Arylamine N-Acetyltransferase Responsible for Acetylation of 2-Aminophenols in *Streptomyces griseus* \(^{\dagger, \ddagger}\)

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An arylamine N-acetyltransferase (NAT) responsible for the N acetylation of exogenous 3-amino-4-hydroxybenzoic acid in *Streptomyces griseus* was identified and characterized. This enzyme was distinct from other eukaryotic and bacterial NATs in that it acetylated various 2-aminophenol derivatives more effectively than it acetylated 5-aminosalicylic acid, and thus it may be involved in the metabolism of xenobiotic compounds.

Arylamine N-acetyltransferases (NATs) (EC 2.3.1.5) are cytosolic enzymes catalyzing the transfer of an acetyl group from acetyl coenzyme A (CoA) to the nitrogen or oxygen atom of arylamines and hydrazines and their N-hydroxylated metabolites (3). Human NAT2 was first identified in the 1960s on the basis of its ability to acetylate and hence inactivate isoniazid (INH), the front-line antitubercular drug (7). NAT was also identified in a bacterium, *Salmonella enterica* serovar Typhimurium, as a factor influencing carcinogen susceptibility in a mutagenicity test (14, 23). Since then, NAT activity has been found in more than 25 bacterial species (6), and genome database analysis has predicted the presence of putative NAT genes in many bacteria (3). Thus, NATs are present in a wide range of eukaryotes and prokaryotes, including *Escherichia coli* (5), *Pseudomonas aeruginosa* (1), *Streptomyces griseus* (24), and the causative agents of tuberculosis, *Mycobacterium tuberculosis* (11) and *Mycobacterium bovis* (1). Importantly, bacterial NATs, like human NAT2, are able to inactivate INH. Overexpression of *nat* from *M. tuberculosis* confers increased resistance to INH in *Mycobacterium smegmatis* (11), which is a nonpathogenic mycobacterium that is used as a model for *M. tuberculosis*, whereas a *nat* knockout strain of *M. smegmatis* has increased sensitivity to the drug (12). Because *nat* is required for synthesis of normal mycolic acid and complex lipids in *M. bovis* BCG (2), NAT, particularly in mycobacteria, has the potential to be a drug target (2). Recent studies have shown that bacterial NATs acetylate not only INH but also numerous arylamines, including important drugs. Of the substrates of bacterial NATs identified so far, 5-aminosalicylate (5-AS), a drug used in the treatment of inflammatory bowel diseases, is one of the most preferred substrates (6, 24). Thus, bacterial NATs have the ability to detoxify xenobiotic compounds, and identification of bacterial NATs and their substrates still receives considerable attention (4).

The filamentous, soil-inhabiting, gram-positive bacterial genus *Streptomyces* is characterized by the ability to produce a wide variety of secondary metabolites. Grixazone is one of the secondary metabolites produced by *Streptomyces griseus*. During our study of grixazone biosynthesis (19, 20), we found that *S. griseus* has distinct NAT activity. In some mutant and recombinant *S. griseus* strains, 3-amino-4-hydroxybenzoic acid (3,4-AHBA) and 3-amino-4-hydroxybenzaldehyde, both of which are intermediates of grixazone synthesis, were acetylated (19, 20). There have been no reports concerning NATs from *Streptomyces* species other than a description of the gene encoding a NAT-like protein found in the rubradirin biosynthesis gene cluster in *Streptomyces achrromagens* (18). In this paper, we describe the N acetylation of exogenous 3,4-AHBA by a NAT in *S. griseus* and properties of the NAT. This enzyme catalyzed the N acetylation of various 2-aminophenol derivatives more effectively than it catalyzed the N acetylation of INH or 5-AS, providing important information that is useful for understanding the role of Streptomyces NATs.

N acetylation of exogenous 3,4-AHBA in *S. griseus*. We recently found that two genes, *griI* and *griH* in the grixazone biosynthesis gene cluster, are responsible for the biosynthesis of 3,4-AHBA from two primary metabolites, L-aspartate-4-semialdehyde and dihydroxyacetone phosphate (20). A recombinant *S. griseus* strain overexpressing *griI* and *griH* produced 3-acetylamino-4-hydroxybenzoic acid (3,4-AcAHBA) in addition to a large amount of 3,4-AHBA. To confirm that 3,4-AcAHBA was produced by acetylation of 3,4-AHBA that had been synthesized by the action of GriI and GriH, we examined bioconversion of exogenous 3,4-AHBA to 3,4-AcAHBA by the wild-type *S. griseus* cells. *S. griseus* IFO13350 (8) was cultured at 30°C for 2 days in 100 ml of YPD liquid medium (19), and then 3,4-AHBA was added to the culture at a final concentration of 1 mM. Under the cultivation conditions used, no detectable 3,4-AHBA was produced endogenously by strain IFO13350. After the cells were incubated for an additional 2 days, compounds in the culture broth were analyzed by reversed-phase high-performance liquid chromatography (HPLC), as described previously (20) (Fig. 1C). As shown in Fig. 1C, the culture broth contained 3,4-AHBA (0.48 mM) and 3,4-AcAHBA (0.49 mM), and the amount of 3,4-AcAHBA produced was stoichiometrically equivalent to the decrease in the amount of 3,4-AHBA. Therefore, the 3,4-

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AHBA added was bioconverted to 3,4-AcAHBA by *S. griseus* cells.

We next examined the acetylation of 3,4-AHBA by using a cell lysate of *S. griseus*. The cell lysate was prepared from *S. griseus* cells cultured in YPD medium at 30°C for 4 days by the procedure described below for preparation of lysate from *Escherichia coli* cells. Incubation of the cell lysate with 1 mM 3,4-AHBA and 1 mM acetyl-CoA at 30°C for 16 h resulted in the N acetylation of 3,4-AHBA (data not shown). Almost all N-acetylation activity was found in the cytoplasmic fraction, since negligible N-acetylation activity was found in the culture supernatant (total activity in the cell lysate from 1 liter of culture, 590 U; total activity in 1 liter of the culture supernatant, 0.8 U). These results showed that the N acetylation of 3,4-AHBA was catalyzed by a cytosolic, acetyl-CoA-dependent acetyltransferase(s). Addition of 3,4-AHBA to the culture did not enhance the N-acetylation activity, showing that the acetyltransferase(s) was not inducible by the substrate. The N-acetylation activity was also observed in a cell lysate of *S. griseus* HH1 (10), a strain deficient in A-factor production. This result suggested that the acetyltransferase gene(s) was expressed independent of A-factor, which is a microbial hormone triggering production of almost all secondary metabolites in *S. griseus* (9). Therefore, it is unlikely that the N-acetylation enzyme is involved in the biosynthesis of a certain secondary metabolite.

**Putative nat gene in *S. griseus***. Because the acetyltransferase(s) responsible for the N acetylation of 3,4-AHBA in *S. griseus* used acetyl-CoA as the acetyl donor and because the acetyl acceptor, 3,4-AHBA, is an arylamine, we expected that the enzyme(s) would be a NAT homologue(s). We searched the genome sequence of *S. griseus*IFO13350 (our unpublished data) for open reading frames encoding a NAT homologue. A single open reading frame, tentatively designated *natA*, which encoded a 270-amino-acid protein exhibiting end-to-end similarity to eukaryotic and bacterial NATs, was found (see Fig. S1 in the supplemental material). We designated the protein SgNAT. SgNAT exhibited 35 to 40% amino acid identity to many bacterial NATs, whereas the sequence of the third domain of SgNAT (amino acids 184 to 270), which is thought to be the key to acetyl acceptor binding and specificity (15), exhibited greater sequence divergence. Cys-69, His-108, and Asp-125 of SgNAT were assumed to form the catalytic triad. The gene organization in the neighborhood of *natA* is shown in Fig. 1A. Although *natA* is located upstream of the gene encoding a putative aspartate aminotransferase in the same orientation, it is likely that *natA* is transcribed alone because the distance between *natA* and the putative aspartate aminotransferase gene is 347 bp.

While the endogenous role of most bacterial NATs remains to be established (16), some bacterial NAT-like proteins are involved in the biosynthesis of macrolide compounds. The rifamycin B biosynthesis gene cluster of the actinomycete *Amycolatopsis mediterranei* contains a gene (*rifF*) encoding a NAT-like protein. However, recombinant RifF lacked any measurable INH acetylation activity (13). RifF catalyzes the release of the completed polyketide from the rifamycin type I polyketide synthase by intramolecular amide formation, yielding proansamycin X (26). Intramolecular amide formation appears to proceed by a reaction mechanism similar to that of the *N* acetylation by NAT. The rubradirin and ansamitocin biosynthesis gene clusters of *Amycolatopsis mediterranei* and *Actinosynema pretiosum* (25), respectively, also contain a gene encoding a NAT-like protein, which is probably responsible for the intramolecular amide formation of the ansamycin precursor. Payton et al. (13) described the possible involvement of Gly-130 corresponding to the highly conserved Gly-126 in *S. enterica* serovar Typhimurium NAT in acetyl-CoA binding. The Gly residue is situated adjacent to the active site Cys-69 residue in the crystal structure of *S. enterica* serovar Typhimurium NAT (17) and is highly conserved in NATs but not in the NAT-like proteins in actinomycetes. Because SgNAT contains Gly-130 corresponding to the highly conserved Gly residue (see Fig. S1 in the supplemental material) and because *natA* is probably not a member of a gene cluster for a certain metabolite, we assumed that SgNAT has NAT activity and uses acetyl-CoA as the acetyl donor, as occurs widely in organisms.

**Disruption of the chromosomal *natA* gene**. To determine whether *natA* is responsible for the *N* acetylation of 3,4-AHBA, we generated a *natA* disruptant by replacing most of *natA* with a kanamycin resistance gene, *aphIII* (Fig. 1A). Two-
kilobase fragments upstream and downstream of natA were amplified by PCR and cloned into pCR4Blunt-TOPO (Invitrogen), resulting in pΔnatA-up and pΔnatA-down, respectively. To amplify the upstream region, primers 5'-GCATAATGGTTGACGGCGACCAC-3' (with an NdeI site underlined) and 5'-GAAGCTTCGGGCAAATACCGTCG-3' (with a HindIII site underlined) were used. To amplify the downstream region, primers 5'-GCATAATGGTTGACGGCGACCAC-3' (with an NdeI site underlined and a HindIII site in boldface) and 5'-GCCCAATTCGGTCG-3' were used. The upstream region was excised with NdeI and HindIII from pΔnatA-up and placed between the NdeI and HindIII sites of pΔnatA-down, together with a 1.1-kb HindIII fragment carrying the kanamycin resistance gene (aphII) from Tn5, resulting in pΔnatA. The resulting plasmid was digested with EcoRI, alkaline denatured, and introduced by protoplast transformation into the wild-type S. griseus strain. A kanamycin-resistant transformant was isolated, and the correct replacement of the deleted natA sequence with the intact chromosomal natA sequence, as a result of double crossover, was checked by Southern hybridization with appropriate probes (Fig. 1B). The natA disruptant grew normally and formed aerial hyphae and spores with the same time course as the wild-type strain. As we expected, no bioconversion of exogenous 3,4-AHBA to 3,4-AcAHBA occurred in the natA disruptant (Fig. 1C). Therefore, we concluded that SgNAT was solely responsible for the N acetylation of exogenous 3,4-AHBA.

There was no difference in sensitivity to 3,4-AHBA between the wild-type and natA-disrupted strains. Both strains exhibited poor growth on YPD agar medium supplemented with 2.5 mM 3,4-AHBA or 3,4-AcAHBA (data not shown), showing that the toxicity of 3,4-AcAHBA for S. griseus is almost the same as that of 3,4-AHBA and that SgNAT makes no apparent contribution to 3,4-AHBA resistance in S. griseus. Because disruption of natA resulted in no apparent phenotypic change, the endogenous role of SgNAT in S. griseus remains unknown, like the endogenous roles of most bacterial NATs. However, it is possible that SgNAT is involved in the detoxification of some other xenobiotic compounds.

**Production of recombinant SgNAT by E. coli**

To elucidate the catalytic properties of SgNAT, decahistidine-tagged SgNAT was purified to homogeneity by Ni²⁺ ion affinity chromatography and gel filtration column chromatography from E. coli BL21(DE3)/pLysS harboring pET-natA, which contained natA under control of the T7/lac promoter. To construct pET-natA, the natA sequence was amplified by PCR using primers 5'-GCATAATGGTTGACGGCGACCAC-3' (with the start codon of natA in boldface and an NdeI site underlined) and 5'-GCCCAATTCGGTCG-3' (with the stop codon of natA in boldface and an XhoI site underlined). The amplified fragment was digested with NdeI and XhoI and placed between the NdeI and XhoI sites of pET-16b (Novagen). The E. coli cells harboring pET-natA were cultured at 26.5°C for 24 h in LB medium supplemented with 50 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 1% lactose, allowing constant expression of the T7/lac promoter. The harvested E. coli cells were suspended in buffer A (50 mM sodium phosphate [pH 8.0], 0.5 M NaCl, 20% glycerol, 7.5 mM 2-mercaptoethanol) containing 10 mM imidazole and 2 mg/ml lysozyme and disrupted by sonication. The cell lysate obtained by centrifugation was applied to a Ni²⁺-nitritriacetic acid spin column (QIAGEN) equilibrated with buffer A containing 10 mM imidazole. The column was washed successively with buffer A containing 50 mM imidazole and buffer B (50 mM sodium phosphate [pH 8.0], 7.5 mM 2-mercaptoethanol) containing 100 mM imidazole. Recombinant SgNAT, having the structure Met-Gly-(His)₉-Ser-Ser-Gly-His-Ile-Glu-Arg-His-SgNAT, was eluted with buffer B containing 250 mM imidazole from the column. The buffer of the enzyme solution was changed by chromatography on a gel filtration column (Superdex 75 10/30; Amersham) with isocratic elution in buffer C (50 mM sodium phosphate [pH 7.0], 0.15 M NaCl, 7.5 mM 2-mercaptoethanol). Proteins were quantified by determining the absorbance of the protein solution at 280 nm using the molar absorbance coefficient 33,000 M⁻¹ cm⁻¹, which was calculated on the basis of the amino acid sequence.

The apparent molecular mass of the recombinant SgNAT was 34 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2A). The NAT of S. enterica serovar Typhimurium forms an active dimer during storage of the purified recombinant protein for 2 weeks in vitro (15). Gel filtration column chromatography of purified SgNAT which had been stored at 4°C for 48 h after preparation of the cell lysate resulted in a single peak at a retention time representing a molecular mass of 40 kDa. This peak was also predominant (>90%) when chromatography was performed after 2 weeks, although some minor peaks were observed at retention times representing molecular masses of 51 to 71 kDa. These results show that, in contrast to the observation for the NAT of S. enterica serovar Typhimurium (15), SgNAT is a stable, enzymatically active monomer for at least 2 weeks.

**Substrate specificity of SgNAT.** We investigated the acetyl transfer activity of the recombinant SgNAT by using several compounds as substrates (Table 1). The standard reaction mixture (100 μl) consisting of 50 mM sodium phosphate (pH 7.0), 0.4 mM acetyl-CoA, 2 mM acyl acceptor, and the enzyme solution was incubated at 30°C for 20 min. The acetylated products were identified by liquid chromatography-electro-
spray mass spectrometry in positive or negative electrospray ionization mode (20). The amount of the arylamine substrate remaining in the reaction mixture was determined by a colorimetric method using 4-dimethylaminobenzaldehyde (24). The reaction was stopped by addition of 100 µl of aqueous trichloroacetic acid (20%, wt/vol) at 0°C. After the mixture had been cleared by centrifugation, 800 µl of 5% 4-dimethylaminobenzaldehyde in acetonitrile-water (9:1, vol/vol) was added, and the absorbance at 450 nm was determined by spectrometry with a SPECTRA MAX plus (Molecular Devices). The amount of INH remaining in the reaction mixture was determined by HPLC.

In most cases, INH and 5-AS have been used as substrates for bacterial NATs. Like most bacterial NATs (6, 13, 24), SgNAT was able to acetylate 5-AS and INH (Table 1). Since we found SgNAT because of its N-acetylation activity with 3,4-AHBA, we analyzed the acetyl transfer activity of SgNAT by using 3,4-AHBA as a substrate. SgNAT acetylated 3,4-AHBA as a substrate that was preferable to 5-AS, and the catalytic constant \( k_{cat} \) for 3,4-AHBA was 60-fold larger than that for 5-AS (Table 1). Therefore, we examined the acetyl transfer activity of SgNAT with various 2-aminophenol derivatives, which contained various functional groups at the para position with respect to the hydroxyl group of 2-aminophenol, although 2-aminophenol derivatives, including 3,4-AHBA, have not been determined to serve as substrates for eukaryotic or prokaryotic NATs. The compounds that we tested were 2-aminophenol, 2-aminophenol, 2-aminophenol, 3-aminophenol, 4-hydroxybenzaldehyde, and 3-amino-4-hydroxybenzensulfonic acid. All these compounds, especially 3,4-AHBA and 2-aminophenol, were good substrates for SgNAT. The other hand, 4-aminophenol, 3-aminophenol, and 4-hydroxybenzoic acid, which contained a carboxyl group at the para position with respect to the amino group of 2-aminophenol, was a poor substrate. These results indicate that the position of the substituent on 2-aminophenol is important for the substrate specificity of SgNAT. The hydroxyl group at the ortho position with respect to the amino group of 2-aminophenol should be particularly important because the NAT showed negligible activity with 3-aminobenzoic acid, aniline, and 5-AS, corresponding to a 3,4-AHBA analogue containing the hydroxyl group at the para position with respect to the amino group. SgNAT showed no detectable activity with 4-aminobenzoic acid, which is a typical substrate for human NAT1 (22) and a poor substrate for most prokaryotic NATs (6, 13), with the notable exception of the NAT of P. aeruginosa (24). Thus, SgNAT exhibited much higher levels of activity with 2-aminophenol derivatives (Fig. 2B) than with other substrates, such as INH and 5-AS. The differences in the catalytic constants for 2-aminophenol derivatives and other substrates are attributable mainly to the differences in the \( k_{cat} \) values and not to the differences in the \( K_m \) values.

Is the extreme preference for 2-aminophenol as acetyl acceptors found in many other NATs or specific to SgNAT? To our knowledge, there has been only one report indicating that 2-aminophenol is used as a substrate for a NAT. Takenaka et al. (21) examined the NAT activity in Bacillus cereus strain PDe-1 by biotransformation of various arylamines by using resting cells. The rate of transformation of 2-aminophenol by the strain in 24 h was reported to be 30.1%, while the rate of transformation of 5-AS was 100%. Although it is necessary to examine the N-acetylation activities of other NATs with 2-aminophenols, we assume that the substrate specificity of SgNAT is very different from the substrate specificities of other bacterial NATs.

In conclusion, we identified the natA gene responsible for the N acetylation of exogenous 3,4-AHBA in S. griseus. SgNAT, encoded by natA, is capable of acetylating various 2-aminophenol derivatives as substrates that are much more preferable than INH and 5-AS. The extreme preference for 2-aminophenols as acetyl acceptors is characteristic of SgNAT. This finding provides new insight into the substrate specificities of eukaryotic and bacterial NATs and should assist in identifying the endogenous roles of bacterial NATs (4).

**Nucleotide sequence accession number.** The nucleotide sequence reported in this paper has been deposited in the DDBJ, EMBL, and GenBank DNA databases under accession number AB280932.
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REFERENCES


