

## Molecular Characterization of Multidrug-Resistant Isolates of *Mycobacterium tuberculosis* from Patients in North India

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Received 17 April 2001/Returned for modification 29 June 2001/Accepted 1 November 2001

The World Health Organization has identified India as a major hot-spot region for *Mycobacterium tuberculosis* infection. We have characterized the sequences of the loci associated with multidrug resistance in 126 clinical isolates of *M. tuberculosis* from India to identify the respective mutations. The loci selected were *rpoB* (rifampin), *katG* and the ribosomal binding site of *inhA* (isoniazid), *gyrA* and *gyrB* (ofloxacin), and *rpsL* and *rrs* (streptomycin). We found known as well as novel mutations at these loci. Few of the mutations at the *rpoB* locus could be correlated with the drug resistance levels exhibited by the *M. tuberculosis* isolates and occurred with frequencies different from those reported earlier. Missense mutations at codons 526 to 531 seemed to be crucial in conferring a high degree of resistance to rifampin. We identified a common Arg463Leu substitution in the *katG* locus and certain novel insertions and deletions. Mutations were also mapped in the ribosomal binding site of the *inhA* gene. A Ser95Thr substitution in the *gyrA* locus was the most common mutation observed in ofloxacin-resistant isolates. A few isolates showed other mutations in this locus. Seven streptomycin-resistant isolates had a silent mutation at the lysine residue at position 121. While certain mutations are widely present, pointing to the magnitude of the polymorphisms at these loci, others are not common, suggesting diversity in the multidrug-resistant *M. tuberculosis* strains prevalent in this region. Our results additionally have implications for the development of methods for multidrug resistance detection and are also relevant in the shaping of future clinical treatment regimens and drug design strategies.

Recent years have witnessed a dramatic upsurge in cases of drug-resistant *Mycobacterium tuberculosis* infections. The acquisition of resistance by the bacterium is a random event, and in a given mycobacterial population, 1 in 10<sup>6</sup> bacteria mutates to develop isoniazid resistance, while 1 in 10<sup>8</sup> mutates to develop rifampin resistance (8). The chance that a bacterium will acquire multidrug resistance (defined as resistance to at least rifampin and isoniazid) is thus 10<sup>-14</sup> (8). The drug-resistant phenotype may get selected due to single-drug therapy, poor patient adherence, and improper diagnosis. With the AIDS pandemic fuelling increasing numbers of multidrug-resistant (MDR) strains of *M. tuberculosis*, urgent measures need to be taken to contain this scourge (2). A recently published World Health Organization report reviewing the global status of tuberculosis has pointed to an increasing incidence of drug-resistant tuberculosis (5). The highest rates of MDR tuberculosis have been reported in Nepal (48.0%), Gujarat, India (33.8%), New York City (30.1%), Bolivia (15.3%), and Korea (14.5%). Furthermore, the report points to the alarming increase in the number of tuberculosis patients in the Indian subcontinent, with India being singled out as having the greatest burden of tuberculosis patients. Three different studies

from North and Northwest India indicate an increasing incidence of acquired MDR tuberculosis (9, 12, 15). Furthermore, the incidence of primary MDR tuberculosis in North India was put at 3.3% in one of the studies (12).

While there is lot of literature on the molecular epidemiology and characterization of MDR isolates from the United States and Europe, the same is not true for the Indian strains. The prevalence of drug-resistant tuberculosis in North India is known, but no serious efforts have been made to identify the drug resistance genotypes or their prevalence in the community. The present study was undertaken to characterize mutations prevalent in patient isolates of *M. tuberculosis* from North India with respect to a few of these drug target loci. We have chosen to look at the drug target genes for the drugs rifampin, isoniazid, streptomycin, and fluoroquinolones, which are commonly prescribed for the treatment of tuberculosis in North India. The first three drugs are the frontline drugs in tuberculosis chemotherapy, while fluoroquinolones are prescribed for drug-resistant cases. The loci studied were *rpoB* (RNA polymerase B subunit), *katG* (catalase-peroxidase), *inhA* (enoyl coenzyme A reductase), *rpsL* (ribosomal protein S12), *rrs* (16S rRNA), and *gyrAB* (DNA gyrase A and B). The present study, in combination with the molecular epidemiology of the drug-resistant strains, will help track the routes of infection and the extent of drug-resistant tuberculosis in this region. The elucidation of common and novel mutations in these loci could form the basis for the creation of new diagnostic tools and the

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TABLE 1. Primers used in the study to amplify and sequence the different loci, amplicon sizes, annealing temperatures, and amplicon positions on the respective genes

Gene (accession no.)	Primer	Sequence	Annealing temp (°C)	Position (nt)	Amplicon size (bp)
<i>rpoB</i> (L27989)	Forward	GGG AGC GGA TGA CCA CCC	60	2266	350
	Reverse	GCG GTA CGG CGT TTC GAT GAA C		2615	
<i>katG</i> (X68081)	Forward	GCC CGA GCA ACA CCC	60	3	237
	Reverse	ATG TCC CGC GTC AGG		239	
	Forward	CGA GGA ATT GGC CGA CGA GTT	55	1187	414
	Reverse	CGG CGC CGC GGA GTT GAA TGA		1600	
<i>inhA</i> regulator sequence	Forward	CCT CGC TGC CCA GAA AGG GA	45	Upstream of <i>inhA</i> gene	248
Reverse	ATC CCC CGG TTT CCT CCG GT				
<i>gyrA</i> (L27512)	Forward	CAG CTA CAT CGA CTA TGC GA	45	2383	320
	Reverse	GGG CTT CGG TGT TAC CTC AT		2702	
<i>gyrB</i> (L27512)	Forward	CCA CCG ACA TCG GTG GAT T	55	1538	428
	Reverse	CTG CCA CTT GAG TTT GTA CA		1965	
<i>rpsL</i> (X70995)	Forward	GGC CGA CAA ACA GAA CGT	54	5' noncoding region	505
	Reverse	GTT CAC CAA CTG GGT GAC			
<i>rrs</i> (Z83862)	Forward	TTG GCC ATG CTC TTG ATG CCC	54	141	1140
	Reverse	TGC ACA CAG GCC ACA AGG GA		1280	

development of novel strategies that can be used to combat the menace of drug-resistant *M. tuberculosis*.

#### MATERIALS AND METHODS

**Sources of *Mycobacterium* isolates.** *Mycobacterium* isolates were collected from patients reporting to the outpatient departments of hospitals in northern India, primarily New Delhi and its neighboring regions. Another source of samples was the National Mycobacterial Repository at the Central Jalma Institute for Leprosy, Agra, India. The samples collected over a 3-year period from 1995 to 1998 were included in the present study. A large number of the patients (75%) had histories of previous treatment and were on antitubercular treatment at the time of collection of their sputa. Most of these patients had been through various degrees of antitubercular drug therapy during the previous 20 months. Rifampin and isoniazid were the most common drugs used in these regimens. Sputum samples collected from patients reporting with pulmonary tuberculosis were processed by standard methods and were streaked onto Lowenstein-Jensen slants. Most of them were coded with ICC numbers (ICC01, ICC201, etc.). The samples were biochemically characterized as belonging to the *M. tuberculosis* complex by nitrate reduction, niacin production, and BACTEC NAP tests. Drug susceptibility profiles were evaluated by the proportion method. The drugs tested were rifampin (Lupin, India), isoniazid (Lupin), ofloxacin (Ranbaxy, India), and streptomycin (Lupin). The MICs at which the isolates were considered resistant were as follows: 10 µg/ml for rifampin, 1 µg/ml for isoniazid, 2 µg/ml for ofloxacin, and 2 µg/ml for streptomycin. The numbers of drug-resistant isolates included in the study were as follows: for rifampin,  $n = 94$ ; for isoniazid,  $n = 74$ ; for streptomycin,  $n = 14$ ; and for ofloxacin,  $n = 68$ . A total of 126 isolates were tested. Thirty-six isolates were resistant to a single drug, 66 isolates were resistant to two drugs, 22 isolates were resistant to three drugs, and 4 isolates were resistant to four drugs.

**DNA isolation and PCR.** The isolates were cultured on Lowenstein-Jensen slants. The colonies were scraped, resuspended in 500 µl of TE (10 mM Tris, 1 mM EDTA [pH 8]), and killed by freezing at  $-70^{\circ}\text{C}$  followed by heating at  $80^{\circ}\text{C}$ . This cycle was repeated thrice to kill all the bacteria. The DNA was isolated (by treatment with cetyltrimethylammonium bromide in the presence of 0.7 M so-

dium chloride) and amplified by standardized protocols as reported previously (21).

Table 1 lists the sequences of the different primers used and their positions on the corresponding genes. It also lists the amplicon sizes generated and the annealing temperatures used for PCR cycling. The temperatures used for all cycles were identical for all PCRs except for that for annealing, the temperature of which varied for each primer pair. Briefly, 35 cycles of  $94^{\circ}\text{C}$  for 1 min,  $45$  to  $60^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 2 min were used to amplify the loci. The samples were resolved in a 2% agarose gel, and the specific bands were excised. DNA was extracted from the gel slices with a QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions. The purified DNA was resuspended in sterile double-distilled water and was used for the sequencing studies.

**DNA sequencing.** Sequencing of the amplicons was carried out with an ABI Prism 377 automated DNA sequencer (ABI Prism). PCR sequencing was carried out with a BigDye terminator kit (ABI Prism) according to the manufacturer's instructions. The Sequencing Analysis (version 3.3) software package was used to analyze the gel information. The sequences generated with the program were compared to their respective wild-type sequences by using MegAlign software (Lasergene; DNASTAR, Inc., Madison, Wis.).

#### RESULTS

Mutations in the hot-spot regions of various loci were characterized. The results are summarized in Table 2. On the basis of the drug susceptibility profile for an isolate, the corresponding loci (representing the drug target gene) were amplified and sequenced. The largest number of samples was obtained from New Delhi, followed by Chandigarh, Ahmedabad, Agra, Bangalore, and Shimla, with a few samples coming from Jaipur and Chennai. Except for Chennai and Bangalore, all the cities are located in North India. We could establish a previous treatment history for patients from whom 94 of the 126 isolates

TABLE 2. Characteristics of *M. tuberculosis* isolates from patients

Strain no.	Geographic location	Treatment history <sup>a</sup>	Drug susceptibility <sup>b</sup>	Polymorphism <sup>c</sup>			
				<i>rpo</i>	<i>katG</i> or <i>inhA</i>	<i>gyrA</i>	<i>rpsL</i>
ICC14	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	D516V	N35D, NA at second locus	S95T	
ICC19	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	L511L, S531L	R463L	S95T	
ICC23	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	L511L, S531L	NA		
ICC98	New Delhi	-	I <sup>r</sup> , O <sup>r</sup>		R463L, Inh (C/T)	S95T	
ICC100	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>	S531L	R463L	S95T	NM
ICC101	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	S531L		S95T	
ICC102	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S531L	NM	S95T	
ICC103	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	L511L, N518T	NM	A90A, S95T	
ICC104	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	D516V	Δ30C, R463L	S95T	
ICC105	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	K527N	R463L		
ICC107	New Delhi	-	R <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>	N518T, R528P		S95T	NM
ICC109	New Delhi	+	I <sup>r</sup>		NM		
ICC111	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , S <sup>r</sup>	S531W	Insertion 185C		K121K
ICC114	New Delhi	+	I <sup>r</sup>		R463L		
ICC115	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	S531W	Insertion 98A, R463L		
ICC123	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	R528P	NA	S95T	
ICC124	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	H526Y	R463L		
ICC125	New Delhi	+	R <sup>r</sup>	L511L, S531L			
ICC128	New Delhi	+	R <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>	H526Y, R528H		S95T	K121K
ICC129	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , S <sup>r</sup>	R528P	R463L		K121K
ICC147	New Delhi	-	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S531W	Δ109G, R463L	S95T	
ICC203	New Delhi	+	R <sup>r</sup>	D516V			
ICC204	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	L521L, K527N	R463L		
ICC205	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	D516V	NA		
ICC206	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	S531W	NM		
ICC208	New Delhi	-	R <sup>r</sup>	D516V	R463L		
ICC209	New Delhi	-	R <sup>r</sup>	D516V			
ICC210	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	L511V, N518T		S95T	
ICC211	New Delhi	+	I <sup>r</sup> , O <sup>r</sup>		R463L	S95T	
ICC212	New Delhi	-	R <sup>r</sup>	S531L			
ICC213	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	R528H, S531W	R463L, Inh (C/T)		
ICC214	New Delhi	+	I <sup>r</sup>		R463L		
ICC215	New Delhi	+	R <sup>r</sup>	S531L			
ICC216	New Delhi	+	R <sup>r</sup> , S <sup>r</sup>	S531L			K121K
ICC217	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S531L	R463L	S95T	
ICC218	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S522Q	R463L	S95T	
ICC219	New Delhi	+	I <sup>r</sup>		T12P, R463L		
ICC220	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>	S531W	R463L, Inh (T/A)	D94G, S95T	NM
ICC221	New Delhi	-	R <sup>r</sup> , O <sup>r</sup>	L521L		D94A, S95T	
ICC222	New Delhi	-	O <sup>r</sup>			S95T	
ICC223	New Delhi	-	R <sup>r</sup> , O <sup>r</sup>	H526Y		S95T	
ICC225	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	S531L		S95T	
ICC237	New Delhi	-	R <sup>r</sup> , O <sup>r</sup>	D516G		S95T	
ICC239	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	D516V	R463L		
ICC240	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	D516V	R463L		
ICC242	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	L511V		S95T	
ICC244	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S531L	A61T, R463L	S95T	
ICC246	New Delhi	+	I <sup>r</sup> , O <sup>r</sup>		Insertion 185C, R463L	S95T	
ICC275	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	H526Y		S95T	
ICC277	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>	H526Y	Δ30C	D94A, S95T	K121K
ICC284	New Delhi	-	O <sup>r</sup>			NM	
ICC286	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	D516G	NM		
ICC287	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	H526Y	NM		
ICC325	New Delhi	+	I <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>		NM	NM	NM
ICC326	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , S <sup>r</sup>	H526L	Insertion 98A, R463L		NM
ICC327	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , S <sup>r</sup>	S509R	R463L		K121K
ICC328	New Delhi	-	O <sup>r</sup>			NM	
ICC408	New Delhi	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup> , S <sup>r</sup>	Q510H, S531W	R463L	S95T	K121K
ICC425	New Delhi	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L		
F4	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	H526Y		D94G, S95T	
F5	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	S531L		S95T	
F7	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	S531L		A90V, S95T	
F8	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	S531L		A90V, S95T	
F9	New Delhi	+	R <sup>r</sup> , O <sup>r</sup>	N518T		S91P, S95T	
N31	New Delhi	+	I <sup>r</sup>		R463L		

Continued on following page

TABLE 2—Continued

Strain no.	Geographic location	Treatment history <sup>a</sup>	Drug susceptibility <sup>b</sup>	Polymorphism <sup>c</sup>			
				<i>rpo</i>	<i>katG</i> or <i>inhA</i>	<i>gyrA</i>	<i>rpsL</i>
N33	New Delhi	+	I <sup>r</sup>		R463L		
N34	New Delhi	+	I <sup>r</sup>		R463L		
N35	New Delhi	+	I <sup>r</sup>		D73N, R463L		
N36	New Delhi	-	I <sup>r</sup>		R463L		
ICC32	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	S531L	NA		
ICC33	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S509R	R463L	S95T	
ICC36	Ahmedabad	+	I <sup>r</sup> , O <sup>r</sup>		R463L	S95T	
ICC37	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	D516G	Insertion 98A, R463L		
ICC131	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	H526Y, R528P	R463L		
ICC132	Ahmedabad	-	O <sup>r</sup>			S95T	
ICC133	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	H526R	Δ30C, R463L		
ICC134	Ahmedabad	-	R <sup>r</sup> , O <sup>r</sup>	S522Q		S95T	
ICC136	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	H526R	Insertion 98A, R463L		
ICC137	Ahmedabad	-	O <sup>r</sup>			S95T	
ICC138	Ahmedabad	-	O <sup>r</sup>			S95T	
ICC226	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	L511L, H526R	Δ109G, R463L		
ICC233	Ahmedabad	+	R <sup>r</sup> , I <sup>r</sup>	D516V, H526Y	Δ30C, R463L		
ICC151	Chandigarh	-	O <sup>r</sup>			S95T	
ICC154	Chandigarh	-	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	S531L	Δ30C, R463L	S95T	
ICC155	Chandigarh	+	I <sup>r</sup>		Δ30C, R463L		
ICC159	Chandigarh	+	R <sup>r</sup> , O <sup>r</sup>	H526Y		S95T	
ICC161	Chandigarh	+	R <sup>r</sup> , O <sup>r</sup>	S522Q		S95T	
ICC162	Chandigarh	-	R <sup>r</sup> , O <sup>r</sup>	S522Q		S95T	
ICC164	Chandigarh	-	O <sup>r</sup>			S95T	
ICC165	Chandigarh	-	O <sup>r</sup>			S95T	
ICC166	Chandigarh	-	O <sup>r</sup>			S95T	
ICC167	Chandigarh	+	I <sup>r</sup> , O <sup>r</sup>		R463L	S95T	
ICC168	Chandigarh	+	O <sup>r</sup>			S95T	
ICC169	Chandigarh	+	R <sup>r</sup> , I <sup>r</sup>	N518T	R463L		
ICC170	Chandigarh	-	O <sup>r</sup>			S95T	
ICC171	Chandigarh	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L		
ICC172	Chandigarh	+	R <sup>r</sup> , O <sup>r</sup>	S522Q		S95T	
ICC173	Chandigarh	-	R <sup>r</sup> , S <sup>r</sup>	H526L			K121K
ICC174	Chandigarh	+	O <sup>r</sup>			S95T	
ICC175	Chandigarh	-	R <sup>r</sup> , O <sup>r</sup>	H526Y		S95T	
ICC247	Chandigarh	-	R <sup>r</sup> , O <sup>r</sup>	D516V		S95T	
ICC248	Chandigarh	-	R <sup>r</sup> , O <sup>r</sup>	D516V		S95T	
ICC249	Chandigarh	-	O <sup>r</sup>			S95T	
ICC251	Chandigarh	-	O <sup>r</sup>			NM	
ICC254	Chandigarh	+	R <sup>r</sup>	S531L			
ICC255	Chandigarh	-	R <sup>r</sup> , O <sup>r</sup>	N518T		NM	
ICC256	Chandigarh	+	R <sup>r</sup> , I <sup>r</sup>	H526Y	NM		
ICC257	Chandigarh	+	R <sup>r</sup> , O <sup>r</sup>	Q510H, L511L		S95T	
ICC262	Chandigarh	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	D516V	R463L	S95T	
ICC95	Bangalore	-	O <sup>r</sup>			S95T	
ICC96	Bangalore	+	R <sup>r</sup> , O <sup>r</sup>	S531L		S95T	
ICC399	Bangalore	+	R <sup>r</sup> , O <sup>r</sup>	S531W		NM	
ICC524	Bangalore	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L		
ICC525	Bangalore	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L		
ICC143	Shimla	-	O <sup>r</sup>			NM	
ICC144	Shimla	+	I <sup>r</sup>		R463L		
ICC145	Shimla	-	O <sup>r</sup>			NM	
A3	Agra	+	R <sup>r</sup> , I <sup>r</sup> , S <sup>r</sup>	S531L	R463L		NM
A4	Agra	+	R <sup>r</sup> , I <sup>r</sup>	D516V	R463L		
A9	Agra	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L		
A11	Agra	+	R <sup>r</sup> , I <sup>r</sup>	D516G	T11A, R463L		
A12	Agra	+	R <sup>r</sup> , I <sup>r</sup>	D516V	N35D, R463L		
A13	Agra	+	R <sup>r</sup> , I <sup>r</sup>	D516V, N518T	R463L		
A14	Agra	+	R <sup>r</sup> , I <sup>r</sup>	D516V	R463L		
A15	Agra	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L		
ICC332	Jaipur	+	R <sup>r</sup> , I <sup>r</sup>	H526R	Insertion 185C, R463L		
ICC337	Jaipur	+	R <sup>r</sup> , I <sup>r</sup>	S531L	R463L	S95T	
ICC85	Chennai	+	R <sup>r</sup> , I <sup>r</sup> , O <sup>r</sup>	H526R	NA	S95T	

<sup>a</sup> History of treatment in the previous 20 months.<sup>b</sup> R<sup>r</sup>, rifampin resistant; I<sup>r</sup>, isoniazid resistant; O<sup>r</sup>, ofloxacin resistant; S<sup>r</sup>, streptomycin resistant.<sup>c</sup> NA, no amplification; NM, no mutation; Inh, mutation in the *inhA* ribosome binding site; Δ, deletion at the indicated nucleotide position.

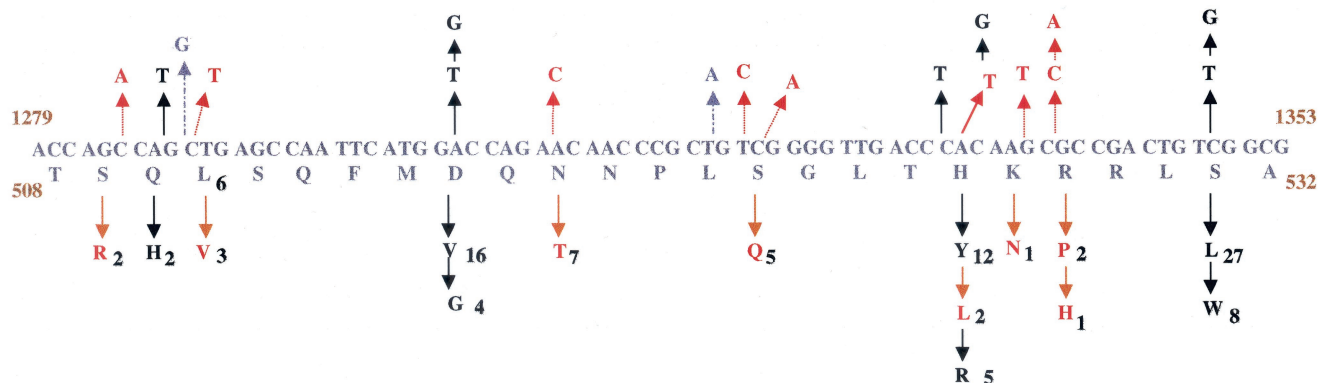


FIG. 1. Summary of mutations at codons 508 to 532 in the *rpoB* gene. The wild-type sequence and amino acids are shown in the middle frame. Nucleotide changes are marked with arrows in the top frame, and the corresponding amino acid changes are denoted in the bottom frame. The amino acids are subscripted with numbers that indicate the number of isolates harboring the change. Changes marked with orange lines (dotted arrows) are novel mutations; silent mutations are marked with blue lines (dashed arrows). Codons 531, 526, and 516 exhibit high degrees of polymorphism. Codons 509, 511, 522, 527, and 528 show novel mutations.

were recovered. These isolates probably represent those with acquired resistance, as the patients had at some time point been given antitubercular drug therapy.

A stretch of 30 amino acids at the center of the amplicon for the *rpoB* locus was studied. Amino acids 432 to 458 comprised the hot-spot region for mutations. For the sake of comparison, we used the corresponding *Escherichia coli* numbering, which is amino acids 507 to 533. We identified previously reported mutations as well as certain novel mutations. Codon 531 seemed to be the most vulnerable to mutations, as most rifampin-resistant isolates had this mutation (Fig. 1). Of the 93 rifampin-resistant strains in our study, 28 had the missense mutation Ser531Leu and 8 had the substitution Ser531Trp. The next most common mutations were the amino acid substitutions Asp516Val or Asp516Gly (20 isolates) and His526Tyr, His526Leu, or His526Arg (19 isolates). We found two isolates with Gln510His changes. While all these mutations have been reported earlier, we also found mutations that have not been reported previously. These included Ser509Arg (isolate ICC33), Leu511Val (isolate ICC242), Asn518Thr (isolate ICC107), Ser522Gln (isolate ICC172), Lys527Asn (isolate ICC105), Arg528Pro (isolate ICC129), and Arg528His (isolate ICC213). Most of these mutations occurred less frequently, comprising about 24% of the total mutations in the 94 isolates studied. Other mutations identified in our study were silent mutations at amino acids Leu511 and Leu521. Interestingly, the mutation at position 511 never occurred alone and was present only in isolates with more than one mutation at the *rpoB* locus.

An important outcome of these studies is the direct correlation of certain mutations to high MICs. Table 3 lists the isolates, their mutations, and the corresponding MICs at which they remained resistant. Mutations in codons 516 and 521 conferred low-level resistance (MIC, <40 µg/ml) to rifampin, whereas mutations in codons 510, 526, 527, 528, and 531 were seen to confer high levels of resistance (MICs, ≥64 µg/ml). Amino acids 526 to 531 appear to be very important in drug target interactions, and mutations in them result in MICs in the range of 64 µg/ml and above. In a few cases (e.g., for

isolates ICC204, ICC257, and ICC128), double mutations were found to have an additive effect on the degree of resistance.

Insertion, deletion, and substitution mutations were mapped in the *katG* locus in 24 isoniazid-resistant isolates. In the present study we looked for mutations in the 5' region (nucleotides [nt] 3 to 239) and the midregion (nt 1187 to 1600) of the *katG* gene, corresponding to amino acid positions 2 to 77 and 395 to 533, respectively. The results are summarized in Fig. 2. A C nucleotide at position 30 was deleted in six of the isolates. This deletion results in chain termination, thereby generating only a short polypeptide of 26 amino acids. Another deletion of a single nucleotide, a G residue at position 109, was observed in two isolates; this deletion would result in the production of

TABLE 3. Correlation of specific mutations with rifampin MICs<sup>a</sup>

Strain	Rifampin MIC (µg/ml)	Mutation	Mutation type	Amino acid change
ICC221	10	G1317A	Novel	L521L
ICC208, ICC205	10	A1304T	Reported	D516V
ICC37	10	A1304G	Reported	D516G
ICC204	40	G1317A	Novel	L521L
ICC204	40	G1336T	Novel	K527N
ICC105	40	G1336T	Novel	K527N
ICC129	40	G1338C	Novel	R528P
ICC131	40	C1331T	Reported	H526Y
ICC131	40	G1338C	Novel	R528P
ICC123	64	G1338C	Novel	R528P
ICC100	64	C1349T	Reported	S531L
ICC213	64	G1340A	Novel	R528H
ICC213	64	C1349G	Reported	S531W
ICC218	64	T1321C	Novel	S522Q
ICC218	64	C1322A	Novel	S522Q
ICC257	64	G1287T	Novel	Q510H
ICC257	64	C1288T	Novel	L511L
ICC275	64	C1333T	Reported	H526Y
ICC220	64	C1349G	Reported	S531W
ICC128	128	C1331T	Reported	H526Y
ICC128	128	G1338A	Novel	R528H

<sup>a</sup> Missense mutations in the RpoB protein at amino acid positions 510, 511, 522, 526, 527, 528, and 531 confer higher levels of resistance (MICs, ≥40 µg/ml) than those at positions 509, 516, and 521 (MICs, ≤10 µg/ml).



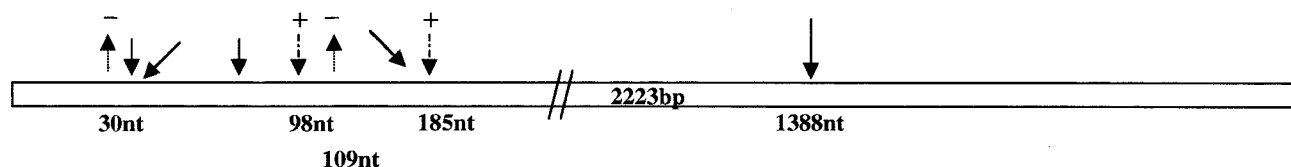


FIG. 2. Summary of mutations in the *katG* gene. Deletions are indicated by lines with a minus sign, while insertions are depicted by dashed lines with a plus sign. Solid lines show the substitutions. Codon 463 exhibited the highest degree of polymorphism, followed by the deletion at nucleotide 30.

a 45-amino-acid truncated polypeptide. Insertions were also observed at nt positions 98 (an A nucleotide) and 185 (a C nucleotide) in four and three isolates, respectively. Both of these insertions cause aberrant chain termination. Ala61Thr, Thr12Pro, Thr11Ala, Asp73Asn, and Asn35Asp missense mutations were observed in this locus in a few of the isolates. These are novel observations, as there are no reports of such mutations occurring in isoniazid-resistant strains from other parts of the world. We were unable to amplify this locus in six of the isolates (isolates ICC14, ICC23, ICC32, ICC85, ICC123, and ICC205), indicating a partial deletion of the gene. A common mutation in all these isolates was Arg463Leu. However, this mutation has been shown to have no direct consequence for drug resistance. To confirm this we sequenced this locus for all 126 isolates included in the study. It was found that the majority of the isolates carried this change. It has been argued previously that this polymorphism in the *katG* locus might be more important as a marker of evolution than as a marker of resistance (22). Three isoniazid-resistant isolates carried mutations in the ribosomal binding site upstream of the *inhA* gene. While two isolates showed a C-to-T transition, one had a T-to-A transversion. These mutations have previously been reported by other groups. The present understanding of these mutations is that they probably confer resistance by a drug titration effect.

Sixty-eight ofloxacin-resistant isolates were analyzed. The hot-spot region of the *gyrA* gene spanning codons 89 to 95 was sequenced to identify mutations. Most of the isolates showed a single mutation corresponding to the amino acid change Ser95Thr (Fig. 3). The second most common mutation, observed in four isolates, was Asp94Gly or Asp94Ala. Two isolates had an Ala90Val substitution, while one had a silent mutation at this codon. Seven isolates had double mutations, with the S95T change being common to all seven. These mu-

tations were present in MDR isolates for which the MICs of the drugs were high, including the frontline drugs used in antituberculosis therapy. All strains were also checked for mutations in the *gyrB* locus, which is associated with low levels of resistance. However, we found no mutations in the *gyrB* loci of these isolates. It has been argued that the S95T mutation does not correlate with drug resistance (22). It therefore appears that the isolates have acquired resistance to ofloxacin via other mechanisms.

We tested 14 isolates resistant to streptomycin for mutations in the *rpsL* and *rrs* loci. In eight strains we found a novel silent mutation at amino acid position 121 in the *rpsL* locus, where the codon AAA (Lys) was changed to AAG (Lys), but we found no mutations in the *rrs* genes. To our knowledge, there are no reports of this mutation. The reported mutations at the *rpsL* locus are generally Leu43Arg, Leu43Thr, or Lys88Arg. We are still not clear about how a mutation at this locus leads to the development of streptomycin resistance. The remaining isolates probably acquired resistance by other means, such as by the development of a permeability barrier or by the production of drug-altering enzymes.

A point to be kept in mind is that the majority of isolates included in the present study were from North India. Our data are therefore inherently biased toward drug-resistant strains from this region and should not be seen as representative for isolates from the whole of India.

## DISCUSSION

The mycobacterium uses various mechanisms to evade killing by drugs, including mutations in genes that code for drug target proteins (20), a complex cell wall which blocks drug entry, and membrane proteins that act as drug efflux pumps (6, 14). The objective of the present study was to identify muta-

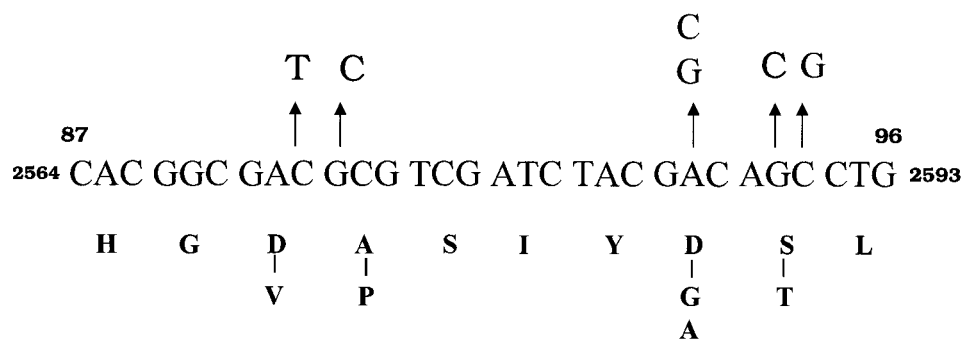


FIG. 3. Summary of missense mutations in the *gyrA* locus. Nucleotide changes are indicated on top of the wild-type sequence, and the corresponding amino acid changes are shown at the bottom. The most common mutation in this locus is Ser95Thr.

tions in drug target loci in Indian strains of *M. tuberculosis* and to identify the different drug resistance genotypes. As in all such studies, the aim was to generate information about the markers associated with drug resistance, polymorphisms in the drug target genes, the association of the level of resistance with particular mutations, etc. Our findings of mutations in the *rpoB*, *katG*, and *rpsL* loci are similar to those reported from other parts of the world, especially the common mutations, which reflect a global pattern (20). Rifampin resistance is often regarded as an excellent surrogate marker for MDR tuberculosis (4, 10), and our study corroborates this hypothesis. The mutation frequency of codon 531 (*rpoB*) was similar to that reported earlier (13, 17, 19, 20, 21, 25). Significantly, the frequency of mutations (relative to those of other mutations) was higher at codon 516 and lower at codon 526 in Indian isolates compared to those reported elsewhere. We found novel mutations that broaden the range of known mutations at this locus. When taken together, these mutations were detected in a significant number of drug-resistant isolates, a fact that needs to be considered when designing tools for the detection of MDR *M. tuberculosis*. We found a definite correlation between MICs and the type of mutation in many isolates. As reported by previous investigators (24), mutations at positions 528 and 531 are important in the development of high MICs. Our findings further strengthen the belief that the degree of resistance to rifampin exhibited by an isolate is related to the type of mutation in the *rpoB* locus.

In isoniazid-resistant isolates, significantly more deletion and insertion mutations than substitution mutations were found, of which a few have been reported previously (11, 19). We observed that almost all isolates studied carried the Arg463Leu substitution, which is also present in isolates that were sensitive to isoniazid. This is in concordance with a report from Sreevatsan et al. (22), who argue that polymorphism at this residue does not contribute to resistance per se but is an important marker for evolutionary genetics. The insertions and deletions in the *katG* locus invariably resulted in chain truncation and termination, leading to the generation of dysfunctional polypeptides. We found changes in the putative ribosomal binding site of the *inhA* gene in three isolates. While the exact mechanism of how these mutations confer resistance to isoniazid is not clear, reports (1, 18, 20) indicate that they probably increase the levels of enoyl-acyl carrier protein reductase which in turn leads to resistance via a drug titration mechanism. In isolates with no mutations in the hot-spot region of the gene, the complete sequencing of the gene is being done. However, resistance to isoniazid can also be due to mutations in the *ahpC-oxvR* and *kasA* gene loci (7, 16).

Fluoroquinolones comprise the secondary drug regimen in the treatment of tuberculosis. A large number of isolates were resistant to ofloxacin, which could be due in part to the inaccurate diagnosis of tuberculosis as a bacterial infection and fluoroquinolone overuse in the population. Codons 89, 90, 91, 94, and 95 in the *gyrA* gene have been shown to be polymorphic (20, 23, 26). The most common mutation in ofloxacin-resistant isolates in the present study was Ser95Thr, which reportedly has no direct role in the development of drug resistance, as it also occurs in drug-sensitive strains (22). It seems likely that ofloxacin resistance possibly results due to mutations elsewhere in the gene or the presence of drug efflux pumps.

Mutations in codons 43 and 88 of the *rpsL* gene generally result in high levels of resistance to streptomycin, while mutations in the loop at codon 530 or the region at codon 915 of the *rrs* locus are associated with low levels of resistance (3). We did not find any of these mutations in the 14 streptomycin-resistant isolates included in our study. However, we did observe a silent mutation at codon 121 that has not been reported by any other group.

Our study provides valuable data on the different kinds of mutations occurring at various target loci in Indian clinical isolates of *M. tuberculosis* that enhance our understanding of the molecular mechanisms of drug resistance. The diversity of the polymorphisms exhibited at these loci by the drug-resistant strains indicates the prevalence of a large numbers of drug-resistant strains in this region. Additionally, our data will also assist in the process of designing new molecular biology-based techniques for the diagnosis of MDR tuberculosis. Such methods promise faster detection rates compared to those achieved by methods based solely on culture of the isolates.

#### ACKNOWLEDGMENTS

This study was supported by research grants from the Department of Biotechnology, Government of India.

We thank Sunder S. Bisht and Mohammed Iliyas Ghazi, who helped with the generation of the sequencing data. We are also grateful to Ram Das, Kiran Srivastava, and V. Chauhan of the Central Jalma Institute of Leprosy, who provided us with the isolate DNAs.

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