diagnosis to death (10). Delayed recognition of drug resistance, which results in delayed initiation of effective therapy, is one of the major factors contributing to MDRTB outbreaks, especially in health-care facilities (11,12). In most countries, MDRTB has increased in incidence and interferes with TB control programs, particularly in developing countries, where prevalence rates are as high as 48% (13,14). The high infection and death rates pose an urgent challenge to rapidly detect cases.

In the past few years, genetic and molecular insights have unraveled the mechanisms involved in the acquisition of drug resistance by *Mycobacterium tuberculosis* (MTB), concomitant with the development of various molecular strategies to rapidly detect MDRTB. In this review, we examine the status of the mechanisms of resistance to antitubercular drugs.

MDRTB and the Mechanisms of Resistance

Currently TB is treated with an initial intensive 2-month regime comprising multiple antibiotics—rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB) or streptomycin (SM)—to ensure that mutants resistant to a single drug do not emerge (15). The next 4 months, only RIF and INH are administered to eliminate any persisting tubercle bacilli. INH and RIF, the two most potent antituberculous drugs, kill more than 99% of tubercule bacilli within 2 months of initiation of therapy (16,17). Along with these two drugs, PZA, with a high sterilizing effect, appears to act on semidormant bacilli not affected by any other antitubercular drugs (18). Using these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months. Therefore, the emergence of strains resistant to either of these drugs causes major concern, as it leaves only drugs that are far less effective, have more toxic side effects, and result in higher death rates, especially among HIV-infected persons.

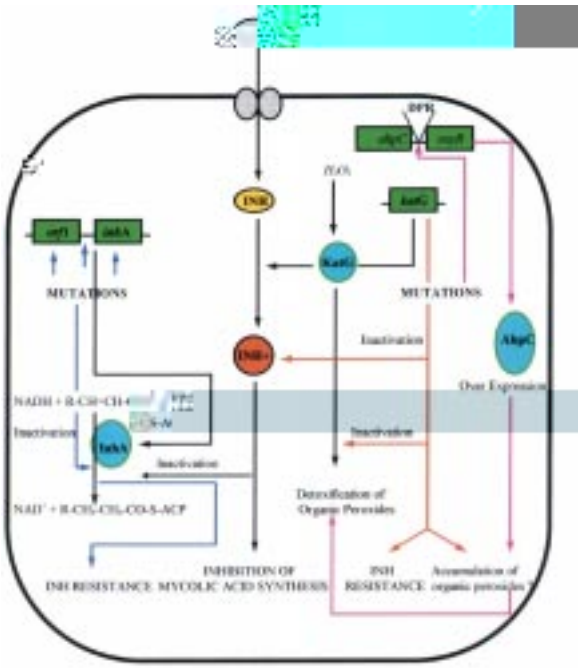
The phrase "MDR state" in mycobacteriology refers to simultaneous resistance to at least RIF and INH (19) (with or without resistance to other drugs). Genetic and molecular analysis of drug resistance in MTB suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation (20) or by titration of the drug through overproduction of the target (21). MDRTB results primarily from accumulation of mutations in individual drug target genes (Table). The probability of resistance is very high for less effective antitubercular drugs such as thiacetazone, ethionamide, capreomycin, cycloserine, and viomycin (10^{-3}); intermediate for drugs such as INH, SM, EMB, kanamycin, and p-amino salicylic acid (10^{-6}); and lowest for RIF (10^{-8}) (22,23). Consequently, the probability of a mutation is directly proportional to the bacterial load. A bacillary load of 10^9 will contain several mutants resistant to any one antitubercular drug (24). Because the mutations conferring drug resistance are chromosomal, the likelihood of a mutant being simultaneously resistant to two or more drugs is the product of individual probabilities; thus the probability of MDR is multiplicative. Resistance to a drug does not confer any selective advantage to the bacterium unless it is exposed to that drug (19). Under such circumstances, the sensitive strains are killed

Table. Gene loci involved in conferring drug-resistance in *Mycobacterium tuberculosis*

Drug	Gene	Product	Reported frequency in resistant strains[a] (%)	Reference
Rifampicin	*rpo*B	B-subunit of RNA polymerase	>95	45-48,68-71
Isoniazid	*kat*G	Catalase-peroxidase	60-70	39-48
	*oxy*R-*ahp*C	Alky hydro-reductase	~20	36
INH-Ethionamide	*inh*A	Enoyl-ACP reductase	<10	46-48
Streptomycin	*rps*L	Ribosomal protein S12	60	46-48
	rrs	16s rRNA	<10	113-117
Fluoroquinolone	*gyr*A	DNA gyrase	>90	107
Pyrazinamide	*pnc*A	Amidase	70-100	92-94
Ethambutol	*emb*CAB	EmbCAB	69	88

[a]Mutation frequencies are as determined by sequencing and polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis.

and the drug-resistant mutants flourish. When the patient is exposed to a second course of drug therapy with yet another drug, mutants resistant to the new drug are selected, and the patient may eventually have bacilli resistant to two or more drugs. Serial selection of drug resistance, thus, is the predominant mechanism for the development of MDR strains; the patients with MDR strains constitute a pool of chronic infections, which propagate primary MDR resistance. In addition to accumulation of mutations in the individual drug target genes, the permeability barrier imposed by the MTB cell wall can also contribute to the development of low-level drug resistance. Studies addressing resistance to SM have found evidence of such a two-step mechanism for the development of drug resistance (119,120).



Resistance to INH

INH (isonicotinic acid hydrazide, 4-pyridinecarboxylic acid hydrazide), highly active against the MTB complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*), has very low MICs (0.02 µg/ml to 0.06 µg/ml) (25). The mechanism of action of INH, as well as mechanisms conferring INH resistance, are complex and not completely understood (Figure 2). However, evidence suggests that INH inhibits the biosynthesis of cell wall mycolic acids (long-chain α-branched ß-hydroxylated fatty acids), thereby making the mycobacteria susceptible to reactive oxygen radicals and other environmental factors. Activation of INH to an unstable electrophilic intermediate requires the enzyme catalase-peroxidase (KatG, coded by *kat*G) and an electron sink (hydrogen peroxide) (26), although hydrazine formed after INH spontaneously decomposes may also mediate activation of INH (27). Nevertheless, KatG is the only enzyme capable of activating INH, and consequently, KatG mutant MTB strains are invariably INH resistant.

Early studies by Middlebrook demonstrated that INH resistance was associated with loss of catalase activity (28). Genetic studies demonstrated that transformation of INH-resistant *M. smegmatis* and MTB strains with a functional KatG restored INH susceptibility and put forth the hypothesis that *kat*G deletion may cause INH

activity did not have this strict linear relationship. Characterization of the *oxy*R-*ahp*C region further demonstrated that mutations responsible for AhpC upregulation occurred at low frequencies and were primarily G>C to A>T transitions localized in the *oxy*R-*ahp*C intervening region (36). Although the sequence alterations in the *oxy*R-*ahp*C region were predominantly restricted to INH-resistant isolates, not all alterations detectably increased the AhpC levels. The apparent rarity of AhpC upregulation among INH-resistant and *kat*G mutant isolates could be attributed partially to the rare occurrence of MTB strains with complete *kat*G deletion (38-41,44). Alternatively, among *kat*G mutant isolates, selection of AhpC upregulatory mutations may be subject to the selective pressure exerted by residual catalase-peroxidase activity (36). However, AhpC upregulation was not observed among MTB isolates with *kat*G315 codon mutations, which reportedly lead to more than a 20-fold decrease in KatG activity and confer high MICs against INH (>90 µg/ml) (42, 43). This inconsistency and rarity of AhpC upregulation among *kat*G mutant INH-resistant isolates indicates a more complex relationship between the two and underlines the need for in-depth studies to determine precisely the conditions regulating AhpC expression.

Clinical studies to validate the paradigm of *kat*G deletions and INH resistance showed that complete deletion rarely occurred (38-41). We constructed a 35-mer oligonucleotide probe specific for *kat*G gene. Southern hybridization demonstrated the presence of *kat*G in all INH-resistant isolates, precluding complete deletion of *kat*G gene as a dominant mechanism for INH resistance (44). Previous studies using polymerase chain reaction (PCR) amplification had also established these findings; sequence analysis of *kat*G from INH-resistant strains showed randomly distributed mutations, including point mutations and deletions and insertions of up to 1 to 3 bases (38-41). These mutations could disrupt the *kat*G gene, leading to the production of an inactive gene product or a gene product with compromised peroxidative activity. PCR amplification of the *kat*G gene followed by single strand conformational polymorphism (SSCP) detected mobility shifts supporting the presence of these mutations and thereby INH resistance.

Our analysis of the *kat*G gene by PCR-SSCP resulted in the amplification of the 237 bp fragment of the *kat*G gene and demonstrated a 67.3% (n = 19) correlation between mutations in the *kat*G gene and INH resistance (45). The results were consistent with those from earlier studies indicating that *kat*G gene mutations had a correlation rate of less than 60% to 70% with INH resistance (46-48). Sequence analysis of INH-resistant strains demonstrating altered SSCP patterns showed that the most common mutation was G>T transversion in codon 463 (42). In this G>T change, Leu is substituted for Arg, and the restriction site for *Nci*I and *Msp*I is lost (40). Polymorphism in the *kat*G locus can then be easily detected by restriction digestion. Recent kinetic and spectroscopic studies have demonstrated striking similarities between KatG from wild-type strains and the R463L mutant isolates (49). Both enzymes had similar visible and electron-paramagnetic-resonance spectra and similar ability to oxidize INH and inactivate InhA. Further, when the INH-resistant *kat*G-defective strains of *M. smegmatis* with wild-type *kat*G or the R463L *kat*G were transformed, INH susceptibility was restored to about the same extent (50). These similarities do not support the contention that the R463L mutation of *kat*G allows discrimination against INH as a substrate and thereby confers resistance to INH. Although the exact role of the R463L mutation of *kat*G requires further scrutiny, this mutation may be a frequent polymorphism and may not affect INH susceptibility.

Other common mutations resulting in an attenuated KatG have been identified primarily as missense mutations that result in single amino acid substitutions (46-48). While the data point towards mutations in the *kat*G gene as the dominant mechanism for INH resistance, they also point to other factors that could mediate MTB acquisition of resistance to INH.

Mutations in the *oxy*R regulon, from which *Ahp*C is divergently transcribed, could explain the acquisition of INH resistance in the remaining INH-resistant isolates (33,51). OxyR confers high-level intrinsic resistance to INH in *Escherichia coli* and *Salmonella* Typhimurium; mutations in the *oxy*R or *Ahp*C restore INH susceptibility in these species (51). The MTB *oxy*R regulon is much smaller than in *M. leprae* and other mycobacteria—because of two important deletions of 29 bp and 372 bp (32,52). In addition to these deletions, the *oxy*R regulon carries many frame shift mutations, which result in low expression of this regulon and eventually lead to low-level expression of AhpC (consistent

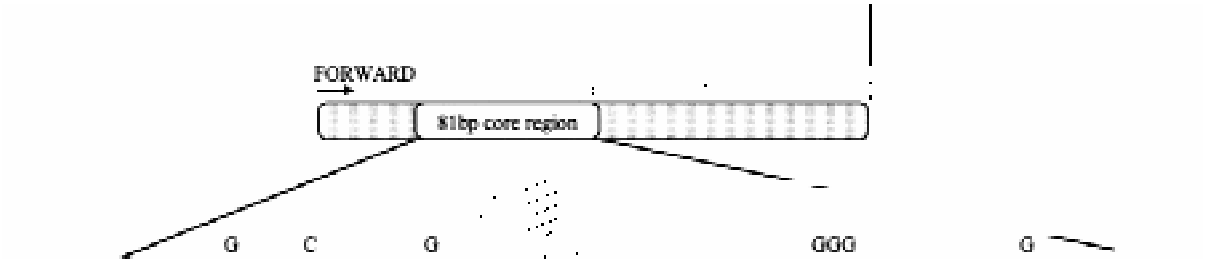
with the finding of low-level expression of AhpC in INH-sensitive strains vs. INH-resistant strains) (33). A related member of the genus resistant to INH, *M. leprae*, however, has a complete *oxy*R-*ahp*C region that is transcriptionally fully active and may play a role in the detoxification of active INH intermediates (52). By analogy, therefore, the loss of the OxyR function, in conjunction with its putative effects on *ahp*C expression, could explain the exquisite specificity of INH for the MTB complex. However, evidence from recent studies does not indicate a direct role for *oxy*R or the *ahp*C genes in determining susceptibility to INH (36,37). Polymorphisms in *oxy*R do not have any preferential predisposition and exist among both INH-resistant and -susceptible isolates with about the same frequency (36). The relationship of AhpC overexpression to INH resistance is more complex. Earlier observations based on transformation of *M. smegmatis* strains suggested a possible involvement of AhpC overexpression in acquiring INH resistance (53). Transformation of *M. smegmatis* isolates with multicopy constructs of *ahp*C led to almost a fivefold increase in the MIC for INH. However, an increasing body of evidence precludes any direct role of AhpC in determining INH susceptibility among MTB isolates. MTB transformants bearing multicopy constructs of *ahp*C did not demonstrate significant increase in the MIC for INH, thus any direct role for AhpC in acquisition of INH resistance was ruled out (37).

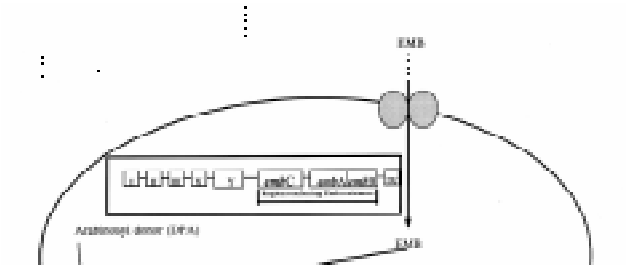
Efforts to determine the factors involved in resistance to INH led to the discovery of the *inh*A locus, which was proposed as the primary target for coresistance to INH and ethionamide (54). This locus is composed of two open reading frames (ORFs), designated *orf*1 and *inh*A, separated by a 21-bp noncoding region. InhA, an enoyl-ACP reductase (55), more than 40% homologous to the EnvM protein, catalyzes an early step in fatty acid synthesis among enterobacteria. Like EnvM, InhA activity is also thought to use NAD(H) as cofactor. INH susceptibility could result from incorporation of iso-NAD, which is formed as a consequence of the action of KatG on INH, and thus hinders the enzymatic activity of InhA and blocking fatty acid synthesis (56). A T>G transversion, observed in few of the resistant strains, at position 280 in the *inh*A gene, results in the ser94 to ala94 replacement (54). This replacement, thought to alter the binding affinity of InhA to NAD(H), ultimately results in INH resistance (57). Alternatively, because of mutations in the putative promoter region, hyperexpression of InhA could result in INH resistance.

Studies conducted in clinical settings to provide corroborating evidence of mutations in the *inh*A locus and INH resistance have shown approximately 10% correlation (46-48). Analysis of 37 INH-resistant isolates by Kapur et al. demonstrated no ser94-ala94 substitution in the resistant isolates. Only one isolate had a missense mutation: ATC>ACC at position 47, resulting in substitution of Ile16 by Thr16. Morris et al. also demonstrated the lack of mutations in the *inh*A gene among 42 INH-resistant MTB isolates. However, five of the INH-resistant isolates showed single nucleotide mutations in the putative *inh*A regulatory region upstream of *orf*1.

Subsequent biochemical characterization of InhA function demonstrated that it catalyzed the reduction of 2-trans-octenoyl-acyl carrier protein and also that protein of enoyl CoA esters (58-590, thereby acting at the final step in chain elongation in fatty acid synthesis (58). This observation contradicted earlier biochemical evidence suggesting that an enzyme involved in the synthesis of an unsaturated 24-carbon fatty acid was the target for activated INH (60,61). Thus, the targets identified biochemically and by complementation of *M. smegmatis* are different. Lipid pulse labeling experiments demonstrated that the lipid biosynthetic response of *M. smegmatis* and MTB after exposure with INH were different (62), indicating a different mechanism of action for the INH intermediate in the two species. Transformation of *M. smegmatis* with single-copy alleles of mutant *inh*A loci did not result in significant resistance to INH, indicating the presence of a different promoter in *M. smegmatis*. Further, the inability of multicopy vector constructs bearing the *inhA* gene to significantly increase the MIC for INH provided substantiating evidence for the limited involvement of this locus in mediating INH resistance among MTB isolates. These data, along with clinical evidence, preclude the likelihood that *inh*A is the primary target for the activated form of INH.

Functional characterization of *inh*A mutations, occurring with *kat*G mutations (as observed in isolates with very high MICs) (46) in relation to lipid metabolism of INH-resistant isolates, could perhaps resolve this discrepancy


FORWARD
81bp core region
G
C
G
GGG
G



genetic basis for EMB resistance. Using target overexpression by a plasmid vector, Belanger et al. cloned the *emb* locus from an EMB-resistant strain of *M. avium* (86). Transformation of this *emb* locus conferred resistance to *M. smegmatis* mc^2155 strain and also demonstrated that the level of resistance conferred depended on the copy number of the gene, which was consistent with the notion of drug resistance due to target overexpression. Site-directed mutagenesis and overlapping clone analysis localized a 9.8-kb EMB resistance locus, subsequently shown to be ubiquitous among mycobacteria. Sequence analysis of this locus revealed three complete ORFs—designated *emb*R, *emb*A, and *emb*B. The *emb*R ORF is separated by a 178 bp divergent promoter region from the *emb*A and *emb*B ORFs. Characterization of the *emb*R ORF showed that the region was strongly homologous with a family of transcriptional activators of *Streptomyces* and thus could play a role in modulating the expression of *emb*A and *emb*B. Importantly, the *emb*B ORF lacks a potential ribosome binding site and is thus translationally coupled to *emb*A, which suggests that a heterodimeric enzyme complex may be the target for EMB. Mapping studies further demonstrated that both *emb*A and *emb*B, along with the divergent promoter region, were essential to EMB resistance.

In contrast to the organization of the emb locus in *M. avium*, molecular genetic approaches applied to MTB revealed a highly conserved 14-kb region comprising three homologous ORFs designated embC, embA, and embB preceded by a predicted coding region and by orfX (which encodes a putative protein belonging to the short chain alcohol dehydrogenase family) (87). Primer extension analysis of the emb region supported the notion of its organization as an operon and further indicated the polycistronic nature of its transcripts. The emb genes are translationally coupled the absence of any untranslated intercistronic region between the emb genes so indicated). However, the presence of a secondary stem loop structure between the embA and the embB genes indicates that the embB gene in MTB could be differentially regulated. The embCAB proteins are believed to be integral membrane proteins, consistent with their role in the synthesis of various arabinan-linkage motifs of the arabinogalactan and lipoarabinomannan (86,87).

Identification of the *emb*CAB genes prompted a detailed analysis of the molecular mechanisms responsible for conferring resistance to EMB in MTB isolates. Preliminary studies documented among EMB-resistant isolates missense substitutions in the conserved *emb*B codon 306 that coded for methionine; their role in conferring resistance to EMB was confirmed by gene tranfer assays (87). Recent analysis of the *emb*CAB region has confirmed the predominance of *emb*B Met306 substitutions among EMB-resistant clinical isolates of MTB (approximately 89% among EMB-resistant isolates with single amino

resistance to PZA (89). Naturally resistant strains of *M. bovis* lack the enzyme Pzase, which hydrolyzes PZA to pyrizinoic acid, the presumed active form of PZA (90,91). PZA in this context is similar to INH; it is transported as a neutral species into the cell, where it is converted into its active form. This notion was strengthened by evidence provided by in vitro studies that demonstrated the susceptibility of PZA-resistant MTB and *M. bovis* to pyrizinoic acid. MTB Pzase has both pyrazinamidase and nicotinamidase activities (90). Using sequence information of *E. coli* nicotinamidase, Scorpio and Zhang isolated the mycobacterial *pnc*A gene, which codes for the amidase (92). Characterization of the *pnc*A gene from *M. bovis* isolates identified a single point mutation that results in the substitution of His to Asp at position 57. This substitution results in the production of an ineffective Pzase in *M. bovis* strains. Point mutations in the *pnc*A gene of PZA-resistant MTB strains were also identified. Substitution of Cys138 with Ser, Gln141 with Pro, and Asp63 with His and deletion G nucleotide at positions 162 and 288 resulted in a defective Pzase. Transformation of Pzase-resistant strains with functional construct of MTB *pnc*A gene restored susceptibility to PZA, providing further evidence that mutations in the *pnc*A gene were responsible in conferring the resistant phenotype. Subsequent characterization of the *pnc*A gene from clinical isolates of MTB confirmed these findings (93,94). Mutations including missense alterations, nucleotide insertions or deletions, and termination mutations have been found in the *pnc*A gene from PZA-resistant MTB isolates. These sequence alterations are interspersed along the entire length of the *pnc*A gene, demonstrate limited degree of clustering, and vary in frequency from 70% to 100% (93,94). The absence of correlating mutations in the *pnc*A gene from PZA- resistant MTB isolates indicates that perhaps at least one additional mechanism mediates resistance to PZA.

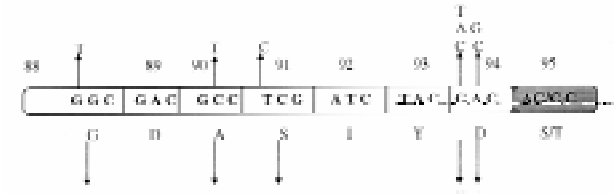
The cellular target for PZA, however, has not been identified, although the apparent similarity of PZA to nicotinamide suggests that enzymes involved in pyridine nucleotide biosynthesis are probable targets. Implication of the *pnc*A gene in conferring PZA-resistant phenotype has profound clinical applications. Application of PCR-SSCP for detection of mutations in the *pnc*A gene could help circumvent the difficulties in determining PZA susceptibilities (96) and rapidly discriminate between MTB and *M. bovis* (96).

Resistance to Fluoroquinolones (FQ)

FQs as antimycobacterial agents were first described in 1984 and have primarily been used as therapeutic alternatives in MDRTB cases (97). DNA gyrase (Gyr), a member of the type II DNA topoisomerases (98), is the primary target for FQ action. Gyr introduces negative supercoils in closed circular DNA molecules and is a heterotetramer (A_2B_2), coded by *gyr*A and *gyr*B respectively (99,100). Quinolone sensitivity is determined by the GyrA protein, which contains the cleavage/religation activity (100), while GyrB contains the intrinsic coumarin-sensitive ATPase activity (101).

FQs, synthetic derivatives of nalidixic acid, act by inhibiting DNA supercoiling and relaxation activity of Gyr without affecting the ATPase activity (102) and enhance the rate of DNA cleavage by Gyr. Quinolone-mediated cleavage of double-stranded DNA results in a 4 bp 5' overhangs on either strand, to which GyrA subunits become attached covalently by O^4 phosphotyrosine bond (103). Gyr catalyzes the cutting of DNA, denaturation of the overhang, and strand separation. The exact mechanism of inhibition of Gyr activity with respect to quinolones remains unknown. However, quinolone drugs bind with a greater affinity to single-stranded DNA than double-stranded DNA and possibly do not bind to Gyr at all (104). Consequently, by binding to the single-stranded DNA, the quinolones may inhibit religation, thereby imposing an effective transcriptional block (105), culminating in cellular death. However, questions about the specific interaction of quinolones and the Gyr/DNA complex remain unsolved (106).

Cloning and expression of the MTB *gyr*A and *gyr*B genes allowed mapping of mutations that confer resistance to FQs (107). Mutations were found to be clustered in a small region in GyrA that is close, approximately 40 residues amino-terminal, in the linear amino acid sequence to the active site tyrosine, Tyr122 (*E. coli* numbering) (108). Other single amino substitutions, for residues 88 to 94, were also identified in ciprofloxacin-resistant MTB isolates (Figure 5). Because polymorphism encountered at codon 95 (Ser95>Thr95) occurred in both resistant and susceptible isolates, it may not be involved in


88
89
90
91
92
93
94
95
GGC
GAC
GCC
TCG
ATC
G
D
A
S
I
Y
D
S/T

suggesting the probable role of cell wall permeability barrier in conferring SM resistance (119). More recently, it has been demonstrated that membrane-active substances augmented the MIC for SM in strains with alterations in the *rrs* genes, thus providing further evidence for a probable role of the MTB-permeability barrier in mediating resistance to SM (120).

Resistance to Other Drugs

Related aminoglycosides such as kanamycin, amikacin, and paromomycin demonstrate no obvious cross-resistance to SM and thus are alternatives in cases of SM resistance. Viomycin and capreomycins are bacteriostatic agents that act by binding to the 50S ribosomal subunit and inhibit the translocation reaction (111). Although cross-resistance between viomycin and capreomycin does occur, the exact mechanism for

possible. Clearly, a concerted global effort is required to defeat TB resurgence.

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Dr. Ashok Rattan is an additional professor, Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India. His research focuses on drug resistance and application of cost-effective methods for surveillance of MDRTB strains.

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