ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1997, p. 540–543 Vol. 41, No. 3
0066-4804/97/$04.00+0

Characterization of *pncA* Mutations in Pyrazinamide-Resistant *Mycobacterium tuberculosis*

ANGELO SCORPIO,[1] PAMELA LINDHOLM-LEVY,[2] LEONID HEIFETS,[2] ROBERT GILMAN,[1] SALMAN SIDDIQI,[3] MICHAEL CYNAMON,[4] AND YING ZHANG[1]*

Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205[1]; National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206[2]; Becton Dickinson Diagnostic Instrument Systems, Sparks, Maryland 21152[3]; and Veterans Affairs Medical Center, Syracuse, New York 13210[4]

Received 5 August 1996/Returned for modification 3 September 1996/Accepted 23 December 1996

Pyrazinamide (PZA) is a first-line drug for short-course tuberculosis therapy. Resistance to PZA is usually accompanied by loss of pyrazinamidase (PZase) activity in *Mycobacterium tuberculosis*. PZase converts PZA to bactericidal pyrazinoic acid, and the loss of PZase activity is associated with PZA resistance. The gene (*pncA*) encoding the *M. tuberculosis* PZase has recently been sequenced, and mutations in *pncA* were previously found in a small number of PZA-resistant *M. tuberculosis* strains. To further understand the genetic basis of PZA resistance and determine the frequency of PZA-resistant strains having *pncA* mutations, we analyzed a panel of PZA-resistant clinical isolates and mutants made in vitro. Thirty-three of 38 PZA-resistant clinical isolates had *pncA* mutations. Among the five strains that did not contain *pncA* mutations, four were found to be falsely resistant and one was found to be borderline resistant to PZA. The 33 PZA-resistant clinical isolates and 8 mutants made in vitro contained various mutations, including nucleotide substitutions, insertions, or deletions in the *pncA* gene. The identified mutations were dispersed along the *pncA* gene, but some degree of clustering of mutations was found at the following regions: Gly132-Thr142, Pro69-Leu85, and Ile5-Asp12. PCR–single-strand conformation polymorphism (SSCP) analysis was shown to be useful for the rapid detection of *pncA* mutations in the PZA-resistant strains. We conclude that a mutation in the *pncA* gene is a major mechanism of PZA resistance and that direct sequencing by PCR or SSCP analysis should help to rapidly identify PZA-resistant *M. tuberculosis* strains.

The emergence of drug-resistant *Mycobacterium tuberculosis* strains seriously compromises our ability to control tuberculosis. This problem has been further compounded in recent years by human immunodeficiency virus coinfection. Multiple-drug-resistant strains of *M. tuberculosis*, defined as resistance to at least isoniazid and rifampin, have already caused several fatal outbreaks (1, 4). This has stimulated a great deal of research aimed at understanding the molecular mechanisms of drug resistance in *M. tuberculosis*. Such knowledge should facilitate the rational design of new antituberculosis drugs and the development of rapid tests for the detection of drug resistance.

The antituberculosis activity of pyrazinamide (PZA) was discovered in 1952 (27), but its unique role in accelerating the effect of therapy when used in combination with isoniazid and rifampin was reported only in the 1980s (2, 8, 22, 23). These observations allowed for the shortening of the therapy for tuberculosis from 9 to 6 months and made PZA the third most important drug in the modern therapy of tuberculosis. The effect of PZA is usually associated with its activity against the semidormant bacterial population persisting in a low-pH environment (5), in early acute-inflammation sites, and possibly, within the phagosomes of infected macrophages (16). The high degree of sterilizing activity of PZA was confirmed in an in vitro model (5) and in mice (13).

PZA, an analog of nicotinamide, is not active against *M. tuberculosis* under normal culture conditions (24), but it is active in acid medium (pH 5.5) (14) and in host macrophages (11). The mode of action of PZA is not understood. It is thought that the bacterial enzyme pyrazinamidase (PZase) is required to convert PZA to pyrazinoic acid (POA), which is toxic to *M. tuberculosis* (10), but the target of PZA or POA is unknown. Resistance to PZA develops readily, and in a fashion analogous to INH resistance (28), PZA-resistant *M. tuberculosis* strains lose both PZase and nicotinamidase activities (5, 10). These two enzyme activities are due to a single enzyme that acts on both nicotinamide and PZA (10). Loss of PZase correlates with resistance to PZA, and negative PZase tests for clinical isolates of *M. tuberculosis* are indicative of PZA resistance (12, 15, 25).

In order to understand the molecular basis of PZA resistance, we have recently cloned the PZase gene (*pncA*) from *M. tuberculosis* and identified mutations in this gene in five PZA-resistant *M. tuberculosis* strains as well as in *M. bovis* strains that are naturally resistant to PZA (19). *M. bovis* strains were found to contain a single characteristic mutation at nucleotide position 169, changing from C to G, which caused amino acid substitution of histidine to aspartic acid at amino acid position 57 (19). This characteristic bovine mutation (C-to-G change) has been shown to be a useful marker for the rapid differentiation of bovine from human tubercle bacilli (20). The identified *pncA* mutations in PZA-resistant *M. tuberculosis* or *M. bovis* strains were shown to be responsible for the PZA resistance by transformation with a functional *pncA* gene (19). In the present study, we analyzed 38 PZA-resistant clinical isolates of *M. tuberculosis* as well as in vitro mutants for potential mutations in the *pncA* gene to gain further insight into the genetic basis of PZA resistance and to address the correlations between PZA resistance, PZase activity, and *pncA* mutations.

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, 615 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 614-2975. Fax: (410) 955-0105. E-mail: yzhang@phnet.sph.jhu.edu.

TABLE 1. Characteristics of PZA-resistant clinical *M. tuberculosis* isolates[a]

Strain	PZA MIC (μg/ml)	*pncA* mutation
T1139	>900	Ile5 → Ser
2923-5	>300	Asp12 → Ala
W6472	>900	Asp12 → Asn
411	>900	Ala26 → Gly
M43548	>900	His51 → Gln
F43948	>300	Pro69 → Arg
2721-1	>300	Pro69 → Arg
T7527	>900	Cys72 → Arg
H3652	>900	Leu85 → Pro
T5721	>900	Leu85 → Pro
27795	>900	Leu85 → Pro
T2878	>900	Lys96 → Asn
VA205	>900	Gly132 → Ser
BD195	>900	Gly132 → Ser
557	>900	His137 → Pro
2957-3	>900	Cys138 → Tyr
CDCBP98	>900	Val139 → Leu
M4812	>300	Val139 → Leu
W57575	>900	Gln141 → Pro
BD200	>900	Thr142 → Lys
DHM4319	>900	Thr142 → Met
M4809	>900	Ala171 → Pro
F6182	>900	Nucleotide C deletion at position 28
8294	>900	Nucleotide G deletion at position 71
H1976	>900	Nucleotide C deletion at position 104
510	>900	Nucleotide G deletion at position 391
EP59	>900	Nucleotide T and G deletions at position 416
W296	>900	Nucleotide G deletion at position 443
EP66	>900	Nucleotide C insertion at position 475
H4171	>900	11-bp deletion at start codon
W5758	>900	11-bp deletion at start codon
576	>900	8-bp deletion at position 446
H5190	>900	5-bp insertion at position 518

[a] All strains lacked PZase activity.

Thirty-three of the 38 clinical isolates and 8 in vitro mutants were found to contain *pncA* mutations, as revealed by DNA sequence analysis. The *pncA* mutations could be rapidly identified by the PCR–single-strand conformation polymorphism (SSCP) technique.

MATERIALS AND METHODS

Mycobacterial genomic DNA. *M. tuberculosis* cultures were grown in 7H9 liquid medium with albumin-dextrose-catalase enrichment (Difco) at 37°C for 2 to 4 weeks. Genomic DNA for PCR was isolated as follows. Bacterial cultures (5 to 10 ml) were concentrated by centrifugation, and the cell pellet was washed twice with distilled water and resuspended in 150 to 250 μl of water. The bacterial cells were heat killed by incubating them at 80°C for 20 min. One-third to one-half volume of glass beads (diameter, 0.1 mm; Sigma) was added to the bacterial suspension, followed by vigorous vortexing at high speed for 5 min. Bacterial lysates were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) three times. Genomic DNA was precipitated with 2 volumes of absolute alcohol, collected by centrifugation, washed with 70% alcohol, and resuspended in 100 μl of sterile distilled water.

Clinical *M. tuberculosis* isolates. The clinical isolates were reported to be PZA resistant by laboratories by using various methods at the time of isolation from 1992 to 1995 (Table 1). All the PZA-resistant *M. tuberculosis* strains were identified by using the BACTEC radiometric method at pH 6.0 (6, 21). Susceptibility was defined as an MIC of no more than 100 μg of PZA per ml. For most PZA-resistant strains analyzed in this study, MICs were more than 900 μg/ml. Retesting of the MIC for PZA-resistant strains without *pncA* mutations was performed by both the BACTEC method (6, 21) and the 7H9 liquid medium method at acid pH (pH 5.6) (14). For PZA susceptibility testing, susceptible strains H37Ra or H37Rv and PZA-resistant strain *M. bovis* BCG were included as sensitive and resistant controls, respectively. PZase activity was assayed as described by Wayne (26). A positive culture (PZA-sensitive *M. tuberculosis* H37Rv) and a negative culture (BCG Pasteur) were included as controls for the PZase assay.

Isolation of in vitro mutants resistant to PZA or POA. About 10^8 to 10^9 tubercle bacilli (H37Rv or Erdman strain) were spread onto 7H11 agar plates containing 500 μg of PZA or POA per ml at acid pH (pH 5.6). The plates were incubated at 37°C for 4 weeks before the plates were examined. Mutant colonies were subcultured into 7H9 liquid medium and were analyzed for PZase activity (26) and *pncA* mutations by DNA sequencing (see below).

PCR-SSCP analysis. For PCR-SSCP analysis, three sets of primers were designed according to the *M. tuberculosis pncA* sequence (558 bp) (GenBank accession number U59967 [19]). These primers were as follows: set 1, primers P1 (5′-GTCGGTCATGTTCGCGATCG-3′; from −105 bp upstream of *pncA*) and P2 (5′-TCGGCCAGGTAGTCGCTGAT-3′; from nucleotide positions 110 to 91 of *pncA*); set 2, primers P3 (5′-ATCAGCGACTACCTGGCCGA-3′; nucleotide positions 91 to 110) and P4 (5′-GATTGCCGACGTGTCCAGAC-3′; nucleotide positions 270 to 251); and set 3, primers P5 (5′-CCACCGATCATTGTGTGC GC3′; 401 to 420 bp) and P6 (5′-GCTTTGCGGCGAGCGCTCCA-3′; from 60 bp downstream of the stop codon). PCR was performed as described by Saiki et al. (18). The PCR cycling parameters were 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. SSCP was carried out as described by Orita et al. (17). Briefly, PCR products (5 μl containing about 0.5 to 1 μg of DNA) were denatured by boiling for 5 to 10 min in formamide dye (95% formamide, 10 mM sodium hydroxide, 20 mM EDTA, and 0.05% bromophenol blue and 0.05% xylene cyanol FF), followed by cooling on ice for 5 to 10 min. The denatured PCR products were loaded onto a 20% polyacrylamide gel (16 by 20 cm; containing 5% glycerol) that had been precooled to 4°C. Electrophoresis was performed in 0.5× TBE (Tris-borate-EDTA) buffer at a constant power of 5 W in a cold room overnight. The SSCP bands in the gel were visualized by silver staining.

DNA sequencing. To determine the sequences of the *pncA* genes from various strains of *M. tuberculosis*, we first amplified the *pncA* genes by PCR using the conditions described above with the forward and reverse primers (P1 and P6, respectively). The expected size of the *pncA* PCR products was about 720 bp. The PCR products from different *M. tuberculosis* strains were cut from the gel and purified with the GeneClean Kit (United States Biochemicals), according to the manufacturer's instructions. The gel-purified PCR products were directly sequenced in an Applied Biosystems Inc. automatic DNA sequencer (model 377) with primers P1 and P6.

RESULTS

Identification of *pncA* mutations in PZA-resistant clinical *M. tuberculosis* isolates. We have recently sequenced the *pncA* gene from *M. tuberculosis* and identified *pncA* mutations in five PZA-resistant *M. tuberculosis* strains (19). In order to further define the molecular basis of PZA resistance and determine the frequency of *pncA* mutations among PZA-resistant *M. tuberculosis* strains, we analyzed 38 PZA-resistant clinical *M. tuberculosis* isolates for potential mutations in the *pncA* gene. The *pncA* genes from various PZA-resistant *M. tuberculosis* strains were amplified by PCR, and the PCR products were subjected to direct DNA sequencing. Mutations in the sequences of *pncA* genes from PZA-resistant strains were identified by comparison with the wild-type *M. tuberculosis pncA* gene sequence (19). Since publication of the report of Scorpio and Zhang (19), we found that the *pncA* sequence contained a sequencing error; i.e., the C at nucleotide position 69 should be T. This error has been corrected, and thus, *pncA* sequences with a T at nucleotide position 69 were not considered to be mutated in this study. The results of the sequence analysis of the *pncA* gene from various PZA-resistant strains are presented in Table 1.

Among 38 PZA-resistant strains analyzed, 33 had *pncA* mutations including nucleotide substitutions (missense mutations) or insertions and small deletions (nonsense mutations), causing amino acid substitutions in most cases or frame shifts leading to nonsense polypeptides. Overall, 26 types of mutations were found in the 33 PZA-resistant strains, and these mutations were dispersed along the *pncA* gene. However, a certain degree of conservation of *pncA* mutations was observed at the following amino acid residues: Asp12 → Ala or Asn, Leu85 → Pro, Gly132 → Ser, and Thr142 → Lys or Met. Some degree of clustering of mutations (including both missense and

nonsense mutations) was found at the following regions: Gly132-Thr142, Pro69-Leu85, and Ile5-Asp12.

Five of 38 PZA-resistant strains did not contain detectable *pncA* mutations. All five strains (strains M39396, T5458, DHM444, 260-93, and 2888-3) were found to have positive PZase activity. Four strains (strains M39396, T5458, 260-93, and 2888-3) were falsely resistant; i.e., they were actually susceptible to PZA (MIC, 100 μg/ml) upon retesting by both methods (6, 14). However, one strain, strain DHM444, was less susceptible to PZA, with a borderline or low-level resistance (MIC, 200 to 300 μg/ml, which is two to three times the MIC used to define susceptible strains). Sequencing of 20 random PZA-susceptible *M. tuberculosis* strains did not reveal any silent mutations in the *pncA* gene. This suggests that mutations in the *pncA* gene are indicative of PZA resistance, an important feature for the detection of PZA resistance on the basis of identifying mutations in the *pncA* gene.

Analysis of PZA-resistant mutants made in vitro. In order to determine whether PZA-resistant mutants made in vitro would also have the same mechanism of resistance as PZA-resistant clinical isolates with *pncA* mutations, we made in vitro mutants of strain H37Rv and strain Erdman resistant to PZA on 7H11 plates containing 500 μg of PZA per ml. We obtained 14 PZA-resistant mutants (10 from H37Rv and 4 from Erdman), and they were all negative for PZase, as judged by Wayne's method (26). This indicates that they may have *pncA* mutations. Sequence analysis of 4 of 10 H37Rv mutants (mutants Rv-P1, Rv-P2, Rv-P3, and Rv-P4) revealed that they contained *pncA* mutations. Rv-P1 had a deletion of 118 bases between positions 98 and 216 of the *pncA* gene. Rv-P2 had a substitution of A to C at nucleotide position 287, leading to an Lys96 → Thr substitution. Rv-P3 had a substitution of T to C at nucleotide position 40, resulting in a Cys34 → Arg amino acid substitution. Rv-P4 had an extra T nucleotide at position 100, causing a frame shift in the open reading frame of PncA.

Analysis of four Erdman mutants (mutants Erd-P1 to Erd-P4) indicated that they had the following *pncA* mutations: Erd-P1 had an extra T at nucleotide position 162, causing a frameshift mutation; Erd-P2 had a C-to-G change at nucleotide position 24, leading to an Asp8 → Glu substitution; Erd-P3 had a G-to-A change at nucleotide position 413, causing a Cys138 → Tyr substitution; and Erd-P4 had a mutation of A to C at position −11 upstream of the start codon of *pncA*. This mutation may weaken the promoter activity and decrease the level of PZase expression in this mutant.

Because the POA derived from conversion of PZA by PZase is thought to be the active bactericidal component, we reasoned that potential mutants resistant to POA would represent strains with mutations in the drug target. It would be of interest to obtain such mutants in order to better understand the mode of action of PZA and to determine if there is any alternative mechanism of PZA resistance in strains with mutations in the potential drug target. However, after repeated attempts we were unable to obtain mutants resistant to POA on 7H11 agar medium.

PCR-SSCP analysis of PZA-resistant *M. tuberculosis* isolates. To test the feasibility of using the PCR-SSCP technique for the rapid detection of point mutations in the *pncA* gene in PZA-resistant strains, we performed PCR-SSCP analysis with selected PZA-resistant clinical isolates. The results indicated that rapid identification of mutations in the *pncA* gene was possible (Fig. 1). Analysis of 10 random PZA-susceptible *M. tuberculosis* strains did not show any abnormal SSCP pattern (data not shown).

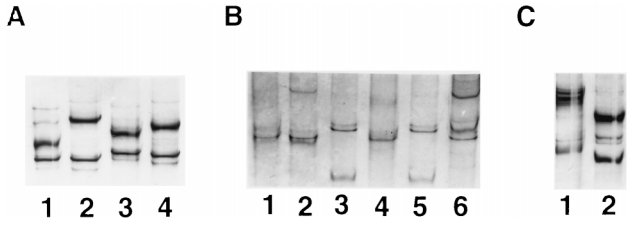

FIG. 1. PCR-SSCP analysis of *pncA* mutations in PZA-resistant *M. tuberculosis* strains. (A) SSCP with primer set 1. Lanes 1 to 3, PZA-resistant clinical isolates H4171, Quirk, and 2923-5, respectively; lane 4, PZA-susceptible control strain Erdman. (B) SSCP analysis with primer set 2. Lanes 1 to 5, PZA-resistant clinical isolates Vertullo, F36946, F43948, M43548, and T2727, respectively; lane 6, PZA-susceptible control strain Erdman. (C) SSCP analysis with primer set 3. Lane 1, PZA-resistant clinical isolate M4809; lane 2, PZA-susceptible control strain Erdman.

DISCUSSION

The present study has shown that 33 of 38 PZA-resistant clinical *M. tuberculosis* isolates and 8 mutants made in vitro had mutations in the *pncA* gene. This indicates that the *pncA* mutation is the major mechanism of PZA resistance in *M. tuberculosis*, a finding consistent with previous observations that most PZA-resistant *M. tuberculosis* strains lack PZase activity (3, 12, 15, 25). The nature of the *pncA* mutations includes substitution of amino acids (26 of 41 total PZA-resistant isolates with *pncA* mutations), insertions or small deletions of nucleotides causing nonsense peptides (14 of 41 isolates), and mutations in the *pncA* promoter (1 of 41 isolates). The distribution of *pncA* mutations is dispersed along the gene. Among the five resistant clinical isolates that did not contain *pncA* mutations, four were due to false resistance (i.e., they were susceptible upon retesting), a common problem of current PZA susceptibility testing (6, 7). The only strain (strain DHM444) without *pncA* mutations was found to have borderline or low-level resistance to PZA. It is interesting that borderline resistant strain DHM444 was still responsive to PZA treatment in mice at a dose comparable to that used for humans (9), indicating that the borderline resistance may not have clinical significance.

The existence of a borderline resistant strain may suggest alternative mechanisms of PZA resistance which may be a result of the following possibilities. One is that mutations in genes involved in the uptake of PZA or in genes encoding an efflux pump may be responsible for the low level of PZA resistance. Work is under way to test these possibilities by [^{14}C] PZA uptake and genetic transformation studies. Another possibility is that mutations in the PZA or POA target (which is unknown) may be the cause. However, our results do not support this possibility, since the borderline resistant strain is susceptible to POA (data not shown), indicating that it does not have mutations in the drug target. In fact, we were unable to isolate mutants resistant to POA on 7H11 agar plates in vitro, suggesting that mutants resistant to POA (i.e., strains with mutations in the drug target) may be lethal or auxotrophic. It is possible that the PZA target mutants may require additional nutrients such as amino acids in order to grow. We are investigating this possibility by selecting in vitro mutants using 7H11 medium supplemented with various amino acids and other nutrients.

While it is clear from this study that *pncA* mutations causing defective PZase account for almost all PZA-resistant strains, the scarcity of PZase-positive, PZA-resistant clinical isolates could be due to the following possibilities. It is likely that mutations in the drug target are lethal so that such mutants

may not be viable in vitro in normal medium or in vivo in patients. Another possibility is that most PZA-resistant *M. tuberculosis* strains analyzed in this study had high-level resistance (MIC >900 μg/ml, a PZA concentration which may preferentially select for mutations in the *pncA* gene). The *pncA* mutations identified in various PZA-resistant strains in this study are presumed to be responsible for the PZA resistance, as previously shown by transformation studies with a functional *pncA* gene (19). Yet, how PZA interacts with the PZase enzyme leading to activation of PZA to POA and how mutations affect PZase activity and thus its inability to activate PZA are unknown. Site-directed mutagenesis along with crystallography studies of both wild-type and mutant PZase enzymes will provide insight into the structure-function relationship of this enzyme. This information will help us to better understand the mechanism of action of PZA and resistance to PZA.

Our finding that most PZA-resistant *M. tuberculosis* strains have mutations in the *pncA* gene has implications for developing a rapid test for detecting PZA-resistant *M. tuberculosis* strains. The diversity of methods currently used in clinical laboratories for the detection of PZA resistance in *M. tuberculosis* isolates causes inconsistent results of PZA susceptibility testing (7). Inconsistent results of PZA susceptibility testing have been reported by a number of laboratories by various methods, including the qualitative BACTEC test (6, 21). On the basis of our analyses of both PZA-resistant clinical isolates and mutants made in vitro, there is a very good correlation between the loss of PZase activity and *pncA* mutations and PZA resistance. This feature is important for designing PCR-based tests for the rapid detection of *pncA* mutations as a correlate of PZA resistance. Analysis of the *pncA* sequence has found that four PZA-resistant strains determined by the BACTEC method are in fact susceptible, indicating that the sequence-based test, e.g., direct sequencing by PCR, may be more accurate or reliable. Further comparative analyses of more strains by the *pncA* sequence-based method and BACTEC method will be necessary to determine which method is more accurate. We have demonstrated in this study that *pncA* mutations in PZA-resistant strains can be readily detected by the PCR-SSCP technique in 1 to 2 days. Thus, detection of *pncA* mutations by direct sequencing by PCR or SSCP not only is fast but also will avoid the problems of current PZA susceptibility testing. Such a test should be useful for directing the treatment of tuberculosis, reducing treatment costs, and potentially limiting the spread of drug-resistant *M. tuberculosis* isolates.

ACKNOWLEDGMENT

This work was supported by a Faculty Development Award (to Y.Z.) from the Johns Hopkins School of Hygiene and Public Health.

REFERENCES

1. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
2. **British Thoracic Association.** 1984. A controlled trial of six months chemotherapy in pulmonary tuberculosis. Final report: results during the 36 months after the end of chemotherapy and beyond. Br. J. Dis. Chest **78:**330–336.
3. **Butler, W. R., and J. O. Kilburn.** 1983. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob. Agents Chemother. **24:**600–601.
4. **Centers for Disease Control.** 1991. Nosocomial transmission of multidrug-resistant tuberculosis among HIV-infected persons—Florida and New York, 1988–1991. Morbid. Mortal. Weekly Rep. **40:**585–591.
5. **Heifets, L., and P. Lindholm-Levy.** 1992. Pyrazinamide sterilizing activity in vitro against semi-dormant *Mycobacterium tuberculosis* bacterial populations. Am. Rev. Respir. Dis. **145:**1223–1225.
6. **Heifets, L. B.** 1991. Drug susceptibility in the chemotherapy of mycobacterial infections, p. 89–122. *In* L. B. Heifets (ed.), Drug susceptibility tests in the management of chemotherapy of tuberculosis. CRC Press, Inc., Boca Raton, Fla.
7. **Hewlett, D., D. L. Horn, and C. Alfalla.** 1995. Drug-resistant tuberculosis: inconsistent results of pyrazinamide susceptibility testing. JAMA **273:**916–917.
8. **Hong Kong Chest Service/British Medical Research Council.** 1981. Controlled trial of four thrice-weekly regimens and a daily regimen all given for 6 months for pulmonary tuberculosis. Lancet **i:**171–174.
9. **Klemens, S. P., C. A. Sharpe, and M. H. Cynamon.** 1996. Activity of pyrazinamide in a murine model against *Mycobacterium tuberculosis* isolates with various levels of in vitro susceptibility. Antimicrob. Agents Chemother. **40:** 14–16.
10. **Konno, K., F. M. Feldman, and W. McDermott.** 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis. **95:** 461–469.
11. **Mackaness, G. B.** 1956. The intracellular activation of pyrazinamide and nicotinamide. Am. Rev. Tuberc. **74:**718–728.
12. **McClatchy, J. K., A. Y. Tsang, and M. S. Cernich.** 1981. Use of pyrazinamidase activity in *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility. Antimicrob. Agents Chemother. **20:** 556–557.
13. **McCune, R. M., R. Tompsett, and W. McDermott.** 1956. The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by administration of pyrazinamide and a companion drug. J. Exp. Med. **104:**763–802.
14. **McDermott, W., and R. Tompsett.** 1954. Activation of pyrazinamide and nicotinamide in acidic environments in vitro. Am. Rev. Tuberc. **70:**748–754.
15. **Miller, M., L. Thibert, F. Desjardins, S. Siddiqi, and A. Dascal.** 1995. Testing of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide: comparison of BACTEC method with pyrazinamidase assay. J. Clin. Microbiol. **33:**2468–2470.
16. **Mitchison, D. A.** 1985. The action of antituberculosis drugs in short course chemotherapy. Tubercle **66:**219–225.
17. **Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi.** 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics **5:**874–879.
18. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
19. **Scorpio, A., and Y. Zhang.** 1996. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nature Med. **2:**662–667.
20. **Scorpio, A., D. M. Collins, D. Whipple, D. Cave, J. Bates, and Y. Zhang.** 1997. Rapid differentiation of bovine and human tubercle bacilli based on a characteristic mutation in the bovine pyrazinamidase gene. J. Clin. Microbiol. **32:**106–110.
21. **Siddiqui, S. H.** 1992. Antimicrobial susceptibility testing: radiometric (BACTEC) tests for slowly growing mycobacteria, p. 14–25. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook. ASM Press, Washington, D.C.
22. **Singapore Tuberculosis Service/British Medical Research Council.** 1981. Clinical trial of six-month and four-month regimens of chemotherapy in the treatment of pulmonary tuberculosis: the results up to 30 months. Tubercle **62:**95–102.
23. **Snider, D. E., J. Rogowski, M. Zierski, E. Bek, and M. W. Long.** 1982. Successful intermittent treatment of smear-positive pulmonary tuberculosis in six months. Am. Rev. Respir. Dis. **125:**265–267.
24. **Tarshis, M. S., and W. A. Weed.** 1953. Lack of significant in vitro sensitivity of *Mycobacterium tuberculosis* to pyrazinamide on three different solid media. Am. Rev. Tuberc. **67:**391–395.
25. **Trivedi, S. S., and S. G. Desai.** 1987. Pyrazinamidase activity of *Mycobacterium tuberculosis*—a test of sensitivity to pyrazinamide. Tubercle **68:**221–224.
26. **Wayne, L. G.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am. Rev. Respir. Dis. **109:**147–151.
27. **Yeager, R. L., W. G. Munroe, and F. I. Dessau.** 1952. Pyrazinamide (Aldinamide) in the treatment of pulmonary tuberculosis. Am. Rev. Tuberc. **65:** 523–534.
28. **Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole.** 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature (London) **358:**591–593.