Cloning and Molecular Characterization of Three Arylamine *N*-Acetyltransferase Genes from *Bacillus anthracis*: Identification of Unusual Enzymatic Properties and Their Contribution to Sulfamethoxazole Resistance†

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ABSTRACT: The arylamine *N*-acetyltransferases (NATs) are xenobiotic-metabolizing enzymes that catalyze the N-acetylation of arylamines and their N-hydroxylated metabolites. These enzymes play a key role in detoxication of numerous drugs and xenobiotics. We report here the cloning, functional expression, and characterization of three new *NAT* genes (termed *banatA*, *banatB*, and *banatC*) from the pathogen *Bacillus anthracis*. The sequences of the corresponding proteins are ∼30% identical with those of characterized eukaryotic and prokaryotic NAT enzymes, and the proteins were recognized by an anti-NAT antibody. The three genes were endogenously expressed in *B. anthracis*, and NAT activity was found in cell extracts. The three NAT homologues exhibited distinct structural and enzymatic properties, some of which have not previously been observed with other NAT enzymes. Recombinant BanatC displayed strong NAT activity toward several prototypic NAT substrates, including the sulfonamide antibiotic sulfamethoxazole (SMX). As opposed to BanatC, BanatB also had acetyl-CoA (AcCoA) and *p*-nitrophenyl acetate (PNPA) hydrolysis activity in the absence of arylamine substrates, indicating that it may act as an AcCoA hydrolase. BanatA was devoid of NAT or AcCoA/PNPA hydrolysis activities, suggesting that it may be a new bacterial NAT-like protein with unknown function. Expression of BanatC in *Escherichia coli* afforded higher-than-normal resistance to SMX in the recombinant bacteria, whereas an inactive mutant of the enzyme did not. These data indicate that BanatC could contribute to the resistance of *B. anthracis* to SMX.

Arylamine *N*-acetyltransferases (NATs)[1] are xenobiotic-metabolizing enzymes (XMEs) that catalyze the acetylCoA (Ac-CoA)-dependent N- and O-acetylation of various arylamines, hydrazines, and their N-hydroxylated metabolites (*1*). These enzymes thus play an important role in the detoxication and bioactivation of numerous drugs and xenobiotics (*2*, *3*).

NAT enzymes have been found and characterized in several vertebrate species, such as human, mouse, and chicken (*4*). In humans, two NAT enzymes have been described (NAT1 and NAT2) (*5*). Although NAT1 and NAT2 are highly similar, their kinetic selectivity toward aromatic substrates differs markedly (*6*, *7*). NAT1 acetylates in vivo several therapeutic drugs, including the antibacterial drugs sulfamethoxazole (SMX) (*8*) and *p*-aminosalicylate (PAS) (*9*). The NAT2 isoform, which also acetylates in vivo numerous xenobiotics and therapeutic drugs, was first identified as the enzyme that inactivates the front-line antitubercular drug isoniazid (INH) (*10*). NATs have also been identified and characterized in several prokaryotes (*11*−*14*). X-ray crystal structures have been reported for four NAT orthologs from *Salmonella typhimurium*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, and *Mesorhizobium loti* (*11*, *13*, *15*, *16*). NAT enzymes are 31−33 kDa proteins (270−290 amino acids). They contain a highly conserved three-domain fold (an N-terminal helical bundle, a central β-barrel, and a C-terminal α/β-lid) and a cysteine protease-like (Cys-His-Asp) catalytic triad. Three-dimensional models of the catalytic domain of human NAT1 and NAT2 isoforms showed a high degree of structural similarity with the bacterial enzymes (*17*, *18*). NAT activity is based on a bi-bi ping-pong mechanism in which the active-site cysteine is initially acetylated by AcCoA to form a thioacetyl ester (*16*, *19*).

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[1] Abbreviations: AcCoA, acetyl-coenzyme A; DMAB, 4-(dimethylamino)benzaldehyde; NAT, arylamine *N*-acetyltransferase; PNPA, *p*-nitrophenyl acetate; SMX, sulfamethoxazole; 5 AS, 5-aminosalicylic acid; 4 AS, 4-aminosalicylic acid; HDZ, hydralazine; 2 AF, 2-aminofluorene.

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Bacterial NATs acetylate various arylamine xenobiotic substrates and therapeutic drugs (*4*, *20*). It has been suggested that bacterial NATs could be employed as a defense weapon against environmental toxins (*4*). Several studies showed that acetylation of INH by *Mycobacterium tuberculosis* NAT contributed to the resistance of this bacterium to INH antibiotic (*12*, *21*). While NAT family members may have common functions, certain NAT homologues may have different roles in the various organisms in which they are present. This is exemplified by the existence of a NAT homologue, rifamycin-amide synthase (RifF), which lacks NAT activity but which catalyzes the last step of the synthesis of the antibiotic rifamycin in *Amycolatopsis mediterranei* (*22*).

Recently, a bioinformatics screen of the bacterial genomes available revealed that *Bacillus anthracis* may possess three different *NAT* genes (*23*). Whereas one of the three genes may encode a typical NAT enzyme of 279 amino acids, the other two *NAT* genes putatively encode two unusually short NAT isoforms (with shorter C-terminal α/β-lid domains) of 217 and 255 amino acids (*4*, *23*). For the NAT enzyme from *S. typhimurium* (Stnat), removal of the C-terminal α/β-lid domain ($\approx$100 amino acids) led to a truncated NAT mutant that was devoid of AcCoA-dependent acetylation activity but that displayed hydrolytic activity toward AcCoA and PNPA (*24*). A truncated Stnat mutant lacking the C-terminal domain undecapeptide was able to catalyze both AcCoA-dependent acetylation of arylamines and AcCoA hydrolysis in the absence of arylamine (*24*).

B. anthracis is a Gram-positive bacterium that causes anthrax disease in animals and humans (*25*). *B. anthracis* has become notorious as a bioweapon because of its tough, environmentally resistant endospore and its ability to cause lethal inhalation anthrax in humans (*26*). Most *B. anthracis* isolates are naturally resistant to several antibiotics, including SMX (*27*). This drug is a substrate for certain bacterial NAT enzymes (*13*, *14*, *28*) and can be inactivated in human liver by NATs (*8*).

Here, we report the molecular cloning, the functional expression, and the primary characterization of three new homologous *NAT* genes from *B. anthracis* (termed *banatA*, *banatB*, and *banatC*). We show that these three NAT homologues exhibit different structural and functional properties, some of which have not been described before. As opposed to other NAT enzymes described to date, BanatB was found to display NAT activity but also AcCoA hydrolysis activity in the absence of arylamine substrates. In addition, expression studies in *Escherichia coli* indicate that the BanatC isoform could contribute to SMX resistance.

EXPERIMENTAL PROCEDURES

Materials. 4-Aminosalicylic acid (4 AS), 5-aminosalicylic acid (5 AS), sulfamethoxazole (SMX), 2-aminofluorene (2 AF), hydralazine (HDZ), isoniazid (INH), *p*-aminobenzoic acid (PABA), acetyl-coenzyme A (AcCoA), 1,4-dithiothreitol (DTT), mutanolysin, and nickel-NTA superflow resin were obtained from Sigma. The bacterial pET28 expression vector was purchased from Novagen. The Bradford protein assay kit was supplied by Bio-Rad. All other reagents were purchased from Sigma. Polyclonal antibodies against NAT from *S. typhimurium* were kindly provided by E. Sim (University of Oxford, Oxford, U.K.) and have been described previously (*12*).

Sequence Alignment, Secondary Structure Predictions, and Phylogenetic Analysis. DNA and protein sequences were retrieved from the NCBI Genbank and Genpept databases. Secondary structure predictions were carried out using the PSI-Pred algorithm (*29*). Protein sequence alignments were conducted with Clustal W1.8 (*30*). For phylogenetic analysis, the neighbor-joining (NJ) method was used with the multiple-amino acid sequence alignments as implemented in the phylogenetics package PHYLIP (*31*). The distance matrices were calculated with the Dayhoff substitution model (*31*).

B. anthracis Strains. Two nonvirulent *B. anthracis* strains were used in this study, *B. anthracis* RTC50 (pXO1$^+$/Tox$^-$, pXO2$^+$/*capE*::Spec) (*32*) and *B. anthracis* 9131 (pXO1$^-$, pXO2$^-$) (*33*). These two strains are derivative of the Sterne strain, the DNA sequence of which is known (Refseq NC_005945). Strains were grown in Luria broth (LB, Difco Laboratories) at 37 °C.

DNA Extraction. Extraction of chromosomal DNA from *B. anthracis* RTC50 was performed as described in ref *33* with the addition of mutanolysin to obtain better peptidoglycan degradation.

Molecular Cloning and Plasmid Construction. The *banatA*, *banatB*, and *banatC* open reading frames (ORFs) were amplified from *B. anthracis* RTC50 strain genomic DNA (*32*) by high-fidelity PCR using the oligonucleotides 5′-CCGGATCCATGACCGACTTTCAAAAACA-3′ (sense) 5′-GGCTCGAGCTACAATAAGAGGTACTTTG-3′ (reverse) for *banatA*, 5′-CCGGATCCATGACAAGTCTACAACAT-AA-3′ (sense) 5′-GGCTCGAGCTATAACTCAATAGCAAA-TA-3′ (reverse) for *banatB*, and 5′-CCGGATCCATGAT-GACCAATTTACAAAA-3′ (sense) 5′-GGCTCGAGCTAA-CCTCTTTCTAGTGTTT-3′ (reverse) for *banatC*. The PCR used Pfu DNA polymerase (Promega) and consisted of a denaturation step (94 °C for 2 min) followed by 40 cycles of annealing (55 °C for 30 s), extension (72 °C for 2 min), and denaturation (94 °C for 1 min), and a final extension step (72 °C for 5 min). PCR products were subcloned into the pET28a expression vector for further six-His-tagged recombinant protein expression. The sequences of the inserts were checked by DNA sequencing.

Site-Directed Mutagenesis of BanatC. An inactive mutant of BanatC (BanatC C69A) was constructed via replacement of the catalytic cysteine residue (Cys69) with an alanine using a cassette mutagenesis strategy employing an overlap extension PCR protocol. The mutagenesis primers were 5′-GGCGGTCTTGCTTATGAATTA-3′ (sense) and 5′-TAAT-TCATAAGCAAGACCGCC-3′ (reverse).

Protein Production and Purification. E. coli BL21(DE3)-pLysS transformed with the pET-28A-based plasmids encoding *banatA*, *banatB*, and *banatC* ORFs were used for the production and purification of the corresponding recombinant proteins. Transformed bacteria were induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), grown for 5 h at 37 °C, and incubated overnight at 4 °C. Bacteria from a 1 L culture were pelleted (6000*g* for 10 min) and washed with PBS (phosphate-buffered saline). Pelleted bacteria (6000*g* for 10 min) were resuspended in 20 mL of PBS containing lysozyme (final concentration of 1 mg/mL) and protease inhibitors. Following incubation (1 h at 4 °C), protease inhibitors and 0.1% Triton X-100 (final concentra-

tion) were added, and the suspension was incubated for a further 1 h at 4 °C. The lysate was then sonicated on ice (five pulses of 30 s each) and pelleted (12000*g* for 30 min). The supernatant was incubated with 1.5 mL of HIS-Select nickel resin (in the presence of 20 mM imidazole) for 2 h at 4 °C. The resin was then poured into a column and washed successively with PBS supplemented with 0.1% Triton X-100 and with PBS. Recombinant BanatA, BanatB, and BanatC were eluted with 300 mM imidazole in PBS. Purified enzymes were reduced by incubation with 10 mM DTT for 10 min at 4 °C and then dialyzed against 25 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Further purification of the His-tagged proteins was also carried out using CM Sephadex (Amersham-Pharmacia) ion exchange chromatography. Ni^{2+}-agarose-purified proteins were batch-bound to CM Sephadex at 4 °C {pre-equilibrated in buffer A [25 mM Tris-HCl (pH 7.4) and 1 mM EDTA]}, and the resin was poured into an empty column. After extensive washes with buffer A, proteins were eluted with a NaCl step gradient in buffer A. Purified proteins were eluted with 150 mM NaCl in buffer A, reduced with DTT, and dialyzed overnight at 4 °C against buffer A. The purity of protein samples was checked by SDS−PAGE and Western blotting as described below. Optimal protein production in *E. coli* was achieved with 100 μM IPTG.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Experiments. Total RNA was extracted from stationary phase cultures (OD_{600} = 0.9) of *B. anthracis* RTC50 and 9131 strains grown in LB medium. TRIzol reagent (Invitrogen) was used for RNA extraction as described for *Bacillus subtilis* by Burguiere et al. (*34*). The RNA preparation was treated with DNAse I (Ambion), and RNA quality was analyzed on a RNA NanoLabChip (Agilent Technologies). The first-strand cDNA was synthesized from 2 μg of total RNA, using the *Carboxydothermus hydrogenoformans* RT kit from Roche and reverse primers for *banatA*, *banatB*, and *banatC* (see above). PCRs were carried with Taq DNA polymerase (Sigma) using the *banatA*, *banatB*, and *banatC* primers used for molecular cloning (see above). The absence of genomic DNA contamination was confirmed in controls to which no reverse transcriptase was added.

Preparation of Protein Extract. Extracts were prepared from stationary phase cultures (150 mL, OD_{600} = 0.9) of *B. anthracis* strain 9131 grown in LB medium. Cells were harvested by centrifugation (5000*g* at 4 °C for 10 min). Pellets were resuspended in 2 mL of PBS supplemented with 0.05% Triton X-100, lysozyme (1 mg/mL), mutanolysin (50 μg/mL), 5 mM DTT, and protease inhibitors. The resulting suspensions were subjected to sonication or to the FastPrep cell disrupter (ThermoSavant) with glass beads. After centrifugation (12000*g* for 15 min), supernatants were used for Western blotting and enzyme assays.

Enzyme Assays. Standard assays were used to characterize the NAT activity of BanatA, BanatB, and BanatC (*35*). Measurements of the AcCoA-dependent acetylation of typical NAT substrates by recombinant BanatA, BanatB, and BanatC were carried out using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) as described by Brooke et al. (*28*). Purified recombinant enzymes and arylamine substrates (final concentration of 500 μM) were mixed and preincubated (37 °C for 5 min) in assay buffer [25 mM Tris-HCl (pH 7.5) and 1 mM EDTA] in a 96-well ELISA plate. AcCoA was added to a final concentration of 400 μM to start the reaction. The total volume of the assay was 100 μL. The reaction was quenched with 25 μL of 5 mM DTNB in a guanidine hydrochloride solution [6.4 M guanidine-HCl and 0.1 M Tris-HCl (pH 7.3)]. The absorbance at 405 nm was measured using an ELISA plate analyzer. All assays were conducted in triplicate under conditions giving a linear initial rate. Controls were carried out in the absence of enzyme, AcCoA, or arylamine. The amount of CoASH produced in the reaction was determined by comparison with a standard curve obtained with DTNB. Data were expressed as the means ± the standard deviation. AcCoA-dependent acetylation of aromatic substrates by the recombinant proteins was also carried out using the 4-(dimethylamino)benzaldehyde (DMAB) assay (*36*). Similar results were obtained using either the DTNB or DMAB assay.

To assess the "pure" hydrolytic activity of the recombinant proteins toward AcCoA and *p*-nitrophenyl acetate (PNPA), the DTNB assay described above was carried out in the absence of arylamine substrates as described by Mushtaq et al. (*24*).

Apparent kinetic parameters (V_m^{app} and K_m^{app}) for SMZ, SMX, 4 AS, 5 AS, HDZ, and 2 AF were estimated from nonlinear regression analysis using the DTNB assay as previously described (*28*). For bi-bi ping-pong mechanisms (as for all NAT enzymes), V_m^{app}/K_m^{app} ratios are equal to true kinetic parameter ratios (V_m/K_m) (*37*). Apparent V_m/apparent K_m ratios were therefore used for comparing catalytic efficiencies (*14*).

The detection of NAT activity in *B. anthracis* extracts was achieved using HPLC assays as described by Dupret et al. (*38*).

All assays described above were performed in triplicate, under conditions giving a linear initial rate.

Sulfamethoxazole (SMX) Resistance Assay. The sulfamethoxazole resistance assays were carried out on LB agar solid medium plates using *E. coli* strain DH5α (SMX minimum inhibitory concentration of ≈500 μM) as modified from Milner et al. (*39*) and Iliades et al. (*40*). To this end, IPTG (final concentration of 100 μM), SMX (final concentration of 500 μM), and kanamycin (final concentration of 30 μg/mL) were freshly prepared and dissolved in LB agar medium. Control plates were also created without SMX and/or without IPTG. DH5α cells transformed with pET28a vector alone, pET28a-*banatC*, and pET28a-*banatC* C69A mutant were grown in LB medium at 37 °C (containing kanamycin). Cells were harvested while in log-phase growth, normalized to an OD_{600} of 0.1, and grown in the presence of 100 μM IPTG for a further 1 h. Cells were again normalized, and 5 μL was spotted onto plates (with or without IPTG and/or SMX) and grown at 37 °C. Experiments were carried out in triplicate. Pictures were taken after incubation for 2−3 days.

Protein Identification, SDS−PAGE, and Western Blotting Analysis. Protein concentrations were determined using the Bradford assay (Bio-Rad). Samples were combined with reducing 4× SDS sample buffer and separated by SDS−PAGE. Gels were stained with Amido black or Coomassie blue. For Western blotting, following separation by SDS−PAGE, proteins were electrotransferred onto a nitrocellulose membrane. The membrane was blocked by incubation with Tris-buffered saline and Tween 20 (TBS) containing 5%

A

BanatA

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ATG ACC GAC TTT CAA AAA CAA TTT TTT GCA AGA TTA AAT ATA GAA GAA AAA CAA AAA GTT TCA TTT GAA GAT TTA TCT AAC ATT ATG TAC      90
 M   T   D   F   Q   K   Q   F   F   A   R   L   N   I   E   E   K   Q   K   V   S   F   E   D   L   S   N   I   M   Y      30

GCA ATG GCA CAA ACT GTC CCA TTT GAA AAC TTA AAT GTT CTC GAA AAG AAT TTT AAG GAA ATA TCA CAA GAA AAT CTA AAA GAA AAA ATT      180
 A   M   A   Q   T   V   P   F   E   N   L   N   V   L   E   K   N   F   K   E   I   S   Q   E   N   L   K   E   K   I      60

TTA GTA AAC AAT CGT GGT GGT CTT TGT TAT GAA CTC AAT CCT ACT ATG TAT TAT TTC CTT AAA GAT GCT GGA TTT GAT GTT TAT CTC GTT      270
 L   V   N   N   R   G   G   L   C   Y   E   L   N   P   T   M   Y   Y   F   L   K   D   A   G   F   D   V   Y   L   V      90

TCA GGA ACA GTG TAT AAC GCC GCA AAT TCC ATA TGG GCC GTT GAT TCC GGA CAT ATC GCA ACC ATT TTA AAA CAT CAT AAT GAG CTT TAT      360
 S   G   T   V   Y   N   A   A   N   S   I   W   A   V   D   S   G   H   I   A   T   I   L   K   H   H   N   E   L   Y      120

TTA ATT GAA GTT GGA TTC GGA TCA TAC TTA CCG CTT GCA CCT GTT CCT TTC TCA GGT GAA GTC GTT CAA TCC GTT ACA GGA GAT TAT CGT      450
 L   I   E   V   G   F   G   S   Y   L   P   L   A   P   V   P   F   S   G   E   V   V   Q   S   V   T   G   D   Y   R      150

ATT CGT AAA GAA ATG ACC GAA AAA GGA AAC TAC ATG TTA GAG ATG CGT AAA AAC GAT GAG TTT TTG GAT CAA TCT TCT ACT GAT GAT TGG      540
 I   R   K   E   M   T   E   K   G   N   Y   M   L   E   M   R   K   N   D   E   F   L   D   Q   S   S   T   D   D   W      180

ACG TTA GGC TAT GCA TTT TAT ATA GAA GAA GTG GAT GAA AAA AAG CAA ATG CAG CAC AAA AAA TTA TTG TTG AAC ACG ATG GCT CAC CAT      630
 T   L   G   Y   A   F   Y   I   E   E   V   D   E   K   K   Q   M   Q   H   K   K   L   L   L   N   T   M   A   H   H      210

TTA ACA AAG TAC CTC TTA TTG TAA                                                                                          654
 L   T   K   Y   L   L   L   *                                                                                           217
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BanatB

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ATG ACA AGT CTA CAA CAT AAG CTG TTT ACA AGA TTG AAT CGT GCA AAC CGT ACC GAA GTA AAG TTT GAA GAA TTA AAT ACA ATT CTC TTT      90
 M   T   S   L   Q   H   K   L   F   T   R   L   N   R   A   N   R   T   E   V   K   F   E   E   L   N   T   I   L   F      30

ACA TTT GCA CAC ACT ATA CCA TTT GAG AAT TTA GAT GTT ATA GCA AGT AAC ACG AAT ACA ATT ACT ATG GAA AAT TTA CAG AAT AAA ATT      180
 T   F   A   H   T   I   P   F   E   N   L   D   V   I   A   S   N   T   N   T   I   T   M   E   N   L   Q   N   K   I      60

TTA AGT AGA TCC CGC GGC GGA CTT TGT TAT GAA TTA AAT ACG CTT TTT TAT TAC TTT TTA AAA GAT TGT GGT TAT GAT GTA CAA CTC GCA      270
 L   S   R   S   R   G   G   L   C   Y   E   L   N   T   L   F   Y   Y   F   L   K   D   C   G   Y   D   V   Q   L   A      90

TTA GGT ACC GTA TAT AAA AAT GAT ATA AAC GCA TGG GCA CTT GAG AAT GGA CAT ATA ACG ATT ATT TTA AAT TAT GAT AAA GTA CGA TAC      360
 L   G   T   V   Y   K   N   D   I   N   A   W   A   L   E   N   G   H   I   T   I   I   L   N   Y   D   K   V   R   Y      120

GTA ATT GAT GTA GGT ATT GCT TCA CTA GTA CCT CTA GTC CCT GTA CCT TTT ACT GGT GAG TCT GTT TCT TCT AAA AAT GGT ACG TAT CGA      450
 V   I   D   V   G   I   A   S   L   V   P   L   V   P   V   P   F   T   G   E   S   V   S   S   K   N   G   T   Y   R      150

GTA AGA CAA AAA GAT ACG AGT AAA GGG AAT TAC GTT CTA GAA AGA AAA GAT ACG AAC GGT GAG TGG AAA GTA TGT CAT GCT TTT TAT AAT      540
 V   R   Q   K   D   T   S   K   G   N   Y   V   L   E   R   K   D   T   N   G   E   W   K   V   C   H   A   F   Y   N      180

CGT ATG ATT GAT GAA ATA GTA GTG AAT GAT GTT CAA AAA AGA GTA GTA GAA GAT GAG AAA TCT ATT TTC AAT AAG GGC CCA ATT GCA GTC      630
 R   M   I   D   E   I   V   V   N   D   V   Q   K   R   V   V   E   D   E   K   S   I   F   N   K   G   P   I   A   V      210

AAA TTG ACG GAC TCT GGC CAT GTC TCT TTA ACC AAT ACT AGT TTC ACT GAA ATA GTT CAT GGT GAA AAA ACG AAA CGT GAA ATT ACA GAA      720
 K   L   T   D   S   G   H   V   S   L   T   N   T   S   F   T   E   I   V   H   G   E   K   T   K   R   E   I   T   E      240

GAT CAA TAT AGA GAG CTT TTA TAT ACT TTA TTT GCT ATT GAG TTA TGA                                                           765
 D   Q   Y   R   E   L   L   Y   T   L   F   A   I   E   L   *                                                           255
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BanatC

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ATG ATG ACC AAT TTA CAA AAG GAG TTT TTT AAA CGA CTA AAA ATT CCT GCA AAA GAA ATA ACA TTT AAT GAT TTA GAT GAA ATT CTC TTA      90
 M   M   T   N   L   Q   K   E   F   F   K   R   L   K   I   P   A   K   E   I   T   F   N   D   L   D   E   I   L   L      30

AAC ATG GGA ATG ATT CTC CCC TAT GAA AAT CTT GAT ATT ATG GCT GGC ACT ATT AAA AAC ATC TCA AAA AAT AAC TTA GTA GAA AAG TTA      180
 N   M   G   M   I   L   P   Y   E   N   L   D   I   M   A   G   T   I   K   N   I   S   K   N   N   L   V   E   K   L      60

CTT ATT CAA AAA CGA GGC GGT CTT TGT TAT GAA TTA AAC TCC TTA CTA TAT TAC TTT TTA ATG GAT TGT GGA TTT CAA GTA TAT AAA GTA      270
 L   I   Q   K   R   G   G   L   C   Y   E   L   N   S   L   L   Y   Y   F   L   M   D   C   G   F   Q   V   Y   K   V      90

GCC GGT ACT GTT TAT GAC CTT TAC GAT AAT AAA TGG AAA CCT GAT GAT GGT CAT GTC ATT ATC ATA TTA CAT CAT AAT AAA AAA GAT TAT      360
 A   G   T   V   Y   D   L   Y   D   N   K   W   K   P   D   D   G   H   V   I   I   I   L   H   H   N   K   K   D   Y      120

GTT ATA GAT GCC GGT TTT GCC TCT CAC CTA CCT TTA CAT CCA GTC CCT TTT AGC GGA GAA GTC ATA TCT TCT CAA ACG GGT GAA TAT CGA      450
 V   I   D   A   G   F   A   S   H   L   P   L   H   P   V   P   F   S   G   E   V   I   S   S   Q   T   G   E   Y   R      150

ATT CGC AAA CGA ACT ACT CAA AAA GGT ACA CAC ATT TTA GAA ATG AGA AAA GGA GCT AAC GGG GAA TCT ACA AAT TTT TTA CAA TCT GAA      540
 I   R   K   R   T   T   Q   K   G   T   H   I   L   E   M   R   K   G   A   N   G   E   S   T   N   F   L   Q   S   E      180

CCT TCA GAT GAA TGG AAA ATA GGC TAT GCC TTC ACT TTA GAT CCA ATA GAT GAG CAA AAG GTG AAT AAC ATT CAA AAA GTC ATT GTA GAA      630
 P   S   D   E   W   K   I   G   Y   A   F   T   L   D   P   I   D   E   Q   K   V   N   N   I   Q   K   V   I   V   E      210

CAT AAA GAA TCT CCT TTT AAT AAA GGA GCT ATC ACT TGT AAA TTA ACT AAT TAT GGT CAC ATA TCA TTA ACA AAT AAA AAT TAT ACA GAA      720
  H   K   E   S   P   F   N   K   G   A   I   T   C   K   L   T   N   Y   G   H   I   S   L   T   N   K   N   Y   T   E      240

ACC TTT AAA GGC ACC AAA AAT AAA CGT CCA ATA GAA TCA AAA GAT TAT GCT CGC ATT CTT CGT GAA TCT TTT GGA ATA ACA CAA GTG AAA      810
 T   F   K   G   T   K   N   K   R   P   I   E   S   K   D   Y   A   R   I   L   R   E   S   F   G   I   T   Q   V   K      270

TAT GTA GGA AAA ACA CTA GAA AGA GGT TAG                                                                                  840
 Y   V   G   K   T   L   E   R   G   *                                                                                   279
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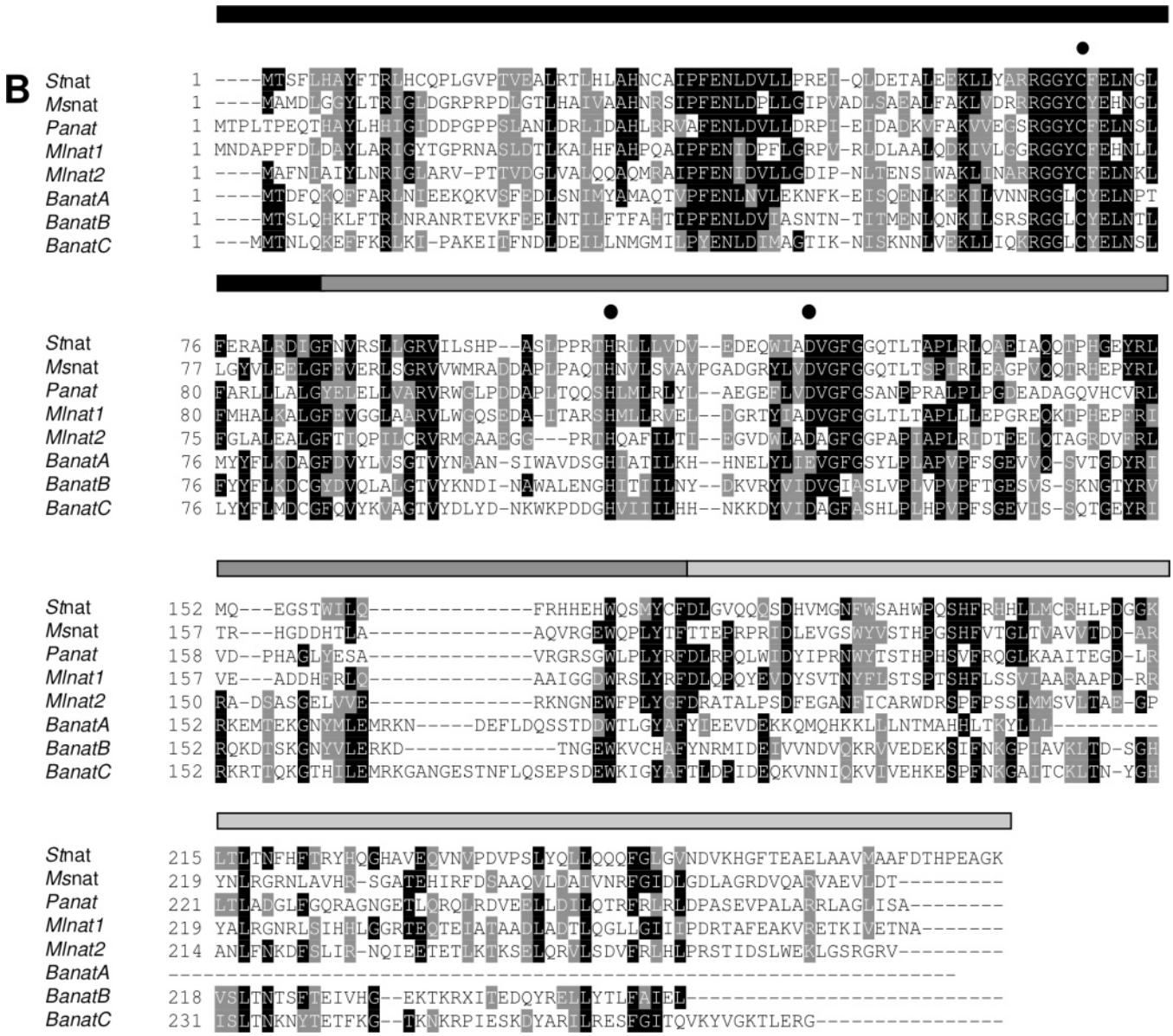

FIGURE 1: Nucleotide and deduced amino acid sequences of the three *B. anthracis* NAT homologues (BanatA, BanatB, and BanatC) and alignment with cloned bacterial NAT enzymes. (A) Complete nucleotide and deduced amino acid sequences of *B. anthracis* NATA (BanatA), NATB (BanatB), and NATC (BanatC). The nucleotide sequences are numbered in the 5′ to 3′ direction, from the initial ATG codon to the stop codon. The three characteristic functional regions conserved in all NAT enzymes (*20*) are highlighted in gray. The putative catalytic triad residues (cysteine, histidine, and aspartate/glutamate) are shown in bold. (B) Multiple alignment of BanatA, BanatB, and BanatC amino acid sequences. *B. anthracis* NAT protein sequences were aligned with the sequences of the cloned and characterized NAT enzymes from *S. typhimurium* (Stnat), *M. smegmatis* (Msnat), *P. aeruginosa* (Panat), and *Me. loti* (Mlnat1 and Mlnat2) using the Clustal W1.8 program. Identical residues are highlighted in black, and conserved substitutions are highlighted in dark gray. Catalytic residues are indicated by a dot. The three characteristic structural domains of NAT enzymes (*16*) are depicted below the BanatC sequence (black for the helical bundle domain, medium gray for the β-barrel domain, and light gray for the α/β-lid domain) and are those deduced from the Stnat structure (PDB entry 1E2T).

nonfat milk powder for 1 h in TBS. An antibody raised against NAT from *S. typhimurium* (*12*) was added (1:10000 dilution), and the membrane was incubated for 1 h in TBS. Secondary conjugated antibody (anti-rabbit at a dilution of 1:100000) was added, and the membrane was incubated for 1 h. The membrane was then washed, and ECL reagent (Amersham-Pharmacia) was used for detection.

RESULTS

Molecular Cloning and Sequence Analysis of the Three NAT Homologues from B. anthracis. A recent screening of more than 300 complete and incomplete bacterial genomes showed that *B. anthracis* possessed three putative open reading frames (ORFs) encoding three NAT homologues (*23*). Two of these *B. anthracis* ORFs could encode unusually short NAT enzymes (Figures 1A,B and 2B). To test whether these three putative *NAT* genes encode functional NAT enzymes, we cloned the three corresponding ORFs [termed *banatA* (GenBank entry YP_029549), *banatB* (GenBank entry YP_028791), and *banatC* (GenBank entry YP_028265)]. The *banatA* (654 nucleotides), *banatB* (768 nucleotides), and *banatC* ORFs (840 nucleotides) encode three polypeptide sequences of 217, 255, and 279 amino acids, respectively (Figure 1A). As shown in panels A and B of Figure 1, these

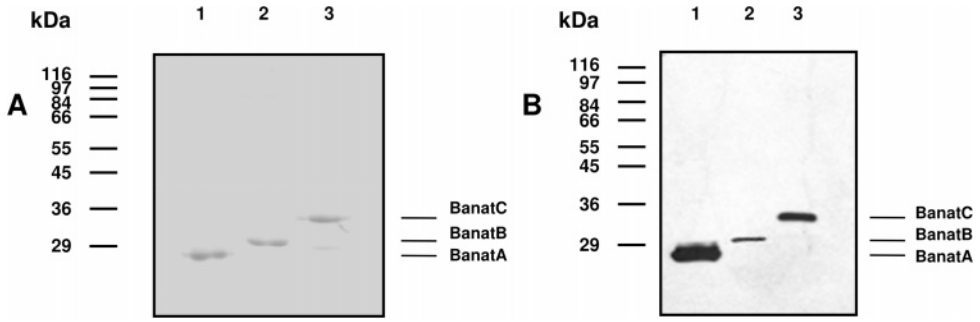

FIGURE 2: Expression of recombinant BanatA, BanatB, and BanatC proteins. (A) SDS−PAGE analysis of recombinant BanatA, BanatB, and BanatC. Two micrograms of purified recombinant proteins wasd subjected to SDS−PAGE under reducing conditions and stained with Coomassie R250 brilliant blue (BanatA in lane 1, BanatB in lane 2, and BanatC in lane 3). (B) Western blot analysis of recombinant BanatA, BanatB, and BanatC proteins. Five hundred nanograms of purified proteins was subjected to SDS−PAGE under reducing conditions, and Western blot analysis was conducted using a polyclonal antibody raised against the Stnat enzyme (1:5000 dilution; BanatA in lane 1, BanatB in lane 2, and BanatC in lane 3).

three protein sequences contain all the characteristic conserved NAT functional motifs (*4*, *41*) found in all NAT enzymes characterized so far (*14*, *20*). The conserved NAT catalytic triad residues (Cys-His-Asp) were also found in the three putative *B. anthracis* NAT enzymes, with BanatA having a glutamate residue instead of an aspartate (Figure 1A,B). Despite their differences in length, the three *B. anthracis* NAT sequences share from 25−35% sequence identity. Multiple-sequence alignments with the well-characterized NAT enzymes from *S. typhimurium*, *M. smegmatis*, *P. aeruginosa*, and *Me. loti* clearly indicate that the three *B. anthracis* sequences are NAT homologues, sharing ∼27% identical sequence with these bacterial NATs (Figure 1B). Phylogenetic analysis (data not shown) further supports the idea that BanatA, BanatB, and BanatC are paralogs, thus confirming that paralogous NAT sequences also exist in prokaryotes (*4*, *41*). Structural analysis of the *B. anthracis* NAT sequences suggests that these proteins have the characteristic NAT secondary structure topology, which consists of three structural domains, an N-terminal α-bundle, a central *β*-barrel, and a C-terminal α/*β*-lid (*16*) (Figure 1B). As stated above, BanatA, BanatB, and BanatC proteins differ in length. Sequence and structural analyses (data not shown) indicate that the length of the BanatC protein (279 amino acids) and, in particular, the sizes of its three structural domains are similar to those found in the bacterial NAT enzymes characterized so far, i.e., three structural domains of the same length (≈90 amino acids) (*4*, *16*). Although BanatA and BanatB possess N-terminal α-bundle and central *β*-barrel domains similar to those found in NAT enzymes, BanatA and BanatB have a C-terminal α/*β*-lid domain shorter than that of BanatC and other well-characterized bacterial NAT enzymes (*4*, *16*). So far, BanatA is the smallest NAT homologue reported.

Expression, Purification, and Characterization of Recombinant BanatA, BanatB, and BanatC Proteins. We obtained highly purified and soluble recombinant BanatA, BanatB, and BanatC proteins with molecular masses consistent with predicted values (27 kDa for BanatA, 31 kDa for BanatB, and 34 kDa for BanatC, 2 kDa His tag included) (Figure 2A). As shown in Figure 2B, the three recombinant proteins were readily detected by a specific antibody against *S. typhimurium* NAT (Stnat) known to cross-react with other bacterial NATs, such as the isoforms of *M. smegmatis*, *M. tuberculosis*, and *Me. loti* (*13*, *14*). These data further supported the idea that BanatA, BanatB, and BanatC belong to the NAT enzyme family. The three recombinant proteins were also detected by a specific anti-six-His antibody (data not shown). In a control, no detection was observed with an anti-human NAT1 known to give no cross-reactivity with bacterial NATs (data not shown).

Table 1: NAT Activity of Purified Recombinant BanatB and BanatC Proteins toward Typical Aromatic NAT Substrates

		rate (nmol min^{-1} mg^{-1})	
compound	short name	BanatB	BanatC
none		168 ± 25	nd[c]
sulfamethoxazole[a]	SMX	*b*	416 ± 16
5-aminosalicylate	5 AS	1360 ± 69	3679 ± 130
4-aminobenzoic acid	pABA	*b*	nd[c]
4-aminosalicylate	4 AS	*b*	3109 ± 50
2-aminofluorene[a]	2 AF	603 ± 49	5852 ± 220
isoniazid	INH	*b*	nd[c]
hydralazine	HDZ	969 ± 36	1744 ± 79

[a] Assay performed in the presence of 5% (v/v) DMSO. [b] No NAT activity measured (identical AcCoA hydrolysis activity measured in the absence or presence of an aromatic substrate). [c] No AcCoA hydrolysis measured either in the presence or in the absence of an arylamine substrate.

Table 2: Steady-State Kinetic Analysis of BanatB and BanatC Enzymes[a]

compound	short name	K_m^{app} (μM)	V_m^{app} (μM min^{-1} mg^{-1})	V_m^{app}/K_m^{app} ($\times 10^3$)
		BanatB		
5-aminosalicylate	5 AS	622 ± 76	22.00 ± 2.8	35
2-aminofluorene	2 AF	84 ± 17	4.7 ± 0.3	56
hydralazine	HDZ	849 ± 96	22.52 ± 1.4	26
		BanatC		
sulfamethoxazole	SMX	662 ± 26	4.61 ± 0.3	7
5-aminosalicylate	5 AS	228 ± 13	43.89 ± 0.8	192
4-aminosalicylate	4 AS	834 ± 147	56.11 ± 2.5	67
2-aminofluorene	2 AF	210 ± 23	64.5 ± 1.9	307
hydralazine	HDZ	1327 ± 56	61.07 ± 1.6	46

[a] Apparent Michaelis−Menten parameters V_{max} and K_m were determined by direct curve fitting to Michaelis−Menten equations (nonlinear regression).

Enzymatic Characterization of Recombinant BanatA, BanatB, and BanatC Proteins. We further characterized the purified recombinant BanatA, BanatB, and BanatC proteins by investigating whether they catalyzed the AcCoA-dependent acetylation of several prototypic arylamine substrates

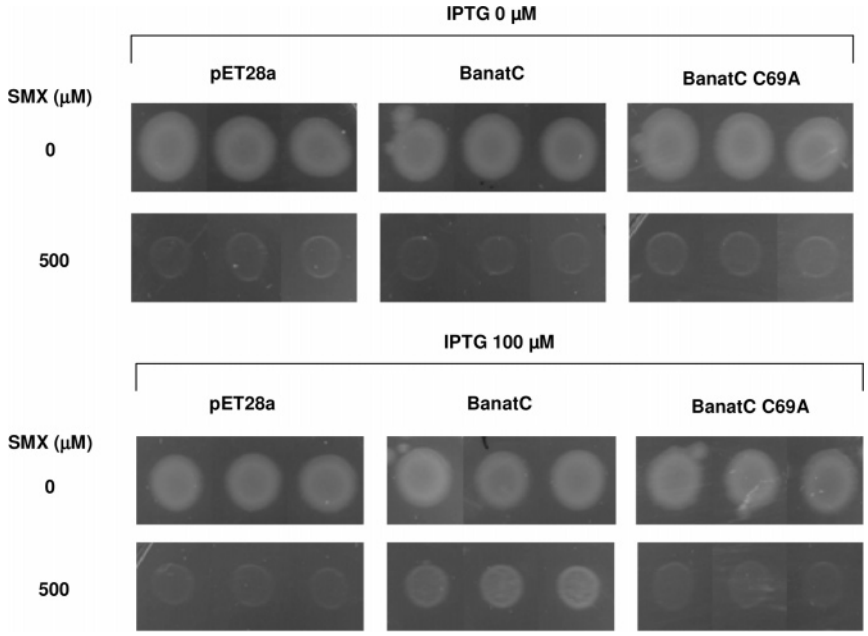

FIGURE 3: SMX resistance assay on solid medium of recombinant *E. coli* (DH5α) cells expressing *banatC*. *E. coli* DH5α cells transformed with pET28a vector, pET28a-*banatC*, or pET28a-*banatC* C69A constructs were spotted (5 μL) on LB solid agar medium (30 μg/mL kanamycin) containing IPTG and/or SMX at the indicated concentrations. The pictures were taken after growth at 37 °C for 72 h.

(NAT activity). Most of these substrates have been used to probe the NAT activity of several bacterial NAT isoforms (*13*, *14*). NAT activity was measured by following the rate of AcCoA hydrolysis in the presence of aromatic substrates, using DTNB as described previously (*13*, *14*, *28*, *42*). As shown in Tables 1 and 2, arylamine *N*-acetyltransferase activity was readily measured for BanatB and BanatC proteins, but with large differences in substrate preference and in acetylation rates. Among the seven prototypic NAT substrates that were tested, BanatB selectively acetylated 2-aminofluorene (2 AF), 5-aminosalicylate (5 AS), and hydralazine (HDZ) with different catalytic efficiencies (V_m^{app}/K_m^{app} ratios ranging from 56×10^{-3} min^{-1} mg^{-1} for 2 AF to 26×10^{-3} min^{-1} mg^{-1} for HDZ) (Table 2). BanatC was also able to acetylate 2 AF, 5 AS, and HDZ but with catalytic efficiencies higher than those found with BanatB (V_m^{app}/K_m^{app} ratios were 5.5 times higher for 2 AF and 5 AS and 1.8 times higher for HDZ). BanatC also significantly acetylated 4-aminosalicylate (4 AS) (V_m^{app}/K_m^{app} ratio of 67×10^{-3} min^{-1} mg^{-1}) and the sulfonamide antibiotic sulfamethoxazole (SMX) (V_m^{app}/K_m^{app} ratios of 7×10^{-3} min^{-1} mg^{-1}). We also determined a V_m^{app}/K_m^{app} ratio of 3×10^{-3} min^{-1} mg^{-1} for human NAT1 (data not shown), this latter value being in agreement with previously published data on the acetylation of SMX by human NAT1 (*8*). No acetylation of PABA was obtained with BanatB and BanatC (Table 1), as observed for most bacterial NATs (*13*). Moreover, contrary to the other bacterial NATs characterized at the molecular level (*12*−*14*, *28*), BanatB and BanatC were unable to acetylate the antitubercular drug INH significantly (Table 1). Thus, the data reported above clearly show that BanatB and BanatC are NAT enzymes with distinct enzymatic properties. This is further supported by the fact that in addition to its NAT activity, we found that BanatB, but not BanatC, displayed AcCoA hydrolysis activity in the absence of an arylamine substrate (Table 1). PNPA hydrolysis was also observed with BanatB (data not shown). Therefore, in addition to its NAT activity, the BanatB isoform may also act as an AcCoA hydrolase. We have not found any NAT or AcCoA/PNPA hydrolysis activities with BanatA.

Effect of BanatC Expression on the Resistance of E. coli Strain DH5α to SMX. The kinetic analysis reported above showed that BanatC has a better efficiency toward SMX than the human NAT1 isoform. In human, SMX is mainly metabolized and subsequently inactivated through NAT1-dependent acetylation. This suggested that BanatC could also contribute to SMX inactivation. We therefore tested whether the expression of BanatC in *E. coli* strain DH5α (minimum inhibitor concentration toward SMX of ≈500 μM) could afford resistance to SMX. BanatC C69A, a BanatC mutant devoid of NAT activity (data not shown), and the plasmid vector pET28a alone were used in control experiments. As shown in Figure 3, when the expression of BanatC was not induced by IPTG, the *E. coli* cells did not grow in the presence of 500 μM SMX. Conversely, with IPTG, only the cells transformed with BanatC were able to grow in the presence of SMX when compared to the other transformants. Overall, our results indicate that BanatC could contribute to SMX resistance in *B. anthracis*. Similar data were obtained in LB liquid cultures where BanatC expression was found to increase the resistance of *E. coli* to 500 μM SMX (data not shown).

Endogenous Expression of BanatA, BanatB, and BanatC in B. anthracis. We investigated whether the three NAT homologues were expressed endogenously in *B. anthracis* (Figure 4). RT-PCR products of expected sizes for *banatA* (667 bp, lanes 1 and 4), *banatB* (781 bp, lanes 2 and 5), and *banatC* (853 bp, lanes 3 and 6) were obtained with RTC50 and 9131 strains, thus indicating that the *banatA*, *banatB*, and *banatC* genes were expressed by *B. anthracis*. We carried out Western blotting experiments on *B. anthracis* protein extracts using the antiserum against Stnat. As shown in Figure 4, two specific bands corresponding to the molecular masses of BanatC (32 kDa) and BanatB (29 kDa)

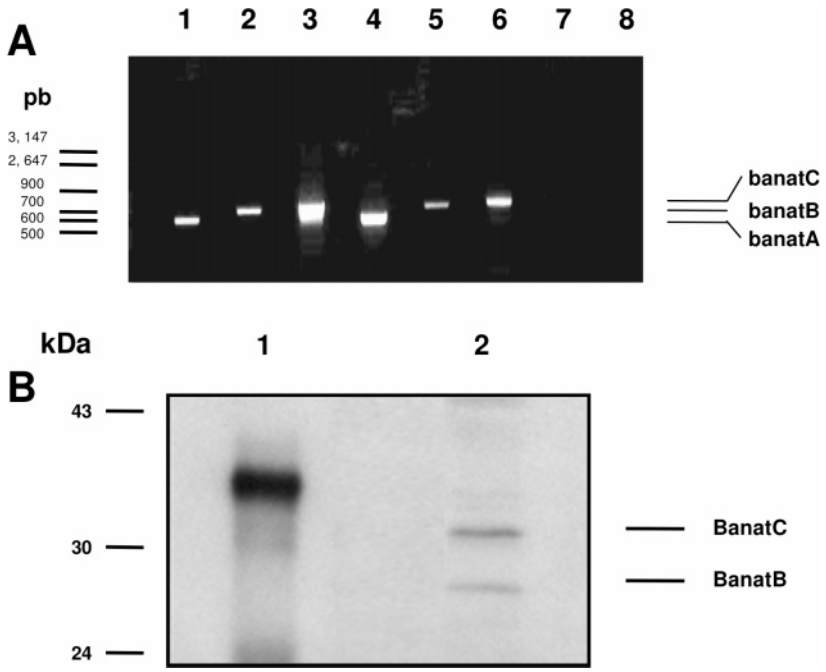

FIGURE 4: Endogenous expression of BanatA, BanatB, and BanatC in *B. anthracis*. (A) Sequence-specific primers for *banatA*, *banatB*, and *banatC* were used for RT-PCR with total RNA from *B. anthracis* strains RTC50 and 9131. RT-PCR products were separated by electrophoresis in 1.8% agarose gels and were detected by ethidium bromide staining. Specific fragments, 667 (*banatA*), 781 (*banatB*), and 853 bp (*banatC*), were obtained with *B. anthracis* (strain RTC50, *banatA* in lane 1, *banatB* in lane 2, and *banatC* in lane 3; strain 9131, *banatA* in lane 4, *banatB* in lane 5, and *banatC* in lane 6). The absence of genomic DNA contamination was confirmed using a negative control with no reverse transcriptase in the RT-PCR experiment (lane 7, negative control for strain RTC50; lane 8, negative control for strain 9131). (B) Western blot detection of NAT proteins in protein extracts of *B. anthracis* strain 9131 (4 μg of total protein) using a polyclonal antibody raised against Stnat (1:5000 dilution). A control (lane 1) was conducted with 500 ng of recombinant Mlnat1 enzyme (36 kDa) (*14*).

were specifically detected by the anti-Stnat antibody. The expression of BanatA protein (25 kDa) was not detectable at the level of sensitivity attained in these experiments. In addition to Western blotting experiments, we investigated whether the *B. anthracis* protein extracts catalyzed the acetylation of the NAT substrates. AcCoA-dependent acetylation occurred with NAT substrates such as 2 AF [30 pmol min^{-1} (mg of protein)$^{-1}$] or 4 AS [20 pmol min^{-1} (mg of protein)$^{-1}$]. Lower activities [<10 pmol min^{-1} (mg of protein)$^{-1}$] were found with the other NAT substrates. Although we cannot distinguish the specific contributions of BanatB and BanatC to the NAT activity of the extracts (separation of the extracts on a chromatography column proved difficult due to the low stability of the proteins in the extracts), our results indicate that the BanatC enzyme, at least, is functional, as 4 AS (not acetylated by recombinant BanatB) was acetylated by the extracts.

DISCUSSION

B. anthracis is a bacterium of significant medical importance since it is the causative agents of food poisoning and anthrax disease (*25*, *43*).

We have described, at the molecular level, three NAT homologues from *B. anthracis* (BanatA, BanatB, and BanatC). Sequence and phylogenetic analyses show that BanatA, BanatB, and BanatC proteins are paralogs that possess all the described NAT-specific functional motifs (*20*, *41*). The cross-reactivity of BanatA, BanatB, and BanatC with an antibody against Stnat further supports the idea that these three proteins are related to bacterial NAT enzymes (*13*, *14*). Functional assays using typical NAT substrates (Table 1) showed that BanatB and BanatC were able to catalyze the AcCoA-dependent acetylation of typical NAT arylamine substrates, thus confirming that these two isoforms are functional NAT enzymes. However, differences in substrate preferences and rates of acetylation were found (Table 1). BanatC acetylated seven of the eight NAT substrates that were tested, whereas BanatB acetylated only three arylamine substrates. Such differences in substrate preferences and acetylation rates have also been reported for mammalian NAT paralogs and for the two *Me. loti* NAT enzymes, where one isoenzyme is catalytically more efficient than the others toward the same aromatic substrates (*4*, *14*, *42*, *44*). Like most bacterial NAT enzymes characterized to date, both BanatB and BanatC were unable to significantly acetylate PABA, an endogenous arylamine that can be used as a growth factor by several prokaryotes (*13*, *45*). However, as opposed to other characterized bacterial NATs (*12*−*14*, *28*), both BanatB and BanatC were unable to significantly acetylate INH, an antitubercular drug (Table 1). Therefore, these results show that BanatB and BanatC have functional differences, in particular with other cloned bacterial NAT enzymes (*13*, *14*). In our hands, BanatA was devoid of any NAT activity toward the eight typical NAT substrates that were tested. The presence of a Glu residue instead of an Asp in the catalytic triad of Banat is unlikely to explain the lack of activity since certain enzymes function with a Glu residue in their triad (*23*). A lack of NAT activity is most likely due to BanatA being a naturally truncated NAT homologue with the smallest C-terminal α/β-lid domain found in any NAT family enzyme (*4*, *23*). This is in agreement with studies using human NAT1 and truncated mutants of Stnat showing that the removal of the C-terminal domain led to enzymes devoid of acetyltransferase activity

(*24*, *46*). These studies also suggested that NAT homologues with shorter or no C-terminal domains could behave as AcCoA hydrolases (*24*). We found no hydrolytic activity toward AcCoA or the nonspecific hydrolase substrate PNPA. These results suggest that BanatA is likely to be a new NAT homologue that lacks NAT and AcCoA hydrolysis activity. The *A. mediterranei* RifF enzyme is a NAT homologue devoid of NAT activity. RifF belongs to the Rif cluster of genes and catalyzes the last step of the synthesis of the rifamycin antibiotic (*22*). Sequence analysis of BanatA (Figure 1) and of the *B. anthracis* genome (*47*) indicates that this NAT homologue is unlikely to be the *B. anthracis* counterpart of RifF. RT-PCR analysis indicates that *banatA* is expressed in *B. anthracis* (Figure 4A), although the BanatA protein could not be detected in *B. anthracis* extracts at the level of sensitivity attained in Western blotting using the anti-Stnat antibody. Overall, it appears that BanatA could be a second example of a bacterial NAT homologue devoid of NAT activity. However, we cannot rule out the possibility that *banatA* is a pseudogene. Future studies will clarify this point.

We found that *banatB* and *banatC* were endogenously expressed at the mRNA and protein levels in *B. anthracis* (Figure 4). The two enzymes were readily detected by Western blotting, and NAT activity could also be readily measured in *B. anthracis* extracts with different NAT substrates. Although we cannot distinguish the specific contribution of BanatB and BanatC to the NAT activity of the extracts, our results show that BanatC, at least, is functional, as 4 AS (not metabolized by recombinant BanatB) was readily acetylated by the extracts. We found that in addition to its NAT activity, BanatB also exhibited AcCoA and PNPA hydrolytic activity at rates higher than those found for truncated Stnat (*24*). No significant hydrolytic activity toward AcCoA or PNPA could be measured with BanatC. As hypothesized by Mushtaq et al. (*24*), it appears therefore that certain NAT enzymes with short C-terminal domains may also behave as AcCoA hydrolases in the absence of an arylamine substrate. BanatB is thus the first "natural" example of such a NAT enzyme.

Our study indicates that BanatC is a functional NAT enzyme. More importantly, we found that BanatC was able to acetylate the sulfonamide antibiotic SMX (Table 1). Further kinetic analysis (Table 2) indicated that, in vitro, this *B. anthracis* NAT enzyme had a higher catalytic efficiency toward SMX than the human NAT1 isoform. Expression studies of BanatC in *E. coli* strain DH5α as modified from refs *39* and *40* clearly indicated that the active enzyme affords higher-than-normal resistance to SMX in recombinant bacteria when compared to the inactive form of the enzyme (Figure 3). Since most of the *B. anthracis* strains isolated to date display resistance to SMX (*27*), the expression of a NAT isoform that efficiently inactivates SMX, such as BanatC, could contribute to the resistance to this antibiotic. Interestingly, *M. tuberculosis* expresses a NAT enzyme that contributes to its resistance against INH (a major antitubercular drug) through acetylation of this antibiotic (*12*, *21*, *48*). It is known that bacterial resistance to SMX involves distinct molecular mechanisms such as point mutations in the dihydropteroate synthase (DHPS) gene of *B. anthracis* (*49*). Like NAT enzymes, DHPS is an enzyme involved in folate metabolism (*49*). Therefore, expression of BanatC could represent an additional mechanism contributing to SMX resistance in *B. anthracis*.

In conclusion, we show that *B. anthracis* expresses new homologous *NAT* genes. These three NAT homologues exhibit structural and functional properties not reported earlier. So far, it is not known why a restricted number of bacteria express more than one *NAT* homologue; however, the presence of two or three *NAT* genes in mammals has been suggested to account for enzymatic specialization (*50*, *51*). This may reflect the need for adaptation to the widely diverse environments bacteria use as their natural habitat (*4*).

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