Evidence towards the role of arylamine 
N-acetyltransferase in Mycobacterium smegmatis and development of a specific antiserum against the homologous enzyme of Mycobacterium tuberculosis

Mark Payton, Claire Gifford, Pamela Schartau, Chris Hagemeier, Adeel Mushtaq, Stuart Lucas, Katalin Pinter and Edith Sim

Arylamine N-acetyltransferase (NAT) in humans inactivates the anti-tubercular drug isoniazid (INH). Homologues of human NAT are present in Mycobacterium tuberculosis and Mycobacterium smegmatis, where they can acetylate, and hence inactivate, INH. The in vivo role of mycobacterial NAT is not known but heterologous expression of the M. tuberculosis gene increases the INH resistance. The 0.85 kb nat gene is part of a gene cluster in M. smegmatis. The gene is transcribed as a large, 7.5 kb mRNA as demonstrated by Northern analysis. A nat knockout strain of M. smegmatis was generated by targeted disruption. The new strain was confirmed to be devoid of NAT activity. The growth of the knockout strain is considerably delayed compared with the wild-type, due to an extended lag phase. The knockout mutant has an increased sensitivity to INH as would be predicted. The NATs from M. smegmatis and M. tuberculosis have a high degree of homology, except in the region of the C terminus. A specific polyclonal antiserum raised against recombinant NAT protein from M. tuberculosis is described that recognizes a stretch of about twenty residues within the C terminus of M. tuberculosis NAT. This highly specific antiserum will enable comparison of nat expression between isolates of M. tuberculosis.

Keywords: mycobacteria, knockout, immunogenic, antibacterial, tuberculosis

INTRODUCTION

Arylamine N-acetyltransferases (NATs, EC 2.3.1.5) are cytosolic enzymes which acetylate arylamines and hydrazines using acetyl-CoA as the acetyl donor (Weber & Hein, 1985). NATs are found in a wide range of eukaryotes and prokaryotes. Many hydrazine- and arylamine-based drugs in common use, including antimicrobial agents, are substrates for NAT. In eukaryotes, isoenzymes of NAT have been described which have distinct but overlapping substrate specificities (Sim et al., 2000). These substrate-specificity profiles are highly similar for homologues from other eukaryotic species. One of the two human isoenzymes, NAT2, was first identified as the enzyme inactivating the front-line anti-tuberculosis drug isoniazid (INH) (Evans et al., 1960).

The distribution of NATs in prokaryotes has recently been described based on activity studies (Delomenie et al., 2001) and genome analysis (Payton et al., 2001). In contrast to eukaryotes, where two or three isoenzymes are often found, only a single nat gene is present in prokaryotes. The substrate specificity of the prokaryotic NATs appears to differ from that of any eukaryotic isoenzyme. A human NAT1 specific substrate, 5-amino salicylate (Delomenie et al., 2001), and a human NAT2 specific substrate, INH, are both acetylated by the same enzyme (Watanabe et al., 1992; Sinclair et al., 1998; Payton et al., 1999).

Mycobacteria are known to infect many animal species (Balasubramanian et al., 1994; Thorel et al., 1997;...
O’Reilly & Daborn, 1995). Moreover, much interest is focused on this genus as it includes major human pathogens such as Mycobacterium tuberculosis and Mycobacterium leprae, and also Mycobacterium avium, the major opportunistic pathogenic agent affecting HIV-infected people in the developed world (Schutt-Gerowitt, 1999). Tuberculosis, caused by M. tuberculosis, has been declared a global emergency and is the most frequent infectious cause of mortality in the world. Incidence of this disease is now reaching epidemic proportions in developing countries, and in some regions of the USA and the UK (World Health Organization, 1999). The emergence of many strains of M. tuberculosis resistant to the currently available chemotherapeutics, particularly INH, is an additional cause of alarm. INH has been used since 1952, but despite extensive research, the endogenous function of the mycobacterial NATs remains to be determined. One of the best-characterized NATs is disrupted. To confirm the orientation of the kanamycin resistance transposon Tn903 (Oka et al., 1981) was generated by using an E. coli–M. smegmatis kan^−/hyg^− shuttle vector (pAGN 40, a gift from Dr T. Parish).

M. smegmatis was grown in Middlebrook 7H9 medium enriched with 10% (v/v) albumin-dextrose-catalase (ADC; Difco) and 0.05% (v/v) Tween 80 (Sigma) until they reached the exponential phase (25–35 h) at 37 °C. E. coli was grown in standard LB medium (Sambrook et al., 1989) supplemented with the required antibiotics.

Where appropriate, M. smegmatis was cultured in medium supplemented with 20 µg kanamycin ml⁻¹ and 50 µg hygromycin ml⁻¹. For E. coli, 30 µg kanamycin ml⁻¹ and 100 µg hygromycin ml⁻¹ were used. Growth was determined by measuring turbidity at 600 nm.

**Transformation of E. coli and M. smegmatis.** E. coli cells were transformed by electroporation according to the manufacturer’s recommendations (Easyjet, Equibio). Transformation of M. smegmatis with the shuttle vector pAGN 40 was carried out as previously described (Parish & Stoker, 1998b). The knockout construct pGEMTnatKO is capable of replacing in E. coli but not in M. smegmatis, making the construct a candidate for integration via a suicide vector approach (Husson, 1998). Plasmid DNA was prepared from transformed E. coli and UV treated at 100 mJ cm⁻² to enhance homologous recombination (Hinds et al., 1999). Competent cells of M. smegmatis were electroporated with 1 µg plasmid DNA. Transformants were selected on 7H11 agar (Difco) supplemented with 10% (v/v) oleic acid-ADC (OADC; Difco) and 20 µg kanamycin ml⁻¹.

**Purification of chromosomal DNA.** Chromosomal DNA from mycobacteria was extracted as described by Wilson et al. (1993). DNA concentration was estimated following agarose gel electrophoresis and also from the A₂₆₀ (Sambrook et al., 1989).

**Construction of the suicide vector.** A chromosomal clone containing the nat gene with flanking sequences at both ends has been isolated from M. smegmatis (accession no. AJ006588; Payton et al., 1999). The 2.5 kb insert was excised from this plasmid (pBS, Stratagene) with Clal and Xbal. It has a single EcoRV site within the nat coding sequence and two more EcoRV sites at each end outside the coding sequence.

A 1-2 kb fragment containing the kanamycin resistance gene, Tn903, was amplified by PCR from pUC4K (Pharmacia). EcoRV sites were also introduced during the reaction. We used Pfu DNA polymerase and the primers 5′-KanR/EcoRV 5′-AGGATATCCGTGCACCTGCAGG-3′ and 3′-KanR/EcoRV 5′-AGGATATCCGATCGCGCCGCGCGG-3′. Cycles were 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2.5 min, for 25 cycles.

The 12 kb fragment was digested with EcoRV then purified from the agarose gel, and ligated between the 1-6 and 0.6 kb EcoRV fragments from the 2.5 kb M. smegmatis chromosomal Xbal/Clal fragment which make up the left and right homology arms. The final product (~3.5 kb) was then purified, adenosine-tagged and ligated into pGEM-T (Promega) to create the suicide construct, pGEMTnatKO in which the ORF of nat is disrupted. To confirm the orientation of the ligated segments of the nat gene and the introduced kanamycin resistance gene, PCR using the transformants as templates, followed by the appropriate restriction digests was carried out.
Fig. 1. Localization of nat within the M. smegmatis genome and schematic representation of the targeted gene disruption. A 16 kb EcoRI M. smegmatis chromosomal fragment contains a 2.5 kb Sau3A fragment. Arrows indicate the three ORFs in this fragment. EcoRV fragments (0.6 kb and 1.6 kb) were isolated and ligated with Tn903 (1.2 kb) into pGEM-T to create the nat gene knockout suicide construct, pGEMNatKO, as shown. The details of construction are in Methods.

Fig. 2. Screening for the knockout mutant by PCR. (a) The nat ORF was amplified from single transformed colonies. The lack of an 840 bp fragment indicates that the gene is interrupted, as in lane KO9. The negative control is without template (C1); positive controls were 100 ng plasmid with M. smegmatis nat (the fainter bands correspond to template DNA) (C2) and pGEMNatKO (C3). Chromosomal DNA (1 µg) from M. smegmatis (mc155) was also used. Lane M shows the molecular mass markers. The PCR product is 2 kb with the insertion of the kanamycin resistance gene. (b) The same templates were used as in (a) to confirm the correct targeting with primers 5’KanrF1600 and SMEG/KO-MINUS. Reaction conditions and primer sequences are in Methods.

Screening for chromosomal integration by PCR. Cells from single colonies were disrupted by heating (5 min at 95 °C) and used as template. As controls, 100 ng plasmid DNA or 1 µg M. smegmatis genomic DNA were used. The nat ORF was amplified using Taq DNA polymerase (Promega) with the primers SMNAT/3’PET28 (5’-CGAGTGCCATATGCGACGACGACGACGAGCACCTCGGC-3’) and SMNAT/3’PET28 (5’-GGAAATTCTCTAGGTCGAGCACCTCGGC-3’). DMSO at 6% (v/v) was added to all reactions. Primers were annealed at 38 °C for 30 s and the elongation was done either for 1 min to amplify the uninterrupted gene (~840 bp) or for 2 min to amplify the nat ORF, now containing the Tn903 kanamycin resistance gene (2-1 kb). The products were denatured at 94 °C for 30 s and the cycle was repeated 30 times.

The correct 3’ integration site was also confirmed by PCR amplification. One primer annealed to the 3’ end of the Tn903 insert (5’KanrF1600, 5’-ATCTTTCTGCAATGTAACCATCAG-3’) and the other annealed to the chromosomal DNA in the 3’ flanking segment of the knockout construct (SMEG/KO-MINUS, 5’-C ACTCTGCAATGTAACCATCAG-3’). Primers were annealed at 56 °C for 30 s and the elongation was done for 2.5 min. The product was then denatured at 94 °C for 30 s and the cycle was repeated 30 times. Following correct integration, a 2 kb fragment was amplified.

RNA preparation and Northern analysis. RNA was prepared from exponential phase cultures of M. smegmatis using the detergent Catrimox 14 as previously described (Payton & Pinter, 1999). Approximately 10 µg total RNA was separated in a 1% agarose gel containing formaldehyde (Sambrook et al., 1989). RNA was transferred onto a nylon membrane by capillary action and cross-linked to the membrane by UV. Hybridization was carried out with digoxigenin (DIG)-labelled DNA probes for detection by chemiluminescence (Roche). Labelled probes were generated by PCR, incorporating the DIG-labelled dUTP into the reaction mix, and then into the final product. The probe is the full coding sequence of M. smegmatis nat. To probe for the kanamycin resistance gene Tn903, the DNA segment was amplified as for the construction of the suicide vector, by using the 5’KanR/EcoRV and 3’ KanR/EcoRV primers.

Generation of recombinant NAT proteins. Recombinant M. smegmatis NAT and M. tuberculosis NAT and its fragments were produced by heterologous expression in E. coli using the pET28b-DE3 expression system. Overexpressed NATs were either purified using the hexa-histidine tag to aid purification (if soluble) or were concentrated in inclusion bodies (if insoluble) as described earlier (Payton et al., 2001).

Analysis of NAT activities. Cell pellets of 10 ml exponential phase M. smegmatis cultures were resuspended in 1 ml 50 mM...
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Tris/HCl, 2 mM EDTA, pH 8.0 containing 1 mM Pefabloc (protease inhibitor; Pentapharm) and disrupted by sonication at 4°C (3 times for 5 min at 7 µm amplitude with 2 min intervals between them). Cell debris was removed by centrifugation (12000 g, 20 min, 4°C) and NAT activity was determined in the supernatant by the detection of N-acetylation of INH as previously described (Payton et al., 1999; Olson et al., 1977).

SDS-PAGE, Western blot analysis and protein preparation for immunization. SDS-PAGE and Western blotting were as described previously (Payton et al., 2001). In each case where purified recombinant proteins were used, equal amounts were loaded onto the gels.

To raise antibodies against *M. smegmatis* and *M. tuberculosis* NATs, the recombinant proteins were generated in *E. coli*. Protein concentration was greater than 5 mg (l culture)⁻¹ including both the soluble and insoluble forms. The proteins were concentrated in inclusion bodies (insoluble fraction) and were subjected to SDS-PAGE. The bands of the proteins were excised from gels after staining with Coomassie brilliant blue and were used for immunization of rabbits as previously described (Stanley et al., 1996), except that only Incomplete Freund’s Adjuvant was used.

RESULTS

**Generation of a nat knockout strain of *M. smegmatis***

Disruption of endogenous *nat* in *M. smegmatis* with pGEMTnatKO (Fig. 1) was confirmed by PCR (Fig. 2). The frequency of correct integration was low, with only one of the 70 kanamycin-resistant colonies analysed (KO9) having the targeted integration as shown by a lack of the 840 bp band representative of the *nat* ORF (Fig. 2a). A 2 kb band is expected when the targeting is correct and was confirmed by using a second primer set.

![Fig. 3. Northern analysis of mRNA from wild-type and nat knockout *M. smegmatis*. Hybridization was with (a) *M. smegmatis* nat DNA and (b) Tn903 DNA. RNA was extracted from the wild-type *M. smegmatis* (lane 1) and from clone KO9 (lane 2).](image)

![Fig. 4. Comparison of growth rates of wild-type and knockout *M. smegmatis*. Growth was monitored by reading the turbidity at 600 nm of wild-type *M. smegmatis* (black circles) and nat knockout KO9 (black squares) and pAGAN-transformed *M. smegmatis* (white circles).](image)

![Fig. 5. The effect of INH on growth of different *M. smegmatis* strains. Mycobacteria from mid-exponential phase cultures were dispensed and grown in media containing INH from 0 to 10 µg ml⁻¹ in 96-well plates until cultures without INH reached the late-exponential phase. The growth rate was followed by measuring the turbidity at 600 nm. The relative growth rate is expressed as a fraction of the optical density of the untreated wild-type cells. Black circles indicate wild-type cells; white circles indicate pAGAN 40 transformed cells; black squares indicate kanamycin-resistant nat knock out KO9. Results are shown as the mean ± SD of four measurements.](image)
annealing to the 3′ end of the kanamycin-resistance cassette and to the nat 3′ flanking region (Fig. 2b). The chromosomal integration in KO9 was stable and no 840 bp fragment was detected after extended growth either on solid medium or in liquid cultures.

Northern analysis demonstrated that nat is transcribed as part of a large message (~7.5 kb) in wild-type M. smegmatis (Fig. 3a). A somewhat larger transcript (~8.5 kb) in KO9 indicates the presence of the kanamycin cassette within the nat sequence (Fig. 3b). When this Northern blot was hybridized with the labelled DNA from the kanamycin cassette, no signal was seen in the wild-type M. smegmatis and a message of approximately 8.5 kb was observed for mutant KO9.

Having confirmed that the nat gene is transcribed in wild-type M. smegmatis, we also demonstrated that the message is translated, and NAT activity is detectable in cell lysates. Enzymic activity of soluble fractions of lysed wild-type M. smegmatis and the nat knockout mutant KO9 was measured using INH as substrate. Lysates of the wild-type M. smegmatis acetylated 10.8 pmol INH min⁻¹ (mg protein)⁻¹ and no activity was detected from the cell lysate of the knockout (Payton et al., 2001). These results confirmed the presence of active endogenous NAT in wild-type M. smegmatis and the loss of nat expression in KO9.

The knockout KO9 appeared to grow more slowly on agar than the parent strain. In liquid cultures, there was an extension of approximately 15 h in the lag phase, in comparison to KO9 to the wild-type M. smegmatis. However, after the lag phase, exponential phase growth rates were very similar. The growth rate was also determined for M. smegmatis transformed with pAGAN 40 alone and this matched the wild-type M. smegmatis with the same length of lag phase (Fig. 4).

The mutant strain KO9 is more sensitive to INH than the parent strain of M. smegmatis (Fig. 5). The growth of the knockout was reduced at a slightly lower INH concentration than the wild-type (IC₅₀ ≈ 4.5 μg ml⁻¹). The nat knockout already displayed a notable decrease in growth at 1 μg INH ml⁻¹. No effect on growth was

**Fig. 6.** NAT recognition by different antibodies. SDS-PAGE analysis was carried out on cell lysates and either stained for protein (a) or used for Western blot analysis (b–d). Western blots were developed with anti-S. typhimurium-NAT at 1:100000 dilution (b), anti-M. smegmatis-NAT at 1:500 dilution (c) or anti-M. tuberculosis-NAT at 1:100000 dilution (d). All antibodies are polyclonal and were raised in rabbits against M. tuberculosis-NAT at 1:100000 dilution (d). Lanes 1–6 correspond to the description given by the scheme in (d). Lane M contains the molecular mass markers.

**Fig. 7.** Identification of the antigenic region recognized by the anti-M. tuberculosis NAT antiserum. (a) SDS-PAGE of the inclusion bodies containing the full length and truncated M. tuberculosis NAT stained for protein. Western blots with anti-M. smegmatis antibody (b) or with anti-M. tuberculosis antibody (c) are shown. Lanes 1–6 correspond to the description given by the scheme in (d). Lane M contains the molecular mass markers. Equal amounts of protein were applied to the gel.
observed for the wild-type and for pAGAN 40 transformed M. smegmatis using INH up to 4 μg ml⁻¹. IC₅₀ is about 5 μg ml⁻¹ for both the wild-type and pAGAN-transformed M. smegmatis.

Antigenic characterization of M. tuberculosis NAT

Polyclonal rabbit antisera generated against purified recombinant NATs from S. typhimurium, M. smegmatis and M. tuberculosis were characterized for their ability to recognize NATs from different sources (Fig. 6). The antibody against S. typhimurium NAT and M. smegmatis NAT cross-reacted to a similar extent with recombinant NATs from Salmonella typhimurium, M. smegmatis and M. tuberculosis (Fig. 6b, c). The antiserum raised against recombinant NAT from S. typhimurium had a much higher titre and could be diluted 1:100 000, whilst the antiserum raised against M. smegmatis NAT could be only used at a dilution of 1:5 000. The antiserum raised against recombinant NAT from M. tuberculosis, in contrast, only recognized M. tuberculosis NAT (Fig. 6d), despite the overall high degree of identity between M. smegmatis and both M. tuberculosis and S. typhimurium NATs at the amino acid level (58% and 40% respectively). This lack of cross-reaction was also observed with other antibodies raised against M. tuberculosis NAT in different rabbits. In addition, the antibody raised against M. tuberculosis NAT did not recognize the recombinant NATs from M. smegmatis or from S. typhimurium even when the proteins had been purified to homogeneity (data not shown).

NATs have three distinct structural domains (Sinclair et al., 2000), and the sequence similarity among NATs from different sources is higher within the first two domains than in the third, C-terminal, domain (Payton et al., 2001). To understand the specificity of the antiserum raised against M. tuberculosis NAT, a series of mutants in the C terminus of M. tuberculosis NAT were generated. These mutants were truncated at their C termini, and one had only the C-terminal half of the molecule as shown in Fig. 7. These deletion mutants were expressed in E. coli. The cell lysates were then subjected to SDS-PAGE and Western blot analysis (Fig. 7a, b, c). All expressed proteins were found exclusively in inclusion bodies.

The antiserum against M. smegmatis NAT recognizes NAT from M. tuberculosis (Fig. 7b) but does not recognize the C-terminal domain. The antiserum raised against recombinant M. tuberculosis NAT recognizes the full-length protein and the C-terminal domain of M. tuberculosis NAT (Fig. 7c, lanes 1 and 6). A lack of the 18 C-terminal amino acids of M. tuberculosis NAT causes a slight reduction in antigen recognition (Fig. 7c, lane 3), and recognition is almost completely abolished with the extension of the truncation to 39 residues (lanes 4 and 5). Therefore the region between residues 39 and 18 from the C terminus of the protein (residues 244–265 as shown underlined in Fig. 8) appear to be highly antigenic and specific to M. tuberculosis NAT. This segment is not highly conserved in M. smegmatis or S. typhimurium NAT (Payton et al., 2001).

The antiserum against M. smegmatis NAT that cross-reacts with the M. tuberculosis NAT recognizes the highly conserved N-terminal domain and not the C-terminal domain of M. tuberculosis NAT which is less similar to M. smegmatis NAT.

DISCUSSION

The role of NAT in mycobacteria still remains to be established. However, we have shown that the nat gene in M. smegmatis is transcribed as part of a gene cluster. Characterization of the transcript with which nat is associated in M. smegmatis may provide an insight into the nature of the genes within the cluster, and may provide further evidence on the possible role that NAT plays. Therefore it also provides a basis for further experimentation, for example knocking out the other genes in this cluster and identifying the effects of this in relation to the effects observed when nat was disrupted.

Targeted disruption of nat resulted in a delayed entry into exponential phase growth, extending the length of the lag phase (Fig. 4). In view of this observation, it may be that the presence of NAT is required during early growth, resulting in the extended lag phase for the knockout. The other possibility is that the other genes in the cluster are also affected by the removal of nat, caused either by a change of message stability or by a lack of transcription depending on the location of the nat gene within the cluster. It would be very important to identify the structure of the cluster in which nat is found for comparison with the putative clusters of M. bovis (Bacille Calmette–Guérin – BCG) and M. tuberculosis, where nat appears to be the last gene (Payton et al., 2001). It is also important to determine the nature of the other genes within the cluster. Nevertheless, the results presented here do suggest that NAT might have an endogenous function associated with growth.

Previous studies have shown that the heterologous expression of M. tuberculosis NAT in M. smegmatis
results in a three-fold increase in resistance to INH (Payton et al., 1999). It was therefore not surprising to observe an increased sensitivity to INH in the \textit{M. smegmatis} nat knockout mutant (Fig. 5), albeit a small difference. INH must be activated by oxidation to be effective against \textit{M. tuberculosis} (Zhang & Young, 1993). It is very unlikely that N-acetyl-INH could be activated, as is the pro-drug INH, through the catalase peroxidase (KatG) pathway (Zhang & Young, 1993; Zhang et al., 1996). The combination of the level of NAT activity, coupled with different rates of KatG activity, for example, may contribute to the effective concentration of active, oxidized INH inside mycobacterial cells. Therefore cells with reduced NAT activity due to lower expression or mutation in the gene may have altered INH sensitivity. To determine whether the nat gene has a similar endogenous role in the pathogenic \textit{M. tuberculosis}, these studies must now be carried out with \textit{M. tuberculosis}.

The lack of a specific antibody recognizing only the \textit{M. tuberculosis} NAT has been a major limitation in this research. The antiserum that we describe here against \textit{M. tuberculosis} NAT is highly specific. We have mapped the predominant epitope to the C-terminal part of the molecule. Since the sequences of \textit{M. tuberculosis} and \textit{M. bovis} BCG NATs are identical, the antibody will also be useful for comparing nat expression in \textit{M. bovis} BCG, another slow growing strain of mycobacteria. In preliminary experiments it has been demonstrated, using this antibody for Western blot analysis, that NAT is found in growing cultures of \textit{M. bovis} BCG and \textit{M. tuberculosis} H37Rv (Upton et al., 2001). This antiserum will also be extremely useful in determining the levels of NAT in clinical isolates of \textit{M. tuberculosis} that differ in their sensitivity to INH.

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\section*{References}


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