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

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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 anti-microbial 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;

Abbreviations: NAT, arylamine *N*-acetyltransferase; INH, isoniazid.

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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, 1995). 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 molecular basis underlying this resistance has not been fully deduced.

We have recently identified genes homologous to *nat* in *M. smegmatis* and *M. tuberculosis*, and have shown that heterologous expression of *M. tuberculosis nat* in *M. smegmatis* results in a concurrent increase in resistance to INH (Payton *et al.*, 1999). It is possible that increased expression of the gene or certain mutations resulting in the increase of NAT activity in some strains of *M. tuberculosis* may contribute to their resistance to INH.

The endogenous function of both eukaryotic and prokaryotic NATs remains to be determined. One approach to investigating their role is to knock out the gene of an organism and to identify the subsequent effects on its phenotype. As a model for *M. tuberculosis*, the non-pathogenic *Mycobacterium smegmatis* has been much studied (Parish & Stoker, 1998a). In this report, we extend these studies by knocking out the *nat* gene of *M. smegmatis* to identify whether this strategy affects the sensitivity of *M. smegmatis* to INH. The knockout was also created with a view to the elucidation of the endogenous function of the mycobacterial NATs.

The *nat* gene in *M. tuberculosis* and in *Mycobacterium bovis* has been predicted to be part of a gene cluster (Payton *et al.*, 2001). Therefore we have also investigated whether the *nat* gene is transcribed as part of a larger message in *M. smegmatis*.

To extend these studies to *M. tuberculosis*, it is necessary to have a specific tool to identify NAT production in these bacteria. We have therefore raised an antiserum against recombinant NAT protein from *M. tuberculosis*, and have identified a major immunogenic peptide in the protein. The antiserum uniquely recognizes *M. tuberculosis* NAT.

METHODS

Bacterial strains and growth conditions. Bacterial stains used were *M. smegmatis* strain mc^{2}155 (Snapper *et al.*, 1990), and *Escherichia coli* strains JM109 and BL21(DE3)pLysS (Promega). The latter was used for heterologous expression of proteins. The expression vector used was pET28b(+) (Novagen) unless stated otherwise.

Genetically modified *M. smegmatis* containing a chromosomal copy of the kanamycin-resistance transposon Tn*903* (Oka *et al.*, 1981) was generated by using an *E. coli*–*M. smegmatis* kan^r/hyg^r shuttle vector (pAGAN 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 (Easyject, Equibio). Transformation of *M. smegmatis* with the shuttle vector pAGAN 40 was carried out as previously described (Parish & Stoker, 1998b). The knockout construct pGEMTnatKO is capable of replicating 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 A_{260} (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 *Cla*I and *Xba*I. It has a single *Eco*RV site within the *nat* coding sequence and two more *Eco*RV sites at each end outside the coding sequence.

A 1·2 kb fragment containing the kanamycin resistance gene, Tn*903*, was amplified by PCR from pUC4K (Pharmacia). *Eco*RV sites were also introduced during the reaction. We used *Pfu* DNA polymerase and the primers 5′KanR/EcoRV 5′-AGGATATCCGTCGACCTGCAGG-3′ and 3′KanR/EcoRV 5′-AGGATATCCCCGGATCCGTCGAC-3′. Cycles were 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2·5 min, for 25 cycles.

The 1·2 kb fragment was digested with *Eco*RV then purified from the agarose gel, and ligated between the 1·6 and 0·6 kb *Eco*RV fragments from the 2·5 kb *M. smegmatis* chromosomal *Xba*I/*Cla*I 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 digestions was carried out.

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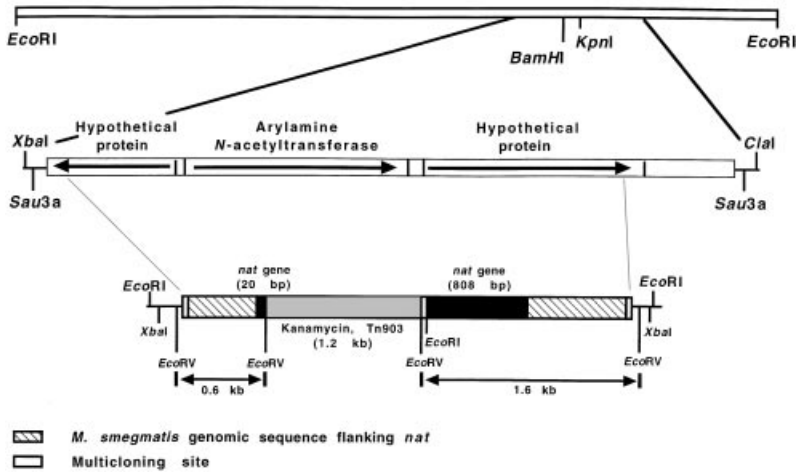

Fig. 1. Localization of *nat* within the *M. smegmatis* genome and schematic representation of the targeted gene disruption. A 16 kb *Eco*RI *M. smegmatis* chromosomal fragment contains a 2·5 kb *Sau*3A fragment. Arrows indicate the three ORFs in this fragment. *Eco*RV fragments (0·6 kb and 1·6 kb) were isolated and ligated with Tn*903* (1·2 kb) into pGEM-T to create the *nat* gene knockout suicide construct, pGEMTnatKO, as shown. The details of construction are in Methods.

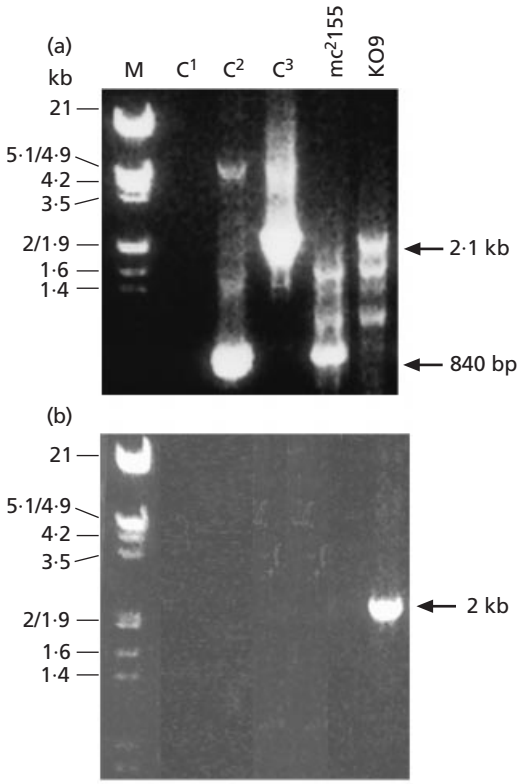

Fig. 2. Screening for the knockout mutant by PCR. (a) The *nat* ORF was amplified from single transformed colonies. The lack of an 840 bp fragment indicates that the gene is interrupted, as in lane KO9. The negative control is without template (C^1); positive controls were 100 ng plasmid with *M. smegmatis nat* (the fainter bands correspond to template DNA) (C^2) and pGEMTnatKO (C^3). Chromosomal DNA (1 μg) from *M. smegmatis* (mc^2155) was also used. Lane M shows the molecular mass markers. The PCR product is 2·1 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/5′PET28 (5′-CGAGTGCCATATGGCGA-TGGACCTCGGC-3′) and SMNAT/3′1PET28 (5′-GGAAT-TCTCAGGTGTCGAGCACCTC-3′). DMSO at 6% (v/v) was added to all reactions. Primers were annealed at 58 °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 Tn*903* 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 Tn*903* insert (5′KanrF1600, 5′-ATCTTGTGCAATGTAACATCA-G-3′) and the other annealed to the chromosomal DNA in the 3′ flanking segment of the knockout construct (SMEG/KO-MINUS, 5′-CACTCGTAGGTGCTCGCAC-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 Tn*903*, the DNA segment was amplified as for the construction of the suicide vector, by using the 5′KanR/*Eco*RV and 3′KanR/*Eco*RV 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-BL21(DE3)plysS 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

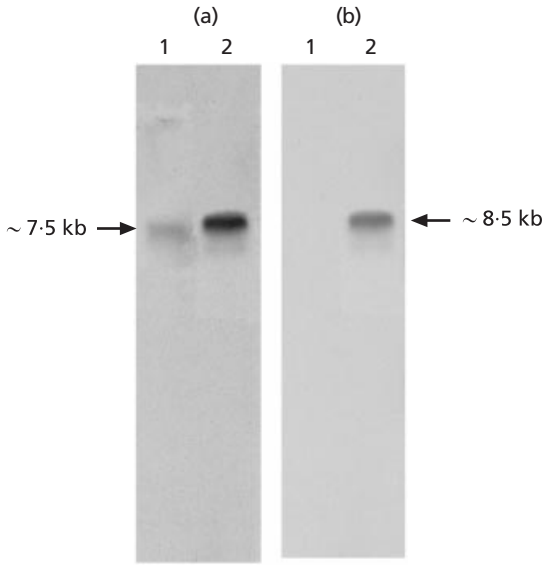

Fig. 3. Northern analysis of mRNA from wild-type and *nat* knockout *M. smegmatis*. Hybridization was with (a) *M. smegmatis nat* DNA and (b) Tn*903* DNA. RNA was extracted from the wild-type *M. smegmatis* (lane 1) and from clone KO9 (lane 2).

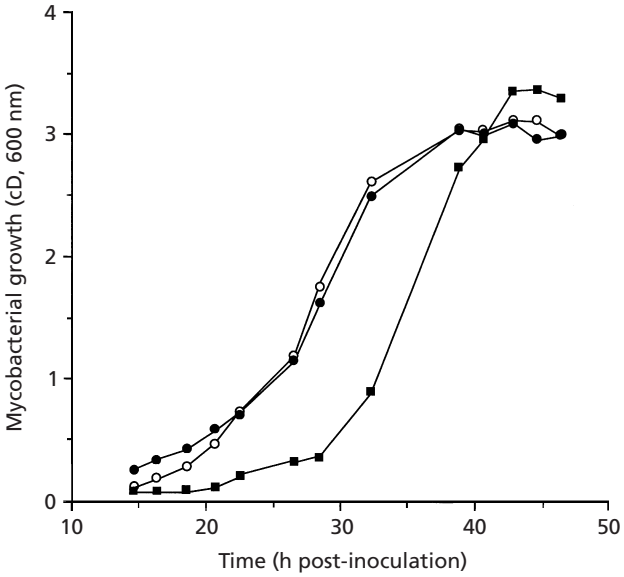

Fig. 4. Comparison of growth rates of wild-type and knockout *M. smegmatis*. Growth was monitored by reading the turbidity at 600 nm of wild-type *M. smegmatis* (black circles) and *nat* knock out KO9 (black squares) and pAGAN-transformed *M. smegmatis* (white circles).

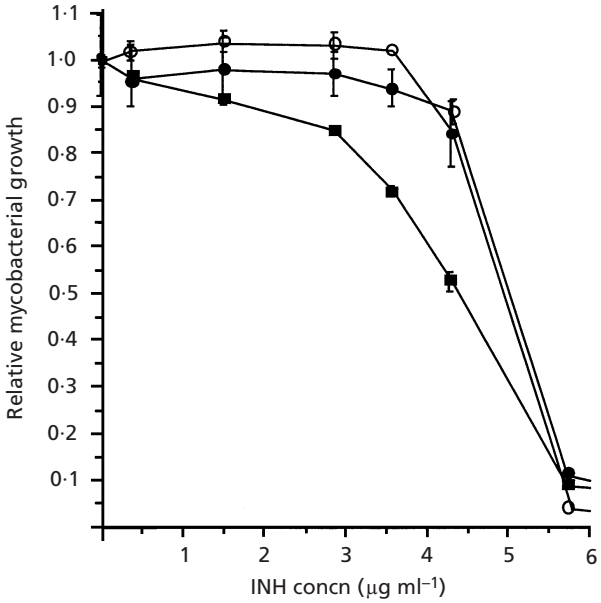

Fig. 5. The effect of INH on growth of different *M. smegmatis* strains. Mycobacteria from mid-exponential phase cultures were dispensed and grown in media containing INH from 0 to 10 μg ml^{-1} in 96-well plates until cultures without INH reached the late-exponential phase. The growth rate was followed 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.

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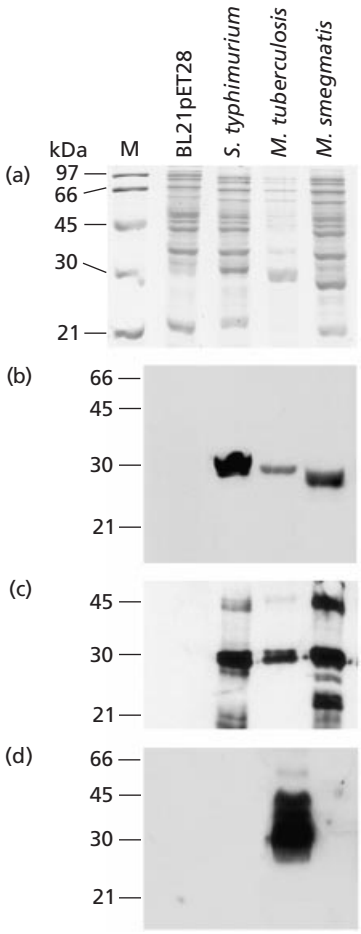

Fig. 6. NAT recognition by different antibodies. SDS-PAGE analysis was carried out on cell lysates and either stained for protein (a) or used for Western blot analysis (b–d). Western blots were developed with anti-*S. typhimurium*-NAT at 1:100 000 dilution (b), anti-*M. smegmatis*-NAT at 1:500 dilution (c) or anti-*M. tuberculosis*-NAT at 1:100 000 dilution (d). All antibodies are polyclonal and were raised in rabbits against recombinant proteins. M indicates the molecular mass markers. BL21pET28b indicates cells transformed with the expression vector alone.

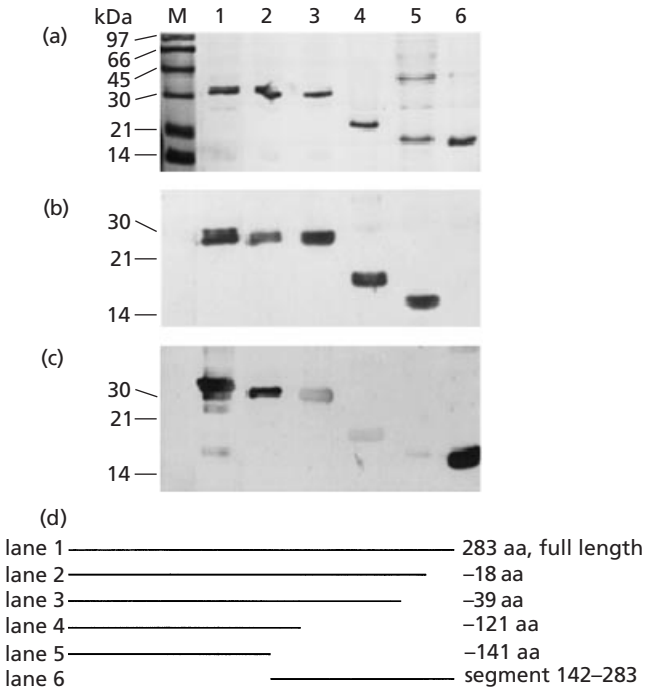

Fig. 7. Identification of the antigenic region recognized by the anti-*M. tuberculosis* NAT antiserum. (a) SDS-PAGE of the inclusion bodies containing the full length and truncated *M. tuberculosis* NAT stained for protein. Western blots with anti-*M. smegmatis* antibody (b) or with anti-*M. tuberculosis* 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.

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 ($IC_{50} \approx 4{\cdot}5$ μg ml^{-1}). The *nat* knockout already displayed a notable decrease in growth at 1 μg INH ml^{-1}. No effect on growth was

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M.tuberculosis    197: STHPASKFVTGLTAAVITDDARWNLSGRDLAVHRAG..GTEKIRLADAAA :244
M.smegmatis       197: STHPGSHFVTGLTVAVVTDDARYNLRGRNLAVHRSG..ATEHIRFDSAAQ :244
S.typhimurium     191: AHVPQSHFRHHLLMCRHLPDGGKLTLTNFHFTRYHQGHAVEQVNVPDVPS :242

M.tuberculosis    245: VVDTLSERFGINVADIGERG..ALETRIDELLARQPGADAP :283
M.smegmatis       245: VLDAIVNRFGIDLGDLAGR...DVQARVAEVLDT....... :275
S.typhimurium     243: LYQLLQQQFGLGVNDVKHGFTEAELAAVMAAFDTHPEAGK  :282
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Fig. 8. Alignment of the amino acid sequences of the C-terminal region of three prokaryotic NATs. The region identified as highly antigenic by the anti-*M. tuberculosis* NAT antiserum is underlined.

observed for the wild-type and for pAGAN 40 transformed *M. smegmatis* using INH up to 4 μg ml^{-1}. IC_{50} 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 1:100000, whilst the antiserum raised against *M. smegmatis* NAT could be only used at a dilution of 1:5000. 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 four other antisera 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*

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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 *M. smegmatis nat* knockout mutant (Fig. 5), albeit a small difference. INH must be activated by oxidation to be effective against *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 *M. tuberculosis*, these studies must now be carried out with *M. tuberculosis*.

The lack of a specific antibody recognizing only the *M. tuberculosis* NAT has been a major limitation in this research. The antiserum that we describe here against *M. tuberculosis* NAT is highly specific. We have mapped the predominant epitope to the C-terminal part of the molecule. Since the sequences of *M. tuberculosis* and *M. bovis* BCG NATs are identical, the antibody will also be useful for comparing *nat* expression in *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 *M. bovis* BCG and *M. tuberculosis* H37Rv (Upton *et al*., 2001). This antiserum will also be extremely useful in determining the levels of NAT in clinical isolates of *M. tuberculosis* that differ in their sensitivity to INH.

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