JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2005, p. 1390–1394 Vol. 43, No. 3
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.3.1390–1394.2005

Genetic Diversity of Multidrug-Resistant *Mycobacterium tuberculosis* Isolates and Identification of 11 Novel *rpoB* Alleles in Taiwan

Ruwen Jou,[1]* Huang-Yau Chen,[1] Chen-Yuan Chiang,[2]
Ming-Chih Yu,[3] and Ih-Jen Su[4]

Reference Laboratory of Mycobacteriology, Center for Disease Control, Department of Health,[1] Taipei Medical University-Municipal Wan Fang Hospital,[3] Taipei, and Division of Clinical Research, National Health Research Institutes, Tainan,[4] Taiwan, Republic of China, and International Union Against Tuberculosis and Lung Disease, Paris, France[2]

Received 22 October 2004/Returned for modification 28 October 2004/Accepted 19 November 2004

Of 162 multidrug-resistant *Mycobacterium tuberculosis* isolates from Taiwan, 60.5% were found to belong to the Beijing family on the basis of spoligotyping results. IS*6110* restriction fragment length polymorphism fingerprinting showed genetic diversity among the multidrug-resistant isolates. Furthermore, 90.1% of the multidrug-resistant isolates had mutations in the *rpoB* gene, and 11 novel alleles were recognized.

The emergence of multidrug resistance in *Mycobacterium tuberculosis* has become a global problem (5). The prevalence rates of multidrug-resistant (MDR) *M. tuberculosis* isolates have been reported to range from 0 to 26.8% (21). An estimated 90% of rifampin (RMP)-resistant isolates are also isoniazid (INH) resistant; therefore, RMP resistance in *M. tuberculosis* can be referred to as a surrogate marker for MDR. The resistance of *M. tuberculosis* to RMP is caused by mutations confined in a short 81-bp-long DNA region in the gene *rpoB* encoding the β-subunit of RNA polymerase (14, 16, 17, 20). Beijing family genotypes strains have been reported to be associated with transmissions of drug-resistant tuberculosis in Germany, Azerbaijan, Cuba, Estonia, Russia, New York, and South Africa (7).

The prevalence of antituberculosis drug resistance in *M. tuberculosis* has been on the increase in Taiwan. The rates of primary anti-tuberculosis drug resistance from 1990 to 2002 were 9.2 to 19% resistance to INH, 5.7 to 10% resistance to streptomycin (SM), 1.5 to 6.1% resistance to RMP, 0.7 to 15.7% resistance to ethambutol (EMB), and 1.2 to 5.1% resistance to MDR (11, 27). Molecular epidemiology of MDR *M. tuberculosis* in Taiwan has not been well known and was thus investigated by using spoligotyping and standard IS*6110* restriction fragment length polymorphism (RFLP) analysis in this study. The prevalence of *rpoB* mutations associated with RMP resistance among MDR isolates was also investigated.

MDR isolates. A total of 162 MDR isolates of *M. tuberculosis* were collected during 1998 to 2003 at the Center for Chest Diseases, Taipei, Taiwan, and 40 susceptible isolates were collected from Taipei Veteran General Hospital, Taipei, Taiwan, in 2003. Among the 162 MDR isolates, 37 (22.8%) isolates were resistant to INH and RMP, 62 (38.3%) isolates were resistant to INH, RMP, and EMB, 15 (9.3%) isolates were resistant to INH, RMP, and SM, and 48 (29.6%) isolates were resistant to all four drugs.

* Corresponding author. Mailing address: Reference Laboratory of Mycobacteriology, Center for Disease Control, Department of Health, Taiwan, 161 Kun-Yang St., Nan-Kang, Taipei 115, Taiwan. Phone: 886 2 26531370. Fax: 886 2 26531387. E-mail: rwj@cdc.gov.tw.

Spoligotyping and RFLP fingerprinting. Spoligotyping was performed with a commercial kit (10) according to the manufacturer's instructions. Standard IS*6110* RFLP fingerprinting was performed as described previously (25).

Computer analysis. The spoligotypes were scanned and analyzed using Bionumerics software, version 2.0 (Applied Maths, Kortijk, Belgium). A statistical analysis was performed using EpiInfo 6.04 (Centers for Disease Control and Prevention, Atlanta, Ga.).

***rpoB* genotyping.** The *rpoB* gene was amplified with primers *rpoB*-F (5′-TCGGCGAGCCCATCACGTCG-3′) and *rpoB*-R (5′-GCGTACACCGACAGCGAGCC-3′), which yielded a 541-bp fragment containing the hot-spot region. PCR products were purified with a commercial kit, and both strands of each product were sequenced.

In the spoligotyping analysis, 39 spoligotypes were resolved, including 17 clusters (Fig. 1). Overall, 98 (60.5%) of the 162 MDR isolates showed the Beijing family genotypes. The proportion of isolates resistant to at least three drugs was higher among the Beijing family genotypes (80%) than among non-Beijing genotypes (73.4%). A sufficient quality of genomic DNA for IS*6110* RFLP fingerprinting was obtained from 155 MDR isolates, and genetic diversity of the MDR isolates was found. Overall, a RFLP dendrogram revealed 139 patterns that included 14 clusters (18.7% of isolates) at a 98% similarity level: 12 clusters had two strains, one cluster had three strains, and one cluster had four strains (Fig. 2). One cluster with a single IS*6110* band was excluded because of distinct spoligotypes.

Of the 162 MDR isolates, 146 (90.1%) had mutations in the 81-bp core region whereas no mutation was found in the 40 susceptible strains. A total of 80 (49.4%), 33 (20.4%), and 14 (8.6%) MDR isolates carried the mutated codons at positions 531, 526, and 516, respectively (Table 1). Overall, 91.8% of the mutated isolates exhibited single site changes. There was no statistical association between Beijing family genotypes and the mutation frequencies of each mutated codon.

Genetic diversities of drug resistance isolates might be attributable to some host factors beside strain evolution in different geographic regions. The frequency of occurrence of

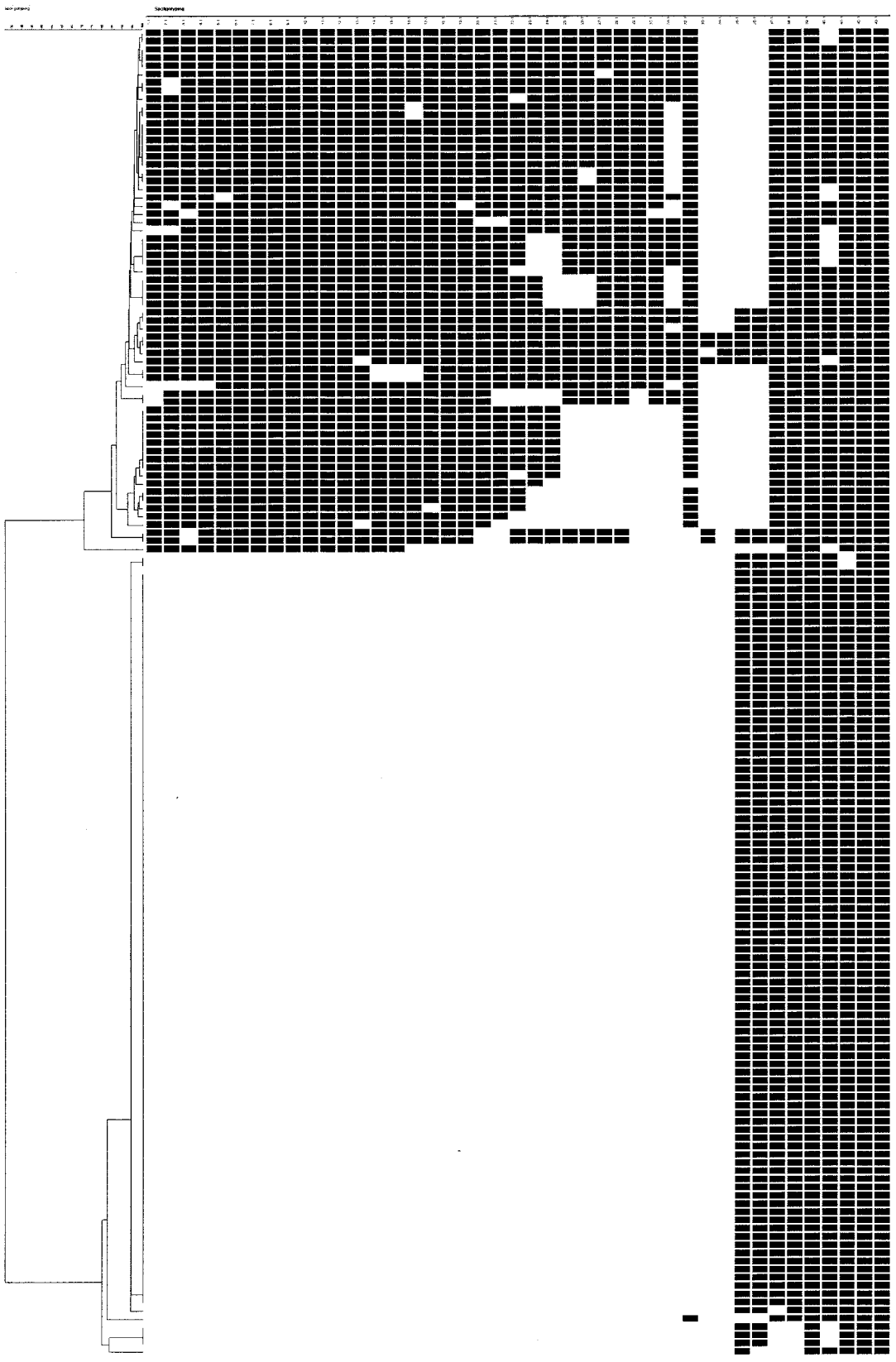

FIG. 1. Dendrogram of 162 MDR-TB isolates analyzed by spoligotyping.

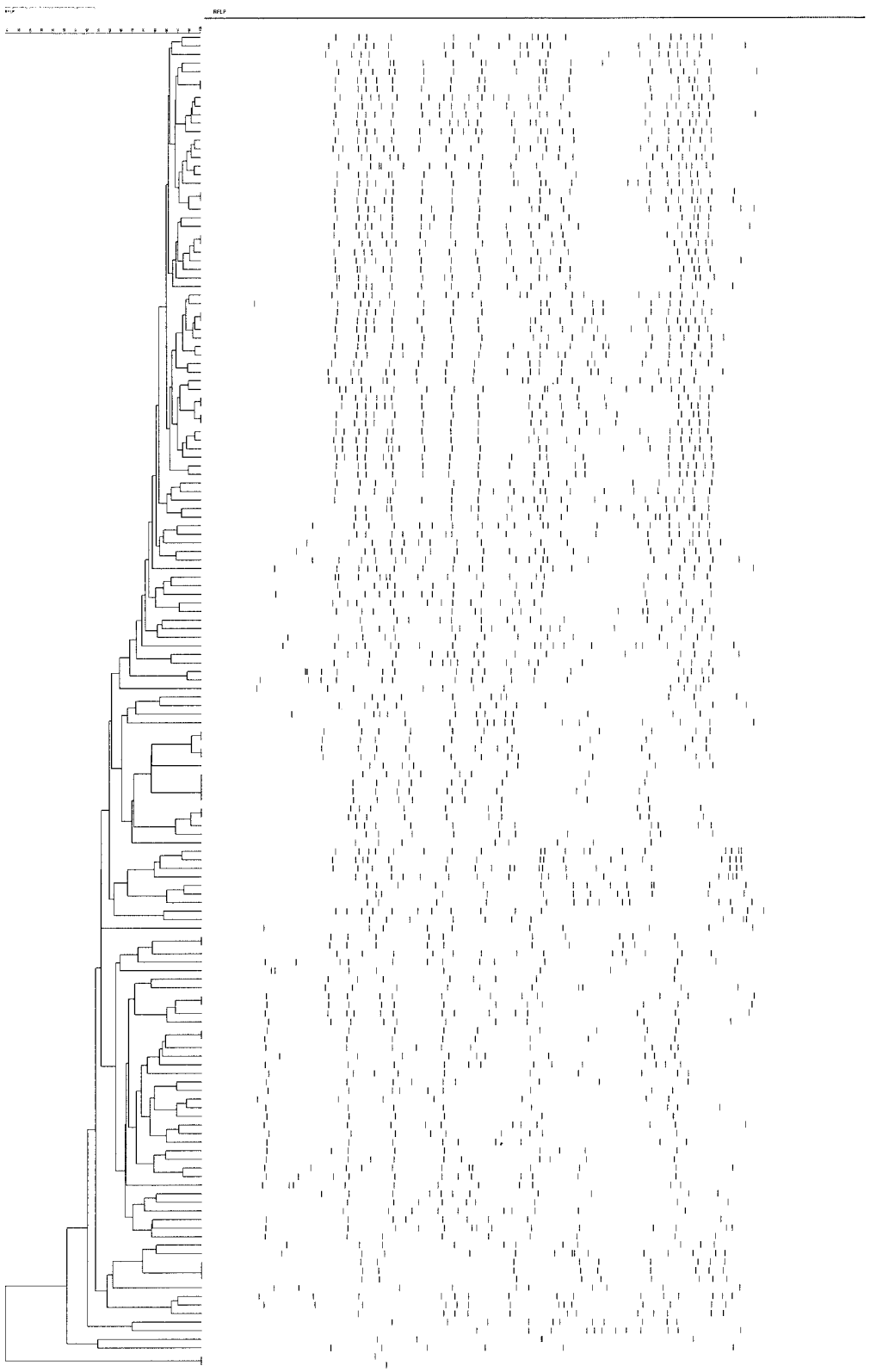

FIG. 2. Dendrogram of 155 MDR-TB isolates analyzed by IS*6110* RFLP genotyping.

TABLE 1. Mutations in an 81-bp region of the *rpoB* gene in 162 *M. tuberculosis* MDR isolates

Mutated codon	Specific mutation	Amino acid change	No. of strains	Frequency 4 (%)
508	**ACC→ATC**	**Thr→Ile**	1	0.6
513	CAA→CCA	Gln→Pro	5	3.1
	CAA→AAA	Gln→Lys	2	1.2
	CAA→CCG	**Gln→Pro**	1	0.6
515	**ATG→GTG**	**Met→Val**	1	0.6
516	GAC→TAC	Asp→Tyr	5	3.1
	GAC→GTC	Asp→Val	4	2.5
	GAC→GGC	Asp→Gly	2	1.2
	GAC→GCC	Asp→Ala	1	0.6
	GAC→TTC	Asp→Phe	1	0.6
	GAC→GAG	**Asp→Glu**	1	0.6
517	**CAG→CCG**	**Gln→Pro**	1	0.6
522	TCG→TTG	Ser→Leu	2	1.2
	TCG→TGG	Ser→Trp	1	0.6
	TCG→TTC	**Ser→Phe**	1	0.6
526	CAC→TAC	His→Tyr	14	8.6
	CAC→GAC	His→Asp	5	3.1
	CAC→CTC	His→Leu	5	3.1
	CAC→CGC	His→Arg	3	1.9
	CAC→TGC	His→Cys	2	1.2
	CAC→AAC	His→Asn	1	0.6
	CAC→ACC	His→Thr	1	0.6
	CAC→CGA	**His→Arg**	1	0.6
	CAC→GGC	**His→Gly**	1	0.6
531	TCG→TTG	Ser→Leu	75	46.3
	TCG→TGG	Ser→Trp	2	1.2
	TCG→CAG	Ser→Gln	1	0.6
	TCG→GTG	**Ser→Val**	1	0.6
	TCG→GGG	**Ser→Gly**	1	0.6
533	CTG→CCG	Ser→Pro	6	3.7
509–511	**DELETION**		1	0.6
513–514	INSERTION	TTC (Phe)	4	2.5

[a] The mutated codons with corresponding amino acids are indicated. New alleles were indicated in boldface characters.

MDR isolates without mutation is comparable to the results seen in studies conducted in some Asia countries (Table 2). It is lower than the results seen in another two studies of RMP-resistant isolates reported from Taiwan but higher than results obtained from other countries. One of the studies analyzing 20 strains revealed four substitutions and one insertion (19), while the other study analyzing 53 RMP-resistant isolates revealed 16 types of mutations and five novel alleles within the 69-bp core region (19). In contrast, our study revealed a total of 32 mutations, 11 new alleles, and the highest frequencies of mutations at codons 513, 526, and 531 (Table 2). Together with results obtained from worldwide studies, these results might be helpful in developing a thorough rapid single-nucleotide-polymorphism detection method, such as microarray, for high-throughput testing. For isolates without mutations, N-terminal codon 146 for low-level resistance and codon 562 might be also involved in RMP drug resistance (30). An additional gene, the *arr* gene (1, 23), found to be associated with RMP resistance in *Pseudomonas aeruginosa* and other mycobacteria, may be also involved in the development of MDR tuberculosis.

In the RFLP analysis, a cluster with three strains belonged to Beijing genotypes; the three isolates had the same drug resistance profiles (they were resistant to INH, RMP, and EMB and susceptible to SM) and had a single mutation at codon 531 (TCG→TTG, Ser→Leu). In the cluster with four strains, which were resistant to all four drugs (except for one isolate, which was susceptible to EMB), all had a universal TTC (Phe) insertion mutation between codons 513 and 514. The epidemiological relatedness of these four isolates was investigated on the basis of the exhibited RFLP patterns, and possible household contact transmission was linked to two of four strains, while the other clusters had no apparent epidemiological links. Besides, no RFLP pattern identical to that of the MDR W strain (3, 15) was observed in this study population. However, 54.9% of the 162 MDR isolates analyzed had the same mutation site as the W strain in *rpoB* of either codon 526(His→Tyr) or 531(Ser→Leu) (3). MDR *M. tuberculosis* could be developed through an acquired resistance or come from an exogenous new infection. These data suggest that the prevalence of RMP resistance among *M. tuberculosis* isolates in Taiwan might be due to the development of mutations in the

TABLE 2. Frequency of codon mutations in RMP-resistant M. tuberculosis isolates from different geographic regions[a]

Country (reference; no. of isolates)	Frequency (%) of mutated codon:								
	533	531	526	522	516	513	511	508	No mutation within hot-spot region
United States (26; $n = 61$)									8.2
Greece (13; $n = 17$)		52.9	17.6		12.0			5.9	5.9
Asia (8; $n = 90$)		53.3	16.7	1.1	14.0	5.6	1.1		6.5
Australia (29; $n = 33$)			30.3	6.1	9.1				3.0
Italy (18; $n = 37$)	2.7	59.4	35.1		8.1		2.7		0.0
Brazil (24; $n = 82$)	1.2	55.7	23.2	2.4	7.4	1.2	1.2		3.6
Hungary (2; $n = 29$)		31.0	6.9		38.0	6.8			10.3
India (12; $n = 44$)	2.2	63.6	22.7		4.5	2.2	6.8	2.2	2.3
Spain (6; $n = 50$)	2.0	48.0	22.0		14.0	2.0	6.0		0.0
East Asia (19; $n = 66$) (China, 20; Japan, 3; Korea, 18)	3.0	51.5	10.6		17.0	6.0			10.6
East Asia (19; $n = 66$) (Taiwan, 20)	**5.0**	**40.0**	**10.0**		**15.0**				**20.0**
Latvia (22; $n = 34$)		41.2	20.6		32.0				
Turkey (4; $n = 41$)	4.8	56.1	19.5	4.9	7.2	2.4			2.4
China (28; $n = 86$)	2.0	41.0	40.0	3.0	4.0	2.0	2.0		10.0
Kaohsiung, Taiwan (9; $n = 63$)	**7.5**	**41.5**	**18.9**	**1.9**	**15.1**		**6.3**		**15.9**
Taiwan (this research) ($n = 162$)	**3.7**	**49.4**	**20.4**	**2.4**	**8.6**	**4.9**	**0.0**	**0.6**	**9.9**

[a] Studies on RMP drug-resistant strains isolated in Taiwan as to the multimutation frequency of the *rpoB* gene are indicated with boldface characters.

rpoB gene in various *M. tuberculosis* strains rather than due to the transmission of MDR clones.

Nucleotide sequence accession numbers. The sequences with mutations in new alleles found in this study were deposited in GenBank under accession numbers AY823310, AY823311, AY823312, AY823313, AY823314, AY823315, AY823316, AY823317, and AY823318.

This work was supported by grant DOH92-DC-2035 from Center for Disease Control Taiwan, Department of Health.

We thank Jing-Jou Yan for helpful discussion; Sharon Liu, Meng-Hsiun Chen, Su-Yin Chang, Pei-Ju Chin, and Wei-Lun Huang for their excellent technical support; and Wei-Jun Su for providing drug-susceptible *M. tuberculosis* isolates.

REFERENCES

1. **Alexander, D. C., J. R. Jones, and J. Liu.** 2003. A rifampin-hypersensitive mutant reveals differences between strains of *Mycobacterium smegmatis* and presence of a novel transposon, IS*1623*. Antimicrob. Agents Chemother. **47:**3208–3213.
2. **Bartfai, Z., A. Somoskovi, C. Kodmon, N. Szabo, E. Puskas, L. Kosztolanyi, E. Farago, J. Mester, L. M. Parsons, and M. Salfinger.** 2001. Molecular characterization of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary by DNA sequencing and the line probe assay. J. Clin. Microbiol. **39:**3736–3739.
3. **Bifani, P. J., B. B. Plikaytis, V. Kapur, K. Stockbauer, X. Pan, M. L. Lutfey, S. L. Moghazeh, W. Eisner, T. M. Daniel, M. H. Kaplan, J. T. Crawford, J. M. Musser, and B. N. Kreiswirth.** 1996. Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. JAMA **275:**452–457.
4. **Cavusoglu, C., S. Hilmioglu, S. Guneri, and A. Bilgic.** 2002. Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. J. Clin. Microbiol. **40:**4435–4438.
5. **Espinal, M., A. Laszlo, L. Simonsen, F. Boulahbal, S. J. Kim, A. Reniero, S. Hoffner, H. L. Rieder, N. Binkin, C. Dye, R. Williams, and M. C. Raviglione.** 2001. Global trends in resistance to antituberculosis drugs. World Health organization—International Union Against Tuberculosis and lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. N. Engl. J. Med. **344:**1294–1303.
6. **Garcia, L., M. Alonso-Sanz, M. J. Rebollo, J. C. Tercero, and F. Chaves.** 2001. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* isolates in Spain and their rapid detection by PCR-enzyme-linked immunosorbent assay. J. Clin. Microbiol. **39:**1813–1818.
7. **Glynn, J. R., J. Whiteley, P. J. Bifani, K. Kremer, and D. van Soolingen.** 2002. Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg. Infect. Dis. **8:**843–849.
8. **Hirano, K., C. Abe, and M. Takahashi.** 1999. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. J. Clin. Microbiol. **37:**2663–2666.
9. **Hwang, H. Y., C. Y. Chang, L. L. Chang, S. F. Chang, Y. H. Chang, and Y. J. Chen.** 2003. Characterization of rifampicin-resistant *Mycobacterium* tuberculosis in Taiwan. J. Med. Microbiol. **52:**239–245.
10. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden.** 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.
11. **Liaw, Y. S., P. R. Hsueh, C. J. Yu, S. K. Wang, P. C. Yang, and K. T. Luh.** 2004. Drug resistance pattern of *Mycobacterium tuberculosis* in a university hospital in Taiwan, 1998–2002. J. Formosa Med. Assoc. **103:**671–677.
12. **Mani, C., N. Selvakumar, S. Narayanan, and P. R. Narayanan.** 2001. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India. J. Clin. Microbiol. **39:**2987–2990.
13. **Matsiota-Bernard, P., G. Vrioni, and E. Marinis.** 1998. Characterization of *rpoB* mutations in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates from Greece. J. Clin. Microbiol. **36:**20–23.
14. **Miller, L. P., J. T., Crawford, and T. Shinnick.** 1994. The *rpoB* gene of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **38:**805–811.
15. **Moss, A. R., D. Alland, E. Telzak, D. Hewlett, Jr., V. Sharp, P. Chiliade, V. LaBombardi, D. Kabus, B. Hanna, L. Palumbo, K. Brudney, A. Weltman, K. Stoeckle, K. Chirgwin, M. Simberkoff, S. Moghazeh, W. Eisner, M. Lutfey, and B. Kreiswirth.** 1997. A city-wide outbreak of a multiple-drug-resistant strain of *Mycobacterium tuberculosis* in New York. Int. J. Tuberc. Lung Dis. **1:**115–121.
16. **Musser, J. M.** 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin. Microbiol. Rev. **8:**496–514.
17. **Ovchinnikov, Y. A., G. S. Monastyrskaya, V. V. Gubanov, V. M. Lipkin, E. D. Sverdlov, I. F. Kiver, I. A. Bass, S. Z. Mindlin, O. N. Danilevskaya, and R. B. Khesin.** 1981. Primary structure of *Escherichia coli* RNA polymerase nucleotide substitution in the beta subunit gene of the rifampicin resistant rpoB255 mutant. Mol. Gen. Genet. **184:**536–538.
18. **Pozzi, G., M. Meloni, E. Iona, G. Orru, O. F. Thoresen, M. L. Ricci, M. R. Oggioni, L. Fattorini, and G. Orefici.** 1999. *rpoB* mutations in multidrug-resistant strains of *Mycobacterium tuberculosis* isolated in Italy. J. Clin. Microbiol. **37:**1197–1199.
19. **Qian, L., C. Abe, T. P. Lin, M. C. Yu, S. N. Cho, S. Wang, and J. T. Douglas.** 2002. *rpoB* genotypes of *Mycobacterium tuberculosis* Beijing family isolates from east Asian countries. J. Clin. Microbiol. **40:**1091–1094.
20. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet **341:**647–650.
21. **The Who/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance.** 2004. Anti-tuberculosis drug resistance in the world, Third Global Report. World Health Organization, Geneva, Switzerland.
22. **Tracevska, T., I. Jansone, L. Broka, O. Marga, and V. Baumanis.** 2002. Mutations in the *rpoB* and *katG* genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia. J. Clin. Microbiol. **40:**3789–3792.
23. **Tribuddharat, C., M. Fennewald.** 1999. Integron-mediated rifampin resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**960–962.
24. **Valim, A. R., M. L. Rossetti, M. O. Ribeiro, and A. Zaha.** 2000. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from Brazil. J. Clin. Microbiol. **38:**3119–3122.
25. **van Embden, J. D. A., Crawford, J. T., Dale, J. W., Gicquel, B., Hermans, P. W. M., McAdam, R., Shinnick, T., and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
26. **Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M. Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis.** 1994. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. **38:**2380–2386.
27. **Yu, M. C., J. Suo, C. Y. Chiang, K. J. Bai, T. P. Lin, and K. T. Luh.** 1997. Initial drug resistance of *Mycobacterium tuberculosis* in Taiwan. J. Formos. Med. Assoc. **96:**890–894.
28. **Yue, J., W. Shi, J. Xie, Y. Li, E. Zeng, and H. Wang.** 2003. Mutations in the *rpoB* of multidrug-resistant *Mycobacterium tuberculosis* isolates from China. J. Clin. Microbiol. **41:**2209–2212.
29. **Yuen, L. K., D. Leslie, and P. J. Coloe.** 1999. Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains isolated in Australia. J. Clin. Microbiol. **37:**3844–3850.
30. **Zhang, Y., A. Telenti.** 2000. Genetics of drug resistance in *Mycobacterium tuberculosis*, p. 235–254. *In* G. F. Hatfull and W. R. Jacobs, Jr. (ed.), Molecular genetics of mycobacteria. ASM Press, Washington, D.C.