proteins with 12 transmembrane domains. It was postulated that the EMB-resistance determining region (ERDR) in EmbB is located in a cytoplasmic loop that is well conserved among different mycobacterial Emb proteins.

Sreevatsan et al.[163] investigated the association of EMB resistance, MICs, and sequence variation in the 10 kbp *embCAB* operon of *M. tuberculosis.* Automated DNA sequencing and SSCP analysis of 19 *M. tuberculosis* strains from diverse localities showed virtually identical sequences for the entire 10 kbp region. However, eight of 16 (50%) EMB^R isolates had mutations producing Met306Ile or Met306Val changes. Automated sequencing of the ERDR region in an additional 69 EMB^R and 30 EMB^S isolates from diverse geographical localities, and representing 70 distinct IS*6110* fingerprints, discovered that 69% of EMB^R isolates had an amino acid substitution in EmbB that was not found in EMB^S strains. The great majority (89%) of strains had a missense mutation in codon 306; moreover, missense mutations were also identified in three additional codons: Phe285Leu, Phe330Val, and Thr630Ile. These mutations were also uniquely represented among EMB^R organisms. For strains with the Met306Leu, Met306Val, Phe330Val, and Thr630Ile replacements, EMB MICs were generally higher (≥ 40 μg/ml) than those for organisms with Met306Ile substitutions (20 μg/ml). The data are consistent with the idea that specific amino acid substitutions in EmbB detrimentally affect the interaction between EMB, a putative arabinose analog, and EmbB, likely to be an arabinosyltransferase. Taken together, many of the amino acid substitutions in EmbB undoubtedly mediate EMB resistance, rather than act simply as surrogate markers for drug-resistant organisms.

To address the potential significance of *embB* mutations and EMB resistance in non-tuberculous mycobacteria, Alcaide et al.[164] characterized the *embB* ERDR region in a panel of strains representing 13 species. High level natural resistance to EMB (MIC ≥ 64 μg/ml) was associated with a variant amino acid motif in the ERDR of *M. abscessus, M. chelonae,* and *M. leprae.* Transfer of the *M. abscessus embB* allele to *M. smegmatis* resulted in a 500-fold increase in MIC, a result supporting the notion that ERDR determines intrinsic and acquired resistance to EMB in some species. This result has been confirmed by Lety et al.[165] in studies with *M. smegmatis.*

To summarize, *embB* mutations are associated with EMB resistance in roughly 70% of EMB^R isolates of *M.*

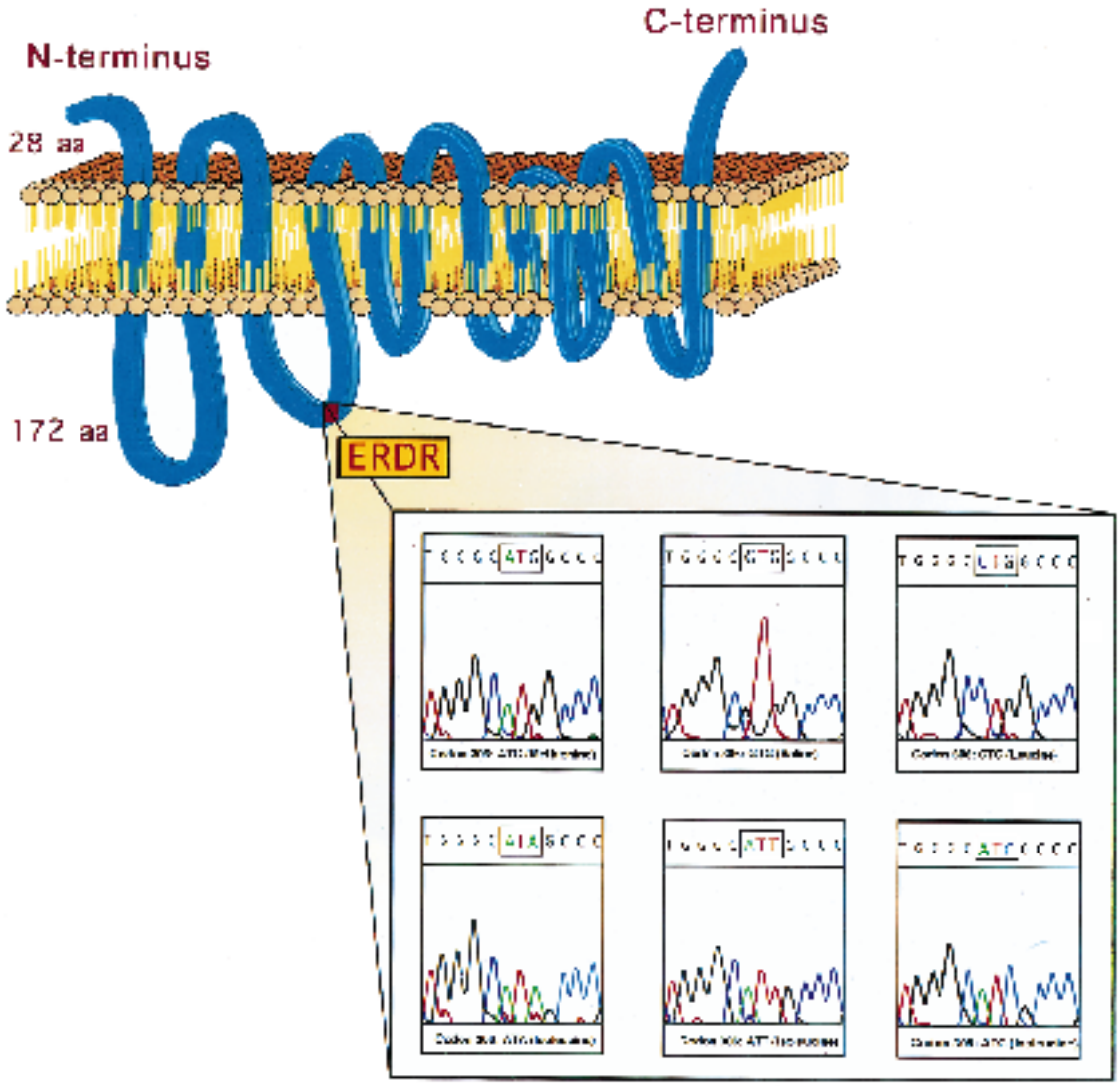

Fig. 8 Schematic representation of polymorphism in *embB* codon 306 in EMB^R *M. tuberculosis.* The DNA sequencing chromatograms show the wild-type and five mutants of *embB* codon 306. The wild-type codon in EMB^S isolates is ATG (Met), and the five mutant codons found in EMB^R strains are GTG, Val; CTG, Leu; ATA, Ile; ATT, Ile; and ATC, Ile. EmbB is presumed to be a transmembrane protein on the basis of computer modeling. The figure is highly schematic. The exact location of amino acid 306 relative to the membrane is unknown.

tuberculosis.[162,163] It is likely that the molecular basis of EMB resistance involves altered interaction of the drug with one or more products of the EmbCAB operon. The cause of EMB resistance in many organisms lacking mutations in the ERDR of EmbB is unknown. It is reasonable to anticipate that the identification of additional altered EmbB variants will contribute to a fuller understanding of structure-function relationships in this putative arabinosyltransferase.

FLUOROQUINOLONES

Ciprofloxacin (CIP) and ofloxacin (OFX) are synthetic derivatives of nalidixic acid that are currently used as second-line or alternative TB drugs.[166,167] These FQ drugs are bactericidal against *M. tuberculosis.* Their target is DNA gyrase, an ATP-dependent type II DNA topoisomerase that catalyzes negative supercoiling of DNA.[168–170] DNA gyrase is a heterotetramer composed of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively.[169] FQs bind to gyrase and inhibit supercoiling, thereby disrupting cellular processes dependent on DNA topology. As with other drugs, FQ resistance develops rapidly when used alone against *M. tuberculosis,* or when added as a single drug to a failing therapy.[171–175] Cross-resistance among FQs is common.[176] Clinical isolates of *M. tuberculosis* with acquired resistance to FQs are usually resistant to RIF and one or more other

first-line drugs because FQs are frequently used to treat MDR-TB.[172]

Cloning and sequencing of the *gyrA* and *gyrB* genes from *M. tuberculosis* resulted in the mapping of mutations that are associated with FQ resistance in many strains.[173–175,177] Mutations associated with high level FQ resistance are clustered in a short region of 40 amino acids in GyrA referred to as the quinolone resistance determining region (QRDR).[168] Sequence analysis of the QRDR of *gyrA* in 39 CIP^S and 15 CIP^R patient isolates of *M. tuberculosis* identified polymorphisms in codons 90, 91, 94, and 95.[177] Missense mutations in codons 90, 91, and 94 were associated with CIP^R (MIC ≥ 4 μg/ml) whereas CIP^S strains lacked mutations in these three codons. There was no simple relationship between a codon 95 polymorphism (AGC, Ser or ACC, Thr) and CIP susceptibility. That is, this polymorphism appears to be naturally-occuring variation that has no known functional participation in FQ resistance. A missense mutation (GAC->CAC, Asp->His) in codon 89 has been associated with ofloxacin resistance in *M. smegmatis,* but, has not been reported for CIP^R *M. tuberculosis.*[178]

Analysis of strains recovered from 22 patients infected with FQ-resistant MDR-TB in New York City found that many distinct amino acid substitutions were uniquely associated with resistant strains.[174] Automated DNA sequencing of the QRDR of *gyrA* identified mutations that would result in amino acid changes in codon 90 (Ala->Val), or codon 94 (Asp->Ala, Asn, Gly, His, or Tyr). Isolates from four patients lacked mutations in this region of *gyrA,* indicating that changes elsewhere in *gyrA* or in other genes were responsible for the resistance phenotype.

To test whether specific GyrA amino acid substitutions were associated with distinct levels of FQ resistance, 13 CIP^R patient isolates were examined for CIP susceptibility relative to control organisms with the same IS*6110* DNA fingerprint.[179] Quantitative estimates of FQ susceptibility were made by determining the antibiotic concentration required to inhibit growth in liquid culture by 50% (ID_{50}). Measurement of ID_{50} provided the same information as the MIC_{50}, but ID_{50} determination was logistically simpler and less expensive. The study found that specific *gyrA* alleles were associated with distinct levels of drug susceptibility for 11 isolates with nucleotide changes that alter the amino acid sequence of the GyrA QRDR. For example, amino acid replacements in residue 90 (Ala->Val) resulted in a 16-fold relative increase in resistance, position 94 changes (Asp->Asn, His, or Tyr) produced a 30-fold relative increase in MIC, and a Asp94Gly replacement resulted in a 60-fold relative increase in MIC.

FQ resistance in other bacteria can involve decreased cell wall permeability to the drug or active drug efflux.[168] However, although some investigation has occurred in *M. smegmatis,*[180] there is no firm evidence that mutations altering an active efflux pump contribute to FQ resistance in *M. tuberculosis* isolates recovered from patients.

Due to the difficulties in treating MDR-TB, newer or modified versions of existing drugs have been tested for their potential use in anti-TB therapy. Lounis et al.[181] compared the activity of ofloxacin, levofloxacin, and sparfloxacin in a murine TB model. Sparfloxacin was far more bactericidal than other FQs. Sparfloxacin sensitive organisms had an MIC of 0.15 μg/ml and no single step resistant mutants were selected at concentrations of 2.5 μg/ml. Two step-wise gyrase mutations were required to achieve high level resistance (MIC ≥ 5 μg/ml) to sparfloxacin. These data are consistent with a report that sparfloxacin was four times as effective as CIP against resistant mutants in vitro.

In summary, acquired resistance to FQs in 42–85% of clinical isolates of *M. tuberculosis* has been shown to be associated with *gyrA* mutations in the QRDR region. Other potential resistance mechanisms include mutations elsewhere in *gyrA*, or in *gyrB,* decreased drug permeability, and active drug efflux.

KANAMYCIN, AMIKACIN, VIOMYCIN AND CAPREOMYCIN

KAN and AMI are aminoglycoside antibiotics that inhibit protein synthesis by inhibiting the normal function of ribosomes.[182,183] VIO and CAP are basic peptide antimicrobial agents that also inhibit protein synthesis.[184,185] These drugs are used as second-line anti-TB agents. Resistance to KAN, AMI, VIO, and CAP is relatively uncommon in clinical isolates, presumably because these drugs are used relatively rarely to treat TB. As is the case for FQs, most organisms resistant to these drugs are also resistant to several first-line agents. A report that only 1% of 466 isolates from New York City were resistant to KAN or CAP illustrates the rarity of resistance to these drugs.[6] Cross-resistance between KAN and VIO and between KAN and CAP is variable among laboratory generated resistant mutants of *M. tuberculosis.*[183,185,186] No cross-resistance occurs between STR and either AMI or KAN, but cross-resistance between AMI and KAN has been reported.[183,185,186]

Recently, Taniguchi et al.[187] examined the molecular basis of resistance to KAN and VIO in *M. smegmatis* and identified an A->G change at position 1389 of 16S rRNA gene (*rrs*) in all laboratory-generated isolates resistant to high levels of KAN. In addition, mutations resulting in G->A or G->T changes at position 1473 of the *M. smegmatis rrs* gene were found in laboratory-generated VIO^R organisms. The results of conjugation experiments showed that these mutations conferred resistance to KAN and VIO.[187]

To determine if analogous mutations occurred in drug-resistant *M. tuberculosis* patient isolates, 10 organisms

variably resistant to STR, KAN, and VIO were analyzed for changes in *rrs* and *rpsL* genes. Importantly, three of four high-level KAN^R mutants (MIC > 200 μg/ml) had the identical A->G change at position 1400 of *rrs* gene.[187] This nucleotide is equivalent to position 1389 in *M. smegmatis* and 1408 in *E. coli,* and mutations located at these nucleotides are known to confer KAN resistance.[188] One strain with high-level resistance to all three drugs (MIC > 200 μg/ml) lacked mutations at positions 1400 and 1483 (equivalent to position 1473 of *M. smegmatis*). This strain had an A->G change at position 705 of the *rrs* gene and also had an Lys43Arg substitution in RpsL. Although the Lys43Arg substitution is strongly associated with STR resistance,[13] it is unclear if the change at *rrs* located at position 705 is responsible for resistance to KAN and VIO. Finally, five strains with low level resistance to KAN and/or VIO (MIC < 50 μg/ml) lacked mutations in the *rpsL* and *rrs* genes, a result indicating that another molecular mechanism is operative in these organisms. Currently, no progress has been made in the understanding of the molecular basis of CAP^R in *M. tuberculosis.*

Building on these studies, Suzuki et al.[189] analyzed a 300-bp region of the *rrs* gene and the intervening sequence between the *rrs* gene and 23S rRNA gene in 43 KAN^R and 71 KAN^S strains from Japan. Nucleotide substitutions were identified in 29 of 43 (67%) KAN^R strains at positions 1400, 1401, or 1483; 26 of the 29 organisms with mutations had an A->G substitution at position 1400. In contrast, all 71 KAN^S organisms had the identical wild-type sequence in the region analyzed. Hence, these data suggest that nucleotide substitutions in the region of *rrs* analyzed are a major cause of resistance to kanamycin in *M. tuberculosis.*

Alangaden et al.[190] recently studied 14 human *M. tuberculosis* strains resistant to KAN and AMI and judged to be distinct by IS*6110* subtyping. All isolates with high-level resistance (MIC > 32 μg/ml) to these antimicrobial agents had the same A->G mutation at position 1400 of *rrs.* In contrast, susceptible strains and organisms with low level resistance to KAN and AMI lacked this mutation. These data, taken together with the findings of Tanaguchi et al.[187] and Suzuki et al.[189] clearly implicate nucleotide substitutions at *rrs* position 1400 as important in high-level resistance to KAN and AMI.

CYCLOSERINE

D-cycloserine is a second-line anti-TB drug. This agent is a cyclic structural analog of D-alanine that inhibits cell wall synthesis.[191–193] Although an effective antimycobacterial agent, its use is limited by adverse side effects, including neurologic reactions.[194] The molecular genetic basis of resistance in *M. tuberculosis* is unknown, but a recent study provided potentially important insights. Caceres et al.[195] found that the D-alanine racemase gene (*alrA*) from *M. smegmatis* was necessary and sufficient to confer cycloserine resistance to this organism. The D-alanine racemase activities of wild-type and recombinant *M. smegmatis* strains were inhibited by D-cycloserine in a concentration-dependent manner. Sequence analysis found that the D-cycloserine resistance phenotype in the recombinant clone was caused by overexpression of the wild-type *alrA* gene on a multicopy vector. Importantly, a spontaneous resistant mutant that overproduced AlrA had a single transversion (G->T) located in the *alrA* promoter. Transformation of *M. bovis* BCG with the *M. smegmatis* wild-type *alrA* gene in a multicopy vector conferred resistance to D-cycloserine. The investigators speculated that AlrA overproduction is a potential mechanism of D-cycloserine resistance in human isolates of *M. tuberculosis.* However, this idea remains to be formally tested.

MULTIDRUG-RESISTANT TUBERCULOSIS

MDR-TB strains are generally considered to be those resistant to at least INH and RIF. These strains have been described worldwide, and their existence poses a serious threat to TB control programs in many countries.[196–220] The frequency of resistance to multiple drugs varies geographically, and acquired (secondary) resistance is more common than primary resistance. High rates of acquired MDR-TB have been reported in Nepal (48.0%); Gujarat, India (33.8%); and in New York City (~30%) early in this decade.[2,210] Although recent studies have shown an overall decrease in number of MDR-TB cases reported in New York City[220] and the USA,[11] the number of states reporting these organisms actually increased substantially since the early 1990s.[210] Outbreaks of MDR-TB among patients with acquired immune deficiency syndrome have been associated with rapid progression, high mortality, and considerable cost to society.[209–211,218] In addition, transmission of MDR-TB on a commercial airplane flight has been documented,[209] thereby contributing to the public concern about these strains. Notwithstanding some reports of limited success,[213,217,221] treatment of patients with MDR-TB is substantially more difficult than individuals infected with drug-sensitive strains.

In principle, MDR-TB strains could arise as a consequence of sequential accumulation of mutations conferring resistance to single therapeutic agents, or by a single-step process such as acquisition of an MDR element, or mutation that alters (for example) cell wall structure. All available evidence indicates that the former process is critical to the emergence of these organisms. Currently, there is no evidence that processes commonly mediating multidrug resistance in other bacteria, such as conjugal transfer of plasmids encoding combinations of

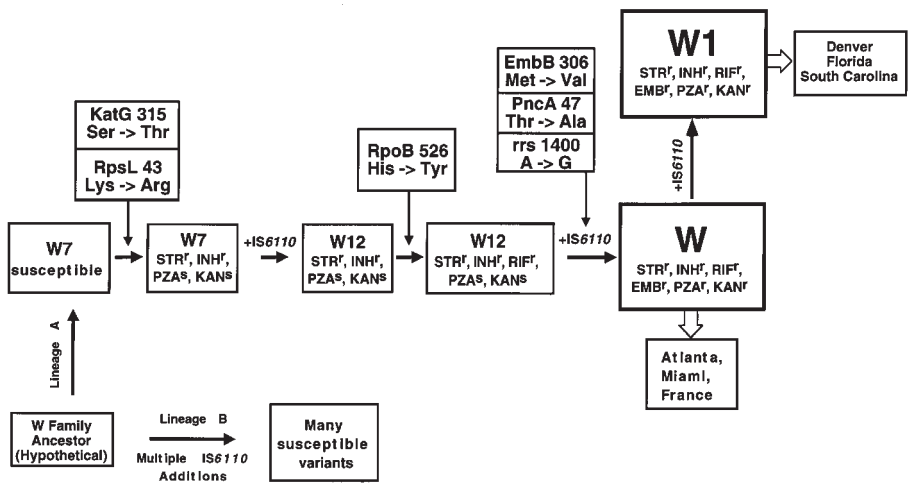

Fig. 9 Schematic representation of a plausible hypothesis to explain the molecular evolution of a clone family of multidrug-resistant *M. tuberculosis* isolates responsible for widespread disease in New York City and elsewhere. The figure is modified from versions published previously.[9,154] Multidrug resistance in these and other *M. tuberculosis* isolates arises by sequential accumulation of mutations in target genes, selected by distinct antibiotics. Many subclones have evolved from the W clone by gain of one or two copies of IS*6110*. In addition, derivatives of the W and W1 clones have developed fluoroquinolone resistance as a consequence of amino acid changes in GyrA.[174]

resistance genes or transfer of transposable elements with resistance genes occur in *M. tuberculosis.*

A well-documented example of how multidrug-resistant *M. tuberculosis* strains arise has been provided by the analysis of the evolution of two closely related subclones in New York City arbitrarily designated as strain W and W1[9] (Fig. 9). Together, these two related organisms have caused greater than 300 cases of tuberculosis in New York City and elsewhere. Automated DNA sequencing of representative organisms defined the exact series of distinct mutations conferring resistance to RIF, INH, STR, ETH, EMB, PZA, KAN, and FQs.[9,154,163,174,190] For example, a His526Tyr amino acid substitution was responsible for conferring RIF resistance and a Ser315Thr replacement was responsible for INH resistance. These drug-resistant strains have spread to other US cities and Europe. In addition, a recent study found that several patients had been infected with the W1 organism by a contaminated bronchoscope.[222] MDR-TB organisms also have been documented to be the cause of a nosocomial outbreak in Argentina.[223] Similarly, clonally-related strains of *M. bovis* resistant to INH, RIF, PZA, ETH, STR, AMI, CAP, para-aminosalicylic acid, clarithromycin, ethionamide, and ofloxacin caused 16 cases of tuberculosis in a nosocomial outbreak involving HIV-positive patients in Spain,[207,224,225] and transmission of this organism to an immunocompetent patient also has been documented.[226] These MDR isolates arise because random mutations in genes that encode targets for the individual antimicrobial agents are selected by subtherapeutic drug levels that can occur due to processes such as treatment errors, poor adherence to treatment protocols, or other factors.[25,214,216,227]

Given the considerable difficulties in successfully treating drug-resistant strains,[25,227] the widespread dissemination of MDR-TB strains poses a serious threat to our global village. Several studies have documented that use of directly observed therapy is a critical factor in limiting the development of drug resistant strains.[228–230] Moreover, it is clear that new therapeutic agents or strategies are needed. Interestingly, the possibility that β-lactam drugs may be useful in the treatment of patients with MDR-TB is being investigated by several groups, although probably there will be a need to circumvent a β-lactamase made by many if not all isolates of *M. tuberculosis.*[231–236]

VIRULENCE AND DRUG RESISTANCE

One important question relating to the study of resistant *M. tuberculosis* is the relative virulence of drug-resistant and -susceptible strains. This issue has been controversial since publication several decades ago of a report that INH^R strains lacking catalase activity had significantly reduced virulence for guinea pigs.[237] In addition, Subbaiah et al.[238] reported that a number of INH^R *M. tuberculosis* strains isolated from patients in India tended to be of low catalase activity, an observation that may explain the low virulence in a guinea pig model of infection. Mouse studies employing a panel of 15 human isolates of *M. tuberculosis,* including several resistant to one or more

anti-TB agents, found that drug-resistant strains had a range of virulence.[239] 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, the exact molecular basis of drug resistance in these organisms was not defined. Hence, it was not possible to correlate relative virulence and specific drug resistance-conferring mutations.

A study conducted by Wilson et al.[240] provided strong evidence that KatG participates in virulence in a guinea pig model of *M. bovis* disease. Isogenic strains were used to demonstrate that KatG was essential for virulence, as assessed by ability to cause gross and microscopic pathology in spleen, liver, and lungs after subcutaneous injection. In contrast, no evidence was generated indicating that InhA directly participated in virulence in this model. The implication from this study is that INH^R *M. tuberculosis* strains with significantly decreased KatG activity may be relatively less virulent for humans. Such a notion may explain the relative lack of INH^R organisms with total *katG* deletions.

Recently Heym et al.[241] compared the virulence of isogenic mutants of *M. tuberculosis* which were either defective in KatG activity, or lacked KatG function but overproduced AhpC. The strains were tested in both normal and immunologically deficient mice. The virulence of the strains was studied in fully immunocompetent, MHC class II-knock out mice with abnormally low levels of CD4 T-cells, and athymic mice lacking a cellular immune response. The results indicated that *M. tuberculosis* strains producing catalase-peroxidase were considerably more virulent in immunocompetent mice than the isogenic *katG* deficient mutants. Restoration of virulence did not occur in the INH^R *M. tuberculosis* strain that overexpressed AhpC, suggesting that AhpC is not a virulence factor in rodent infections.

Li et al.[242] have recently examined the role of catalase-peroxidase as a virulence factor for *M. tuberculosis* in mice and guinea pigs. The growth and persistence of drug susceptible *M. tuberculosis* strain H37Rv in BALB/c mice infected intravenously were compared with a *katG*-deleted (isoniazid-resistant) derivative of this bacterial strain. Transformation of the *M. tuberculosis* H37Rv, *katG* deleted strain with the *katG* gene obtained from the parent strain of this organism restored catalase-peroxidase activities and the ability of the recombinants to persist in spleens of guinea pigs and mice. Similarly, transformation of the *katG*-deleted H37Rv strain with the *katG* allele found in *M. bovis* and principal genetic group 1 *M. tuberculosis* isolates (KatG 463Leu)[85] also restored catalase-peroxidase activities and enhanced persistence. In contrast, transformants containing a mutant *katG* gene (Thr275Pro) expressed low levels of enzymatic activity, survived in mouse tissues, but failed to persist in guinea pig spleens.[242] The Thr275Pro KatG variant was used because it is associated with dramatic reduction in catalase-peroxidase activity and high-level resistance to INH.[87] Taken together, these important results add to the evidence that KatG is an *M tuberculosis* virulence factor.

RAPID DETECTION OF DRUG-RESISTANT STRAINS

Delineation of the molecular mechanisms of antimicrobial agent resistance has resulted in the development and application of several PCR-based strategies designed to rapidly detect mutations associated with resistance. These methods include direct sequencing of PCR products; SSCP analysis; heteroduplex analysis; dideoxy fingerprinting; an RNA/RNA duplex, base pair-mismatch assay; a luciferase mycobacteriophage strategy; a rRNA/DNA-bioluminescence-labelled probe method; a reverse hybridization-based line probe assay; and other strategies.[13,243–261] These methods are all designed to exploit the observation that specific polymorphisms found in resistant strains are absent in susceptible organisms. The fact that natural populations of drug-susceptible *M. tuberculosis* complex isolates recovered globally have remarkably few polymorphisms in structural genes greatly simplifies interpretation of these assay[15,88,262,263] (Table 2). The restricted allelic variation in structural genes means that virtually all susceptible organisms will have the same wild-type allele of the target gene. Hence, one generally needs to differentiate between a single wild-type sequence and mutant sequences. This situation is in striking contrast to other bacteria in which many distinct naturally occurring alleles are usually found in the drug-susceptible condition.[88,264–267] In essence, the *M. tuberculosis* complex is an ideal situation for application of certain kinds of molecular diagnostic testing strategies.

Each molecular strategy has advantages and disadvantages, and a full discussion of this important topic is beyond the scope of this review. Among the many techniques used to identify drug resistance-associated mutations, automated DNA sequencing of PCR products has been the most widely applied. One important advantage of sequence-based approaches is that the resulting data are virtually unambiguous because the resistance-associated mutation is either present or absent. Until recently, the considerable cost required to purchase a sequencing instrument was a major disadvantage. Automated sequencing has been used by several groups in routine clinical settings and been found to give excellent benefit to patient care activities. For example, Pai et al.[243] showed that *rpoB* data bearing on rifampin-resistance could be generated within a few days from growth obtained in an early BACTEC-positive culture. Similarly,

Table 2 Levels of allelic polymorphism recorded in genes of pathogenic bacteria. (Reprinted from ref. 89 with permission.)

Organism	No. of genes or gene segments characterized	D_s[1]	Relative variation[2]
Mycobacterium tuberculosis complex[3]	36	< 0.01	1
Shigella sonnei	2	0.01	1
Vibrio cholerae	1	0.41	41
Streptococcus pyogenes	3	1.02	102
Neisseria meningitidis	4	6.18	618
Mycobacterium avium-intracellulare	1	10.10	1,010
Escherichia coli	11	11.77	1,177
Borrelia burgdorferi	3	~20	~2,000
Salmonella enterica	5	42.08	4,208

[1]Average number of synonymous substitutions per 100 synonymous sites.
[2]Relative to the *M. tuberculosis* complex D_s value.
[3]A total of ~3.6 Mb sequenced from 900 strains.

Telenti's group demonstrated good performance of SSCP-based interrogation of target sequences.[128] In contrast, one group demonstrated that proper selection of oligonucleotide primer sequences is critical to the success of the SSCP approach.[78] The line-probe assay strategy has the advantages of relatively reliable performance, and potential commercial availability. All strategies suffer from the fact that for no antimycobacterial agent do we understand the molecular mechanism of resistance for 100% of organisms. Hence, identification of a resistance-associated mutation is clinically informative whereas lack of a mutation in the target sequence must be interpreted with considerable caution.

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REFERENCES

1. Bloom B R, Murray C J L. Tuberculosis: commentary on a reemergent killer. Science 1992; 257: 1055–1064.
2. Raviglione M C, Snider D E Jr, Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. JAMA 1995; 273: 220–226.
3. Cohn D L, Bustreo F, Raviglione M C. Drug-resistant tuberculosis: review of the worldwide situation and the WHO/IUATLD global surveillance project. Clin Infect Dis 1997; 24: S121–S130.
4. Daley C L, Small P M, Schecter G F et al. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. An analysis using restriction-fragment-length polymorphisms. N Engl J Med 1992; 326: 231–235.
5. Fischl M A, Daikos G L, Uttamchandani R B et al. Clinical presentation and outcome of patients with HIV infection and tuberculosis caused by multiple-drug-resistant bacilli. Ann Intern Med 1992; 117: 184–190.
6. Frieden T R, Sterling T, Pablos-Mendéz A, Kilburn J O, Cauthen G M, Dooley S W. The emergence of drug-resistant tuberculosis in New York City. N Engl J Med 1993; 328: 521–526.
7. Sepkowitz K A, Telzak E E, Recalde S, Armstrong D, and the New York City Area Tuberculosis Working Group. Trends in the susceptibility of tuberculosis in New York City, 1987–1991. Clin Infect Dis 1994; 18: 755–759.
8. Shafer R W, Small P M, Larkin C et al. Temporal trends and transmission patterns during the emergence of multidrug-resistant tuberculosis in New York City: a molecular epidemiologic assessment. J Infect Dis 1995; 171: 170–176.
9. Bifani P J, Plikaytis B B, Kapur V et al. Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. JAMA 1996; 275: 452–457.
10. Ritacco V, Di Lonardo M, Reniero A et al. Nosocomial spread of human immunodeficiency virus-related multidrug-resistant tuberculosis in Buenos Aires. J Infect Dis 1997; 176: 637–642.
11. Moore M, Onorato I M, McCray E, Castro K G. Trends in drug-resistant tuberculosis in the United States, 1993–1996. JAMA 1997; 278: 833–837.
12. Shafer R W, Edlin B R. Tuberculosis in patients infected with human immunodeficiency virus: perspective on the past decade. Clin Infect Dis 1996; 22: 683–704.
13. Musser J M. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin Microbiol Rev 1995; 8: 496–514.
14. Inderlied C B, Nash K A. Antimycobacterial agents: in vitro susceptibility testing, spectra of activity, mechanisms of action and resistance, and assays for activity in biological fluids. In: Lorian V (ed). Antibiotics in Laboratory Medicine. Baltimore, MD: Williams & Wilkins, 1996: pp 127–175.
15. Heifets L B. Antituberculosis drugs: antimicrobial activity in vitro. In: Heifets L B (ed). Drug Susceptibility in the Chemotherapy of Mycobacterial Infections, 1st Ed. Boca Raton: CRC Press, 1991: pp 14–57.
16. Vareldzis B P, Grosset J, de Kantor I et al. Drug-resistant tuberculosis: laboratory issues. World Health Organization recommendations. Tuber Lung Dis 1994; 75: 1–7.
17. Blanchard J S. Molecular mechanisms of drug resistance in

Mycobacterium tuberculosis. Annu Rev Biochem 1996; 65: 215–239.
18. Chopra I, Brennan P. Molecular action of antimycobacterial agents. Tuber Lung Dis 1998; 78: 89–98.
19. Mitchison D A. The action of antituberculosis drugs in short-course chemotherapy. Tubercle 1985; 66: 219–225.
20. Stratton M A, Reed M T. Short-course drug therapy for tuberculosis. Clin Pharm 1986; 5: 977–987.
21. The World Health Organization. Report on the Tuberculosis Epidemic, 1997. Geneva, Switzerland. World Health Organization Global TB Programme.
22. Chaulk C P, Kazandjian V A, for the Public Health Tuberculosis Guidelines Panel. Directly observed therapy for treatment completion of pulmonary tuberculosis. Consensus statement of the Public Health Tuberculosis Guidelines Panel. JAMA 1998; 279: 943–948.
23. Grosset J H. Present status of chemotherapy for tuberculosis. Rev Infect Dis 1989; 11: S347–S352.
24. Iseman M D. Treatment of multidrug-resistant tuberculosis. N Engl J Med 1993; 329: 784–791.
25. Mahmoudi A, Iseman M D. Pitfalls in the care of patients with tuberculosis: common errors and their association with the acquisition of drug resistance. JAMA 1993; 270: 65–68.
26. Bradford W Z, Martin J N, Reingold A L, Schecter G F, Hopewell P C, Small P M. The changing epidemiology of acquired drug-resistant tuberculosis in San Francisco, USA. Lancet 1996; 348: 928–931.
27. Nolan C M, Williams D L, Cave M D et al. Evolution of rifampicin resistance in human immunodeficiency virus-associated tuberculosis. Am J Resp Crit Care Med 1995; 152: 1067–1071.
28. Luftey M, Della-Latta P, Kapur V et al. Independent origin of mono-rifampin-resistant *Mycobacterium tuberculosis* in patients with AIDS. Am J Resp Crit Care Med 1996; 153: 837–840.
29. March F, Garriga X, Rodriguez P et al. Acquired drug resistance in *Mycobacterium tuberculosis* isolates recovered from compliant patients with human immunodeficiency virus-associated tuberculosis. Clin Infect Dis 1997; 25: 1044–1047.
30. Munsiff S S, Joseph S, Ebrahimzadeh A, Frieden T R. Rifampin-monoresistant tuberculosis in New York City, 1993–1994. Clin Infect Dis 1997; 25: 1465–1467.
31. Choudhri S H, Hawken M, Gathua S et al. Pharmacokinetics of antimycobacterial drugs in patients with tuberculosis, AIDS, and diarrhea. Clin Infect Dis 1997; 25: 104–111.
32. Cole S T, Brosch R, Parkhill J et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998; 393: 537–544.
33. Gale E F, Cundliffe E, Reynolds P E, Richmond M H, Waring M J. The Molecular Basis of Antibiotic Action. New York: John Wiley & Sons, 1981.
34. McClure W R, Cech C L. On the mechanism of rifampicin inhibition of RNA synthesis. J Biol Chem 1978; 253: 8949–8956.
35. Levin M E, Hatfull G F. *Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of rifampicin resistance. Mol Microbiol 1993; 8: 277–285.
36. Lisitsyn N A, Sverdlov E D, Moiseyeva E P, Danilevskaya O N, Nikiforov V G. Mutation to rifampin resistance at the beginning of the RNA polymerase β subunit gene in *Escherichia coli.* Mol Gen Genet 1984; 196: 173–174.
37. Jin D J, Gross C A. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. J Mol Biol 1988; 202: 45–58.
38. Ovchinnikov Y A, Monastyrskaya G S, Gubanov V V et al. Primary structure of *Escherichia coli* RNA polymerase. Nucleotide substitution in the β subunit gene of the rifampicin resistant *rpoB255* mutant. Mol Gen Genet 1981; 184: 536–538.
39. Ovchinnikov Y A, Monastyrskaya G S, Guriev S O et al. RNA polymerase rifampicin resistance mutations in *Escherichia coli.* Sequence changes and dominance. Mol Gen Genet 1983; 190: 344–348.
40. Miller L P, Crawford J T, Shinnick T M. The *rpoB* gene of *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1994; 38: 805–811.
41. Telenti A, Imboden P, Marchesi F et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis.* Lancet 1993; 341: 647–650.
42. Donnabella V, Martiniuk F, Kinney D et al. Isolation of the gene for the β subunit of RNA polymerase from rifampicin-resistant *Mycobacterium tuberculosis* and identification of new mutations. Am J Respir Cell Mol Biol 1994; 11: 639–643.
43. Kapur V, Li L-L, Iordanescu S et al. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase β subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. J Clin Microbiol 1994; 32: 1095–1098.
44. Heym B, Honoré N, Truffot-Pernot C et al. Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. Lancet 1994; 344: 293–298.
45. Williams D L, Waguespack C, Eisenach K et al. Characterization of rifampin resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 1994; 38: 2380–2386.
46. Kapur V, Li L-L, Hamrick M R et al. Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antibiotic resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch Pathol Lab Med 1995; 119: 131–138.
47. Morris S, Han Bai G, Suffys P, Portillo-Gomez L, Fairchok M, Rouse D. Molecular mechanisms of multiple drug resistance in clinical isolates of *Mycobacterium tuberculosis.* J Infect Dis 1995; 171: 954–960.
48. Caugant D A, Sandven P, Eng J, Jeque J T, Tønjum T. Detection of rifampin resistance among isolates of *Mycobacterium tuberculosis* from Mozambique. Microbial Drug Resist 1995; 4: 321–326.
49. Rinder H, Dobner P, Feldmann K et al. Disequilibria in the distribution of *rpoB* alleles in rifampicin-resistant *M. tuberculosis* isolates from Germany and Sierra Leone. Microbial Drug Resist 1997; 3: 195–197.
50. Escalante P, Ramaswamy S, Sanabria H et al. Genotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Peru. Tuber Lung Dis 1998; in press.
51. Matsiota-Bernard P, Vrioni G, Marinis E. Characterization of *rpoB* mutations in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates from Greece. J Clin Microbiol 1998; 36: 20–23.
52. Kim B-J, Kim S-Y, Park B-H et al. Mutations in the *rpoB* gene of *Mycobacterium tuberculosis* that interfere with PCR-single-strand conformation polymorphism analysis for rifampin susceptibility testing. J Clin Microbiol 1997; 35: 492–494.
53. Bodmer T, Zürcher G, Imboden P, Telenti A. Mutation position and type of substitution in the β-subunit of the RNA polymerase influence in-vitro activity of rifamycins in rifampicin-resistant *Mycobacterium tuberculosis.* J Antimicrob Chemother 1995; 35: 345–348.
54. Ohno H, Koga H, Kohno S, Tashiro T, Hara K. Relationship between rifampin MICs for and *rpoB* mutations of

Mycobacterium tuberculosis strains isolated in Japan. Antimicrob Agents Chemother 1996; 40: 1053–1056.
55. Taniguchi H, Aramaki H, Nikaido Y et al. Rifampicin resistance and mutation of the *rpoB* gene in *Mycobacterium tuberculosis.* FEMS Microbiol Lett 1996; 144: 103–108.
56. Moghazeh S L, Pan X, Arain T, Stover C K, Musser J M, Kreiswirth B N. Comparative antimicrobial activities of rifampin, rifapentine, and KRM-1648 against a collection of rifampin-resistant *Mycobacterium tuberculosis* isolates with known *rpoB* mutations. Antimicrob Agents Chemother 1996; 40: 2655–2657.
57. Luna-Herrera J, Reddy M V, Gangadharam P R. In vitro activity of the benzoxazinorifamycin KRM-1648 against drug-susceptible and multidrug-resistant tubercle bacilli. Antimicrob Agents chemother 1995; 38: 440–444.
58. Reddy M V, Luna-Herrera J, Daneluzzi D, Gangadharum P R. Chemotherapeutic activity of benzoxazinorifamycin KRM-1648 against *Mycobactertium tuberculosis* in C57BL/6 mice. Tuber Lung Dis 1996; 77: 154–159.
59. Williams D L, Spring L, Collins L et al. Contribution of *rpoB* mutations to the development of rifamycin cross-resistance in *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1998; 42: 1853–1857.
60. Zhou Y N, Jin D J. The *rpoB* mutants destabilizing initiation complexes at stringently controlled promoters behave like 'stringent' RNA polymerase in *Escherichia coli.* Proc Natl Acad Sci USA 1998; 95: 2908–2913.
61. Quan S, Venter H, Dabbs E R. Ribosylative inactivation of rifampin by *Mycobacterium smegmatis* is a principal contributor to its low susceptibility to this antibiotic. Antimicrob Agents Chemother 1997; 41: 2456–2460.
62. Cohn M L, Kovitz C, Oda U, Middlebrook G. Studies on isoniazid and tubercle bacilli. II. The growth requirements, catalase activities, and pathogenic properties of isoniazid-resistant mutants. Am Rev Tuberc 1954; 70: 641–664.
63. Middlebrook G. Isoniazid-resistance and catalase activity of tubercle bacilli. A preliminary report. Am Rev Tuberc 1954; 69: 471–472.
64. Middlebrook G, Cohn M L, Schaefer W B. Studies on isoniazid and tubercle bacilli. III. The isolation, drug-susceptibility, and catalase-testing of tubercle bacilli from isoniazid-treated patients. Am Rev Tuberc 1954; 70: 852–872.
65. Hedgecock L W, Faucher I O. Relation of pyrogallol-peroxidative activity to isoniazid resistance in *Mycobacterium tuberculosis.* Am Rev Tuberc 1957; 75: 670–674.
66. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis.* Nature 1992; 358: 591–593.
67. Heym B, Zhang Y, Poulet S, Young D, Cole S T. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis.* J Bacteriol 1993; 175: 4255–4259.
68. Zhang Y, Garbe T, Young D. Transformation with *katG* restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. Mol Microbiol 1993; 8: 521–524.
69. Stoeckle M Y, Guan L, Riegler N et al. Catalase-peroxidase gene sequences in isoniazid-sensitive and -resistant strains of *Mycobacterium tuberculosis* from New York City. J Infect Dis 1993; 168: 1063–1065.
70. Altamirano M, Marostenmaki J, Wong A, FitzGerald M, Black W A, Smith J A. Mutations in the catalase-peroxidase gene from isoniazid-resistant *Mycobacterium tuberculosis* isolates. J Infect Dis 1994; 169: 1162–1165.
71. Goto M, Oka S, Tachikawa N et al. *katG* sequence deletion is not the major cause of isoniazid resistance in Japanese and Yemeni *Mycobacterium tuberculosis* isolates. Mol Cell Probes 1995; 9: 433–439.
72. Cockerill F R III, Uhl J R, Temesgen Z et al. Rapid identification of a point mutation of the *Mycobacterium tuberculosis* catalase-peroxidase (*katG*) gene associated with isoniazid resistance. J Infect Dis 1995; 171: 240–245.
73. Heym B, Alzari P M, Honoré N, Cole S T. Missense mutations in the catalase-peroxidase gene, *katG,* are associated with isoniazid-resistance in *Mycobacterium tuberculosis.* Mol Microbiol 1995; 15: 235–245.
74. Musser J M, Kapur V, Williams D L, Kreiswirth B N, van Soolingen D, van Embden J D A. Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. J Infect Dis 1996; 173: 196–202.
75. Haas W A, Schilke K, Brand J et al. Molecular analysis of *katG* gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. Antimicrob Agents Chemother 1997; 41: 1601–1603.
76. Dobner P, Rüsch-Gerdes S, Bretzel G et al. Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes *katG* and *inhA* for the prediction of isoniazid resistance. Int J Tuberc Lung Dis 1997; 1: 365–369.
77. Pretorius G S, van Helden P D, Sirgel F, Eisenach K D, Victor T C. Mutations in *katG* gene sequences in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* are rare. Antimicrob Agents Chemother 1995; 39: 2276–2281.
78. Victor T C, Pretorius G S, Felix J V, Jordaan A M, van Helden P D, Eisenach K D. *katG* mutations in isoniazid-resistant strains of *Mycobacterium tuberculosis* are not infrequent. Antimicrob Agents Chemother 1996; 40: 1572.
79. Marttila H J, Soini H, Huovinen P, Viljanen M K. *katG* mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates recovered from Finnish patients. Antimicrob Agents Chemother 1996; 40: 2187–2189.
80. Rouse D A, Li Z, Bai G-H, Morris S L. Characterization of the *katG* and *inhA* genes of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1995; 39: 2472–2477.
81. Ferrazoli L, Palaci M, ds Silva Telles M A et al. Catalase expression, *katG,* and MIC of isoniazid for *Mycobacterium tuberculosis* isolates from Sao Paulo, Brazil. J Infect Dis 1995; 171: 237–240.
82. Marttila H J, Soini H, Eerola E et al. A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates originating from the St Petersburg area in Russia. Antimicrob Agents Chemother 1998; 42: 2443–2445.
83. Wengenack N L, Uhl J R, St Amand A L et al. Recombinant *Mycobacterium tuberculosis* KatG(S315T) is a competent catalase-peroxidase with reduced activity toward isoniazid. J Infect Dis 1997; 176: 722–727.
84. Lee A S-G, Tang L L-H, Lim I H-K, Ling M-L, Tay L, Wong S-Y. Lack of clinical significance for the common arginine-to-leucine substitution at codon 463 of the *katG* gene in isoniazid-resistant *Mycobacterium tuberculosis* in Singapore. J Infect Dis 1997; 176: 1125–1126.
85. Musser J M. Reply. J Infect Dis 1997; 176: 1126–1127.
86. Johnsson K, Froland W A, Schultz P G. Overexpression, purification, and characterization of the catalase-peroxidase KatG from *Mycobacterium tuberculosis.* J Biol Chem 1997; 272: 2834–2840.
87. Rouse D A, DeVito J A, Li Z, Byer H, Morris S L. Site-directed

mutagenesis of the *katG* gene of *Mycobacterium tuberculosis:* effects on catalase-peroxidase activities and isoniazid resistance. Mol Microbiol 1996; 22: 583–592.
88. Kurepina N E, Sreevatsan S, Plikaytis B B et al. Characteristics of the phylogenetic distribution and chromosomal insertion sites of five IS*6110* elements in *Mycobacterium tuberculosis:* nonrandom integration in the *dnaA-dnaN* region. Tuber Lung Dis 1998; in press.
89. Sreevatsan S, Pan X, Stockbauer K E et al. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci USA 1997; 94: 9869–9874.
90. Loewen P C, Switala J, Smolenski M, Triggs-Raine B L. Molecular characterization of three mutations in *katG* affecting the activity of hydroperoxidase I of *Escherichia coli.* Biochem Cell Biol 1990; 68: 1037–1044.
91. Pelletier H, Kraut J. Crystal structure of a complex between electron transfer partners, cytochrome c peroxidase and cytochrome c. Science 1992; 258: 1748–1755.
92. Winder F G, Collins P B, Whelan D. Effects of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis* BCG. J Gen Microbiol 1971; 66: 379–380.
93. Canetti G. The J Burns Amberson lecture. Present aspects of bacterial resistance in tuberculosis. Am Rev Respir Dis 1965; 92: 687–703.
94. Hok T T. A comparative study of the susceptibility to ethionamide, thiosemicarbazone, and isoniazid of tubercle bacilli from patients never treated with ethionamide or thiosemicarbazone. Am Rev Respir Dis 1964; 90: 468–469.
95. Lefford M J. The ethionamide sensitivity of British pre-treatment strains of *Mycobacterium tuberculosis.* Tubercle Lond 1966; 47: 198–206.
96. Banerjee A, Dubnau E, Quémard A et al. *inhA,* a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis.* Science 1994; 263: 227–230.
97. Dessen A, Quémard A, Blanchard J S, Jacobs Jr W R, Sacchettini J C. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis.* Science 1995; 267: 1638–1641.
98. Quémard A, Sacchettini J C, Dessen A et al. Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis.* Biochemistry 1995; 34: 8235–8241.
99. Mdluli K, Sherman D R, Hickey M J et al. Biochemical and genetic data suggest that InhA is not the primary target for activated isoniazid in *Mycobacterium tuberculosis.* J Infect Dis 1996; 174: 1085–1090.
100. Wheeler P R, Anderson P M. Determination of the primary target for isoniazid in mycobacterial mycolic acid biosynthesis with *Mycobacterium aurum* A^+. Biochem J 1996; 318: 451–457.
101. Basso L A, Zheng R, Musser J M, Jacobs Jr W R, Blanchard J S. Mechanisms of isoniazid resistance in *Mycobacterium tuberculosis:* enzymatic characterization of enoyl reductase mutants identified in isoniazid-resistant clinical isolates. J Infect Dis 1998; 178: 769–775.
102. Johnsson K, Schultz P G. Mechanistic studies of the oxidation of isoniazid by the catalase peroxidase from *Mycobacterium tuberculosis.* J Am Chem Soc 1994; 116: 7425–7426.
103. Johnsson K, King D S, Schultz P G. Studies on the mechanism of action of isoniazid and ethionamide in the chemotherapy of tuberculosis. J Am Chem Soc 1995; 117: 5009–5010.
104. Quémard A, Dessen A, Sugantino M, Jacobs Jr W R, Sacchettini J C, Blanchard J S. Binding of catalase-peroxidase-activated isoniazid to wild-type and mutant *Mycobacterium tuberculosis* enoyl-ACP reductases. J Am Chem Soc 1996; 118: 1561–1562.
105. Basso L A, Zheng R, Blanchard J S. Kinetics of inactivation of WT and C243S mutant of *Mycobacterium tuberculosis* enoyl reductase by activated isoniazid. J Am Chem Soc 1996; 118: 11301–11302.
106. Zabinski R F, Blanchard J S. The requirement for manganese and oxygen in the isoniazid-dependent inactivation of *Mycobacterium tuberculosis* enoyl reductase. J Am Chem Soc 1997; 119: 2331–2332.
107. Rozwarski D A, Grant G A, Barton D H R, Jacobs W R Jr, Sacchettini J C. Isoniazid modifies the NADH of its target enzyme (InhA) from *Mycobacterium tuberculosis.* Science 1998; 279: 98–102.
108. Mdluli K, Slayden R A, Zhu Y et al. Inhibition of a *Mycobacterium tuberculosis* β-ketoacyl ACP synthase by isoniazid. Science 1998; 280: 1607–1610.
109. Huang W, Jia J, Edwards P, Dehesh K, Schneider G, Lindqvist Y. Crystal structure of β-ketoacyl-acyl carrier protein synthase II from *E. coli* reveals the molecular architecture of condensing enzymes. EMBO J 1998; 17: 1183–1191.
110. Christman M F, Morgan R W, Jacobson F S, Ames B N. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium.* Cell 1985; 41: 753–762.
111. Storz G, Tartaglia L A, Ames B N. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 1990; 248: 189–194.
112. Farr S B, Kogoma T. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium.* Microbiol Rev 1991; 55: 561–585.
113. Tartaglia L A, Storz G, Ames B N. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. J Mol Biol 1989; 210: 709–719.
114. Toledano M B, Kullik I, Trinh F, Baird P T, Schneider T D, Storz G. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell 1994; 78: 897–909.
115. Rosner J L. Susceptibilities of *oxyR* regulon mutants of *Escherichia coli* and *Salmonella typhimurium* to isoniazid. Antimicrob Agents Chemother 1993; 37: 2251–2253.
116. Deretic V, Phillipp W, Dhandayuthapani S et al. *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. Mol Microbiol 1995; 17: 889–900.
117. Sherman D R, Sabo P J, Hickey M J et al. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc Natl Acad Sci USA 1995; 92: 6625–6629.
118. Dhandayuthapani S, Zhang Y, Mudd M H, Deretic V. Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis.* J Bacteriol 1996; 178: 3641–3649.
119. Dhandayuthapani S, Mudd M, Deretic V. Interactions of OxyR with the promoter region of the *oxyR* and *ahpC* genes from *Mycobacterium leprae* and *Mycobacterium tuberculosis.* J Bacteriol 1997; 179: 2401–2409.
120. Schell M A. Molecular biology of the LysR family of transcriptional regulators. Annu Rev Microbiol 1993; 47: 597–626.
121. Zhang Y, Dhandayuthapani S, Deretic V. Molecular basis for the exquisite sensitivity of *Mycobacterium tuberculosis* to isoniazid. Proc Natl Acad Sci USA 1996; 93: 13212–13216.
122. Deretic V, Pagán-Ramos E, Zhang Y, Dhandayuthapani S, Via L E. The extreme sensitivity of *Mycobacterium tuberculosis* to the front-line antituberculosis drug isoniazid. Nature Biotechnol 1996; 14: 1557–1561.

123. Deretic V, Song J, Pagán-Ramos E. Loss of *oxyR* in *Mycobacterium tuberculosis.* Trends Microbiol 1997; 5: 367–372.
124. Sherman D R, Mdluli K, Hickey M J et al. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis.* Science 1996; 272: 1641–1643.
125. Wilson T M, Collins D M. *ahpC*, a gene involved in isoniazid resistance of the *Mycobacterium tuberculosis* complex. Mol Microbiol 1996; 19: 1025–1034.
126. Sreevatsan S, Escalante P, Pan X et al. Identification of a polymorphic nucleotide in *oxyR* specific for *Mycobacterium bovis.* J Clin Microbiol 1996; 34: 2007–2010.
127. Kelley C L, Rouse D A, Morris S L. Analysis of *ahpC* gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1997; 41: 2057–2058.
128. Telenti A, Honoré N, Bernasconi C et al. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis:* a blind study at reference laboratory level. J Clin Microbiol 1997; 35: 719–723.
129. Sreevatsan S, Pan X, Zhang Y, Deretic V, Musser J M. Analysis of the *oxyR-ahpC* region in isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob Agents Chemother 1997; 41: 600–606.
130. Song J, Pagán-Ramos E, Mudd M, Deretic V. Cotranscription of the ferric uptake homolog *furA* and *katG* in *Mycobacterium tuberculosis* and conservation of their linkage in mycobacteria: implications for regulation of oxidative stress response. Submitted.
131. Moazed D, Noller H F. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature 1987; 327: 389–394.
132. Noller H F. Structure of ribosomal RNA. Annu Rev Biochem 1984; 53: 119–162.
133. Douglass J, Steyn L M. A ribosomal gene mutation in streptomycin-resistant *Mycobacterium tuberculosis* isolates. J Infect Dis 1993; 167: 1505–1506.
134. Finken M, Kirschner P, Meier A, Wrede A, Böttger E C. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis:* alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol 1993; 9: 1239–1246.
135. Nair J, Rouse D A, Bai G-H, Morris S L. The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis.* Mol Microbiol 1993; 10: 521–527.
136. Meier A, Kirschner P, Bange F-C, Vogel U, Böttger E C. Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis:* mapping of mutations conferring resistance. Antimicrob Agents Chemother 1994; 38: 228–233.
137. Honoré N, Cole S T. Streptomycin resistance in mycobacteria. Antimicrob Agents Chemother 1994; 38: 238–242.
138. Sreevatsan S, Pan X, Stockbauer K E, Williams D L, Kreiswirth B N, Musser J M. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. Antimicrob Agents Chemother 1996; 40: 1024–1026.
139. Cooksey R C, Morlock G P, McQueen A, Glickman S E, Crawford J T. Characterization of streptomycin resistance mechanisms among *Mycobacterium tuberculosis* isolates from patients in New York City. Antimicrob Agents Chemother 1996; 40: 1186–1188.
140. Meier A, Sander P, Schaper K-J, Scholz M, Böttger E C. Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1996; 40: 2452–2454.
141. Bercovier H, Kafri O, Sela S. *Mycobacteria* possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem Biophys Res Commun 1986; 136: 1136–1141.
142. Suzuki Y, Yoshinaga K, Ono Y, Nagata A, Yamada T. Organization of rRNA genes in *Mycobacterium bovis* BCG. J Bacteriol 1987; 169: 839–843.
143. Stern S, Powers T, Changchien L M, Noller H F. Interaction of ribosomal proteins S5, S6, S11, S12, S18 and S21 with 16S rRNA. J Mol Biol 1988; 201: 683–695.
144. Hull S I, Wallace R J Jr, Bobey D G et al. Presence of aminoglycoside acetyltransferase and plasmids in *Mycobacterium fortuitum.* Lack of correlation with intrinsic aminoglycoside resistance. Am Rev Respir Dis 1984; 129: 614–618.
145. Konno K, Feldmann F M, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis 1967; 95: 461–469.
146. Yeager R L, Munroe W G C, Dessau F I. Pyrazinamide (Aldinamide) in the treatment of pulmonary tuberculosis. Am Rev Tuberc 1952; 65: 523–534.
147. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity *in vitro* against semi-dormant *Mycobacterium tuberculosis* bacterial populations. Am Rev Respir Dis 1992; 145: 1223–1225.
148. McDermott W, Tompsett R. Activation of pyrazinamide and nicotinamide in acidic environments in vitro. Am Rev Tuberc 1954; 70: 748–754.
149. Cynamon M H, Klemens S P. Antimycobacterial activity of a series of pyrazinoic acid esters. J Med Chem 1992; 35: 1212–1215.
150. Butler W R, Kilburn J O. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob Agents Chemother 1983; 24: 600–601.
151. McClatchy J K, Tsang A Y, Cernich M S. Use of pyrazinamidase activity in *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility. Antimicrob Agents Chemother 1981; 20: 556–557.
152. Scorpio A, Zhang Y. Mutations in *pncA,* a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nature Med 1996; 2: 662–667.
153. Jerlström P G, Bezjak D A, Jennings M P, Beacham I R. Structure and expression in *Escherichia coli* K-12 of the L-asparaginase I-encoding *ansA* gene and its flanking regions. Gene 1989; 78: 37–46.
154. Sreevatsan S, Pan X, Zhang Y, Kreiswirth B N, Musser J M. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. Antimicrob Agents Chemother 1997; 41: 636–-640.
155. Scorpio A, Lindholm-Levy P, Heifets L et al. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1997; 41: 540–543.
156. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis.* Tuber Lung Dis 1998; 78: 117–122.
157. Takayama K, Armstrong E L, Kunugi K A, Kilburn J O. Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis.* Antimicrob Agents Chemother 1979; 16: 240–242.
158. Takayama K, Kilburn J O. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis.* Antimicrob Agents Chemother 1989; 33: 1493–1499.

159. Wolucka B A, McNeil M R, de Hoffmann E, Chojnacki T, Brennan P J. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J Biol Chem 1994; 269: 23328–23335.
160. Wilkinson R G, Shepherd R G, Thomas J P, Baughn C. Stereospecificity in a new type of synthetic antituberculous agent. J Am Chem Soc 1961; 83: 2212–2213.
161. Belanger A E, Besra G S, Ford M E et al. The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci USA 1996; 93: 11919–11924.
162. Telenti A, Philipp W J, Sreevatsan S et al. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nature Med 1997; 3: 567–570.
163. Sreevatsan S, Stockbauer K E, Pan X et al. Ethambutol resistance in *Mycobacterium tuberculosis:* critical role of *embB* mutations. Antimicrob Agents Chemother 1997; 41: 1677–1681.
164. Alcaide F, Pfyffer G E, Telenti A. Role of *embB* in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob Agents Chemother 1997; 41: 2270–2273.
165. Lety M A, Nair S, Berche P, Escuyer V. A single point mutation in the *embB* gene is responsible for resistance to ethambutol in *Mycobacterium smegmatis.* Antimicrob Agents Chemother 1997; 41: 2629–2633.
166. Tsukamura M, Nakamura E, Yoshii S, Amano H. Therapeutic effect of a new antibacterial substance ofloxacin (DL8280) on pulmonary tuberculosis. Am Rev Respir Dis 1985; 131: 352–356.
167. Kennedy N, Berger L, Curram J et al. Randomized controlled trial of a drug regimen that includes ciprofloxacin for the treatment of pulmonary tuberculosis. Clin Infect Dis 1996; 22: 827–833.
168. Hooper D C, Wolfson J S. Quinolone Antimicrobial Agents. Washington DC: American Society for Microbiology Press, 1993.
169. Wentland M. Structure-activity relationships of fluoroquinolones. In: Siporin C, Heifetz C L, Domagala J M (eds). The New Generation of Quinolones. New York: Dekker, 1990: pp 1–44.
170. Wang J C. DNA topoisomerases. Annu Rev Biochem 1985; 54: 665–697.
171. Hong Kong Chest Service/British Medical Research Council. A controlled study of rifabutin and an uncontrolled study of ofloxacin in the retreatment of patients with pulmonary tuberculosis resistant to isoniazid, streptomycin and rifampicin. Tuber Lung Dis 1992; 73: 59–67.
172. Cambau E, Sougakoff W, Besson M, Truffot-Pernot C, Grosset J, Jarlier V. Selection of a *gyrA* mutant of *Mycobacterium tuberculosis* resistant to fluoroquinolones during treatment with ofloxacin. J Infect Dis 1994; 170: 479–483.
173. Alangaden G J, Manavathu E K, Vakulenko S B, Zvonok N M, Lerner S A. Characterization of fluoroquinolone-resistant mutant strains of *Mycobacterium tuberculosis* selected in the laboratory and isolated from patients. Antimicrob Agents Chemother 1995; 39: 1700–1703.
174. Sullivan E A, Kreiswirth B N, Palumbo L et al. Emergence of fluoroquinolone-resistant tuberculosis in New York City. Lancet 1995; 345: 1148–1150.
175. Williams K S, Chan R, Piddock L S. *gyrA* of ofloxacin-resistant clinical isolates of *M. tuberculosis* from Hong Kong (letter). J Antimicrob Chemother 1996; 37: 1032–1034.
176. Kocagöz T, Hackbarth C J, Ünsal I, Rosenberg E Y, Nikaido H, Chambers H F. Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of *Mycobacterium tuberculosis* H37Ra. Antimicrob Agents Chemother 1996; 40: 1768–1774.
177. Takiff H E, Salazar L, Guerrero C et al. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother 1994; 38: 773–780.
178. Revel V, Cambau E, Jarlier V, Sougakoff W. Characterization of mutations in *Mycobacterium smegmatis* involved in resistance to fluoroquinolones. Antimicrob Agents Chemother 1994; 38: 1991–1996.
179. Xu C, Kreiswirth B N, Sreevatsan S, Musser J M, Drlica K. Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis.* J Infect Dis 1996; 174: 1127–1130.
180. Takiff H E, Cimino M, Musso M C et al. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis.* Proc Natl Acad Sci USA 1996; 93: 362–366.
181. Lounis N, Ji B, Truffot-Pernot C, Grosset J. Which aminoglycoside or fluoroquinolone is more active against *Mycobacterium tuberculosis* in mice? Antimicrob Agents Chemother 1997; 41: 607–610.
182. Allen B W, Mitchison D A, Chan Y C, Yew W W, Allan W G L, Girling D J. Amikacin in the treatment of pulmonary tuberculosis. Tubercle 1983; 64: 111–118.
183. McClatchy J K, Kanes W, Davidson P T, Moulding T S. Cross-resistance in *M. tuberculosis* to kanamycin, capreomycin, and viomycin. Tubercle 1977; 58: 29–34.
184. Herr E B Jr, Redstone M O. Chemical and physical characterization of capreomycin. Ann N Y Acad Sci 1966; 135: 940–946.
185. Sutton W B, Gordee R S, Wick W E, Standfield L V. In vitro and in vivo laboratory studies on the antituberculous activity of capreomycin. Ann N Y Acad Sci 1966; 135: 947–959.
186. Tsukamura M, Mizuno S. Cross-resistance relationships among the aminoglucoside antibiotics in *Mycobacterium tuberculosis.* J Gen Microbiol 1975; 88: 269—274.
187. Taniguchi H, Chang B, Abe C, Nikaido Y, Mizuguchi Y, Yoshida S-I. Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J Bacteriol 1997; 179: 4795–4801.
188. Fourmy D, Recht M I, Blanchard S C, Puglisi J D. Structure of the A site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science 1996; 274: 1367–1371.
189. Suzuki Y, Katsukawa C, Tamaru A et al. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol 1998; 36: 1220–1225.
190. Alangaden G J, Kreiswirth B N, Aouad A et al. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1998; 42: 1295–1297.
191. David H L, Goldman D S, Takayama K. Inhibition of the synthesis of wax D peptidoglycolipid of *Mycobacterium tuberculosis* by D-cycloserine. Infect Immun 1970; 1: 74–77.
192. David H L, Takayama K, Goldman D S. Susceptibility of mycobacterial D-alanyl-D-alanine synthetase to D-cycloserine. Am Rev Respir Dis 1969; 100: 579–581.
193. David H L. Resistance to D-cycloserine in the tubercle bacilli: mutation rate and transport of alanine in parental cells and drug-resistant mutants. Appl Microbiol 1971; 21: 888–892.

194. Yew W W, Wong C F, Wong P C, Lee J, Chau C H. Adverse neurological reactions in patients with multidrug-resistant pulmonary tuberculosis after coadministration of cycloserine and ofloxacin. Clin Infect Dis 1993; 17: 288–289.
195. Cáceres N E, Harris N B, Wellehan J F, Feng Z, Kapur V, Barletta R G. Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. J Bacteriol 1997; 179: 5046–5055.
196. Frieden T R, Sherman L F, Maw K L et al. A multi-institutional outbreak of highly drug-resistant tuberculosis. Epidemiology and clinical outcomes. JAMA 1996; 276: 1229–1235.
197. Valway S E, Richards S B, Kovacovich J, Greilfinger R B, Crawford J T, Dooley S W. Outbreak of multidrug-resistant tuberculosis in a New York State prison. Am J Epidemiol 1994; 140: 113–122.
198. Fischl M A, Uttamchandani R B, Daikos G L et al. An outbreak of tuberculosis caused by multiple-drug-resistant tubercle bacilli among patients with HIV infection. Ann Intern Med 1992; 117: 177–183.
199. Edlin B R, Tokars J I, Grieco M H et al. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with acquired immunodeficiency syndrome. N Engl J Med 1992; 326: 1514–1521.
200. Whalen C, Horsburgh C R, Hom D, Lahart C, Simberkoff M, Ellner J. Accelerated course of human immunodeficiency virus infection after tuberculosis. Am J Respir Crit Care Med 1995; 151: 129–135.
201. Wenger P N, Otten J, Breeden A, Orfas D, Beck-Sague C, Jarvis W R. Control of nosocomial transmission of multidrug-resistant *Mycobacterium tuberculosis* among health care workers and HIV-infected patients. Lancet 1995; 345: 235–240.
202. Ridzon R, Kent J H, Valway S et al. Outbreak of drug-resistant tuberculosis with second-generation transmission in a high school in California. J Pediatr 1997; 131: 863–868.
203. Kenyon T A, Ridzon R, Luskin-Hawk R et al. A nosocomial outbreak of multidrug-resistant tuberculosis. Ann Intern Med 1997; 127: 32–36.
204. Reves R, Blakey D, Snider D E Jr, Farer L S. Transmission of multiple drug-resistant tuberculosis: report of a school and community outbreak. Am J Epidemiol 1981; 113: 423–435.
205. Bradford W Z, Daley C L. Multiple drug-resistant tuberculosis. Emerg Infect Dis 1998; 12: 157–172.
206. Salomon N, Perlman D C, Friedmann P, Buchstein S, Kreiswirth B N, Mildvan D. Predictors and outcome of multidrug-resistant tuberculosis. Clin Infect Dis 1995; 21: 1245–1252.
207. Nolan C M. Editorial: nosocomial multidrug-resistant tuberculosis – global spread of the third epidemic. J Infect Dis 1997; 176: 748–751.
208. Rullán J V, Herrera D, Cano R et al. Nosocomial transmission of multidrug-resistant *Mycobacterium tuberculosis* in Spain. Emerging Infect Dis 1996; 2: 125–129.
209. Kenyon T A, Valway S E, Ihle W W, Onorato I M, Castro K G. Transmission of multidrug-resistant *Mycobacterium tuberculosis* during a long airplane flight. N Engl J Med 1996; 334: 933–938.
210. Bloch A B, Cauthen G M, Onorato I M et al. Nationwide survey of drug-resistant tuberculosis in the United States. JAMA 1994; 271: 665–671.
211. Dooley S W, Jarvis W R, Martone W J, Snider D E Jr. Multidrug-resistant tuberculosis. Ann Intern Med 1992; 117: 257–259.
212. Small P M, Shafer R W, Hopewell P C et al. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. N Engl J Med 1993; 328: 1137–1144.
213. Telzak E E, Sepkowitz K, Alpert P et al. Multidrug-resistant tuberculosis in patients without HIV infection. N Engl J Med 1995; 333: 907–911.
214. Sepkowitz K A, Raffalli J, Riley L, Kiehn T E, Armstrong D. Tuberculosis in the AIDS era. Clin Microbiol Rev 1995; 8: 180–199.
215. Hopewell P C. Impact of human immunodeficiency virus infection on the epidemiology, clinical features, management, and control of tuberculosis. Clin Infect Dis 1992; 15: 540–547.
216. Pablos-Méndez A, Sterling T R, Frieden T R. The relationship between delayed or incomplete treatment and all-cause mortality in patients with tuberculosis. JAMA 1996; 276: 1223–1228.
217. Turett G S, Telzak E E, Torian L V et al. Improved outcomes for patients with multidrug-resistant tuberculosis. Clin Infect Dis 1995; 21: 1238–1244.
218. Weltman A C, Rose D N. Tuberculosis susceptibility patterns, predictors of multidrug resistance, and implications for initial therapeutic regimens at a New York City hospital. Arch Intern Med 1994; 154: 2161–2167.
219. Pablos-Mendez A, Raviglione M C, Laszlo A et al. Global surveillance for antituberculosis-drug resistance, 1994–1997. N Engl J Med 1998; 338: 1641–1649.
220. Fujiwara P I, Cook S V, Rutherford C M et al. A continuing survey of drug-resistant tuberculosis, New York City, April 1994. Arch Intern Med 1997; 157: 531–536.
221. Park M M, Davis A L, Schluger N W, Cohen H, Rom W N. Outcome of MDR-TB patients, 1983–993. Prolonged survival with appropriate therapy. Am J Respir Crit Care Med 1996; 153: 317–324.
222. Agerton T, Valway S, Gore B et al. Transmission of a highly drug-resistant strain (strain W1) of *Mycobacterium tuberculosis*. Community outbreak and nosocomial transmission via a contaminated bronchoscope. JAMA 1997; 278: 1073–1077.
223. Weinbaum C, Ridzon R, Joglar O et al. Multidrug resistant tuberculosis (MDR-TB) among AIDS patients in an infectious disease hospital, Buenos Aires. Infect Control Hosp Epidemiol 1997; 18: 47.
224. Blázquez J, Espinosa DE Los Monteros L E E, Samper S et al. Genetic characterization of multidrug-resistant *Mycobacterium bovis* strains from a hospital outbreak involving human immunodeficiency virus-positive patients. J Clin Microbiol 1997; 35: 1390–1393.
225. Samper S, Martin C, Pinedo A et al. Transmission between HIV-infected patients of multidrug-resistant tuberculosis caused by *Mycobacterium bovis*. AIDS 1997; 11: 1237–1242.
226. Palenque E, Villena V, Jose Rebollo M, Soledad Jimenez M, Samper S. Transmission of multidrug-resistant *Mycobacterium bovis* to an immunocompetent patient. Clin Infect Dis 1998; 995–996.
227. Goble M, Iseman M D, Madsen L A, Waite D, Ackerson L, Horsburgh C R Jr. Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. N Engl J Med 1993; 328: 527–532.
228. Weis S E, Slocum P C, Blais F X et al. The effect of directly observed therapy on the rates of drug resistance and relapse in tuberculosis. N Engl J Med 1994; 330: 1179–1184.
229. Chaulk C P, Moore-Rice K, Rizzo R, Chaisson R E. Eleven years of community-based directly observed therapy for tuberculosis. JAMA 1995; 274: 945–951.
230. Frieden T R, Fujiwara P I, Washko R M, Hamburg M A. Tuberculosis in New York City-turning the tide. N Engl J Med 1995; 333: 229–333.
231. Chambers H F, Moreau D, Yajko D et al. Can penicillins and other β-lactam antibiotics be used to treat tuberculosis? Antimicrob Agents Chemother 1995; 39: 2620–2624.

232. Chambers H F, Kocagoz T, Sipit T, Turner J, Hopewell P C. Activity of amoxicillin/clavulanate in patients with tuberculosis. Clin Infect Dis 1998; 26: 874–877.
233. Yew W W, Wong C F, Lee J et al. Do β-lactam-β-lactamase inhibitor combinations have a place in the treatment of multidrug resistant tuberculosis? Tuber Lung Dis 1995; 76: 90–91.
234. Segura C, Salvado M, Collado I, Chaves J, Coira A. Contribution of β-lactam susceptibilities of susceptible and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrob Agents Chemother 1998; 42: 1524–1526.
235. Hackbarth C J, Unsal I, Chambers H F. Cloning and sequence analysis of a class A beta-lactamase from *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1997; 41: 1182–1185.
236. Voladri R K R, Lakey D L, Hennigan S H, Menzies B E, Edwards K M, Kernodle D S. Recombinant expression and characterization of the major β-lactamase of *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 1998; 42: 1375–1381.
237. Middlebrook G, Cohn M L. Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli. Science 1953; 118: 297–299.
238. Subbaiah T V, Mitchison D A, Selkon J B. The susceptibility to hydrogen peroxide of Indian and British isoniazid-sensitive and isoniazid-resistant tubercle bacilli. Tubercle 1960; 41: 323–333.
239. Ordway D J, Sonnenberg M G, Donahue S A, Belisle J T, Orme I M. Drug-resistant strains of *Mycobacterium tuberculosis* exhibit a range of virulence for mice. Infect Immun 1995; 63: 741–743.
240. Wilson T M, de Lisle G W, Collins D M. Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium tuberculosis.* Mol Microbiol 1995; 15: 1009–1015.
241. Heym B, Stavropoulos E, Honoré N et al. Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis.* Infect Immun 1997; 65: 1395–1401.
242. Li Z, Kelley C, Collins F, Rouse D, Morris S. Expression of *katG* in *Mycobacterium tuberculosis* is associated with its growth and persistence in mice and guinea pigs. J Infect Dis 1998; 177: 1030–1035.
243. Pai S, Esen N, Pan X, Musser J M. Routine rapid *Mycobacterium* species assignment based on species-specific allelic variation in the 65-kilodalton heat shock protein gene (*hsp65*). Arch Pathol Lab Med 1997; 121: 859–864.
244. Nachamkin I, Kang C, Weinstein M P. Detection of resistance to isoniazid, rifampin, and streptomycin in clinical isolates of *Mycobacterium tuberculosis* by molecular methods. Clin Infect Dis 1997; 24: 894–900.
245. Nash K A, Gaytan A, Inderlied C B. Detection of rifampin resistance in *Mycobacterium tuberculosis* by use of a rapid, simple, and specific RNA/RNA mismatch assay. J Infect Dis 1997; 176: 533–536.
246. Goldrick M M, Kimball G R, Liu Q, Martin L A, Sommer S S, Tseng J Y-H. NIRCA™: A rapid robust method for screening for unknown point mutations. BioTechniques 1996; 21: 106–112.
247. Nash K A, Inderlied C B. Rapid detection of mutations associated with macrolide resistance in *Mycobacterium avium* complex. Antimicrob Agents Chemother 1996; 40: 1748–1750.
248. Scarpellini P, Braglia S, Brambilla A M et al. Detection of rifampin resistance by single-strand conformation polymorphism analysis of cerebrospinal fluid of patients with tuberculosis of the central nervous system. J Clin Microbiol 1997; 35: 2802–2806.
249. De Beenhouwer H, Lhiang Z, Jannes G et al. Rapid detection of rifampin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. Tuber Lung Dis 1995; 76: 425–430.
250. Jacobs Jr W R, Barletta R G, Udani R et al. Rapid assessment of drug susceptibilities by means of luciferase reporter phages. Science 1993; 260: 819–822.
251. Wilson S M, Al-Suwaidi Z, McNerney R, Porter J, Drobniewski F. Evaluation of a new rapid bacteriophage-based method for the drug susceptibility testing of *Mycobacterium tuberculosis.* Nature Med 1997; 3: 465–468.
252. Riska P F, Jacobs Jr W R, Bloom B R, McKitrick J, Chan J. Specific identification of *Mycobacterium tuberculosis* with the luciferase reporter mycobacteriophage: use of *p*-nitro-α-acetylamino-β-hydroxy propiophenone. J Clin Microbiol 1997; 35: 3225–3231.
253. Carrière C, Riska P F, Zimhony O et al. Conditionally replicating luciferase reporter phages: improved sensitivity for rapid detection and assessment of drug susceptibility of *Mycobacterium tuberculosis.* J Clin Microbiol 1997; 35: 3232–3239.
254. Martin-Casabona N, Xairó Mimó D, González T, Rosselló J, Arcalis L. Rapid method for testing susceptibility of *Mycobacterium tuberculosis* by using DNA probes. J Clin Microbiol 1997; 35: 2521–2525.
255. Rossau R, Traore H, de Beenhouwer H et al. Evaluation of the INNO-LiPA Rif. TB Assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother 1997; 41: 2093–2098.
256. Cooksey R C, Morlock G P, Glickman S, Crawford J T. Evaluation of a line probe assay kit for characterization of *rpoB* mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from New York City. J Clin Microbiol 1997; 35: 1281–1283.
257. Miyamoto J, Koga H, Kohno S, Tashiro T, Hara K. New drug susceptibility test for *Mycobacterium tuberculosis* using the hybridization protection assay. J Clin Microbiol 1996; 34: 1323–1326.
258. Cangelosi G A, Brabant W H, Britschgl T B, Wallis C K. Detection of rifampin- and ciprofloxacin-resistant *Mycobacterium tuberculosis* by using species-specific assays for precursor rRNA. Antimicrob Agents Chemother 1996; 40: 1790–1795.
259. Williams D L, Spring L, Gillis T P, Salfinger M, Persing D H. Evaluation of a polymerase chain reaction – based universal heteroduplex generator assay for direct detection of rifampin susceptibility of *Mycobacterium tuberculosis* from sputum specimens. Clin Infect Dis 1998; 26: 446–450.
260. Pretorius G S, Sirgel F A, Schaaf H S, van Helden P D, Victor T C. Rifampin resistance in *Mycobacterium tuberculosis* – rapid detection and implication in chemotherapy. South Afric Med J 1996; 86: 50–55.
261. Goyal M, Shaw R J, Benerjee D K, Coker R J, Robertson B D, Young D B. Rapid detection of multidrug-resistant tuberculosis. Eur Resp J 1997; 10: 1120–1124.
262. Kapur V, Whittam T S, Musser J M. Is *Mycobacterium tuberculosis* 15,000 years old? J Infect Dis 1994; 170: 1348–1349.
263. Feizabadi M M, Robertson I D, Cousins D V, Hampson D J. Genomic analysis of *Mycobacterium bovis* and other members of the *Mycobacterium tuberculosis* complex by isoenzyme analysis and pulsed-field gel electrophoresis. J Clin Microbiol 1996; 34: 1136–1142.

264. Go M F, Kapur V, Graham D Y, Musser J M. Population genetic analysis of *Helicobacter pylori* by multilocus enzyme electrophoresis: extensive allelic diversity and recombinational population structure. J Bacteriol 1996; 178: 3934–3938.
265. Maiden M C J, Bygraves J A, Feil E et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 1998; 95: 3140–3145.
266. Nelson K, Wang F-S, Boyd E F, Selander R K. Size and sequence polymorphism in the isocitrate dehydrogenase kinase/phosphatase gene (*aceK*) and flanking regions in *Salmonella enterica* and *Escherichia coli.* Genetics 1997; 147: 1509–1520.
267. Nelson K, Selander R K. Evolutionary genetics of the proline permease gene (*putP*) and the control region of the proline utilization operon in populations of *Salmonella* and *Escherichia coli.* J Bacteriol 1992; 174: 6886–6895.