ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2000, p. 2291–2295 Vol. 44, No. 9
0066-4804/00/$04.00+0

Phenotypic Characterization of *pncA* Mutants of *Mycobacterium tuberculosis*

GLENN P. MORLOCK,[1]* JACK T. CRAWFORD,[1] W. RAY BUTLER,[1] SUZANNE E. BRIM,[1] DAVID SIKES,[1] GERALD H. MAZUREK,[2] CHARLES L. WOODLEY,[1] AND ROBERT C. COOKSEY[1]

Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases,[1] and Division of Tuberculosis Elimination, National Center for HIV, STD, and TB Prevention,[2] Centers for Disease Control and Prevention, Atlanta, Georgia 30333

Received 11 June 1999/Returned for modification 27 August 1999/Accepted 5 June 2000

We examined the correlation of mutations in the pyrazinamidase (PZase) gene (*pncA*) with the pyrazinamide (PZA) resistance phenotype with 60 *Mycobacterium tuberculosis* isolates. PZase activity was determined by the method of Wayne (L. G. Wayne, Am. Rev. Respir. Dis. *109:*147–151, 1974), and the entire *pncA* nucleotide sequence, including the 74 bp upstream of the start codon, was determined. PZA susceptibility testing was performed by the method of proportions on modified Middlebrook and Cohn 7H10 medium. The PZA MICs were ≥100 μg/ml for 37 isolates, 34 of which had alterations in the *pncA* gene. These mutations included missense substitutions for 24 isolates, nonsense substitutions for 3 isolates, frameshifts by deletion for 4 isolates, a three-codon insertion for 1 isolate, and putative regulatory mutations for 2 isolates. Among 21 isolates for which PZA MICs were <100 μg/ml, 3 had the same mutation (Thr47→Ala) and 18 had the wild-type sequence. For the three Thr47→Ala mutants PZA MICs were 12.5 μg/ml by the method of proportions on 7H10 agar; two of these were resistant to 100 μg of PZA per ml and the third was resistant to 800 μg of PZA per ml by the BACTEC method. In all, 30 different *pncA* mutations were found among the 37 *pncA* mutants. No PZase activity was detected in 35 of 37 strains that were resistant to ≥100 μg of PZA per ml or in 34 of 37 *pncA* mutants. Reduced PZase activity was found in the three mutants with the Thr47→Ala mutation. This study demonstrates that mutations in the *pncA* gene may serve as a reliable indicator of resistance to ≥100 μg of PZA per ml.

Two essential elements of a tuberculosis control program are the early identification of infectious patients and the rapid implementation of an effective treatment regimen. The first-line drugs used to treat tuberculosis include rifampin, isoniazid, pyrazinamide (PZA), and either ethambutol or streptomycin (1). Because drug resistance can have a serious negative impact on both patient outcome and control of transmission, it is critical that any drug resistance be accurately detected as soon as possible. The timeliness of in vitro drug susceptibility testing of *Mycobacterium tuberculosis* is constrained by the organism's relatively slow growth. Conventional drug susceptibility testing of *M. tuberculosis* can take from 7 to 28 days, depending on the culture system used (23). For most antituberculosis drugs, these conventional methods produce reliable results. In contrast, in vitro testing for susceptibility to PZA is hampered by poor growth of the bacilli under the acidic conditions (pH 5.5 to 6.0) required for optimal drug activity (16, 19).

PZA is an important component of short-course chemotherapy against tuberculosis because of its activity against semidormant bacilli sequestered within macrophages (10, 21). The intracellular sterilizing activity of PZA allows the treatment period to be reduced to 6 months, whereas 9 months of treatment is required when PZA is not used (26, 27). While the mode of action of PZA is not fully understood, there is substantial evidence that the drug must first be converted to pyrazinoic acid (POA) by the enzyme pyrazinamidase (PZase) for it to have antimycobacterial activity (13). Strains of *M. tuberculosis* susceptible to PZA typically have PZase activity, whereas PZA-resistant strains frequently lack PZase activity (3, 18). Recently, the *M. tuberculosis* PZase gene (*pncA*) was identified (24). The involvement of the product of this gene in the mode of action of PZA was convincingly demonstrated through experiments in which the naturally PZA-resistant strain *Mycobacterium bovis* BCG was rendered PZA susceptible by transformation with a cosmid that expressed a functional *M. tuberculosis* PncA enzyme (24).

DNA sequencing studies of the *pncA* gene from PZA-resistant and PZA-susceptible strains of *M. tuberculosis* have established a strong association between mutations in this gene and PZA resistance (6, 11, 15, 17, 20, 25, 29). These studies identified a diverse group of mutations widely dispersed throughout the gene. The majority of these are missense mutations, but insertions, deletions, and putative regulatory mutations were also described. This diversity of mutations highlights the need for further studies for characterization of *pncA*. For example, little is known about the effect of specific mutations on the level of PZA resistance. To increase our understanding of the molecular basis of PZA resistance, we determined the nucleotide sequence of the *pncA* genes of 60 clinical isolates of *M. tuberculosis* initially found to be resistant to 25 μg of PZA per ml. Sequencing results were then compared to the MICs and PZase activities for all isolates.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA isolation. The isolates of *M. tuberculosis* examined in this study were submitted to the Centers for Disease Control and Prevention Mycobacteriology Laboratory for routine drug susceptibility testing. Each isolate was resistant to 25 μg of PZA per ml when it was tested by the method of proportions (4) with Middlebrook and Cohn 7H10 agar. Isolates were stored frozen at −70°C until selected for this study. A total of 60 isolates were selected. Genomic DNA was prepared by a minibead cell disruption protocol. Briefly, 1 ml of a 2-week-old 7H9 broth culture was added to a 1.5-ml screw-cap

* Corresponding author. Mailing address: 1600 Clifton Rd., Mail Stop F08, Atlanta, GA 30333. Phone: (404) 639-1280; Fax: (404) 639-1287. E-mail: gpm0@cdc.gov.

microcentrifuge tube containing approximately 250 mg of siliconized zirconia or silica beads (diameter, 0.1 mm), 200 μl of chloroform, and 300 μl of Tris-EDTA (TE) buffer. This mixture was vigorously agitated for 2 min with a Mickle cell disrupter (Brinkman Instruments, Inc., Westbury, N.Y.) and was then centrifuged at 10,000 rpm for 5 min. The aqueous phase, which contained genomic DNA, was collected and stored at 4°C.

Amplification and sequencing of *pncA* gene. The entire *pncA* open reading frame, as well as 124 bp of the upstream sequence and 59 bp of the downstream sequence, was amplified by PCR. A 744-bp PCR product was generated with primers *pncA*-8 (5′-GGTTGGGTGGCCGCCGGTCAG-3′) and *pncA*-11 (5′-GCTTTGCGGCGAGCGCTCCA-3′). The *pncA* open reading frame (561 bp) begins at nucleotide 125 of the 744-bp PCR product and ends at nucleotide 685. Each 50-μl PCR mixture contained 1.0 μl of template DNA, 2.5 U of *Taq* DNA polymerase (Boehringer-Mannheim, Indianapolis, Ind.), deoxynucleotide triphosphates (concentrations, 200 μM each), and each primer at a concentration of 0.5 μM in 1× PCR buffer. Amplification was performed in a Gene-Amp PCR System 2400 thermal cycler (Perkin-Elmer, Inc., Foster City, Calif.) by a "touchdown" amplification approach in which the primer annealing temperature was decreased 0.5°C per cycle for the first 20 cycles, from 68°C for the first cycle to 58°C for cycles 20 to 35. The amplification profile consisted of an initial 5 min of denaturation at 94°C; 35 cycles of 94°C for 30 s, annealing for 30 s, and elongation at 72°C for 30 s; and a final 8-min elongation. Unincorporated primers and deoxynucleotide triphosphates were removed from the reaction mixtures with QIAquick PCR purification columns (Qiagen Inc., Santa Clarita, Calif.).

Automated DNA sequencing was performed by use of rhodamine DyeDeoxy Terminator chemistry by the protocol supplied by the manufacturer (Perkin-Elmer, Inc.). The fluorescent products were electrophoresed on an ABI model 373A instrument (Perkin-Elmer, Inc.). The *pncA* amplicons were sequenced with four internal primers. Primers *pncA*-10 (5′-GCTGGTCATGTTCGCGATCG-3′) and *pncA*-2R (5′-GAACACCGCCTCGATTGCCG-3′) were used to sequence nucleotide residues 20 to 406 of the 744-bp amplicon. Primers *pncA*-6 (5′-CCTCGTCGTGGCCACCGC-3′) and *pncA*-9 (5′-CGCCAACAAGTTCAA TCCCGG-3′) were used to sequence the region from residues 318 to 720. All postrun analyses were performed with Sequence Navigator, version 1.0.1, software (Perkin-Elmer, Inc.). Each sequencing run included the PZA-susceptible strain *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) as a wild-type control. Each sequence was compared both to the sequence of the control strain and to the published *pncA* sequence (GeneBank accession number U59967).

PZA susceptibility testing. PZA susceptibility testing was performed by a modification of the method of proportions on solid medium (4). Modified Middlebrook and Cohn 7H10 agar was prepared from basic ingredients as described previously (14) and was supplemented with albumin-dextrose-catalase enrichment (Difco Laboratories, Detroit, Mich.). The final pH of the medium was 5.5. A twofold series of PZA (Sigma Chemical Co., St. Louis, Mo.) concentrations ranging from 12.5 to 800 μg/ml was tested. The test media with one of the seven concentrations of PZA were dispensed into two four-quadrant Felsen plates (Falcon Plastics Co., Franklin Lakes, N.J.). The eighth quadrant contained control medium without PZA. The *M. tuberculosis* strains were cultured in Middlebrook 7H9 broth for 2 weeks (approximately 10^7 to 10^9 CFU/ml), after which 10^{-2} and 10^{-4} dilutions were prepared in 0.1% Tween 80 (Fisher Scientific Co., Fairlawn, N.J.). A set of plates was inoculated for each inoculum dilution. Each individual quadrant received 0.1 ml of the inoculum. The plates were incubated for 4 weeks at 37°C, and both sets of plates were read. The reading for the set that received the 10^{-2} inoculum was used to determine the MIC. The PZA-susceptible strain *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) was used as a control. The MIC was defined as the lowest PZA concentration which inhibited more than 99% of the growth seen on the PZA-free control quadrant. The recommended critical concentration of PZA for determination of resistance by the method with 7H10 agar is 25 μg/ml (12). The PZA susceptibilities of 10 of the 60 study isolates were also tested by the BACTEC method. PZA susceptibility testing by the BACTEC method was done by the standard procedure (S. H. Siddiqi, BACTEC 460TB system product and procedure manual, Becton Dickinson and Co., Sparks, Md., p. 1–7, 1996) except that PZA concentrations of 25, 50, 200, 400, and 800 μg/ml were tested in addition to the standard concentration of 100 μg/ml. The recommended critical concentration of PZA for determination of resistance by the BACTEC method is 100 μg/ml (Siddiqi, BACTEC manual).

PZase testing. PZase activity was assayed by a modification of the method developed by Wayne (31). The medium used for PZase testing was inoculated with a mixture of cells both from a 7H9 broth culture and from a Lowenstein-Jensen culture. The mixture consisted of 0.5 ml of cell sediment drawn from the bottom of a 2-week stationary broth culture combined with a visible amount of growth scraped from the surface of a 3-week Lowenstein-Jensen slant. Two tubes of medium used for PZase testing were inoculated for each strain, and the tubes were incubated at 37°C. One tube was examined after 7 days of incubation, and the other was examined after 14 days of incubation. A tube inoculated with an *M. avium* strain was included as a positive control, as called for in the protocol of the Centers for Disease Control and Prevention (12). Test medium that contained 100 μg of POA (Aldrich Chemical Co., Milwaukee, Wis.) per ml and no PZA was used as a positive control for the assay system. Uninoculated test medium was used as a negative control. After incubation, 1.0 ml of 1% ferrous ammonium sulfate (Sigma Chemical Co.) was added to each tube. The tubes were set at room temperature for 1 h. A strain was considered positive for PZase if a diffuse pink band was seen in the agar. Each strain was compared to the positive and negative controls. All tubes were examined independently by three individuals.

RESULTS

***pncA* sequencing of *M. tuberculosis* isolates.** The *pncA* genes of 60 *M. tuberculosis* isolates were sequenced (Table 1). A *pncA* mutation was identified in 37 of these isolates, while 23 isolates lacked mutations. A total of 30 different mutations, 14 of which have not been previously reported, were found among the 37 *pncA* mutants. The majority of these (25 of 30) were point mutations that resulted in either an amino acid substitution or the insertion of a stop codon. Three isolates with the same IS*6110* fingerprint type (isolates 01, 02, and 19) had a frameshift mutation that resulted from the deletion of a guanine at position 71. One isolate (isolate 20) was missing the entire *pncA* gene downstream of nucleotide 106 as the result of the deletion of an approximately 5.3-kb fragment. The size of the deleted fragment was determined by comparing the sequence of an approximately 300-bp PCR product, fortuitously generated with primers *pncA*-1 (5′-ATGCGGGCGTTGATCATCG T-3′) and *pncA*-11, to that of cosmid MTV018 in the Sanger Centre mycobacterial database. A 9-bp in-frame insertion occurred in one strain (strain 09). Mutations within the *pncA* open reading frame were distributed in the region from nucleotide 11 through nucleotide 515. Two putative regulatory mutations were identified. One mutation (in isolate 26) resulted from the insertion of an adenosine and a change in 4 of 6 nucleotides in the region from positions −11 to −16 upstream of the start codon. The second mutation (in isolate 32) resulted from a T-to-C transition at nucleotide −7.

PZA MICs. We determined the MICs of PZA for all isolates in order to compare specific *pncA* mutations with PZA resistance levels (Table 1). Among the 37 isolates that possessed *pncA* mutations, the PZA MICs were ≥100 μg/ml for 34 isolates on solid medium. The remaining three mutant isolates (isolates 51 to 53) all had the same A-to-G transition at nucleotide 139, resulting in a Thr47→Ala amino acid substitution. The PZA MICs for these three isolates were 12.5 μg/ml, and the isolates shared an identical pattern by restriction fragment length polymorphism analysis with IS*6110* (these strains were designated strain W) (7). Among the 23 isolates that lacked a *pncA* mutation, the PZA MIC was <100 μg/ml for 18 of them. For two isolates (isolates 36 and 37) with wild-type *pncA* sequences, the MIC was 100 μg/ml. For a third such isolate (isolate 25), the MIC was 400 μg/ml, and this high-level resistance was confirmed by the BACTEC method. The remaining two wild-type isolates (isolates 59 and 60) failed to grow on the low-pH control medium. Seventeen of the isolates (28.3%) initially found to be resistant to 25 μg of PZA per ml were susceptible to the drug at this concentration. Table 2 compares the *pncA* sequencing results to those of MIC testing on solid medium by use of a PZA concentration of 100 μg/ml as a breakpoint.

While most of the *pncA* mutants were resistant to a relatively high concentration of PZA (MICs, ≥400 μg/ml), six showed intermediate-level resistance (MICs, 100 to 200 μg/ml) and the MICs for three isolates (isolates 51 to 53) with the same mutation (Thr47→Ala) were very low (12.5 μg/ml). These last three isolates were additionally tested by the BACTEC method, and two were determined to be borderline resistant to 100 μg of PZA per ml, while the third one was resistant to >800 μg of PZA per ml. Sequencing of the *pncA* gene of this third strain W isolate was repeated with DNA extracted from bacilli recovered from a PZA-containing BACTEC bottle, and only the

TABLE 1. MICs of PZA, PZase activities, and changes in the *pncA* gene for 60 clinical *M. tuberculosis* isolates

Isolate no.	PZA MIC (μg/ml)[a]	PZase activity		Change in[b]	
		7 days	14 days	Nucleotide no.	Amino acid
01–02	>800	−	−	G dln at position 71	Frameshift
03	>800	−	−	C→G at position 185	P62→R
04	>800	−	−	A→G at position 212	H71→R
05	>800	−	−	G→A at position 233	G78→D
06–07	>800	−	−	T→C at position 254	L85→P
08	>800[c]	−	−	C→G at position 297	Y99→AMB
09	>800	−	−	GAGGTCGAT at position 388, ins	E-V-D ins.
10	>800	−	−	T→G at position 389	V130→G
11	>800	−	−	C→T at position 401	A134→V
12	>800	−	−	A→C at position 403	T135→P
13	>800	−	−	A→G at position 410	H137→R
14	>800	−	−	G→A at position 413	C138→Y
15	>800	−	−	T→G at position 416	V139→G
16	>800	−	−	G→A at position 511	A171→T
17	800	−	−	T→C at position 11	L4→S
18	800	−	−	A→C at position 29	Q10→P
19	800	−	−	G dln. at position 71	Frameshift
20	800	−	−	≅5.3 kb dln at position 106	NA
21	800	−	−	A→C at position 152	H51→P
22	800	−	−	T→A at position 398	I133→N
23	800	−	−	A→G at position 410	H137→R
24	800	−	−	C→A at position 458	T153→N
25	400[c]	−	−	None	NA
26	400	−	−	$_{-16}$AACGTA$_{-11}$→GGCAGTT	NA
27	400	−	−	A→C at position 226	T76→P
28	400	−	−	G→A at position 289	G97→S
29	400	−	−	G→A at position 290	G97→D
30	200[c]	−	−	T→C at position 515	L172→P
31	200	−	−	T→C at position 515	L172→P
32	100	−	−	T→C at position −07	NA
33	100[c]	−	−	C→A at position 309	Y103→OCH
34	100[c]	−	−	C→G at position 309	Y103→AMB
35	100	−	−	G→C at position 415	V139→L
36–37	100[d]	+	+	None	NA
38–41	50	+	+	None	NA
42–50	25	+	+	None	NA
51–53	12.5[e]	−	+	A→G at position 139	T47→A
54–58	12.5	+	+	None	NA
59–60	No growth	+	+	None	NA
$H_{37}Rv$	12.5	+	+	None	NA

[a] As tested on Middlebrook and Cohn 7H10 agar (pH 5.5).
[b] Abbreviations: dln, deletion; ins, insertion; NA, not applicable.
[c] MIC, >800 μg/ml in BACTEC.
[d] MIC, 50 μg/ml by BACTEC, which is considered PZA susceptible.
[e] In BACTEC isolates 51 and 52 were borderline resistant to 100 μg/ml and isolate 53 was resistant to 800 μg/ml.

Thr47→Ala mutation was identified. The MICs for two strains with different mutations within the same codon were very different. The MIC for strain 35, which had a Val139→Leu substitution, was 100 μg/ml, while the MIC for strain 15, which had a Val139→Gly substitution, was >800 μg/ml. The PZA MICs for all four deletion mutants were ≥800 μg/ml, as was that for a strain with a three-codon insertion. For two isolates (isolates 32 and 26) with putative regulatory mutations the MICs were 100 and 400 μg/ml, respectively.

PZase activity. The modified Wayne (31) PZase test was performed to assess each isolate's ability to hydrolyze PZA into POA (Table 1). For 57 of the 60 isolates, the consensus result for the 14-day reading agreed with that of the 7-day reading. Three isolates (isolates 51 to 53) were PZase negative at 7 days but became positive after 14 days of incubation. These three isolates all had the same amino acid substitution (Thr47→Ala), and the PZA MICs were 12.5 μg/ml for all three isolates. The remaining 34 *pncA* mutants all lacked PZase activity. One of the 23 isolates with a wild-type *pncA* sequence (isolate 25) was PZase negative, and the MIC for this isolate was 400 μg/ml. This high-level resistance was verified by the BACTEC method, and the lack of a *pncA* mutation was confirmed by sequencing of DNA extracted from bacilli recovered

TABLE 2. Comparison of the presence of mutations in the *pncA* gene to PZA MICs for 60 clinical *M. tuberculosis* isolates

MIC (μg/ml)[a]	No. of isolates with:	
	pncA mutation	No *pncA* mutation[b]
>100	30	1
100	4	2
<100	3	18

[a] As tested on Middlebrook and Cohn 7H10 agar (pH 5.5).
[b] Two isolates failed to grow on the test medium.

from a PZA-containing BACTEC bottle. Among the isolates for which MICs were ≥100 μg/ml, 35 of 37 were PZase negative. Results for five *M. bovis* isolates, unknowingly included in the study, were as expected (i.e., no PZase activity and PZA MICs of >800 μg/ml). Identification of these five isolates was subsequently confirmed by the finding of an *M. bovis*-associated *pncA* polymorphism (C→G at position 169) and by restriction fragment length polymorphism analysis of the *oxyR* gene (28).

DISCUSSION

We found that the presence of a mutation in the *pncA* gene correlates well with a PZA MIC of ≥100 μg/ml (for 34 of 37 isolates) and with a loss of PZase activity (for 34 of 37 isolates). A total of 30 different mutations were identified, all but one of which correlated with an MIC of ≥100 μg/ml. These mutations were distributed throughout the open reading frame or in a region immediately upstream of the start codon. Seventeen of the mutations have not previously been reported. The finding of 30 different mutations among the 37 strains that possessed a mutation indicates that a high degree of genetic diversity occurs within the *pncA* genes of PZA-resistant *M. tuberculosis* isolates. This conclusion is supported by previous reports which describe more than 120 mutations not seen in this study (6, 11, 15, 17, 20, 24, 25, 29). This diversity of *pncA* mutations may prove useful as a strain-typing marker in investigations of outbreaks caused by PZA-resistant *M. tuberculosis* isolates.

Three isolates had a Thr47→Ala mutation, and all of them had the same strain W pattern by restriction fragment length polymorphism analysis with IS*6110*. Strain W is multidrug resistant and has been involved in several tuberculosis outbreaks (8). Others have reported that the Thr47→Ala mutation is characteristic of the strain W family of strains and that these strains are PZA resistant (29). However, there are a number of conflicting reports concerning the PZA susceptibilities of isolates of this strain (5, 7, 30; D. J. Hewlett, D. L. Horn, and C. Alfalla, letter, JAMA **273:**916–917, 1995). The inconsistency of these reports suggests that the strain may be borderline resistant at the PZA concentrations routinely tested. From 1992 to 1995, our laboratory tested the PZA susceptibilities of 104 strain W isolates. Of these, 41 (39.4%) did not grow on the pH 5.5 control medium. Of the remaining 63 isolates, 53 (84.1%) were susceptible to 25 μg/ml. All three strain W isolates included in this study were susceptible to 12.5 μg of PZA per ml by the agar proportion method. When tested by the BACTEC method, two of these isolates were borderline resistant to 100 μg of PZA per ml, while the third was resistant to >800 μg of PZA per ml. While PZA susceptibility testing of *M. tuberculosis* is, in general, difficult, testing of strain W appears to be especially problematic. Nevertheless, our results indicate that, in vitro, this strain is substantially less PZA resistant than strains that harbor any of the other *pncA* mutations identified. A probable explanation for the modest (if any) increase in PZA resistance associated with the Thr47→Ala mutation is that this is a mutation which substantially reduces, but does not eliminate, PZase expression. This explanation is supported by our finding that the isolates that harbor this mutation were PZase negative at 7 days but became PZase positive at 14 days. The unique nature of the Thr47→Ala mutation is further supported by our recent discovery (subsequent to completion of this study) of a strain that has a different mutation within this codon (Thr47→Asn) and that is highly PZA resistant by both the agar proportion method (>100 μg/ml) and the BACTEC method (>800 μg/ml). The problematic nature of PZA susceptibility testing of strain W may result from genetic changes outside the *pncA* gene or very subtle differences in culture conditions. The discovery of a *pncA* mutation in a strain with borderline in vitro PZA susceptibility demonstrates the need for further investigation of the relationship between specific *pncA* mutations and PZA resistance. In particular, PZA-susceptible strains need to be examined for the possible existence of other *pncA* mutations with little or no association with in vitro PZA resistance.

Although our MIC testing was done with Middlebrook 7H10 agar adjusted to pH 5.5, most clinical laboratories currently use a modified 7H12 broth medium at pH 6.0 (BACTEC PZA test medium) for PZA susceptibility testing (2). Because of differences in medium composition and pH, drug concentrations are not directly comparable between the two systems (22). This difference between the two PZA susceptibility testing methods required that critical test concentrations be determined independently for each system. A PZA concentration of 25 μg/ml is recommended for Middlebrook 7H10 agar, while a PZA concentration of 100 μg/ml is used in BACTEC 12B broth medium (12; Siddiqi BACTEC manual). Regardless of the method used, testing of susceptibility to PZA is significantly more problematic than testing of susceptibility to the other first-line antituberculosis drugs. Problems with reproducibility and discordant results between laboratories are common. We encountered such a reproducibility problem, finding in this study that 17 isolates that were initially found to be resistant to 25 μg of PZA per ml were susceptible to this concentration. These results are now considered false resistance. One possible explanation for this discrepancy is lot-to-lot variation in the PZA test medium. The finding that individual lots of the components used in Middlebrook 7H10 medium may vary significantly in their suitability for drug susceptibility testing has been reported (9). In light of the results of this study, the appropriateness of the currently tested concentration of PZA in 7H10 medium is being reconsidered.

The absence of detectable PZase activity correlated well with a PZA MIC of ≥100 μg/ml (for 35 of 37 isolates) and with the occurrence of a *pncA* mutation (for 34 of 37 isolates). It has long been recognized that PZA susceptibility in *M. tuberculosis* could be indirectly detected by measuring a strain's ability to produce PZase (13). The inherent difficulties associated with the in vitro testing of PZA have even led some investigators to propose use of the Wayne (31) PZase assay as a qualitative screening method for determination of susceptibility to PZA (18). Another study showed that most strains susceptible to ≤150 μg of PZA per ml retained PZase activity, whereas the majority of strains resistant to >150 μg of PZA per ml lacked enzyme activity (3). In our study, the PZA MIC was ≥100 μg/ml for 37 isolates, and 35 of these isolates lacked PZase activity. For one PZase-negative strain (strain 25) the PZA MIC was 400 μg/ml, and it had no *pncA* mutation. Similar highly PZA-resistant strains of *M. tuberculosis* that lack both PZase activity and *pncA* mutations have been reported previously (15). Two PZase-positive strains for which the MIC was 100 μg/ml had no *pncA* mutation. All 21 isolates for which the PZA MIC was <100 μg/ml were PZase positive within 14 days. Three of these had the Thr47→Ala mutation. Overall, we found a strong correlation between the loss of PZase activity and a PZA MIC of ≥100 μg/ml, supporting the contention that PZase activity may be exploited as an indirect assay for PZA susceptibility. However, routine detection of PZase activity would require the development of a rapid, accurate, and technically easy enzymatic assay for the detection of PZase activity.

The identification of mutations within the *pncA* gene has been proposed as a surrogate marker for PZA resistance in *M. tuberculosis* (25, 29). Although the results of our study gener-

ally support this proposition, we found one highly resistant isolate with no *pncA* mutation, which suggests that phenotypic tests are still necessary for the detection of PZA resistance when no *pncA* mutations are identified. The existence of at least one *pncA* mutant with questionable in vitro PZA resistance demonstrates the importance of the precise identification of the mutations involved. This caveat may also apply to the other *M. tuberculosis* drug resistance markers. Currently, the best available technology for the identification of specific nucleotide changes is direct DNA sequencing. In our study, sequencing of the DNA of the *pncA* gene correctly identified 92% of the *M. tuberculosis* strains resistant to ≥100 μg of PZA per ml and 86% of the strains susceptible to 100 μg of PZA per ml.

ACKNOWLEDGMENT

We thank Beverly Metchock for advice provided in the preparation of the manuscript.

REFERENCES

1. **Bass, J. B. J., L. S. Farer, P. C. Hopewell, R. O'Brien, R. F. Jacobs, F. Ruben, D. E. J. Snider, and G. Thornton.** 1994. Treatment of tuberculosis and tuberculosis infection in adults and children. American Thoracic Society and the Centers for Disease Control and Prevention. Am. J. Respir. Crit. Care Med. **149:**1359–1374.
2. **Bird, B. R., M. M. Denniston, R. E. Huebner, and R. C. Good.** 1996. Changing practices in mycobacteriology: a follow-up survey of state and territorial public health laboratories. J. Clin. Microbiol. **34:**554–559.
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. **Canetti, G., W. Fox, A. Khomenko, H. T. Mahler, N. K. Menon, D. A. Mitchison, N. Rist, and N. A. Smelev.** 1969. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bull. W. H. O. **41:**21–43.
5. **Centers for Disease Control.** 1992. Transmission of multidrug-resistant tuberculosis among immunocompromised persons in a correctional system—New York, 1991. Morb. Mortal. Wkly. Rep. **41:**507–509.
6. **Cheng, S., L. Thibert, T. Sanchez, L. Heifets, and Y. Zhang.** 2000. *pncA* mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: spread of a monoresistant strain in Quebec, Canada. Antimicrob. Agents Chemother. **44:**528–532.
7. **Coronado, V. G., C. M. Beck-Sague, M. D. Hutton, B. J. Davis, P. Nicholas, C. Villareal, C. L. Woodley, J. O. Kilburn, J. T. Crawford, and T. R. Frieden.** 1993. Transmission of multidrug-resistant *Mycobacterium tuberculosis* among persons with human immunodeficiency virus infection in an urban hospital: epidemiologic and restriction fragment length polymorphism analysis. J. Infect. Dis. **168:**1052–1055.
8. **Frieden, T. R., L. F. Sherman, K. L. Maw, P. I. Fujiwara, J. T. Crawford, B. Nivin, V. Sharp, D. Hewlett, K. Brudney, D. Alland, and B. N. Kreiswirth.** 1996. A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. JAMA **276:**1229–1235.
9. **Guthertz, L. S., M. E. Griffith, E. G. Ford, J. M. Janda, and T. F. Midura.** 1988. Quality control of individual components used in Middlebrook 7H10 medium for mycobacterial susceptibility testing. J. Clin. Microbiol. **26:**2338–2342.
10. **Heifets, L., and P. Lindholm-Levy.** 1992. Pyrazinamide sterilizing activity in vitro against semidormant *Mycobacterium tuberculosis* bacterial populations. Am. Rev. Respir. Dis. **145:**1223–1225.
11. **Hirano, K., M. Takahashi, Y. Kazumi, Y. Fukasawa, and C. Abe.** 1997. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuber. Lung Dis. **78:**117–122.
12. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology: a guide for the level III laboratory, p. 108 and 164. U.S. Department of Health and Human Services, Washington, D.C.
13. **Konno, K., F. M. Feldmann, and W. McDermott.** 1967. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis. **95:**461–469.
14. **Kubica, G. P., R. E. Beam, and K. D. Stottmeier.** 1967. Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. Am. Rev. Respir. Dis. **96:**1072–1075.
15. **Lemaitre, N., W. Sougakoff, C. Truffot-Pernot, and V. Jarlier.** 1999. Characterization of new mutations in pyrazinamide-resistant strains of *Mycobacterium tuberculosis* and identification of conserved regions important for the catalytic activity of the pyrazinamidase *pncA*. Antimicrob. Agents Chemother. **43:**1761–1763.
16. **Mackaness, G.** 1956. The intracellular activation of pyrazinamide and nicotinamide. Am. Rev. Tuberc. **74:**718–728.
17. **Marttila, H. J., M. Marjamaki, E. Vyshnevskaya, B. I. Vyshnevskiy, T. F. Otten, A. V. Vasilyef, and M. K. Viljanen.** 1999. *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates from northwestern Russia. Antimicrob. Agents Chemother. **43:**1764–1766.
18. **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.
19. **McDermott, W., and R. Tompsett.** 1954. Activation of pyrazinamide and nicotinamide in acidic environments in vitro. Am. Rev. Tuberc. **70:**748–754.
20. **Mestdagh, M., P. A. Fonteyne, L. Realini, R. Rossau, G. Jannes, W. Mijs, K. A. L. De Smet, F. Portaels, and E. Van den Eeckhout.** 1999. Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the *pncA* locus in multidrug-resistant isolates of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemether. **43:**2317–2319.
21. **Mitchison, D. A.** 1985. The action of antituberculosis drugs in short-course chemotherapy. Tubercle **66:**219–225.
22. **Salfinger, M., L. B. Reller, B. Demchuk, and Z. T. Johnson.** 1989. Rapid radiometric method for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*. Res. Microbiol. **140:**301–309.
23. **Salfinger, M., and G. E. Pfyffer.** 1994. The new diagnostic mycobacteriology laboratory. Eur. J. Clin. Microbiol. Infect. Dis. **13:**961–979.
24. **Scorpio, A., and Y. Zhang.** 1996. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat. Med. **2:**662–667.
25. **Scorpio, A., P. Lindholm-Levy, L. Heifets, R. Gilman, S. Siddiqi, M. Cynamon, and Y. Zhang.** 1997. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **41:**540–543.
26. **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.
27. **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: a cooperative study in Poland. Am. Rev. Respir. Dis. **125:**265–267.
28. **Sreevatsan, S., P. Escalante, X. Pan, D. A. Gillies II, S. Siddiqi, C. N. Khalaf, B. N. Kreiswirth, P. Bifani, L. G. Adams, T. Ficht, V. S. Perumaalla, M. D. Cave, J. D. A. van Embden, and J. M. Musser.** 1996. Identification of a polymorphic nucleotide in *oxyR* specific for *Mycobacterium bovis*. J. Clin. Microbiol. **34:**2007–2010.
29. **Sreevatsan, S., X. Pan, Y. Zhang, B. N. Kreiswirth, and J. M. Musser.** 1997. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. Antimicrob. Agents Chemother. **41:**636–640.
30. **Valway, S. E., S. B. Richards, J. Kovacovich, R. B. Greifinger, J. T. Crawford, and S. W. Dooley.** 1994. Outbreak of multi-drug resistant tuberculosis in a New York State prison, 1991. Am. J. Epidemiol. **140:**113–122.
31. **Wayne, L. G.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am. Rev. Respir. Dis. **109:**147–151.