Reduced Pyrazinamidase Activity and the Natural Resistance of *Mycobacterium kansasii* to the Antituberculosis Drug Pyrazinamide

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Received 9 February 1998/Returned for modification 9 April 1998/Accepted 14 December 1998

Pyrazinamide (PZA), an analog of nicotinamide, is a prodrug that requires conversion to the bactericidal compound pyrazinoic acid (POA) by the bacterial pyrazinamidase (PZase) activity of nicotinamidase to show activity against *Mycobacterium tuberculosis*. Mutations leading to a loss of PZase activity cause PZA resistance in *M. tuberculosis*. *M. kansasii* is naturally resistant to PZA and has reduced PZase activity along with an apparently detectable nicotinamidase activity. The role of the reduction in PZase activity in the natural PZA resistance of *M. kansasii* is unknown. The MICs of PZA and POA for *M. kansasii* were determined to be 500 and 125 μg/ml, respectively. Using 

\[ ^{14}\text{C}]PZA and 

\[ ^{14}\text{C}]\text{nicotinamide, we found that }M.\ kansasii\ \text{had about 5-fold-less PZase activity and about 25-fold-less nicotinamidase activity than }M.\ tuberculosis.\ The\ M.\ kansasii\ pncA\ gene\ was\ cloned on a 1.8-kb BamHI DNA fragment, using *M. avium\ pncA* probe. Sequence analysis showed that the *M. kansasii\ pncA* gene encoded a protein with homology to its counterparts from *M. tuberculosis* (69.9%), *M. avium* (65.6%), and *Escherichia coli* (28.5%). Transformation of naturally PZA-resistant *M. bovis* BCG with *M. kansasii\ pncA* conferred partial PZA susceptibility. Transformation of *M. kansasii* with *M. avium\ pncA* caused functional expression of PZase and high-level susceptibility to PZA, indicating that the natural PZA resistance in *M. kansasii* results from a reduced PZase activity. Like *M. tuberculosis*, *M. kansasii* accumulated POA in the cells at an acidic pH; however, due to its highly active POA efflux pump, the naturally PZA-resistant species *M. smegmatis* did not. These findings suggest the existence of a weak POA efflux mechanism in *M. kansasii*.

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Materials and Methods

*Mycobacterium kansasii* is a slow-growing opportunistic human pathogen that often causes pulmonary lesions similar to those caused by *M. tuberculosis* in immunocompromised patients, such as those with AIDS (24). Unlike *M. tuberculosis*, *M. kansasii* is naturally resistant to the antituberculosis drug pyrazinamide (PZA), an analog of nicotinamide, at 50 μg/ml, a concentration at which *M. tuberculosis* is susceptible (4). In *M. tuberculosis*, the susceptibility to PZA correlates with the presence of a single enzyme, nicotinamidase, that also has pyrazinamidase (PZase) activity (8, 9, 11, 22). We recently identified the *M. tuberculosis* PZase/nicotinamidase gene (*pncA*) (13) and showed that mutation of *pncA* is a major mechanism of PZA resistance in *M. tuberculosis* (14, 17). *M. bovis* and *M. bovis* BCG, whose genomes are almost identical to that of *M. tuberculosis*, are naturally resistant to PZA. We have demonstrated that the natural PZA resistance of *M. bovis* is due to a single point mutation of C to G at nucleotide position 169 of the *M. bovis\ pncA* gene, causing an amino acid substitution of aspartate for histidine at amino acid position 57 compared with the *M. tuberculosis\ pncA* sequence (13, 14). In this sense, *M. bovis* strains can be regarded as a special case of PZA-resistant *M. tuberculosis*. In contrast, the natural PZA resistance in nontuberculous mycobacteria such as *M. smegmatis* and *M. avium* is not caused by a defective PZase as in *M. tuberculosis* with acquired PZA resistance, since these mycobacteria have significant PZase activity (5, 20). Natural PZA resistance, at least in the case of *M. smegmatis*, is due to a highly active efflux mechanism that rapidly extrudes pyrazinamide (POA), the active form of PZA, from the cell after conversion of PZA by the bacterial PZase (27).

The basis for the natural resistance of *M. kansasii* to PZA is unknown. *M. kansasii* is known to have reduced PZase activity along with a detectable nicotinamidase activity (5, 20). The role of the reduced PZase activity in the natural PZA resistance of *M. kansasii* is uncertain. In this study using radioactive 

\[ ^{14}\text{C}]PZA and 

\[ ^{14}\text{C}]\text{nicotinamide, we found that }M.\ kansasii\ while having a reasonable amount of nicotinamidase activity, had a very low level of PZase activity that was undetectable by a conventional PZase assay (23). In addition, we cloned the *M. kansasii\ PZase gene (pncA) and showed that it could partially restore PZA susceptibility in naturally PZA-resistant BCG. Furthermore, transformation of *M. kansasii* with *M. avium\ pncA* (known to restore complete susceptibility to BCG) conferred a high degree of susceptibility to PZA in *M. kansasii*. These results suggest that the natural resistance of *M. kansasii* to PZA is due to a deficient PZase activity of its nicotinamidase enzyme.

Materials and Methods

Growth of mycobacteria and isolation of genomic DNA. Mycobacterial strains were grown in 7H9 liquid medium with albumin-dextrose-catalase enrichment (Difco) at 37°C for about 2 weeks for *M. kansasii* ATCC type strain 12478 and for 3 to 4 weeks for *M. tuberculosis* or *M. bovis* BCG-Pasteur. Mycobacterial genomic DNA was isolated as described previously (25).

PZA and POA susceptibility testing. A PZA stock solution (25 mg/ml) was prepared in water. POA was dissolved in dimethyl sulfoxide (1) at a concentration of 100 mg/ml. The susceptibility of *M. kansasii*, *M. kansasii* transformants, and BCG transformants to PZA or POA was determined on 7H11 agar plates of acidic pH (pH 5.5) (10, 21) containing 31, 62.5, 125, 250, 500, or 1,000 μg of PZA or POA/ml. Two dilutions (10^-2 and 10^-3) of early-stationary-phase mycobac-
terial cultures diluted in 7H9 liquid medium were plated onto the 7H11 plates, which were then incubated at 37°C for about 10 to 14 days for M. kansasii or for 21 to 28 days for BCG. The MICs were determined by the proportional method (indirect) (16). The MIC was defined and calculated according to the manufacturer’s protocol. The transformed M. kansasii cells were plated on 7H11 plates containing 100 µg of hygromycin/ml, and the plates were incubated at 37°C for 2 to 4 weeks.

**Results**

MICs of PZA and POA for M. kansasii. Previous studies have shown that M. kansasii is resistant to PZA at concentrations higher than 50 µg/ml (4), a concentration at which M. tuberculosis is susceptible (10). However, the exact MIC of PZA for M. kansasii was not reported. In this study, the MIC of PZA for M. kansasii was found to be about 500 µg/ml. The MIC of POA (the active derivative of PZA) for M. kansasii was about 125 µg/ml. As controls, M. bovis BCG and M. tuberculosis H37Ra were both found to have similar POA MICs of about 62 µg/ml.

**Relative nicotinamidase and PZase activities of the M. kansasii pncA enzyme.** By the conventional Wayne agar method (23), M. kansasii has been shown to have nicotinamidase activity but no detectable PZase activity (5, 20), but some PZase activity was detectable by a more sensitive high-performance liquid chromatography method (16). We compared the relative PZase and nicotinamidase activities of M. kansasii by using [14C]PZA and [14C]NAm, with M. tuberculosis as a control. As shown in Fig. 1A, M. kansasii converted virtually all [14C]NAm to NA at 2 h, whereas conversion of [14C]PZA to POA was hardly seen even by 5 h. However, there was definite conversion of PZA to POA by M. kansasii at 16 h. In contrast, while M. tuberculosis H37Ra converted [14C]NAm to NA by 1 h, M. tuberculosis had no detectable PZase activity but converted PZA to POA at 1 to 2 h (Fig. 1B). PZA alone, in the absence of PZase/nicotinamidase (as a control), did not degrade spontaneously into POA even after incubation at 37°C for several days (data not shown). It is worth noting that when [14C]PZA was added to M. tuberculosis or M. kansasii, only two spots were seen on TLC plates; one was PZA itself, and the other was POA. The identities of the spots were confirmed by running “cold” PZA and POA as controls in parallel with samples containing “hot” PZA and POA by TLC, after which the chromatogram was then examined under UV light. Cold PZA and POA gave fluorescence spots, which were marked with pencil, and the TLC plate was then subjected to autoradiography. Under the given set of experimental conditions, no radioactive compounds except PZA or POA were seen.

To determine the relative amount of nicotinamidase and PZase activities in M. kansasii more quantitatively, we used a more sensitive radioassay to measure the enzyme activities in the M. kansasii protein extract, employing [14C]PZA and [14C]NAm, with an equal amount of M. tuberculosis H37Ra protein extract being utilized as a control. The specific activity of the M. kansasii nicotinamidase was about 18-fold higher than its PZase activity (means ± standard deviations, 0.1 ± 0.03 and 1.8 ± 0.12 U/mg of protein, respectively), whereas the M. tuberculosis nicotinamidase activity was about 94-fold higher than its PZase activity (47.4 ± 5.3 and 0.5 ± 0.04 U/mg

**Nucleotide sequence accession number.** The coding sequence of the M. kansasii pncA gene has been deposited in the GenBank database under accession no. AF002663.
of protein, respectively). On the other hand, the *M. kansasii* PZase activity was about 5-fold less than that of the *M. tuberculosis* PZase, and the *M. kansasii* nicotinamidase activity was about 25-fold less than that of the *M. tuberculosis* enzyme.

**Cloning and sequence analysis of the *M. kansasii pncA* gene.**

Southern blotting analysis of *M. kansasii* genomic DNA indicated that a 1.8-kb *BamHI* fragment hybridized with the 391-bp PCR fragment from the *M. avium pncA* gene (Fig. 2, lane 6). To clone the *M. kansasii pncA* gene, a partial genomic-DNA library was constructed by cloning 1.5- to 2-kb *BamHI* genomic-DNA fragments of *M. kansasii* into pUC19, and the library was screened with the *M. avium pncA* gene probe. A positive plasmid clone containing the 1.8-kb *BamHI* fragment was identified and sequenced. The complete *M. kansasii pncA* gene was found to be 561 bp long and to encode a protein of about 19.8 kDa (GenBank accession no. AF002663). The *M. kansasii* PncA showed 69.9%, 65.6%, and 28.5% amino acid identity with its counterparts from *M. tuberculosis*, *M. avium*, and *E. coli*, respectively (Fig. 3).

**Transformation of BCG with the *M. kansasii pncA* gene partially restored PZA susceptibility.** BCG is naturally resistant to PZA (MIC > 500 μg/ml) due to the presence of a single point mutation in the *pncA* gene (13). To assess the relative contribution of the *pncA* genes of *M. kansasii* and BCG to PZA susceptibility, we transformed BCG with the *M. kansasii pncA* gene on the 1.8-kb *BamHI* fragment cloned into p16R1 and also with the *M. tuberculosis pncA* construct as a positive control. While the *M. tuberculosis pncA* gene conferred full susceptibility to PZA in BCG (MIC, ca. 31 to 62 μg/ml), the *M. kansasii pncA* gene could only partially restore PZA susceptibility to BCG (MIC, ca. 125 μg/ml) (Table 1). This indicates that the *M. kansasii* PncA enzyme indeed has some level of PZase activity, which can enhance the conversion of PZA to POA, and thus is involved in partial restoration of PZA susceptibility to BCG. Transformation with the *pncA* gene from either *M. tuberculosis* or *M. kansasii* did not alter the POA MIC for BCG (Table 1).

**Transformation of *M. kansasii* with *M. avium pncA* conferred a high degree of PZA susceptibility.** *M. avium pncA* has previously been shown to confer to BCG a level of PZA susceptibility similar to that of the PZA-susceptible bacterium *M. tuberculosis*, indicating that *M. avium* has a functional PZase/nicotinamidase enzyme capable of potentiating PZA action. To determine whether the natural PZA resistance of *M. kansasii* is due to the reduced PZase activity of its nicotinamidase enzyme, we transformed *M. kansasii* (ATCC type strain) with the *M. avium pncA* gene in the hygromycin vector p16R1. Transformation of the *M. kansasii* with *M. avium pncA* caused functional overexpression of the *M. avium* PZase enzyme, as revealed by an increased conversion of [14C]PZA to [14C]POA compared to that of the vector control (data not shown). The overexpression of the *M. avium* PZase enzyme rendered the *M. kansasii* strain more susceptible to PZA (MIC, 31 to 62 μg/ml [a concentration to which the susceptible species *M. tu-
berculosis is sensitive). This suggests that the natural PZA resistance of M. kansasii (at least in the case of the ATCC type strain) results from its reduced PZase activity, which does not result in efficient conversion of the prodrug PZA to the bactericidal compound POA.

Weak POA efflux activity in M. kansasii. The natural PZA resistance of fast-growing M. smegmatis correlates with a highly active POA efflux mechanism which does not allow POA to accumulate in cells of this species at an acidic pH (27). In contrast, the PZA-susceptible species M. tuberculosis has been found to have a much weaker POA efflux mechanism, as revealed by the increasing accumulation of POA by this organism at an acidic pH (5.0 to 5.5) (27). To determine the potential POA efflux activity in M. kansasii, we compared the POA accumulation patterns of M. tuberculosis, M. smegmatis, and M. kansasii at pH 5.0 and pH 7.0. As shown in Fig. 4, M. kansasii behaved like the susceptible species M. tuberculosis in accumulating POA at pH 5.0 (Fig. 4A and B); in contrast, M. smegmatis cells did not accumulate a significant amount of POA even at an acidic pH (5.0) (Fig. 4C).

DISCUSSION

M. kansasii is known to have a deficient PZase activity in conventional PZase testing by the Wayne agar method, despite having an apparently normal nicotinamidase activity (5, 20). In this study, using a more sensitive radioactive method involving the use of [14C]PZA as a substrate, we showed that M. kansasii definitely has a weak PZase activity that is undetectable by the Wayne method. This finding is in agreement with the results of Speirs et al., who found weak PZase activity in M. kansasii by a sensitive high-performance liquid chromatography analytical method (16). The M. kansasii nicotinamidase activity was about 18-fold higher than its PZase activity, whereas the M. tuberculosis nicotinamidase activity was 94-fold higher than its

| Strain | MIC (μg/ml) of: | PZA | POA |
|--------|----------------|-----|-----|
| M. kansasii | 500 | 125 |
| M. tuberculosis H37Ra | 31–62 | 62 |
| M. bovis BCG | >500 | 62 |
| BCG + p16R1 vector control | >500 | 62 |
| BCG + p16R1-M. kansasii pncA | 125 | 62 |
| BCG + p16R1-M. tuberculosis pncA | 31–62 | 62 |
| M. kansasii + p16R1+ vector control | 500 | 125 |
| M. kansasii + p16R1-M. avium pncA | 31–62 | 128 |
The PZase activity of M. kansasii was about fivefold lower than that of the M. tuberculosis enzyme. To identify the basis for the separation of PZase activity from nicotinamidase activity of M. kansasii PncA and to assess the role of the reduced PZase activity in the natural resistance of M. kansasii to PZA, we cloned the pncA gene from M. kansasii. Sequence analysis showed that the M. kansasii PncA exhibited a high degree of homology to the M. tuberculosis (69.9%) and M. avium (65.6%) enzymes, which have both PZase and nicotinamidase activities (Fig. 3). However, sequence comparisons did not allow direct identification of the amino acid residues responsible for the weak PZase activity of the M. kansasii nicotinamidase enzyme, because there are several residues in the M. kansasii PncA that are different from the corresponding residues of other mycobacterial PncAs (Fig. 3). The physiological role of the nicotinamidase enzyme (PncA) is to degrade nicotinamide to NA, which can be recycled to NAD via the Preiss-Handler pathway of the pyridine nucleotide cycle in most prokaryotes (2). Because PZase converts PZA to the bactericidal compound POA, the amount of PZase activity, by affecting the rate of conversion of PZA to POA, would be important in determining the susceptibility of mycobacteria to PZA. The presence as well as the amount of PZase activity of the nicotinamidase enzymes from various bacterial species appears to be purely coincidental, because PZA, as an analog of nicotinamide, is an artificial compound that does not exist in nature. It so happens that M. tuberculosis has a nicotinamidase enzyme with a reasonable amount of PZase activity, whereas M. kansasii has a nicotinamidase enzyme with much weaker PZase activity.

Because defective PZase activity caused by pncA mutations correlates with PZA resistance in M. tuberculosis (13, 15, 17), we determined whether the reduced level of PZase activity in M. kansasii is responsible for its natural resistance to PZA. Transformation of M. kansasii with the M. avium pncA gene caused overexpression of PZase and concomitantly conferred a high level of susceptibility to PZA (MIC, 31 to 62 μg/ml); in a previous study, the same M. avium pncA construct was shown to make BCG fully susceptible to PZA (18). This indicates that the reduced PZase activity of M. kansasii is the cause of its natural PZA resistance. On the other hand, transformation of BCG with M. kansasii pncA made BCG more susceptible to PZA (MIC, 125 μg/ml); however, the degree of susceptibility to PZA was not as high as that achieved by transformation with the M. tuberculosis pncA gene (MIC, 31 to 62 μg/ml). This suggests that while M. kansasii PncA has some PZase activity, its PZase activity is lower than that of the M. tuberculosis PncA enzyme, a conclusion also supported by the enzyme assays using [14C]PZA (see Results).

In addition to the level of PZase activity, the activity of efflux pumps (7) could also affect the susceptibility of mycobacteria to PZA. We have recently shown that the natural PZA resistance of M. smegmatis (MIC, >2,000 μg/ml) is not due to a defective PZase but rather is attributable to a highly active efflux pump that extrudes POA from the cell very rapidly (27). Likewise, the natural PZA resistance of M. avium (MIC, 500 μg/ml) also appears to involve an active efflux pump with an efficiency in between those of M. smegmatis and M. tuberculosis (unpublished observation), since the M. avium pncA gene, when transformed into BCG, completely restored PZA susceptibility (18), indicating that the M. avium PZase is fully functional and the natural PZA resistance of M. avium is not due to an inability of its PZase to convert PZA to POA. In this study, M. kansasii, like the PZA-susceptible species M. tuberculosis, could also accumulate POA in the cells at an acidic pH (5.0) (Fig. 4A). The accumulation of POA at an acidic pH, along with the observation that the M. avium pncA gene can confer a high degree of PZA susceptibility to M. kansasii, indicates that M. kansasii has a weak PZA efflux mechanism that is unlikely to contribute to its natural PZA resistance. We conclude that the natural PZA resistance of M. kansasii is due to the somewhat-deficient PZase activity of its nicotinamidase enzyme. Site-directed mutagenesis and comparative structural and enzymatic analyses are needed to determine the amino acid residues in PncA that underlie the weak PZase activity of the M. kansasii nicotinamidase.

ACKNOWLEDGMENTS

This work was supported by research grants from the American Lung Association, Potts Memorial Foundation, and the NIH (RO1 AI40584 [to Y.Z.]).

We thank Diane Griffin and Barbara Laughon for encouragement, Salman Siddiqi for M. kansasii strains, and the NIH AIDS Reagents Program for [14C]PZA. We also thank the reviewers for helpful suggestions.

REFERENCES

1. Cynamon, M. H., R. Gimé, F. Gyenes, C. A. Sharpe, K. E. Bergherrmann, H. J. Han, L. B. Gregor, R. Rapolo, G. Luciano, and J. T. Welch. 1995. Pyrazinamide acid esters with broad spectrum in vitro antimycobacterial activity. J. Med. Chem. 38:3902–3907.
2. Foster, J. W., and A. G. Moat. 1980. Nicotinamide adenine dinucleotide biosynthesis and pyridine nucleotide cycle metabolism in microbial systems. Microbiol. Rev. 44:83–105.

3. Garbe, T., J. Barathé, S. Barnini, Y. Zhang, C. Abou-Zeid, D. Tang, R. Mukherjee, and D. Young. 1994. Transformation of mycobacterial species using hygromycin resistance as selectable marker. Microbiology 140:133–138.

4. Good, R. C., V. A. Sileo, J. O. Kilburn, and B. D. Plikaytis. 1985. Identification and drug susceptibility test results for Mycobacterium spp. Clin. Microbiol. Newslett. 7:133–136.

5. Helbecque, D. M., V. Handzel, and L. Eidus. 1975. Simple amide test for identification of mycobacteria. J. Clin. Microbiol. 1:50–53.

6. Inderlie, C. B., and K. A. Nash. 1996. Antimycobacterial agents: in vitro susceptibility testing, spectra of activity, mechanism of action and resistance, and assays for activity in biological fluids. In V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, Md.

7. Jarlier, V., and H. Nikaido. 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol. Lett. 122:11–18.

8. Kommo, K., F. M. Feldman, and W. McDermott. 1967. Pyrazinamide susceptibility and amide activity of tubercle bacilli. Am. Rev. Respir. Dis. 95:461–469.

9. McClatchy, J. K., A. V. Tsang, and M. S. Cornich. 1981. Use of pyrazinamide activity in Mycobacterium tuberculosis as a rapid method for determination of pyrazinamide susceptibility. Antimicrob. Agents Chemother. 20:556–557.

10. McDermott, W., and R. Tompsett. 1954. Activation of pyrazinamide and nicotinamide in acidic environment in vitro. Am. Rev. Tuberc. 70:748–754.

11. Miller, M. A., L. Thibert, F. Desjardins, S. H. 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.

12. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

13. Scorpio, A., and Y. Zhang. 1996. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antitubercular drug pyrazinamide in tubercle bacilli. Nat. Med. 2:662–667.

14. Scorpio, A., D. 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. 35:106–110.

15. Scorpio, A., P. Lindholm-Levy, L. Heifets, R. Gilman, S. Siddiqi, M. Cyna-