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Identification, Cloning, and Expression of the *Escherichia coli* Pyrazinamidase and Nicotinamidase Gene, *pncA*

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Pyrazinamide (PZA) is one of the three most important drugs for treatment of *Mycobacterium tuberculosis* infections. The antibacterial activity of PZA requires a bacterial enzyme, pyrazinamidase (PZAase), which hydrolyzes PZA to form pyrazinoic acid and ammonia. Most PZA-resistant clinical *M. tuberculosis* isolates lack PZAase activity. With the goal of eventually identifying and characterizing the *M. tuberculosis* PZAase gene, we began with the more tractable organism, *Escherichia coli*, which also has PZAase activity. We screened a transposon-generated *E. coli* insertion mutant library, using a qualitative PZAase assay. Two PZAase-negative mutants out of 4,000 colonies screened were identified. In each mutant, the transposon interrupted the same 639-bp open reading frame (ORF), ORF1. The expression of ORF1 on a multicopy plasmid complemented a PZAase-negative mutant, leading to PZAase activity levels approximately 10-fold greater than those of the wild type. PZA has a structure similar to that of nicotinamide, a pyridine nucleotide cycle intermediate, so we tested our strains for nicotinamidase activity (EC 3.5.1.19) (genetic locus *pncA*). The construct with multiple plasmid copies of ORF1 had an approximately 10-fold increase in levels of nicotinamidase activity. This overexpressing strain could utilize nicotinamide as a sole nitrogen source, though wild-type *E. coli* cannot. We conclude that a single *E. coli* enzyme accounts for both PZAase and nicotinamidase activities and that ORF1 is the *E. coli* PZAase and nicotinamidase gene, *pncA*.

Pyrazinamide (PZA) is an important drug for the treatment of *Mycobacterium tuberculosis* infections. The addition of PZA to isoniazid (INH) and rifampin allowed the conventional 9-month tuberculosis treatment regimen to be shortened to 6 months (30). These three drugs form the cornerstone of initial tuberculosis therapy as recommended by the Centers for Disease Control and Prevention (9). PZA is also useful in the treatment of patients infected with multidrug-resistant *M. tuberculosis* strains (18).

The precise mechanism of action of PZA is not known. A bacterial enzyme, pyrazinamidase (PZAase) (synonym, PZA amidohydrolase), is necessary for PZA activity (21). PZAase hydrolyzes PZA to form pyrazinoic acid and ammonia (Fig. 1). *M. tuberculosis* strains which lose PZAase are uniformly resistant to PZA, and the loss of PZAase accounts for most PZA-resistant clinical isolates (25). As expected, pyrazinoic acid is active in vitro against both PZA-resistant and PZA-susceptible *M. tuberculosis* strains (21); its cellular target is not known.

PZA is structurally similar to nicotinamide (NAM), a pyridine nucleotide cycle intermediate (35). Nicotinamidase (NAMase) (synonyms, NAM amidohydrolase and NAM deamidase) catalyzes a reaction very similar to that of PZAase (Fig. 1) as a part of NAD recycling. The *Escherichia coli* NAMase gene (*pncA*) (synonym, *nam*) has been mapped to 39 min (4, 5, 10). Circumstantial evidence suggests that a single *M. tuberculosis* enzyme has both PZAase and NAMase activities (21). Although many bacteria produce PZAase (see data below), only members of the *M. tuberculosis* complex are known to be inhibited by PZA (16, 21). The present study was directed at the identification of the *E. coli* PZAase gene, with the eventual goal of identifying the homologous gene in *M. tuberculosis*. In addition, *E. coli* may provide a model for understanding the mechanism of action of PZA. There are numerous advantages to working with *E. coli* (e.g., rapid growth, well-developed genetic tools, substantial sequence information). Several *M. tuberculosis* genes, including three genes responsible for drug resistance (8, 33, 40), have been identified by using the sequences of their *E. coli* homologs. This report describes the identification, cloning, and expression of the *E. coli* PZAase gene.

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MATERIALS AND METHODS

Strains and genetic elements. The *E. coli* strains and genetic elements used in this study are described in Table 1. *Mycobacterium tuberculosis* ATCC 27294 (H37Rv [type strain]), *M. tuberculosis* ATCC 25177 (H37Ra), *Mycobacterium bovis* ATCC 35734 (BCG Pasteur), *Mycobacterium intracellulare* ATCC 13950 (type strain), *E. coli* ATCC 25922, *Proteus vulgaris* ATCC 49132, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 13880 (type strain), *Haemophilus influenzae* ATCC 10211, and a *Xanthomonas maltophilia* clinical isolate were obtained from the Clinical Microbiology Laboratory, Duke University Medical Center. *E. coli* N1624 was obtained from Paul L. Modrich, Department of Biochemistry, Duke University. *E. coli* DH5α was obtained from Steven W. Matson, Department of Biology, University of North Carolina, Chapel Hill, N.C. *Mycobacterium smegmatis* mc^2155 was obtained from W. R. Jacobs, Jr., Albert Einstein College of Medicine, Bronx, N.Y. *Mycobacterium avium* ATCC 35714 was obtained from the American Type Culture Collection, Rockville, Md.

Generation of an insertion mutant library. Derivative 103 of transposon Tn*10* was delivered by using λNK1316 to generate insertion mutants in *E. coli* N1624 (Table 1) (14, 20). λNK1316 contains a mutant transposase with a low specificity of insertion. The phage vector was lost after infection, because the phage has an amber mutation that is not suppressed by *E. coli* N1624. The integrated transposons are stable even in the absence of antibiotic selection, because the transposase gene is lost with the phage vector. The library was estimated to have about 10^6 independent members, and the vast majority should contain only a single transposon insert. Individual insertion mutant colonies were selected on LB agar (LB Agar, Miller; Difco Laboratories, Detroit, Mich.) with kanamycin (25 μg/ml).

Qualitative PZAase assay for mycobacteria. The bulk of a large mycobacterial

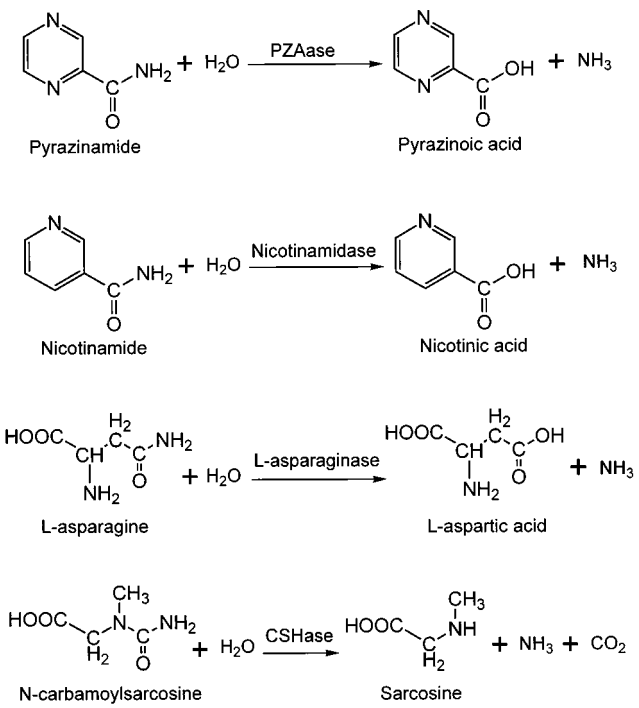

FIG. 1. Reactions catalyzed by PZAase, NAMase (EC 3.5.1.19), L-asparaginase (EC 3.5.1.1), and *N*-carbamoylsarcosine amidohydrolase (CSHase) (EC 3.5.1.59).

colony was transferred to the top of an agar butt containing PZA substrate as described by Wayne (37). After 7 days of incubation, pyrazinoic acid product was detected by the formation of a red-colored complex with ferrous ammonium sulfate. The positive and negative controls were *M. tuberculosis* ATCC 25177 and *M. bovis* ATCC 35734, respectively (26).

Qualitative PZAase assay for *E. coli* and other gram-negative bacteria. Wayne's PZAase assay (37) was modified to screen the *E. coli* insertion mutant library. Each well of a 96-well microtiter plate was filled with 180 μl of LB agar containing 4 mM PZA and inoculated from a single colony by stabbing with a sterile toothpick. After 2 or 3 days of incubation at 37°C, 100 μl of 25 mM ferrous ammonium sulfate was added to each well to detect pyrazinoic acid product. Wild-type PZAase-positive *E. coli* colonies, which form an intense red color, were used as positive controls. Uninoculated wells (no red color) were used as negative controls until the first PZAase-negative *E. coli* mutant was identified (21C8). This strain served as the negative control in subsequent assays. The same assay was also used to test a variety of gram-negative bacteria, all of which grew well on LB agar except *H. influenzae* ATCC 10211. *H. influenzae* ATCC 10211 was tested by transferring the bulk of a large colony to a well filled with 1.5% (wt/vol) agar (Bacto-Agar; Difco) containing 4 mM PZA and comparing the pyrazinoic acid production after 3 days at 37°C to that of an *E. coli* colony of a similar size.

Quantitative *E. coli* PZAase assays. Each *E. coli* strain was grown overnight in LB broth (LB Broth, Miller; Difco) containing 8 mM PZA and appropriate antibiotics (kanamycin [25 μg/ml] for the insertion mutants and ampicillin [500 μg/ml] for the strain containing pEAST2). This ampicillin concentration is higher than that commonly used. The optical density at 600 nm (OD_{600}) was measured, and the samples were diluted to obtain OD measurements between 0.2 and 0.6. Bacteria were washed twice in assay buffer (150 mM NaCl, 100 mM TrisCl, pH 7.5) and then resuspended at an OD_{600} of 40 (insertion mutants and wild-type strains) or 2 (strain containing pEAST2). Assays were performed both on whole cells and on a crude extract. The extract was generated by mixing 1.7 ml of bacterial suspension with 0.2 ml of glass beads (diameter, 25 to 50 μm) and agitating the mixture at the highest speed in the MiniBeadbeater-8 (Biospec Products, Bartlesville, Okla.) for 30 s twice and 60 s once. Each agitation was separated by 2 min on ice. The glass beads were pelleted by centrifugation (2 min at 16,000 × *g* at 4°C), washed with 0.3 ml of assay buffer, and centrifuged again. The two supernatants were combined as the crude extract. PZA substrate (8 mM final concentration) was added to each bacterial suspension or crude extract. Extracts were incubated at 37°C for 0, 15, 30, and 60 min. Bacterial suspensions were incubated at 37°C for 0, 15, 30, 60, and 120 min and then centrifuged for 3 min at 13,800 × *g* at room temperature. Pyrazinoic acid product concentrations in the extracts and in the supernatants of the bacterial suspensions were determined by adding 0.05 volume of 500 mM ferrous ammonium sulfate and comparing the change in OD_{480} with a standard curve. Samples were diluted as necessary to obtain an OD measurement between 0.2 and 1.0. PZAase activities were expressed as nanomoles of pyrazinoic acid produced per minute per OD_{600} of bacterial cells. All enzyme determinations were performed at least three times with independently grown bacterial cultures.

PZAase production in the presence of potential effectors. *E. coli* N1624 was grown overnight in minimal medium M9 (3) and then was diluted 1/100 into fresh M9 and incubated in a shaker at 37°C. When the OD_{600} was 0.1 to 0.4 (logarithmic growth phase), the culture was divided into eight aliquots. Two aliquots were kept as negative controls, and 10 mM (each) six potential effectors was added to the other aliquots. The aliquots were incubated for 2 h more and then centrifuged and assayed for PZAase activity by the quantitative assay described above. The experiment was repeated on four separate days.

Quantitative *E. coli* NAMase assay. The NAMase assay conditions were similar to those described above for PZAase. Bacterial suspensions were incubated in LB broth with either 0 or 8 mM NAM for 30, 60, 120, and 180 min. Ammonia product concentrations in the supernatants were determined with the Sigma ammonia diagnostic kit (Sigma Chemical Co., St. Louis, Mo.). We corrected for baseline ammonia production by subtracting the ammonia produced at each time point in cells incubated without NAM substrate.

Utilization of NAM as a sole nitrogen source. Minimal agar M63 was prepared with no $(NH_4)_2SO_4$ and with 30 mM NAM as a nitrogen source, as described by Pardee et al. (27). Appropriate antibiotics (kanamycin and/or ampicillin) were included. Overnight cultures of various strains of *E. coli* were spread on this agar, and growth was observed at 18 and 40 h.

Cloning transposon 103 with flanking DNA. Chromosomal DNA from *E. coli* 21C8 was purified, digested with various restriction enzymes, and ligated into the multiple cloning site of plasmid pSU18 (Table 1). The ligation products were transformed into *E. coli* DH5α (Table 1) by standard methods (3). Transformants containing transposon 103 inserted into plasmid pSU18 were selected on

TABLE 1. Strains and genetic elements used in this study

Strain or genetic element	Relevant characteristics	Reference or source
Strains		
E. coli N1624	F⁻ *relA1 rpsL150*(Str^r) *spoT1 bglR6*, PZAase positive	14
E. coli 15C5	N1624 with arbitrary insertion of transposon 103, PZAase positive	This work
E. coli 21C8	N1624 with insertion of transposon 103 in *pncA*, PZAase negative	This work
E. coli 44D7	N1624 with insertion of transposon 103 in *pncA*, PZAase negative	This work
E. coli DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, efficient transformation	15
Genetic elements		
Phage λNK1316	P_{am}80 λ hop phage vehicle; *ats1 ats2* (low-specificity transposase)	20
Transposon 103	Derivative of Tn*10*, small size (1,800 bp), Kan^r	20
Plasmid pSU18	Derivative of pSU2718 without undesired *Eco*RI and *Acc*I sites, Cam^r	6
Plasmid pWAMO1	pSU18 with insert of transposon 103 and flanking *E. coli* 21C8 DNA, Cam^r Kan^r	This work
Plasmid pCRII	High copy number, *lac* promoter, multiple cloning sites, Kan^r Amp^r	Invitrogen[a]
Plasmid pEAST2	pCRII with insert containing wild type *E. coli pncA* gene, Kan^r Amp^r	This work

[a] Original TA cloning kit (Invitrogen).

FIG. 2. Transposon insertion sites in two PZAase-negative insertion mutants. This figure includes the 3′ end of the *ansA* gene and the complete 639-bp coding sequence of *pncA* (ORF1). The figure shows deduced amino acid sequences for both genes, sites for primers A and B, the *pncA* ribosomal binding site (RBS), and a region of hairpin symmetry which may terminate transcription (double underlined) (19). The sites of transposon insertion in PZAase-negative mutants 21C8 and 44D7 are shaded. These insertion sites were determined by sequencing flanking DNA on both sides of the transposon in both strains. As expected, the transposon produced duplications of the 9-bp insertion site in each mutant (20). Both transposon insertions interrupt translation of *pncA*. The figure includes nucleotides 1019 to 1859 from the GenBank sequence with accession number M26934 (19), and the sequence is numbered from the start codon of *pncA*.

LB agar with 25 μg of kanamycin per ml (transposon 103) and 25 μg of chloramphenicol per ml (pSU18).

PCR primers and conditions. Four primers were used in the PCR: M13R (5′ AGCGGATAACAATTTCACACAGGA 3′), TRANSP (5′ TGTTCCGTTGCG CTGCCCGG 3′), A (5′ GAAGCCACGCTAACCAAACTGC 3′), and B (5′ GCATCAATTGCCAGATGTGCC 3′). Primer M13R anneals at a location adjacent to the multiple cloning site of pSU18. Primer TRANSP is complementary to inverted repeat DNA about 100 bp from both ends of transposon 103. Primers A and B are complementary to *E. coli* chromosomal DNA about 100 bp upstream and downstream from *pncA*. About 5 ng of plasmid DNA or 5 μl of bacterial lysate containing chromosomal DNA (13) was used in each 50-μl reaction mixture. Multiple interspersed negative controls (reagents only, no DNA) were included each time that PCR was performed. For primer pair M13R + TRANSP, the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% (wt/vol) gelatin, 1.2 mM $MgCl_2$, 100 nM each primer, 200 μM (each) the four deoxynucleoside triphosphates, and 1.25 U of *Taq* DNA polymerase (Amplitaq DNA polymerase; Perkin-Elmer Cetus, Norwalk, Conn.) in a total volume of 50 μl. The mixture was incubated at 95°C for 3 min, subjected to 25 temperature cycles (95°C for 1 min [denaturation], 55°C for 2 min [annealing], and 72°C for 1 min [extension]), incubated at 72°C for 10 min, and cooled to room temperature, all in the TwinBlock System TCX20A temperature cycler (Ericomp, San Diego, Calif.). For primer pairs TRANSP + A, TRANSP + B, and A + B the reaction mixture had 2.0 mM $MgCl_2$ and no gelatin, but was otherwise identical to the mixture described above. The temperature cycles were also identical except that an annealing temperature of 60°C was used. The presence and size of each PCR product was determined by electrophoresis on an agarose gel containing ethidium bromide.

DNA sequencing and analysis. Plasmids and PCR products were sequenced directly with the *Taq* Dye Deoxy Terminator cycle sequencing kit and the 373A DNA Sequencer (both from Applied Biosystems, Foster City, Calif.) as previously described (12). The computer program BLAST (2) was used to search the nucleotide and protein databases at the National Center for Biotechnology Information. GenBank nucleotide and protein accession numbers are M26934 and P21369, respectively (19).

RESULTS

Qualitative PZAase activities of various bacteria. The following bacteria were strongly PZAase positive: *M. tuberculosis* ATCC 27294 and ATCC 25177; *M. intracellulare* ATCC 13950; *M. avium* ATCC 35714; *M. smegmatis* mc^2155; *E. coli* DH5α, ATCC 25922, and N1624; *Proteus vulgaris* ATCC 49132; *P. aeruginosa* ATCC 27853; *S. typhimurium* ATCC 14028; *Serratia marcescens* ATCC 13880; and the *X. maltophilia* clinical isolate. Only two bacteria tested were PZAase negative: *M. bovis* ATCC 35734 (BCG Pasteur) and *H. influenzae* ATCC 10211.

Identification of the *E. coli* PZAase gene by insertion mutagenesis. A library of transposon insertion mutants in *E. coli* N1624 (PZAase positive) was screened for PZAase activity. By screening over 4,000 insertion mutants, two PZAase-negative mutants were identified (21C8 and 44D7). In order to identify the site of insertion, the transposon and adjoining chromosomal DNA were cloned into a recombinant plasmid, by using the kanamycin resistance gene of the transposon to select for the appropriate plasmid insert. An 8.4-kb *Eco*RI-*Sac*I fragment of *E. coli* 21C8 chromosomal DNA, which contained transposon 103, was cloned in plasmid pSU18, generating plasmid pWAMO1 (Table 1). Flanking DNA from one side of the transposon was isolated from pWAMO1 by PCR using the primer pair TRANSP + M13R (Materials and Methods). The PCR product was sequenced directly by using the transposon-specific primer TRANSP, and the resulting sequence was used to search the National Center for Biotechnology Information databases. The site of transposon insertion in *E. coli* 21C8 was identified in the sequence with accession number M26934 and fell between the ribosomal binding site and start codon of open reading frame 1 (ORF1) (Fig. 2). ORF1 is a 639-bp ORF with no known function. It is part of an operon with *ansA*, the gene for L-asparaginase I (Fig. 1) (19).

Chromosomal DNA was then amplified to confirm the site of transposon insertion in *E. coli* 21C8 and to identify the site

TABLE 2. PZAase and NAMase activities of various *E. coli* strains used in this study

E. coli strain	Qualitative PZAase phenotype	Enzyme activity (nmol/min · OD_{600})[a]		
		PZAase in whole cells	PZAase in extract	NAMase in whole cells
N1624	Positive	0.82 ± 0.02	0.05 ± 0.02	0.10 ± 0.06
15C5 (insertion mutant of N1624)	Positive	0.66 ± 0.06	Not done	Not done
21C8 (insertion mutant of N1624)	Negative	<0.02	Not done	Not done
44D7 (insertion mutant of N1624)	Negative	<0.02	<0.02	<0.02
44D7 plus pEAST2	Positive	6.5 ± 0.5	0.62 ± 0.05	0.90 ± 0.65

[a] Each determination was performed in triplicate at least, with independently grown bacterial cultures. Values are means ± standard deviations. PZAase and NAMase activities were obtained by different methods and may not be directly comparable (see Materials and Methods).

in *E. coli* 44D7. Chromosomal DNA from each strain was amplified with the transposon-specific primer TRANSP plus either primer A or B, which both anneal just outside the two ends of ORF1 (Fig. 2). The PCR products from *E. coli* 21C8 (about 250 and 800 bp, respectively) and those from *E. coli* 44D7 (about 500 and 525 bp, respectively) were sequenced to determine the exact site of transposon insertion in each strain (Fig. 2). Transposon insertion interrupted the translation of ORF1 in both PZAase-negative mutants. We have renamed ORF1 *pncA* on the basis of these results and those described below.

Cloning and expression of *pncA*. Chromosomal DNA from *E. coli* N1624 (PZAase positive) was amplified with primer pair A + B (Fig. 2), yielding the expected 841-bp product containing the 639-bp *pncA* gene with flanking DNA. This PCR product was ligated into plasmid pCRII (Table 1) with the TA cloning kit (Invitrogen, San Diego, Calif.). A resulting plasmid, pEAST2 (Table 1), contained *pncA* in the correct orientation with respect to the plasmid *lac* promoter. The PZAase-negative insertion mutant *E. coli* 44D7 was transformed with pEAST2, converting its qualitative phenotype to PZAase positive.

Quantitative PZAase activities. Table 2 lists the qualitative and quantitative PZAase activities of the strains described above and those of an arbitrary insertion mutant strain, 15C5, with the wild-type phenotype (PZAase positive). Transposon insertion alone had no meaningful effect on PZAase production (compare whole-cell assay results for strains N1624 and 15C5). The two PZAase-negative insertion mutants (21C8 and 44D7) had no detectable PZAase activity in the whole-cell assay, representing at least a 40-fold reduction from wild-type levels. The plasmid pEAST2 restored the PZAase-positive phenotype to 44D7 and actually resulted in an approximately 10-fold-increased level of PZAase activity over wild-type levels. Overexpression was probably due to multiple plasmid copies and the strong plasmid *lac* promoter. The extracts had less PZAase activity than did the whole cells, but the same relative activities were observed. Plasmid pEAST2 also resulted in an approximately 10-fold increase in levels of PZAase activity when assayed in a crude extract, providing the strongest argument that the *pncA* gene is the structural gene for PZAase.

Quantitative NAMase activities. We tested the same strains for NAMase activities and found a similar pattern (Table 2). Wild-type *E. coli* N1624 produces a low level of NAMase, similar to that found by previous investigators (27). The PZAase-negative mutant 44D7 had no measurable NAMase activity. The plasmid construct 44D7 with pEAST expressing multiple copies of *pncA* had levels of NAMase activity that were about 10-fold increased over wild-type levels.

Utilization of NAM as sole nitrogen source. Ammonia is one of the products of NAMase and is also the preferred nitrogen source for *E. coli* (Fig. 1). Wild-type *E. coli* does not produce enough NAMase to utilize NAM as a sole nitrogen source, but NAMase-hyperproducing mutants can use NAM as a sole nitrogen source (27). We plated both 44D7 and 44D7 plus pEAST2 on minimal agar with 30 mM NAM as a sole nitrogen source. As expected, 44D7 (NAMase negative) did not grow but 44D7 with pEAST2 grew to confluence.

PZAase production in the presence of potential effectors. We grew *E. coli* N1624 for 2 h in minimal media with 10 mM (each) six potential effectors (L-asparagine, L-aspartic acid, PZA, pyrazinoic acid, NAM, and nicotinic acid.). These are the substrates and products of the three enzymatic activities attributed to the *ansA-pncA* operon, namely, L-asparaginase, encoded by *ansA*, and PZAase and NAMase, encoded by *pncA* (Fig. 1). None of these compounds inhibited the growth of this *E. coli* strain under the conditions of the assay. We found no meaningful change in PZAase activity in the presence of any potential effector. The effectors and relative enzyme activities (mean ± standard deviation), based on quadruplicate determinations, were as follows: no effector, 1.00 ± 0.04; L-asparagine, 0.97 ± 0.24; L-aspartic acid, 1.06 ± 0.24; PZA, 0.93 ± 0.07; pyrazinoic acid, 0.76 ± 0.09; NAM, 1.06 ± 0.12; and nicotinic acid, 0.88 ± 0.12.

Comparison of PncA sequence with those in National Center for Biotechnology Information databases. The *pncA* nucleotide and PncA deduced amino acid sequences were used to search the National Center for Biotechnology Information databases. The search included the complete nucleotide sequence of PZAase-negative *H. influenzae* (accession number, L42023), and the available nucleotide sequences of *Mycobacterium leprae* (about 30% of the entire genome). Four proteins with significant similarity to PncA were found, and all were similar in the same region (PncA amino acids 132 to 170). Three of these are hypothetical *Bacillus subtilis* and *E. coli* proteins with no known function (accession numbers, P37532, P21367, and P37347). The fourth protein is the *N*-carbamoylsarcosine amidohydrolase (Fig. 1) of an *Arthrobacter* species (28) (accession number, P32400). The conserved regions of *E. coli* PncA and the four related proteins are aligned in Fig. 3. The probable active site of the *Arthrobacter N*-carbamoylsarcosine amidohydrolase (cysteine 177) is in this conserved region and is itself conserved in PncA (cysteine 156) and in two of the three hypothetical proteins. Translation of *pncA* was interrupted before the cysteine 156 codon in both PZAase-negative insertion mutants (21C8 and 44D7).

DISCUSSION

***E. coli pncA*.** We conclude that a single *E. coli* enzyme accounts for both PZAase and NAMase activities and that ORF1 is the *E. coli* PZAase and NAMase gene (*pncA*) on the basis of the following evidence. (i) Two of two PZAase-negative inser-

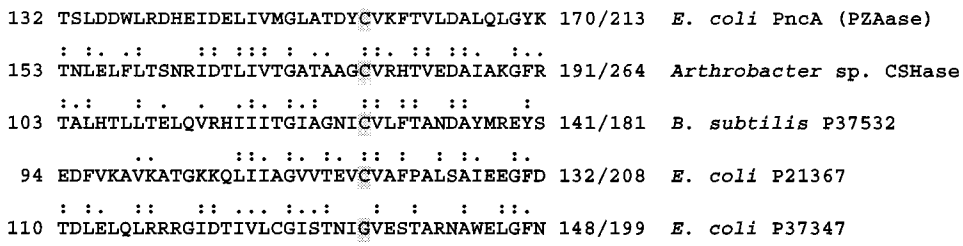

FIG. 3. Alignment of partial amino acid sequences of *E. coli* PncA (PZAase) and related proteins. Related proteins include an *Arthrobacter* species carbamoylsarcosine amidohydrolase (CSHase) and hypothetical proteins from *B. subtilis* and *E. coli*. The beginning and ending amino acid positions of these segments are indicated, and the latter is followed by the total number of amino acids in each protein sequence. Each sequence is compared with the *E. coli* PncA sequence. Identical amino acids are indicated by two dots, and similar amino acids are indicated by one dot. The following amino acids are defined as similar: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, and W. The probable active site of the *Arthrobacter* amidase, cysteine 177 (28), is shaded as are the corresponding residues in the other proteins. This cysteine is conserved in PncA and in two of the three other protein sequences shown.

tion mutants had mutations which interrupted the translation of *pncA*. (ii) Expression of *pncA* on a multicopy plasmid complemented a PZAase- and NAMase-negative mutant, resulting in levels of PZAase and NAMase activity about 10-fold increased over wild-type levels. Importantly, increased PZAase activity was detected in crude extracts, eliminating the possibility that the increased activity was due to changes in the permeability of the substrate or the product. (iii) The construct with multiple plasmid copies of *pncA* was able to grow with NAM as a sole nitrogen source. (iv) The *E. coli* NAMase gene was previously mapped to 39 min, the same location as *pncA* (4, 5, 10, 19). (v) The deduced PncA amino acid sequence has a region similar to the active site of a known amidohydrolase.

PZAase and NAMase catalyze very similar reactions (Fig. 1), so it is not surprising to find that the same *E. coli* enzyme has both activities. NAMase is probably the natural function of PncA (27), as PZA is not known to occur in nature. A single *M. tuberculosis* enzyme may also have both PZAase and NAMase activities (21). Although it is unlikely, we did not exclude the possibility that *E. coli pncA* is a positive regulator of a separate gene or genes encoding PZAase and NAMase.

Operon organization. *ansA* (coding for L-asparaginase I) and *pncA* (coding for NAMase) form an operon. Both genes are transcribed from a common promoter upstream from *ansA* (19), and a likely transcriptional terminator follows *pncA*. L-Asparaginase I and NAMase catalyze similar reactions (Fig. 1). Their respective products are part of known pathways for NAD synthesis (aspartic acid) and cycling (nicotinic acid) (35). The two genes do not appear to be products of gene duplication, since there is no meaningful homology between the DNA sequences of *ansA* and *pncA* or between their deduced amino acid sequences.

Both L-asparaginase I and NAMase are considered to be constitutive enzymes in *E. coli* (19, 27). Pardee et al. (27) added various nicotinic acid derivatives to minimal media and found no effect on NAMase production. In a similar experiment, we tested six potential effectors (the substrates and products of L-asparaginase I, NAMase, and PZAase) and found no change in PZAase activity. Our results are consistent with the constitutive production of PZAase, though it is possible that these substances might influence PZAase production under different growth conditions (e.g., in a chemostat, on different media) or that other substances are effectors.

Implications for *M. tuberculosis*. *M. tuberculosis* normally loses its PZAase activity when it acquires PZA resistance. The loss of an enzyme activity is a relatively rare mechanism for the acquisition of drug resistance. Nonetheless, *M. tuberculosis* also develops resistance to INH by a similar mechanism, namely the loss of catalase activity (40).

Identification of the *E. coli* PZAase gene is a first step toward the identification of the *M. tuberculosis* PZAase gene. Several antibiotic resistance genes in *M. tuberculosis* have recently been identified on the basis of the homologous *E. coli* genes. Zhang et al. (40) identified the *M. tuberculosis* catalase gene, *katG*, using probes based on conserved amino acid sequences in the homologous *E. coli* and *Bacillus stearothermophilus* enzymes. Deletions and other mutations in the *M. tuberculosis katG* gene are associated with INH resistance in both laboratory and clinical *M. tuberculosis* strains (1, 31, 40). Similarly, Cambau et al. (8) identified the *M. tuberculosis gyrA* gene by PCR using primers derived from nucleic acid sequences of the homologous *E. coli* and *Staphylococcus aureus* genes. A mutation in this gene was associated with the acquisition of ofloxacin resistance in sequential *M. tuberculosis* isolates from a patient treated with this drug (7). Lastly, Telenti et al. used an *E. coli* probe containing a partial *rpoB* sequence to identify the homologous gene region in *M. tuberculosis* (33). Mutations in this region are associated with rifampin resistance in both species.

In a similar fashion, we are pursuing several strategies to identify the *M. tuberculosis* PZAase gene on the basis of the *E. coli* PZAase sequence. PZA-resistant PZAase-negative *M. tuberculosis* strains are likely to have PZAase gene mutations. The detection of those mutations may provide a rapid assay for PZA resistance in *M. tuberculosis*. Similar rapid molecular assays for rifampin resistance in *M. tuberculosis* have been developed (34, 38). Rapid susceptibility testing for PZA and other antibiotics would allow early appropriate antibiotic therapy. Patients with multidrug-resistant *M. tuberculosis* who receive active drugs within 2 weeks of diagnosis have reduced mortality (11, 36). Unfortunately, drug susceptibility testing for *M. tuberculosis* often takes more than 4 weeks (17).

Potential application to *M. bovis* BCG. *M. bovis* BCG is a live vaccine given to infants to prevent tuberculosis. BCG is PZAase negative and PZA resistant, as are essentially all *M. bovis* strains (26). Complications of BCG vaccination are rare, but disseminated infections and fatalities do occur. Lotte et al. (22, 23) gathered case reports of 39 cases of fatal disseminated BCG disease, and at least 20 additional fatal cases have been reported since those reviews. Fatalities have been caused by BCG strains with acquired resistance to multiple drugs (24, 29). An ideal live tuberculosis vaccine should be susceptible to all first-line drugs, including PZA. We are currently attempting to express the *E. coli pncA* gene in *M. bovis* BCG with the goal of making BCG sensitive to PZA. In a similar fashion, expression of *katG* restores INH sensitivity in INH-resistant *M. tuberculosis* isolates (39). Work is under way to construct a recombinant BCG strain expressing multiple vaccine antigens

(32). The inclusion of *pncA* or other antibiotic susceptibility genes might enhance the safety of this vaccine.

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