The *pncA* gene from naturally pyrazinamide-resistant *Mycobacterium avium* encodes pyrazinamidase and confers pyrazinamide susceptibility to resistant *M. tuberculosis* complex organisms

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The antituberculosis drug pyrazinamide (PZA) needs to be converted into pyrazinoic acid (POA) by the bacterial pyrazinamidase (PZase) in order to show bactericidal activity against *Mycobacterium tuberculosis*. *M. avium* is naturally resistant to PZA. To investigate whether this natural resistance to PZA is due to inability of the *M. avium* PZase to convert PZA to bactericidal POA, the *M. avium* PZase gene (*pncA*) was cloned by using the *M. tuberculosis pncA* gene as a probe. Sequence analysis showed that the *M. avium pncA* gene is 561 bp long, encoding a protein with a predicted size of about 19.8 kDa; but Western blotting showed that the *M. avium* PZase migrated as a 24 kDa band when expressed in *M. bovis* BCG and *Escherichia coli*. Sequence comparison revealed that *M. avium* PZase has 67.7% and 32.8% amino acid identity with the corresponding enzymes from *M. tuberculosis* and *E. coli*, respectively. Southern blot analysis with the *M. avium pncA* gene as a probe showed that *M. terrae*, *M. gastri*, *M. marinum*, *M. fortuitum*, *M. xenopi*, *M. goodii*, *M. szulgai*, *M. celatum* and *M. kansasii* have close *pncA* homologues, whereas *M. chelonae* and *M. smegmatis* did not give significant hybridization signals. Transformation with the *M. avium pncA* gene conferred PZA susceptibility to PZA-resistant *M. tuberculosis* complex organisms, indicating that the nonsusceptibility of *M. avium* to PZA is not due to an ineffective PZase enzyme, but appears to be related to other factors such as transport of POA.

Keywords: pyrazinamide susceptibility, pyrazinamidase, *pncA*, *Mycobacterium avium*

INTRODUCTION

Mycobacterium avium, a slow-growing saprophytic mycobacterium, is an important opportunistic pathogen in immunocompromised conditions such as HIV infection (Ellner *et al.*, 1991). Like many other mycobacteria, *M. avium* is naturally resistant to many antibiotics. The nonsusceptibility of mycobacteria to antibiotics has been suggested to be due to the impermeability of the mycobacterial cell wall (Jarlier & Nikaido, 1994). While

M. avium has a similar lipid-rich cell wall structure to *Mycobacterium tuberculosis* (Jarlier & Nikaido, 1994), it is nevertheless resistant to some drugs that are active against *M. tuberculosis*, such as isoniazid and pyrazinamide (PZA). In a study by Heifets *et al.* (1986), *M. avium* was found to be resistant to at least 100 µg PZA ml⁻¹. In contrast, *M. tuberculosis* is uniquely susceptible to PZA, with a minimum inhibitory concentration (MIC) of 50–100 µg ml⁻¹ (McDermott & Tompsett, 1954); other mycobacteria or bacterial species are usually completely insensitive to PZA.

In *M. tuberculosis*, the susceptibility to PZA is correlated with the activity of a single amidase enzyme with both pyrazinamidase (PZase) and nicotinamidase activities (Konno *et al.*, 1967). PZase in *M. tuberculosis*

Abbreviations: POA, pyrazinoic acid; PZA, pyrazinamide; PZase, pyrazinamidase.

The GenBank accession number for the *M. avium pncA* gene sequence determined in this work is U80820.

converts PZA to bactericidal pyrazinoic acid (POA) inside the bacterial cell (Bonicke & Lisboa, 1959; Scorpio & Zhang, 1996), and it is conceivable that loss of PZase activity could cause PZA resistance in this organism. Many studies have documented the correlation between defective PZase activity and PZA resistance in clinical isolates of *M. tuberculosis* (McClatchy *et al.*, 1981; Trivedi & Desai, 1987; Miller *et al.*, 1995). We have recently cloned the *M. tuberculosis* gene (*pncA*) encoding an enzyme with both PZase and nicotinamidase activity (Scorpio & Zhang, 1996) and have confirmed, by transforming resistant bacilli with a functional *M. tuberculosis pncA* gene, that defective PZase activity resulting from *pncA* mutations is the cause of PZA resistance in *M. tuberculosis* complex organisms (Scorpio & Zhang, 1996). Furthermore, we have shown that mutation in *pncA* is the major mechanism of PZA resistance in *M. tuberculosis* (Scorpio *et al.*, 1997b; Sreevatsan *et al.*, 1997). Interestingly, *M. bovis* and *M. bovis* BCG – a member of the *M. tuberculosis* complex – were found to have a single point mutation of C to G at nucleotide position 169 of the *pncA* gene compared with the *M. tuberculosis pncA* sequence, causing a substitution of histidine to aspartic acid at amino acid position 57 of the PncA sequence (Scorpio & Zhang, 1996; Scorpio *et al.*, 1997a). This single point mutation in the *M. bovis pncA* gene is the cause of the defective PZase activity and natural resistance to PZA in *M. bovis* and BCG, and has been recently shown to be useful for rapid differentiation of *M. bovis* from *M. tuberculosis* (Scorpio *et al.*, 1997a).

However, the correlation between presence of PZase activity and susceptibility to PZA does not hold for other mycobacterial species. For example, unlike *M. tuberculosis*, *M. avium* has PZase activity (Tarnok & Rohrscheidt, 1976) but it is not susceptible to PZA (Heifets *et al.*, 1986). It was not clear whether the natural resistance of *M. avium* to PZA is due to insufficient ability of the *M. avium* PZase to potentiate the PZA activity, or to other potential differences between these organisms in properties such as cell wall permeability or the transport of PZA. To distinguish between the above possibilities, we cloned the *M. avium pncA* homologue by using the *M. tuberculosis pncA* gene as a probe and transformed the *M. avium pncA* gene into PZA-resistant strains of *M. tuberculosis* complex organisms. We found that the *M. avium pncA* gene conferred a high degree of PZA susceptibility upon PZA-resistant *M. tuberculosis* strains. Our result suggests that the nonsusceptibility of *M. avium* to PZA is not due to its having an inefficient PZase enzyme in terms of potentiating PZA activity, but rather to other factors such as the relative impermeability of the *M. avium* cell wall or the transport of PZA/POA.

METHODS

Mycobacterial strains and DNA. Mycobacteria were grown in 7H9 liquid medium with ADC (albumin-dextrose-catalase) enrichment (Difco) at 37 °C for 3–5 d for fast growers and 3–4

weeks for slow growers. The mycobacterial species *M. terrae*, *M. kansasii*, *M. gastri*, *M. marinum*, *M. fortuitum*, *M. chelonae*, *M. xenopi*, *M. gordonae*, *M. szulgai* and *M. celatum* were kindly provided by Dr Salman Siddiqi, Becton Dickinson Diagnostic Instrument Systems Inc. Genomic DNA from various mycobacterial species was isolated using glass beads as described previously (Zhang *et al.*, 1992).

PZase enzyme assay and PZA susceptibility testing. PZase activity was assayed according to Wayne (1974). Briefly, a heavy bacterial inoculum was overlaid on the surface of Dubos agar (Difco) in a test tube containing 100 µg PZA ml⁻¹. The inoculated tubes were incubated at 37 °C for 3–5 d, then 2 ml 1% ferrous ammonium sulfate was added to the tubes. After about 2 h incubation at 4 °C, the results were recorded. Positive PZase activity was indicated if a brownish band appeared on the agar surface. A positive culture (PZA-sensitive *M. tuberculosis* strain H37Rv) and a negative culture (BCG Pasteur, defective in PZase) were included as controls. The susceptibilities to PZA of different bacterial species or strains were determined on 7H11 agar (Difco) plates in the case of mycobacteria, and on LB agar plates in the case of *Escherichia coli*. The agar media, which were adjusted to pH 5.5, contained 50, 100, 250, 500, 1000 or 2000 µg PZA ml⁻¹. Two dilutions (about 10⁴–10⁶ bacterial cells) of stationary-phase bacterial cultures were spread onto the agar plates, which were then incubated at 37 °C for 1 d for *E. coli*, 3–5 d for fast-growing mycobacteria, or 3–4 weeks for slow-growing mycobacteria. The minimum inhibitory concentration (MIC) was determined using the absolute concentration method based on no bacterial growth on plates with the lowest concentration of PZA.

Cloning of the *M. avium pncA* gene. The *M. avium pncA* gene was cloned by screening an *M. avium* λZAPII phage library (NIH AIDS Reagent Program) as described by Sambrook *et al.* (1989) with a ³²P-labelled 329 bp PCR product containing part of the *M. tuberculosis pncA* gene (Scorpio & Zhang, 1996) as a probe. The 329 bp *M. tuberculosis pncA* probe was obtained by PCR amplification (Saiki *et al.*, 1988) using the following forward and reverse primers. The forward primer (5'-ATCA-GCGACTACCTGGCCG3') was taken from 91–109 bp and the reverse primer (5'-GCGCACACAATGATCGGTGG3') from 420–400 bp of the *M. tuberculosis pncA* coding sequence (GenBank accession number U59967; Scorpio & Zhang, 1996). A positive phage clone containing the *M. avium pncA* gene was isolated, amplified, and the phage DNA was extracted as described by Sambrook *et al.* (1989). To locate the *M. avium pncA* gene, the phage DNA was subjected to restriction enzyme mapping followed by Southern blot analysis with the 329 bp *M. tuberculosis pncA* PCR product as a probe. A 1.6 kb *Bam*HI hybridizing fragment from the *pncA*-containing phage DNA was subcloned into pUC19. Standard molecular cloning techniques were carried out as described by Sambrook *et al.* (1989).

DNA sequence analysis. The complete *M. avium pncA* gene sequence was determined for both strands from the 1.6 kb *Bam*HI-pUC19 construct by primer walking in an automatic DNA sequencer (ABI model 377) at Johns Hopkins Genetic Core Facility. The multiple sequence homology alignment among *M. avium*, *M. tuberculosis* and *E. coli* PncA sequences was performed using the CLUSTAL method.

Southern blotting analysis. Southern blotting analysis of mycobacterial genomic DNA was performed as described previously (Zhang *et al.*, 1992). Briefly, genomic DNA from various mycobacterial species was isolated and digested with

*Bam*HI, and run on 0.8% DNA agarose gel. Genomic DNA fragments from the gel were transferred onto nylon membrane by vacuum blotting, and the membrane was fixed by UV. The DNA probe for the Southern blotting analysis was prepared by a PCR approach using primers derived from within the *M. avium* *pncA* gene. The forward primer (5'-GCATCAACGC-CTACCTGGAC3') was taken from 87–107 bp, and the reverse primer (5'-TGCACCAGCACCCGGGTGGT3') from 474–455 bp of the *M. avium* *pncA* coding sequence. The 391 bp PCR fragment was labelled with [³²P]dCTP using the Random Primers DNA Labelling System (Gibco-BRL) according to the manufacturer's protocol. The blot was probed with the ³²P-labelled PCR fragment as a probe, washed under low stringency, and subjected to autoradiography.

Transformation of mycobacteria. The *pncA* plasmid construct for transformation of BCG and PZA-R (ATCC 35828, a PZA-resistant derivative of *M. tuberculosis* strain H37Rv) was made as follows. The 1.6 kb *Bam*HI fragment containing the *M. avium* *pncA* gene was cloned into the *Kpn*I site of the hygromycin mycobacterial shuttle vector p16R1 (Garbe *et al.*, 1994) as described by Sambrook *et al.* (1989). The p16R1–1.6 kb *M. avium* *pncA* construct and the same vector harbouring the *M. tuberculosis* *pncA* gene on a 3.2 kb *Eco*RI–*Pst* I fragment, along with the vector control, were transformed by electroporation into the naturally PZA-resistant *M. bovis* BCG and PZA-R using the method described previously (Zhang *et al.*, 1993). The PZA-R isolate has been shown in a previous study to contain a single G deletion at nucleotide position 288 of the *M. tuberculosis* *pncA* sequence (Scorpio & Zhang, 1996).

Western blot analysis. This was performed as described previously (Zhang *et al.*, 1991). The rabbit polyclonal antibody used was raised against the purified *M. tuberculosis* PZase protein overexpressed in *E. coli* (unpublished data), and this antibody was found to cross-react with the *M. avium* PZase protein.

Overexpression of *M. avium* PZase protein in *E. coli*. The *M. avium* PZase protein was overexpressed by a PCR approach using the following primers. The forward primer (5'-GCTCG-TTGTCGAGCAAACCG3') was taken from 93 bp upstream of the *M. avium* *pncA* gene start codon, and the reverse primer (5'-TGTCCGATCCAGGGCGCCA3') was taken from 23 bp downstream of the stop codon of the *M. avium* *pncA* gene. The PCR was performed as described by Saiki *et al.* (1988) with the cloned 1.6 kb *Bam*HI–pUC19 construct as a template using the following parameters: 95 °C for 5 min, followed by 30 cycles of 95 °C 1 min, 55 °C 1 min and 72 °C 1 min. The PCR product containing the *M. avium* *pncA* gene was cloned into pCR2.1 vector using the TA Cloning Kit (Invitrogen). The recombinant *E. coli* clone overexpressing the PZase enzyme was identified by restriction digestion of the plasmid DNA and by assaying for enhanced PZase enzyme activity conferred by the insert DNA using the PZase assay as described above. The *M. tuberculosis* *pncA* gene was similarly overexpressed and included as a control for comparison with the *M. avium* *pncA*.

RESULTS

PZA susceptibilities of various mycobacterial species

Heifets *et al.* (1986) found that *M. avium* was naturally resistant to more than 100 µg PZA ml⁻¹, a concentration to which *M. tuberculosis* is susceptible; however, the exact MIC for *M. avium* was not determined. We

therefore determined the MIC of *M. avium* along with those of some fast-growing mycobacteria (*M. smegmatis*, *M. vaccae*) and *E. coli*, to shed some light on the relationship between susceptibility to PZA and PZase activity. The MIC of PZA for *M. avium* was found to be about 500 µg ml⁻¹, whereas *M. smegmatis*, *M. vaccae* and *E. coli* were all resistant to more than 2000 µg PZA ml⁻¹. Thus the MIC for *M. avium* is intermediate between those for *M. tuberculosis* and fast-growing mycobacteria.

Cloning of the *M. avium* *pncA* gene

In a preliminary Southern blot analysis, genomic DNA from *M. avium* was found to hybridize with the *M. tuberculosis* *pncA* probe (data not shown). To clone the *M. avium* *pncA* gene, we screened an *M. avium* λZAPII DNA library using the 329 bp PCR product containing part of the *M. tuberculosis* *pncA* gene as a probe. Because the *M. avium* phage library was constructed by ligating into the vector 4–8 kb genomic DNA fragments which were sonicated, blunt-ended and added with *Eco*RI linker, the insert DNA can be released with *Eco*RI. The DNA from the identified positive phage clone was digested with *Eco*RI and a 5.8 kb *Eco*RI insert was obtained. Restriction mapping and Southern blotting analysis of the phage DNA localized the *M. avium* *pncA* gene on a 1.6 kb *Bam*HI subfragment within the 5.8 kb insert using the *M. tuberculosis* *pncA* probe. This 1.6 kb *Bam*HI fragment was subcloned into pUC19 for DNA sequence analysis.

Sequence analysis of the *M. avium* *pncA* gene

The *M. avium* *pncA* sequence was determined on both strands for the 1.6 kb *Bam*HI pUC19 construct using universal forward and reverse primers as well as primers derived from within the 1.6 kb *Bam*HI fragment. The *M. avium* *pncA* gene, which is located at 248 bp downstream from the 5' end of the 1.6 kb fragment, was found to be 561 bp long; this would encode a polypeptide of 187 amino acids with a predicted molecular mass of 19785.91 Da. The predicted *M. avium* PncA protein is only one amino acid longer than the corresponding *M. tuberculosis* enzyme (186 amino acids, GenBank accession number U59967). Sequence comparisons showed that the *M. avium* PncA has about 67.7% and 32.8% amino acid identity with the corresponding *M. tuberculosis* and *E. coli* enzymes, respectively (Fig. 1).

Translation of both upstream and downstream regions of the *pncA* did not reveal significant open reading frames with homology to known proteins in the databases. It is noteworthy that the upstream and downstream DNA sequence of the *M. avium* *pncA* did not show any significant homology with the corresponding regions in *M. tuberculosis* (data not shown). In *E. coli*, *pncA* is located immediately downstream of the L-asparaginase I gene (*asnA*) and forms an operon with *ansA* (Jarlstrom *et al.*, 1989). However, the

	1	15 16	30 31	45 46	60
1 <i>M. avium</i> PncA	M -- RALII VDVQ NDF	CEGGS VPVAGGA AVA	PPSTPT TWTTLP GYDY	VVVAT QDFHID PGDH	
2 <i>M. tb</i> PncA	M -- RALII VDVQ NDF	CEGGS LAVTGGA AALA	RAISDY LAEAA DYHH	-VVAT KDFHID PGDH	
3 <i>E. coli</i> PncA	MPPRAL LLVDLQ NDF	CAGGAL AVPEG DSTV	DVANRL IDWCQ SRGE	AVIASQ DWEPAN HGS	
	61	75 76	90 91	105 106	120
1 <i>M. avium</i> PncA	FS ----- DRP ---	D -- YSSSW PAHCL AG	SAGAD FRPELD TTRV	DAVFR KG -- AYAAG Y	
2 <i>M. tb</i> PncA	FS ----- GTP ---	D -- YSSSW PPECV S	TPGAD FHPSLD TSAI	EAVFY KG -- AYTG A	
3 <i>E. coli</i> PncA	FASQH GVEPY TFG QL	DGLP QTFW PDHC VQN	SEGAQ LHPLL HQKAI	AAVFH KGENP LVD SY	
	121	135 136	150 151	165 166	180
1 <i>M. avium</i> PncA	SGFEG VDD -- NGT PLL	EWLRR RGVDE VDV G	IATDH CVRRTA EDAA	RAGLT TTRVL VHLTA A	
2 <i>M. tb</i> PncA	SGFEG VDE -- NGT PLL	NWLRQ RGVDE VDV G	IATDH CVRQTA EDAV	RNGLA TRVL VDLT AG	
3 <i>E. coli</i> PncA	SAFFD NGRRQ KTS LD	DWLRD HEIDEL IVM G	LATDY CVKFT VLDA L	QLGYK VNVIT DGCR G	
	181	195 196	210 211		
1 <i>M. avium</i> PncA	AAEDS -- AARAL DEM	RSAGI ELV GAR ----	----	187	
2 <i>M. tb</i> PncA	VSADT -- TVAAL EEM	RTASV ELV CSS ----	----	186	
3 <i>E. coli</i> PncA	VNIQP QDSAH AFM EM	SAAGAT LYTL ADWEE	TQG	213	

Fig. 1. Comparison of the *M. avium* PZase (PncA) amino acid sequence with those of *M. tuberculosis* (*M. tb*) and *E. coli*. Gaps in the sequence alignment were introduced automatically by the CLUSTAL W method to maximize the sequence homology. Identical amino acid residues in all three sequences are in bold type.

upstream sequence of *M. avium* *pncA* did not have any significant homology to the *E. coli* *ansA*, indicating that the organization of the *M. avium* *pncA* and its upstream sequence is different from that in *E. coli*.

PZase expression in *M. avium*

To determine the level of PZase expression and investigate its possible regulation, we performed Western blot analysis with protein extracts of *M. avium* and *M. tuberculosis* grown under various conditions, i.e. acid pH (5.5) and with the enzyme substrates nicotinamide and PZA, using a rabbit polyclonal antibody raised against the *M. tuberculosis* PZase protein. Both *M. avium* and *M. tuberculosis* were found to constitutively express a very low amount of PZase protein, hardly detectable by Western blotting (data not shown). The level of PZase expression in *M. avium* was comparable to that in *M. tuberculosis*. Acid pH (5.5) or growth in presence of 50 µg ml⁻¹ of the PZase substrates nicotinamide and PZA did not significantly induce the production of PZase in *M. avium* or in *M. tuberculosis* as judged by Coomassie staining of SDS-PAGE gels and Western blot analysis (data not shown).

Presence of *pncA* homologues in other mycobacteria

To investigate whether there are *pncA* homologues in other mycobacteria, a Southern blot was performed with a portion of the *M. avium* *pncA* gene as a probe on *Bam*HI-digested genomic DNA from a panel of mycobacterial species (Fig. 2). The *M. avium* *pncA* probe hybridized significantly with genomic DNA from *M. terrae* (6.4 kb, lane 4), *M. gastri* (1.6 kb, lane 5), *M. marinum* (1.6 kb, lane 6), *M. fortuitum* (1.2 kb, lane 7), *M. gordonae* (1 kb, lane 10), *M. szulgai* (6.2 kb, lane 11),

Fig. 2. Presence of *pncA* homologues in mycobacteria detected by Southern blotting. Genomic DNA from various mycobacterial species was digested with *Bam*HI, transferred to nylon membrane, and the blot was probed with the ³²P-labelled PCR fragment containing part of the *M. avium* *pncA* gene (see Methods). Lanes: 1, *M. avium*; 2, *M. bovis* BCG; 3, *M. tuberculosis* H37Rv; 4, *M. terrae*; 5, *M. gastri*; 6, *M. marinum*; 7, *M. fortuitum*; 8, *M. chelonae*; 9, *M. xenopi*; 10, *M. gordonae*; 11, *M. szulgai*; 12, *M. celatum*.

M. celatum (3.8 kb, lane 12) and *M. kansasii* (1.8 kb, not shown); it hybridized less intensely with *M. xenopi* (6.4 kb, lane 9), and only poorly with DNA from *M. chelonae*. *M. smegmatis* did not give significant hybridization with the *M. avium* *pncA* probe (data not shown).

M. avium *pncA* confers PZA susceptibility to PZA-resistant *M. tuberculosis* complex organisms

Naturally PZA-resistant BCG and *M. tuberculosis* strains with acquired PZA-resistance lack or have

Table 1. Susceptibilities of mycobacterial strains to PZA

Strain	MIC of PZA ($\mu\text{g ml}^{-1}$)			
	No plasmid	p16R1	<i>M. avium</i> <i>pncA</i>	<i>M. tb. pncA</i>
<i>M. avium</i>	500	ND	ND	ND
BCG	> 500	> 500	50–100	50–100
<i>M. tb.</i> PZA-R	> 500	> 500	50–100	50–100*
<i>M. tb.</i> H37Rv	50–100	ND	ND	ND

ND, Not done. *M. tb.*, *M. tuberculosis*.

* Data from Scorpio & Zhang (1996).

reduced PZase activity due to *pncA* mutations (Scorpio & Zhang, 1996; Scorpio *et al.*, 1997a, b; Sreevatsan *et al.*, 1997). To determine if the *pncA* gene from *M. avium* would be functionally expressed and confer PZA sensitivity, we transformed BCG and PZA-R, a PZA-resistant *M. tuberculosis* isolate (PZA-R) derived from strain H37Rv, with a hygromycin plasmid construct harbouring the 1.6 kb *Bam*HI fragment containing the *M. avium pncA* gene. The *M. avium pncA* gene in the 1.6 kb *Bam*HI fragment has 248 bp upstream sequence from the start codon, which should contain the promoter for the *M. avium pncA*. The *M. tuberculosis pncA* gene on a 3.2 kb *Eco*RI–*Pst*I fragment (Scorpio & Zhang, 1996) which was cloned into the same hygromycin vector was used as a positive control, and the vector alone as a negative control. Interestingly, the *M. avium pncA* construct gave functional expression of PZase activity in both BCG and PZA-R, and conferred PZA susceptibility in both organisms. Both BCG and PZA-R are resistant to more than 500 $\mu\text{g PZA ml}^{-1}$, and transformation with the *M. avium pncA* gene made them susceptible to 50–100 $\mu\text{g PZA ml}^{-1}$ (Table 1). The above data indicate that the promoter of the *M. avium pncA* gene is recognized by the *M. tuberculosis* and *M. bovis* transcriptional and translational apparatus and that the *M. avium* PZase has the ability to potentiate PZA action. The level of PZA susceptibility conferred by the *M. avium pncA* gene was comparable to that conferred by the *M. tuberculosis pncA* construct (MIC 50–100 $\mu\text{g PZA ml}^{-1}$). Western blot analysis of the recombinant BCG strains revealed that the *M. tuberculosis pncA* gave a protein band of about 19.8 kDa as expected; however, the *M. avium pncA* produced a protein band with a size of about 24 kDa (Fig. 3), bigger than the 19.8 kDa predicted from the DNA sequence.

Investigation of anomalous mobility of the *M. avium* PZase protein on Western blots

Because the *M. avium* PZase migrated as a 24 kDa band on Western blots, as opposed to a 19.8 kDa band as expected, we wondered if this was due to glycosylation of the *M. avium* PZase. However, incubation of the protein extracts containing *M. avium* PZase with the enzyme Endo H (Sigma), which removes carbohydrates

Fig. 3. Western blot analysis, using a rabbit polyclonal antibody raised against the *M. tuberculosis* PZase protein, of the expression of the *M. avium* PZase in BCG. Lanes: 1, BCG transformed with vector control p16R1; 2, BCG transformed with the *M. tuberculosis pncA* gene; 3, BCG transformed with *M. avium pncA* gene; 4, BCG transformed with vector control p16R1.

from glycoproteins, did not alter the mobility of this protein on Western blots (data not shown), indicating that the slower migration of *M. avium* PZase on SDS-PAGE gels is not due to glycosylation of this protein.

To obtain more direct evidence that the slower migration of *M. avium* PZase on SDS-PAGE gels is not due to glycosylation, we overexpressed the *M. avium pncA* gene as well as the *M. tuberculosis pncA* gene as a control in *E. coli*, a host that is not known to glycosylate proteins. Both the overexpressed *M. avium* and *M. tuberculosis* PZase proteins gave functional enzyme activity in *E. coli* (data not shown). The same migration pattern as in Fig. 3 was observed, i.e. the overexpressed *M. avium* PZase still migrated more slowly than the corresponding overexpressed *M. tuberculosis* enzyme included as a control on a Western blot (data not shown). This strongly suggests that the retarded mobility of the *M. avium* PZase is not due to glycosylation.

DISCUSSION

In this study, we have cloned and characterized the *M. avium pncA* gene encoding PZase. We have demonstrated that *M. avium*, which is naturally resistant to PZA, has a functional *pncA* gene that confers PZA susceptibility to PZA-resistant *M. tuberculosis* complex organisms. Sequence analysis showed that the *M. avium*

PZase has a high degree of homology (67.7% amino acid identity) with the *M. tuberculosis* enzyme, a finding consistent with the strong hybridization of *M. avium* *pncA* with the corresponding *M. tuberculosis* gene. Southern blot analysis indicated that there are close *pncA* homologues in the following mycobacterial species: *M. terrae*, *M. gastri*, *M. marinum*, *M. fortuitum*, *M. goodii*, *M. celatum*, *M. xenopi*, *M. szulgai* and *M. kansasii*. However, *M. chelonae* and *M. smegmatis* did not produce significant hybridization with the *M. avium* *pncA* gene; the lack of significant hybridization in these organisms is probably due to poor homology of their *pncA* genes with the *M. avium* *pncA*, rather than to the absence of a *pncA* gene, since these mycobacteria have been shown to have functional PZase activity (Tarnok & Rohrscheidt, 1976; Koneman *et al.*, 1988). It is interesting to note that *M. celatum* gave strong hybridization signals with the *M. avium* *pncA* gene, a finding consistent with the similarity of these two organisms in terms of 16S rRNA sequence homology and biochemical properties used to identify the species (Butler *et al.*, 1993). An unexpected finding is that *M. xenopi*, which is taxonomically close to *M. avium* and *M. celatum* (Butler *et al.*, 1993), gave a relatively weak hybridization signal with the *M. avium* *pncA* gene. On the other hand, *M. fortuitum*, a fast-growing mycobacterium, produced a relatively strong hybridization signal. It is worth noting that unlike many other mycobacteria, *M. kansasii* has an amidase with only nicotinamidase activity but no PZase activity (Tarnok & Rohrscheidt, 1976), yet it produced a 1.8 kb *Bam*HI fragment which hybridized with the *M. avium* *pncA* probe by Southern blot analysis. Work is under way to characterize the *M. kansasii* nicotinamidase gene and to determine the genetic basis for the dissociation of PZase and nicotinamidase activities in the *M. kansasii* enzyme.

A somewhat unexpected finding is that *M. avium* PZase when overexpressed in BCG gave a higher molecular mass band (24 kDa) than that predicted from its DNA sequence (19.8 kDa) on Western blots; by contrast, the *M. tuberculosis* PZase, which is only one amino acid shorter than the *M. avium* PZase, gave a 19.8 kDa band as expected based on its DNA coding sequence. The retarded mobility of the *M. avium* PZase cannot be due to glycosylation for two reasons: (1) overexpression of the *M. avium* PZase in *E. coli*, which is not known to glycosylate proteins, still gave the same mobility pattern as in BCG; and (2) digestion of the *M. avium* PZase with Endo H, which removes carbohydrates from glycoproteins, did not change the mobility of the protein on Western blots. The possibility that the *M. avium* PZase used an alternative upstream start codon and encoded a larger protein can also be excluded, because there is a stop codon immediately upstream of the start codon of the *M. avium* *pncA* gene. Very basic proteins such as histones with considerably more positively charged amino acids migrate more slowly on SDS-PAGE gels than expected from their molecular mass (Panyim & Chalkley, 1971). The *M. avium* PZase has more overall positively charged amino acid residues than the *M.*

tuberculosis enzyme: 21 positively charged (with 32 negatively charged) versus 17 positively charged (with 34 negatively charged). The more positive overall charge of the *M. avium* PZase is likely to be the cause of its slower migration on the Western blots.

In the present study, we have shown that the *M. avium* *pncA* gene conferred PZA susceptibility in *M. tuberculosis* complex organisms. This is surprising because *M. avium* itself is not susceptible to PZA (MIC 500 µg PZA ml⁻¹ versus 50 µg PZA ml⁻¹ in *M. tuberculosis*); and the fact that the *M. avium* *pncA* conferred PZA susceptibility to resistant *M. tuberculosis* strains suggests that the relative nonsusceptibility to PZA of the *M. avium* species is not because it has an ineffective PZase enzyme. The low-level expression of the *M. avium* PZase could not have contributed to its natural resistance to PZA, as its level of expression is comparable to that in susceptible *M. tuberculosis* (data not shown). There are three possible reasons, which are not necessarily mutually exclusive, for the nonsusceptibility of *M. avium* to PZA. The first possibility is that *M. avium* has a more active efflux mechanism that pumps out POA than *M. tuberculosis*. *M. smegmatis* has been shown to have an efflux pump LfrA (Takiff *et al.*, 1996), and it is not clear whether *M. avium* would also have a similar efflux mechanism involved in pumping out certain antibiotics including POA. Further studies will be necessary to determine whether the natural resistance of *M. avium* to PZA is due to a more active efflux mechanism for pumping out POA. The second possibility is that *M. avium*, unlike *M. tuberculosis*, may not have a sensitive target for POA or have a target that is less susceptible to POA than that in *M. tuberculosis*; however, because the target for POA in *M. tuberculosis* has not yet been identified, this possibility cannot be addressed at this time.

The third possibility is that the cell wall of *M. avium* may not take up PZA or its derivative POA as efficiently as that of *M. tuberculosis*. Indeed, we have some data indicating that the *M. avium* cell wall does not take up as much [¹⁴C]POA as that of *M. tuberculosis* under acid pH conditions (unpublished). This difference in the cell wall permeability to POA is consistent with the finding that *M. avium* is less susceptible than *M. tuberculosis* to certain antibiotics such as POA esters (Cynamon *et al.*, 1995; Yamamoto *et al.*, 1995) and aminoglycosides (Jarvier & Nikaido, 1994). Another example showing that significant differences exist among the cell walls of different mycobacteria in terms of their permeability to certain antibiotics is that the cell wall of *M. chelonae* is much less permeable to the β-lactam antibiotic cephalosporins than that of *M. tuberculosis* (Jarvier & Nikaido, 1994). Our data on the differential permeability of mycobacterial cell walls to POA are consistent with these findings. It will be interesting to analyse further the relationship between cell wall permeability and susceptibility to PZA/POA using reagents that alter the cell wall permeability, and to identify the structural basis of the cell wall differences between *M. avium* and *M. tuberculosis* that may be involved in the uptake of POA.

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