

# Evidence for arylamine N-acetyltransferase activity in *Klebsiella pneumoniae*

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Arylamine N-acetyltransferase (NAT) enzymes have been found in laboratory animals, humans, microorganisms (fungi, bacteria and parasites), and in plants. But the characteristics of NAT from *Klebsiella pneumoniae* are not clear. NAT activities with *p*-aminobenzoic acid (PABA) and 2-aminofluorene (AF) as substrates were examined in the cytosol of *K. pneumoniae*. NAT activity (N-acetylation of substrates) was determined using an acetyl coenzyme A recycling assay and high performance liquid chromatography for determining the amounts of acetylated or non-acetylated PABA or AF. NAT activities from a number of *K. pneumoniae* isolates were found to be  $0.72 \pm 0.08$  nmol/min/mg protein for AF, and  $0.49 \pm 0.04$  nmol/min/mg protein for PABA. The kinetic parameters of apparent Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) obtained were  $2.92 \pm 0.48$  mM and  $7.89 \pm 0.82$  nmol/min/mg protein, respectively, for AF and  $2.42 \pm 0.28$  mM and  $9.87 \pm 0.64$  nmol/min/mg protein, respectively, for PABA. The optimal pH value for the NAT activity was 7.0 for AF and PABA. The optimal temperature for NAT activity was 37°C for both substrates. The NAT activity was inhibited by 50% with 0.25 mM iodoacetamide, and by more than 90% at 1.0 mM. Among a series of divalent cations and salts,  $Cu^{2+}$  and  $Zn^{2+}$  were the most potent inhibitors of NAT activity.

**Key words:** N-acetyltransferase, 4-aminobenzoic acid, 2-aminofluorene, *Klebsiella pneumoniae*, mRNA

Species differences in the bioactivation and detoxification of many arylamine drugs and carcinogens are well known [1-3]. The involvement of arylamine carcinogens in the initiation of neoplasia requires activation steps for generating electrophilic species with the capacity to bind to tissue macromolecules such as DNA [4].

N-acetylation plays an important role in the metabolic pathway of arylamine carcinogens [5]. The N-acetylation is catalyzed by cytosolic arylamine N-acetyltransferase (NAT), which requires acetyl coenzyme A (acetyl CoA) as an acetyl group donor. A polymorphism in the activity of NAT has been extensively studied in a number of species including humans [6,7].

Humans can be divided into rapid and slow acetylators, and susceptibility to aromatic amine toxicity has been associated with acetylator phenotypes [5]. In humans, the epidemiological statistics have shown that slow acetylators had high risk for bladder cancer [8] and rapid acetylators had high risk for colorectal cancer

[9,10]. Therefore, the genetically mediated variation in NAT activities within target tissue may indicate differential risks among human populations.

NAT has been found in tissues of laboratory animals [11-13], and in birds [14], frogs [15], insects [16,17], nematodes [18], fish [19], and bacteria [20-22]. *Klebsiella pneumoniae* can cause community-acquired primary lobar pneumonia. Pneumonia due to *Klebsiella* species frequently involves the necrotic destruction of alveolar spaces, formation of cavities, and the production of blood-tinged sputum.

It had been showed that *K. pneumoniae* can acetylate 2-aminofluorene (AF) and contain NAT activity [23-25]. Therefore, 2 questions occur: 1) what is the difference between N-acetylation of AF and *p*-aminobenzoic acid (PABA) from *K. pneumoniae*, and 2) what are the characteristics of NAT from *K. pneumoniae*? Although N-acetylated AF had been demonstrated in *K. pneumoniae*, the NAT character is not reported. Thus, the purposes of this study were to determine the differences of N-acetylation of AF and PABA, and the determination of the kinetic constants of *K. pneumoniae* NAT, NAT examination by polymerase chain reaction (PCR), and the characterization of the enzyme.

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## Materials and Methods

### Chemicals and reagents

Ethylenediaminetetraacetic acid (EDTA), PABA, acetyl-PABA, leupeptin, Tris, AF, 2-acetyl-aminofluorene, carnitine acetyltransferase, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), acetyl carnitine, dithiothreitol (DTT), and acetyl CoA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetic acid, sodium azide, acetonitrile, and potassium phosphate were obtained from Merck & Co. (Darmstadt, Germany). All of the chemicals used were reagent grade.

### *Klebsiella pneumoniae*

*K. pneumoniae* was clinically isolated from patients at the China Medical University Hospital and identified by the VITEK GNI kit. Subcultures were performed weekly by using nutrient agar plates and stored at 4°C.

### Preparation of bacteria cytosols

About  $3 \times 10^9$  bacterial cells were washed twice in cold phosphate buffered saline, then placed immediately in 1 mL of lysis buffer [20 mM Tris-HCl, pH 7.5 (at 4°C), 1 mM DTT, 1 mM EDTA, 50  $\mu$ M PMSF, and 10  $\mu$ M leupeptin], and then disrupted by sonication followed by centrifugation for 30 minutes at  $10,000 \times g$ . The supernatants were kept on ice until assayed for NAT activity (N-acetylation of substrates).

### NAT activity determination

The determination of acetyl CoA-dependent N-acetylation of PABA and AF were performed as described by Chung et al [21].

### Protein determination

Protein concentrations of the cytosols from the bacterium *K. pneumoniae* were determined by the method of Bradford [26] with BSA as the standard. All of the samples were assayed in triplicate.

### Effects of incubation conditions on NAT activity

The enzyme cytosols were assayed at different pH values (4.0-10.5) in lysis buffer to determine the NAT activity. The enzyme cytosols were first prepared in lysis buffer, then kept on ice until the substrates were added, and then assayed at various temperatures. Second, the enzyme solutions were mixed with the various concentrations of iodoacetamide, adjusted to pH 6.0, and incubated at 37°C for 10 minutes. Third,

the enzyme solutions were mixed with various salts (KCl, Tris-HCl, ammonium acetate, Tris-acetate, ammonium citrate,  $\text{Na}_2\text{SO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{CoCl}_2$ ,  $\text{FeSO}_4$ ,  $\text{CaCl}_2$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{CuSO}_4$ ,  $\text{MnCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{ZnSO}_4$ ) to a final concentration of 0.5 mM, adjusted to pH 6.0, and incubated at 37°C for 10 minutes. Fourth, the enzyme solutions were incubated with each reagent (1 mM EDTA, 1 mM iodoacetate, 1 mM PMSF, 0.1 mM leupeptin, 0.1 mM pepstatin A, and 1 mg/mL trypsin inhibitor) at 4°C for 2, 4, and 6 days. Fifth, the enzyme solutions were mixed with the various salts to a final concentration of 0.5 mM, adjusted to pH 6.0, and incubated at 37°C for 10 minutes. The kinetic constants were calculated with the Cleland HYPER Program [27] that performs linear regression using a least-squares method.

### Partial purification of NAT from *Klebsiella pneumoniae*

A 5-step procedure was performed for this experiment.

- Step 1: finely ground ammonium sulfate (10 g/5 mL) was added to the cytosol which contained approximately 2031 mg protein. The precipitate was subtracted by centrifugation at  $10,000 \times g$  for 30 minutes. The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.4):50 mM KCl: 5 mM  $\text{MgCl}_2$ :20% glycerol overnight and was then centrifuged at  $10,000 \times g$  for 10 minutes to remove the precipitate.
- Step 2: the enzyme fraction from the first step was applied to a DEAE-Sephacel column (5 cm  $\times$  11 cm) equilibrated with 20 mM Tris-HCl (pH 7.4), containing 1 mM EDTA, 2 mM DTT, and 50 mM PMSF. Enzyme activity was subsequently eluted at 0.5 mL/min with a linear gradient (500 mL) of NaCl (0-0.5 M). Fractions (10 minutes each) were collected, and those containing NAT activity were pooled and concentrated by ultrafiltration (Amicon PM 10 membrane, 1.5 atm).
- Step 3: the enzyme from the second step was applied to a hydroxylapatite column (3  $\times$  90 cm) equilibrated with the same buffer (as described in step 2) and those containing NAT activity were pooled and concentrated by ultrafiltration (Amicon PM 10 membrane, 1.5 atm).
- Step 4: the enzyme from step 3 was separated at a flow rate of 0.25 mL/min on a Sephadex G-100 column (3  $\times$  120 cm) equilibrated with the same buffer as in step 2 but containing sodium azide. Fractions (10 minutes each) containing NAT activity

were concentrated by ultrafiltration as described in step 2.

Step 5: purification, involving anion exchange chromatography using a high performance liquid chromatography system comprising 2-solvent delivering pump and column (waters protein-pak DEAE-5pw, 7.5 mm internal diameter  $\times$  7.5 cm). The column was equilibrated with buffer as described in step 4.

### SDS-polyacrylamide gel electrophoresis

Samples of column fractions and pools from the above steps were routinely analysed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after reduction and alkylation of samples at pH 6.8, as described previously [22,28]. To assign a band to NAT, 25  $\mu$ L of each fraction across the appropriate region of columns was treated identically and the stained gels [22,28] were compared with the activity profiles from high performance liquid chromatography.

### Reverse transcriptase polymerase chain reaction

The total RNA was extracted from *K. pneumoniae* using Qiagen RNeasy Mini Kit. Total RNA (1.5  $\mu$ g), 0.5  $\mu$ g of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined into a microcentrifuge tube to a final volume of 12.5  $\mu$ L. The procedures for conducting reverse transcription were exactly as in the instruction manual (First-strand cDNA synthesis kit, Novagen, Madison, WI, USA). The products from the reverse transcription served as a template for PCR. When amplifying target cDNA, components in 50  $\mu$ L of solution were as follows: 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 20 pmol of each primer (B-MDIEA-NAT1 and VPKHGD-X-NAT1 for NAT1; FP1-NAT2 and RP1-NAT2 for NAT2; and Act b1 and Act b2 for beta-actin), and cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase. The sequence of primers was as follows: B-MDIEA-NAT1, 5'-CACCCGGATCCGGGATCATGGACATTGAAGC-3', nt 435-454, GenBank accession number X17059; VPKHGD-X-NAT1, 5'-GGTCCTCGAGTCAATCACCATGTTTGGGCAC-3', nt 1295-1278, GenBank accession number X17059; FP1-NAT2, 5'-CTAGTTCCTGGTTGCTGGCC-3', nt 79-98, GenBank accession number NM-000015; RP1-NAT2, 5'-TAACGTGAGGGTAGAGAGGA-3', nt 1073-1054, GenBank accession number NM-000015; Act b1, 5'-GCTCGTCGTCGACAACGGCTC-3', nt 94-114, GenBank accession number

NM-001101; Act2 b2, 5'-CAAACATGATCTGGGTCATCTTCTC-3', nt 446-422, GenBank accession number NM-001101 [29-31].

### Statistical treatment of data

Statistical analysis of the data was performed by use of unpaired Student's *t* test.

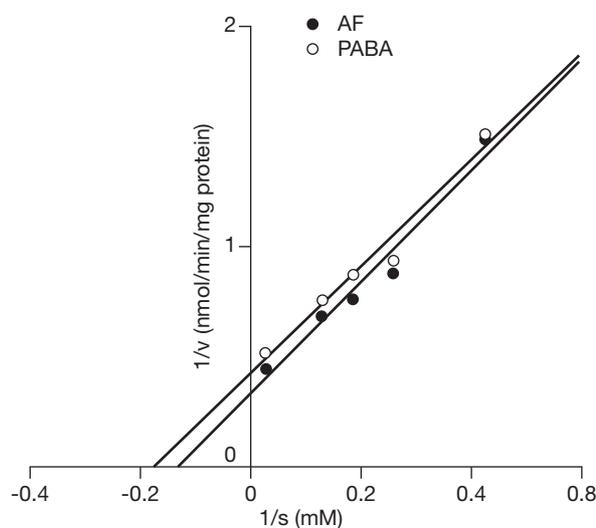
## Results

### Kinetic constants of NAT activity

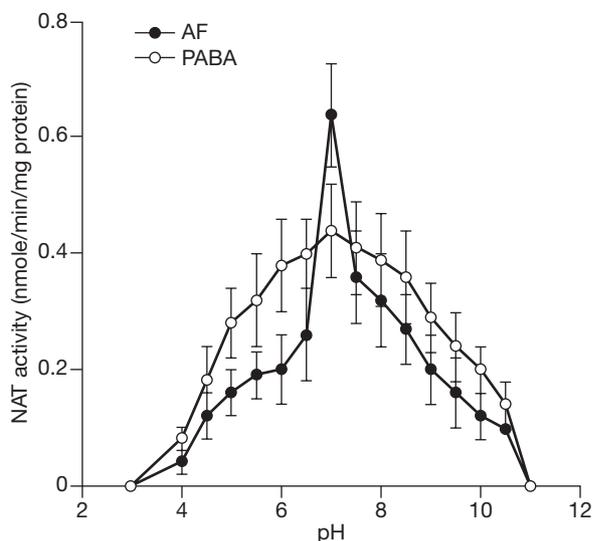
NAT activity was found in the cytosol of *K. pneumoniae*. The activities found for 0.1 mM substrate and 0.5 mM acetyl CoA were (mean  $\pm$  standard deviation)  $0.72 \pm 0.08$  nmol/min/mg protein for AF and  $0.49 \pm 0.04$  nmol/min/mg protein for PABA. The kinetic constants were determined for *K. pneumoniae* NAT using AF and PABA as substrates are shown in Fig. 1. The apparent values of the kinetic parameters Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) were  $2.92 \pm 0.48$  mM and  $7.89 \pm 0.82$  nmol/min/per mg protein using AF, and  $2.42 \pm 0.28$  mM and  $9.87 \pm 0.64$  nmol/min/mg protein using PABA as substrate.

### Effect of pH and temperature on the activity of *Klebsiella pneumoniae* NAT

The effect of pH on *K. pneumoniae* NAT activity was investigated with both AF and PABA as substrates. As



**Fig. 1.** Lineweaver-Burk double reciprocal plot of *Klebsiella pneumoniae* N-acetyltransferase activity as a function of 2-aminofluorene (AF) and *p*-aminobenzoic acid (PABA) concentration. The cytosols were prepared as described in Materials and Methods. The acetyl coenzyme A concentration was 0.1 mM. Values are mean.



**Fig. 2.** Effect of pH on *Klebsiella pneumoniae* N-acetyltransferase (NAT). The NAT activity of *K. pneumoniae* was determined at different pH values using 2-aminofluorene (AF) and *p*-aminobenzoic acid (PABA) as substrates. Values are mean  $\pm$  SD.

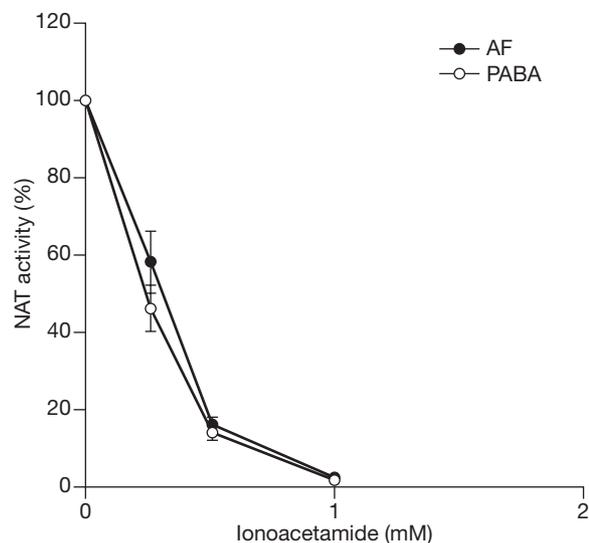
shown in Fig. 2, the enzyme was active in a range from pH 4.0 to 11.0, with maximal activity observed at pH 7.0. The enzyme was inactive above pH 11 or below pH 4.0. The temperature-activity profile of *K. pneumoniae* NAT was determined. The enzyme was active in a range from 4 to 60°C, with maximal activity observed at 37°C.

#### Effect of iodoacetamide on the activity of *Klebsiella pneumoniae* NAT

The effect of iodoacetamide on *K. pneumoniae* NAT activity was investigated with both AF and PABA as substrates. As shown in Fig. 3, for both substrates, the enzyme activity was decreased by over 50% at 0.25 mM iodoacetamide. Over 90% decrease in NAT activity was observed at 1.0 mM iodoacetamide.

#### Effect of salts on enzyme activity of *Klebsiella pneumoniae* NAT

The effect of various of salts on the bacterial enzyme activity was examined. As shown in Table 1, the NAT activity enzyme was inhibited by a number of anions at 0.5 mM concentration, including several chlorides, citrate, several sulphates, and phosphate. However, it was unaffected by Tris-HCl and only partially affected by citrate and acetates. On the other hand, Tris-acetate increased NAT activity. Overall, the divalent cations, such as  $Zn^{++}$ ,  $Ca^{++}$ ,  $Cu^{++}$ ,  $Mn^{++}$ ,  $Fe^{++}$ , and  $Mg^{++}$  produced more inhibition than the monovalent cations at 0.5 mM concentration. Pronounced concentration-dependent



**Fig. 3.** Inhibition of N-acetyltransferase (NAT) from *Klebsiella pneumoniae* by iodoacetamide. Enzyme solutions were incubated with various concentrations of iodoacetamide and activity with 2-aminofluorene (AF) and *p*-aminobenzoic acid (PABA) was determined. Percentage activity compared to a control sample with no iodoacetamide is shown.

inhibition occurred in the presence of other divalent ions ( $Zn^{++} > Ca^{++} > Fe^{++} > Mg^{++} > Cu^{++} > Mn^{++} > NH_4^+ > Co^{++}$ ).  $Zn^{++}$  completely abolished enzyme activity at  $>0.5$  mM, but only partially inactivated enzyme activity at 0.01 mM (data not shown). Further, it was demonstrated that the inhibitory effects observed with  $CuSO_4$  and  $ZnSO_4$  were not due to the  $SO_4^-$  anion, since  $MgSO_4$  did not affect enzyme activity as compared with the activity in the presence of  $MgCl_2$ .

**Table 1.** Effect of monovalent and divalent cations on enzyme activity of N-acetyltransferase from *Klebsiella pneumoniae*

Salt	Enzyme activity (%)
None	100
KCl	82
Tris-HCl	100
Ammonium acetate	96
Tris-acetate	124
Ammonium citrate	72
$Na_2SO_4$	72
$KH_2PO_4$	64
$CoCl_2$	60
$FeSO_4$	8
$CaCl_2$	4
$(NH_4)_2SO_4$	58
$CuSO_4$	22
$MnCl_2$	44
$MgCl_2$	18
$ZnSO_4$	0

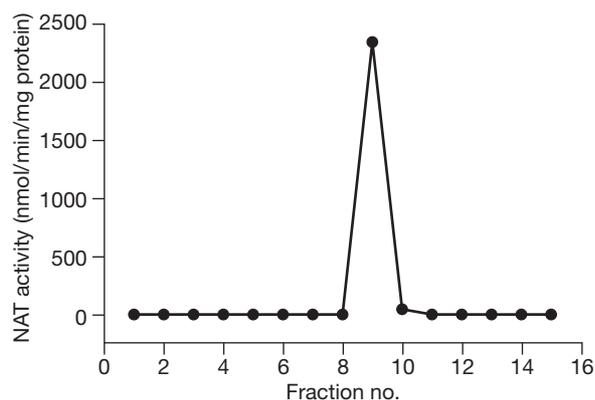
**Table 2.** Purification of N-acetyltransferase from *Klebsiella pneumoniae*

	Total protein (mg)	Specific activity (nmol/min/mg protein)	Relative purity
Cytosol	9080.0	0.50	1.00
Ammonium sulphate precipitation	4002.0	0.84	1.78
DEAE-Sephacel	1208.0	6.42	11.40
Sephadex G-100	218.0	20.90	40.90
Hydroxylapatite	11.4	543.00	1086.00
DEAE-5pw	1.4	3506.00	7028.00

### Purification of NAT from *Klebsiella pneumoniae*

Ammonium sulfate precipitation of the cytosol of *K. pneumoniae* showed that approximately 200% of the activity was recovered. Ion exchange chromatography on DEAE-Sephacel of the NAT recovered from ammonium sulphate precipitation showed that NAT was 3-fold higher than that of ammonium sulfate precipitation. Subsequent gel filtration of the NAT was about 14-fold higher than that of the DEAE-Sephacel procedure. The procedure using the treatment of hydroxylapatite increased the NAT activity 5-fold higher than with the Sephadex G-100 treatment. The final purification step using DEAE-5pw increased NAT activity 6-fold higher than the hydroxylapatite treatment (Table 2).

SDS-polyacrylamide gel electrophoresis showed a clear protein band after the purification. The band on SDS-polyacrylamide gels corresponding to NAT had an apparent molecular weight 44.9 kDa and was identified from SDS-polyacrylamide gel electrophoresis of all chromatographic fractions (Fig. 4).

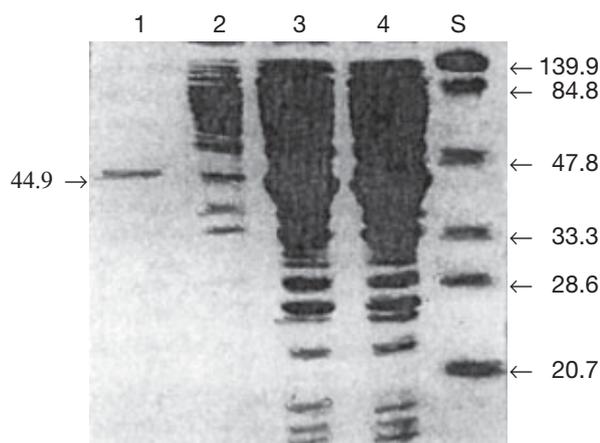
**A**

### NAT mRNA expression in *Klebsiella pneumoniae*

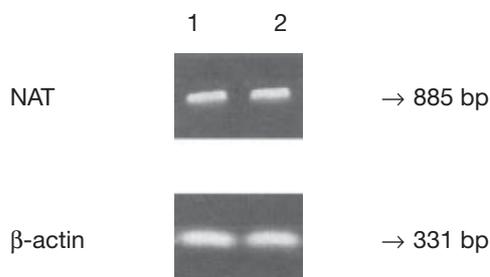
The NAT1 mRNA expression in examined *K. pneumoniae* is presented in Fig. 5. No NAT2 mRNA expression was found in this bacterium.

### Discussion

The existence of acetyl CoA-dependent arylamine NAT has been reported previously in human blood and tissues, frogs, fishes, fungi, nematodes, bacteria, general foods and vegetables and a number of laboratory animals. But comprehensive studies of NAT activity, kinetic constants for arylamine substrates, NAT mRNA expression from PCR product and the characterization of NAT from *K. pneumoniae* have not been previously reported. Therefore, the purpose of this experiment was to determine the character of NAT from *K. pneumoniae*. The present study chose AF and PABA as substrates to determine *K. pneumoniae* N-acetylation and kinetic constant, as have been used for NAT activity

**B**

**Fig. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis fractions from the peak region of N-acetyltransferase (NAT) separated on Mono Q. **(A)** Activity profile for a preparation from *Klebsiella pneumoniae* cytosol. The gel is 12% (w/v) acrylamide and the equivalent of 25 mL of each fraction was further electrophoresed in each track shown in **(B)**. Lane 1, DEAE-5pw pool; lane 2, hydroxylapatite pool; lanes 3 and 4, Sephadex G-100 pool; lane s, molecular weight standards. The assigned NAT band is indicated with an arrow.



**Fig. 5.** The expression of N-acetyltransferase (NAT) mRNA in *Klebsiella pneumoniae*. The extracted RNA was subjected to reverse transcriptase polymerase chain reaction (PCR) analysis using specific primers for NAT and  $\beta$ -actin, and then PCR-amplified cDNA derived from mRNA was subjected to agarose gel electrophoresis.

determination in other species. The N-acetylation (NAT activity) found for AF and PABA in *K. pneumoniae* was similar to that found in *Helicobacter pylori* and *Escherichia coli* [21,22], but lower than that of certain mammals [5,6,32]. The kinetic constants were also determined from *K. pneumoniae* NAT using AF and PABA as substrates (Fig. 1). The apparent values of  $K_m$  and  $V_{max}$  from N-acetylation of AF and PABA by NAT from *K. pneumoniae* cytosols was lower than that reported in mammals [32], but similar to that reported for *H. pylori* and *E. coli* [21,22].

NAT protein was measured in the *K. pneumoniae* cytosols from  $3 \times 10^9$  bacteria. The data clearly demonstrated that *K. pneumoniae* had NAT protein in examined cytosol (Fig. 4A and 4B), the NAT being about 34 kD. Samples from many *K. pneumoniae* isolates were pooled together in this study, and it was not known whether the genetic polymorphism in NAT activity seen in humans and laboratory animals also occurred in *K. pneumoniae*. It is well known that NAT polymorphisms in human and laboratory animals and NAT can be divided into NAT1 and NAT2 that play an important role in the first step of activation of arylamine carcinogens. The results also show that NAT activity for AF was significantly higher from that for PABA. This is reasonable, because PABA is the substrate for NAT1 and AF is a common substrate for NAT1 and NAT2 [5]. It was also demonstrated that NAT1 and NAT2 may occur in this examined bacteria. This needs further investigation. It has been reported that N-acetylation (NAT activity) is associated with disease occurrence after human exposure to arylamine carcinogens and that attenuation of NAT activity is associated with disease processes such as breast and bladder cancer in humans [5,6]. A more interesting point is that promoted

levels of NAT activity are associated with increased sensitivity to the mutagenic effects of many arylamines [33]. Therefore, the N-acetylation of carcinogenic arylamines may be involved in determining tissue-specific susceptibility to cancer.

The effect of pH on the N-acetylation from *K. pneumoniae* cytosols was also examined with both AF and PABA as substrates for NAT activity. The data from Fig. 2 demonstrate that the NAT enzyme was active in a range from pH 4 to 10, with the maximal activity observed at pH 7.0. The enzyme was inactive (no N-acetylation of substrates detected) above pH 10.5 or below 4.0. Our previous studies demonstrated that optimal activity for pH of NAT from *H. pylori* was pH 6.0 [21]. There may be different amino acids in catalytic sites in NAT of these 2 bacteria. However, the pH profiles of *K. pneumoniae* NAT are similar to those of mammalian NAT [34,35]. The temperature-activity profile of the *K. pneumoniae* NAT was active in a range from 4°C to 50°C with maximum activity observed at 37°C. The temperature profiles for *K. pneumoniae* NAT activity were similar to those of mammalian NAT [34,35] and those of other bacteria *H. pylori* [21] and *E. coli* [22].

After 0.25 mM iodoacetamide was added to the reaction mixture, the NAT activity was decreased approximately 50% with either AF or PABA as substrates (Fig. 3). When the iodoacetamide concentration was increased to 1.0 mM, there was an over 90% decrease in NAT activity. The inhibition of NAT activity by iodoacetamide was similar to that reported for other NATs [34,35]. Iodoacetamide was chosen to elicit NAT activity in *K. pneumoniae* since iodoacetamide is an inhibitor of NAT enzyme [34,35]. Data of the effects of various salts on *K. pneumoniae* NAT activity are given in Table 1, the NAT enzyme was inhibited by a number of anions at 0.5 mM including several chlorides, citrate, several sulphates, and phosphate.

This result is in agreement with our previous studies on certain nematodes and *H. pylori* and *E. coli* [21,22]. Apparently the mechanism of generation of NAT polymorphism differs in different species. Thus, it has been reported that NAT from the rabbit has been cloned and sequenced and in slow acetylators rabbits it appears that the gene is deleted [34]. However, this is not the case in mice [35]. However, pigeon [34], rabbit [35] and chicken liver [36] all appear to express a single NAT isozyme. The actual degree of similarity of the *K. pneumoniae* to other NAT enzymes is best determined through nucleotide (and thereby amino acid) sequence and PCR analysis.

In the present study, we used PCR for this examined bacteria and showed the product of NAT1 mRNA but we did not examine NAT2 mRNA. Therefore, other methods such as molecular biological techniques are needed to determine whether NAT probes for avian, mouse, rat, or mammalian NATs will hybridize with *K. pneumoniae* NAT sequences. If the *K. pneumoniae* NAT is not similar to vertebrate NAT, it may resemble insect NATs that are of lower molecular weight, and do not contain the conservative amino acid sequence of mammalian NAT, and thus differ in substrate specificity [37]. Further characterization of the *K. pneumoniae* arylamine NAT protein(s) as to active, catalytic, and inhibitory sites, and additional substrate specificities would also help to determine the extent of similarities between *K. pneumoniae* and mammalian enzymes.

It is not known from the present work whether *K. pneumoniae* would be subject to arylamine toxicities or carcinogenesis. It also cannot address possible association of inhibition of the NAT activity of *K. pneumoniae* and cancer occurrence and the possibility for clinical application. Nevertheless, we have shown that *K. pneumoniae* contains unique NAT. Currently, we are working to determine in which class of NAT the *K. pneumoniae* activity should be placed, as the knowledge would be of interest and importance in the evolutionary development of NAT, and may provide insights into the purpose and natural substrate of NAT.

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