An acetyltransferase conferring tolerance to toxic aromatic amine chemicals: molecular and functional studies

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ABSTRACT

Aromatic amines (AA) are a major class of environmental pollutants which have been shown to have genotoxic and cytotoxic potentials towards most living organisms. Fungi are able to tolerate a diverse range of chemicals compounds including certain AA and have long been used as models to understand general biological processes. Deciphering the mechanisms underlying this tolerance may improve our understanding of the adaptation of organisms to stressful environments and pave the way for novel pharmaceutical and/or biotechnological applications. We have identified and characterized two arylamine N-acetyltransferase (NAT) enzymes (PaNAT1 and PaNAT2) from the model fungus Podospora anserina that acetylate a wide range of AA. Targeted gene disruption experiments revealed that PaNAT2 was required for the growth and survival of the fungus in the presence of toxic AA. Functional studies using the knock-out strains and chemically-acetylated AA indicated that tolerance of P. anserina to toxic AA was due to the N-acetylation of these chemicals by PaNAT2. Moreover, we provide proof-of-concept remediation experiments where P. anserina, through its PaNAT2 enzyme, is able to detoxify the highly toxic pesticide residue 3,4-dichloroaniline in experimentally contaminated soil samples. Overall, our data show that a single xenobiotic-metabolizing enzyme (XME) can mediate tolerance to a major class of pollutants in a eukaryotic species. These findings expand the understanding of the role of XME and in particular of NATs in the adaptation of organisms to their chemical environment and provide a basis for new systems for the bioremediation of contaminated soils.

INTRODUCTION

Aromatic amines (AA) represent one of the most important classes of occupational or environmental pollutants. Many AA are toxic to most living organisms due to their genotoxic or cytotoxic properties (1). AA account for 12% of the 415 chemicals which are either known or strongly suspected to be carcinogenic in humans (2). AA are common by-products of chemical manufacturing (pesticides, dyestuffs, rubbers or pharmaceuticals), coal and gasoline combustion or pyrolysis reactions (3). Moreover, the presence of AA in groundwater or soil samples subject to industrial, agricultural or urban pollution is of increasing concern, particularly for persistent toxic AA contaminants, such as pesticide-derived anilines (4).

The identification of mechanisms by
which living organisms can tolerate harmful chemicals, such as AA, is of prime importance to understand their adaptation to stressful environments. In addition, deciphering the molecular mechanisms underlying this tolerance may lead to novel biotechnological and pharmaceutical applications.

Fungi are environmentally ubiquitous and are found with great diversity in both terrestrial and aquatic environments. Fungi are known to tolerate a large range of chemicals of natural or anthropogenic origin by developing mechanisms to act on xenobiotic and natural compounds (5,6). Fungi are therefore good models to identify and to understand tolerance mechanisms to xenobiotics (7,8). Moreover, characterization of the mechanisms by which fungi tolerate certain toxic xenobiotics can potentially lead to the identification of new targets for the treatment of fungal infections in vertebrates (7,8) or plants and to the development of new bioremediation tools for cleaning up contaminated environments (5,9).

Using the common ascomycete Podospora anserina as a model, we provide here the demonstration that a single enzyme can mediate tolerance to toxic AA chemicals in a eukaryotic species. This enzyme was identified and characterized as an arylamine N-acetyltransferase (NAT), a xenobiotic-metabolizing enzyme (XME) that acetylates efficiently several toxic AA. Targeted-disruption of this NAT gene led to the complete loss of tolerance to AA thus confirming that this enzyme enables the fungus to detoxify AA that would otherwise prove toxic. These findings will help to understand the enzymatic mechanisms contributing to adaptation of living organisms to their environment. In particular, our data demonstrate that the NAT-dependent detoxification mechanisms may provide a eukaryotic organism with tolerance to toxic AA. Moreover, we provide proof-of-principle experiments using soils contaminated with the highly toxic pesticide residue 3,4-dichloroaniline, that fungal NAT-dependent detoxification pathway may represent a novel model with reasonable cost and a low environmental impact for the bioremediation of AA-contaminated environments.

**EXPERIMENTAL PROCEDURES**

**Strains, culture conditions, basic protocols and phenotypic characterization** - The S strain of P. anserina was used for all experiments. The culture conditions for this organism have been described elsewhere (10). The methods currently used for genetic analysis, the extraction of nucleic acids and proteins, and genetic transformation are available from http://podospora.igmors.u-psud.fr. P. anserina was grown in the well-defined M2 synthetic medium providing the wild-type (WT) strain with optimal growth conditions. Phenotypic analyses were performed on WT and mutant (ΔPaNat1, ΔPaNat2 single mutants and ΔPaNat1/2 double mutants) P. anserina strains. The vegetative part of the life cycle was investigated in laboratory conditions, by assessing growth rate and mycelial morphology — presence or absence of aerial hyphae and accumulation of pigments, as described for other mutants (11). Senescence and other types of cell degeneration were evaluated by measuring life span and investigating "crippled growth" as previously described (12). Hyphal interference was evaluated by placing P. anserina mycelia in the presence of Penicillium chrysogenum and Coprinopsis cinerea (13). Completion of the sexual cycle was investigated by measuring male and female fertility, perithecium maturation and content, and the timing of ascospore germination (11).

**Chemical synthesis of N-acetylated AA** - The acetylated forms of 3,4-DCA, 2-AF, and 4-BOA were synthesized from 3,4-DCA, 2-AF and 4-BOA (Sigma) using acetic acid chloride in presence of triethylamine (base). Column chromatography purification was carried out on silica gel 60 (70-230 mesh ASTM, Merck). The structure of all compounds was confirmed by IR and NMR spectra. IR spectra were obtained in paraffin oil with a ATI Mattson Genesis Series FTIR spectrometer; 1H NMR spectra were recorded at 200 MHz and 50 MHz respectively, in CDCl3, on a BRUKER AC 200 spectrometer using hexamethyldisiloxane (HMDS) as an internal standard. Yields of acetylated products were around 80%.

**Production and purification of recombinant proteins** - E. coli C41 (DE3) cells containing pET-28A-based plasmids encoding PaNat1 or PaNat2 were used to produce and purify 6xHis-tagged recombinant proteins. The purified PaNat1 and PaNat2 enzymes were reduced with 10 mM dithiothreitol (DTT) before dialysis against 25 mM Tris–HCl, pH 7.5, 1 mM EDTA. Yields of recombinant proteins, basic protocols and phenotypic characterization are described elsewhere.
proteins were in general 15-20 mg per liter of bacterial culture.

SDS-PAGE and western blot analysis- Protein samples were separated by SDS/PAGE and transferred to a nitrocellulose membrane for western blot analysis. An antibody raised against the Salmonella typhimurium NAT enzyme was used to detect the purified recombinant PaNat1 and PaNat2 proteins (14).

Deletion of PaNat1 and PaNat2 and complementation of the ΔPaNat2 mutation by the PaNat2′ gene- The null ΔPaNat1 and ΔPaNat2 alleles were constructed separately, using the approaches indicated in supplementary Fig. 1. The native PaNat1 and PaNat2 genes were replaced by recombinant defective alleles in which all or most of the coding sequences were replaced by the hygromycin B and phleomycin selectable markers, respectively. Tests carried out with the hygromycin B- and phleomycin-resistant strains obtained after transformation of the WT with the marker genes showed that neither of these genes interfered with resistance/sensitivity to AA. Independent hygromycin B- or phleomycin-resistant transformants were tested for homologous integration of the defective alleles at the PaNat1 and PaNat2 loci, by appropriate PCR and Southern blot analyses (see supplementary Fig. 1). ΔPaNat1/2 double mutants were recovered from the progenies of crosses between ΔPaNat1 mutants and ΔPaNat2 mutants of opposite mating types. The ΔPaNat2 mutation was complemented by cotransforming the ΔPaNat2 mutant with a PCR-amplified DNA fragment encompassing the PaNat2 gene and the pBC-hygro vector (15). Six of the 16 transformants tested were resistant to AA, whereas none was resistant in the control transformation carried out with the vector only, in the absence of the PaNat2 gene. Genetic analysis of four randomly picked transformants showed that resistance to AA cosegregated with the hygromycin B resistance marker, confirming that the restoration of AA resistance was due to the introduced PaNat2 gene.

Extract preparation and enzyme assays- Fungi were grown for two days at 27°C in M2 liquid medium supplemented with 2.5 mg/ml yeast extract. The fungal mass was then harvested under sterile conditions by filtration. Typically ~1.5 g of fungal dry mass was obtained from each 100 ml culture. Total extracts were prepared by grinding fungal pellets in Tris-HCl 25 mM, pH 7.5 supplemented with 0.1 % Triton X-100, 1 mM DTT and protease inhibitors. The resulting suspensions were subjected to ultracentrifugation at 100,000 g for 1 hour. The supernatants were removed and used for enzyme assays.

Remediation studies- WT or ΔPaNat1/2 mutant P. anserina (1.5 g of fungal dry mass) was grown in 25 ml of M2 medium broth in the presence or absence of DCA (400 µM), for 2 days at 27°C. The medium was centrifuged and filtered (0.22 µM pores) and used for the preparation of agar plates (50% conditioned medium). Sterilized Lactuca sativa seeds were germinated on agar plates and photographed after seven days (room temperature, illumination for 12 h per day). For the remediation of 3,4-DCA (25 mg/kg) contamination in soil (Terre Végétale NF-U44-551, Truffaut, Paris), soil samples (3g) were inoculated with WT or ΔPaNat1/2 mutant P. anserina (0.3 g or 0.9 g of fungal dry mass) and incubated for 2 days at 25°C. Aliquots (0.5 g) of soil were mixed with absolute ethanol at different time points, for the quantification of 3,4-DCA and acetyl-3,4-DCA by HPLC. For the germination and growth of L. sativa seeds in soil, contaminated soil samples (20 g per pot, 80 mg/kg 3,4-DCA) were inoculated three times (every 24 h) with 0.5 g of WT or ΔPaNat1/2 strains and incubated for 72 h at 25°C. Seeds (20) were then sown in soil samples and allowed to germinate and grow at 25°C for 8 days (illumination for 12 h per day). Controls were carried out with acetyl-3,4-DCA (80 mg/kg) and H2O.

Enzyme assays and detection of acetylated aromatic amines by HPLC- NAT activity was measured in the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay, as previously described (16). Recombinant enzymes and aromatic amine substrates (500 µM final concentration) in assay buffer (25 mM Tris-HCl, pH 7.5) were incubated for 5 minutes at 37°C in a 96-well plate. AcCoA (400 µM final concentration) was added and the plate was incubated at 37°C (for up to 30 minutes). The reaction (100 µl total volume) was quenched with 25 µl of guanidine hydrochloride solution (6.4 M guanidine/HCl, 0.1 M Tris-HCl, pH 7.5) supplemented with 5 mM DTNB and
absorbance was measured at 405 nm. Kinetic analyses were performed by varying the aromatic amine substrate concentrations. Kinetic constants were determined by nonlinear regression analysis, with the Kaleidagraph program (Synergy Software, USA). The rate of acetylation of aromatic amines by fungal extracts was measured by HPLC, as previously described (17). N-acetylated aromatic amines were detected in samples (M2 liquid medium) or ethanol eluates of soil, by HPLC on a C18 column, using 60% sodium perchlorate (20 mM, pH 3) and 40% acetonitrile as the mobile phase. Chemically-acetylated AAs (acetylated-3,4-DCA; acetylated-2AF and acetylated-BOA) were synthesized as reported above and used as HPLC standards to identify enzymatically-acetylated AAs in samples.

Statistical analysis: Data are expressed as the mean±SD of three independent experiments, with quadruplicate assays for each experiment. Student's t-test was used to determine the statistical significance of differences between means. Significance was defined as a P ≤ 0.05.

RESULTS AND DISCUSSION

Identification and characterization of P. anserina NAT enzymes and their activity towards aromatic amine substrates: AA are an important class of chemicals that are used in manufacturing of pesticides, dyes and pharmaceuticals. AA are also found in tobacco smoke and food pyrolysis products (18). Many AA are toxic for living organisms and several AA are recognized as carcinogens. It has been suggested that certain soil fungi can detoxify and tolerate toxic AA (19). Therefore, fungal models are of interest to identify and understand mechanisms responsible for tolerance to toxic AA in eukaryotic organisms (7,8).

Four well-known fungal species (Fusarium graminearum, Phycomyces blakesleeanus, Podospora anserina and Rhizopus oryzae) from different ecosystems, for which complete genome sequences were available were screened for radial growth in the presence of three toxic AA: 3,4-dichloroaniline (3,4-DCA: pesticide residue), 2-aminofluorene (2-AF: carcinogen) and 4-butoxyaniline (4-BOA: chemical intermediate) (14). The growth of R. oryzae and P. blakesleeanus was almost completely abolished by these three AA at concentrations of 100 to 250 µM in standard minimal growth medium. In the same conditions, little effect on growth was observed in the other two species studied (P. anserina and F. graminearum) (data not shown), suggesting that these two species have mechanisms of tolerance to AA. We aimed to decipher the mechanisms underlying fungal survival in AA-contaminated environments.

Tolerance to potentially toxic xenobiotics often depends on specific biotransformation pathways catalyzed by endogenous enzymes and in particular XME, such as cytochromes P450 or glutathione S-transferases. AA may be biotransformed via several routes, involving different types of XME, in particular arylamine N-acetyltransferases (NAT) (20). In eukaryotes, NAT enzymes have long been known to biotransform AA (20). However, it is still unclear what role NAT enzymes actually play in either preventing or enhancing toxic response to AA (21). So far the studies on the knockout (KO) NAT mouse models (21-23) have not demonstrated clearly the relevance of this pathway to AA tolerance in living organisms.

BLAST analysis indicated that P. anserina had two putative NAT enzymes and that F. graminearum had three such enzymes, whereas the two sensitive fungi had no genes encoding NAT enzymes (see Supplementary Table 1 and supplementary Fig. 2). A broader BLAST screening of the complete genome sequences of eumycete fungi showed that many of these fungi had NAT genes (see Supplementary Table 1 and supplementary Fig. 2). We investigated the possible role of these genes in AA tolerance, focusing on the filamentous ascomycete P. anserina, which is highly tolerant to 2-AF, 3,4-DCA and 4-BOA and amenable to reverse genetics techniques. The two NAT genes present in the genome of P. anserina (24) encode two putative NAT enzymes, PaNat1 and PaNat2 (CDS numbers: Pa_2_13150 and Pa_4_4860, respectively). These genes are expressed, as an expressed sequence tag was identified for each of these genes in the database (http://podospora.igmors.u-psud.fr). The P. anserina PaNat1 and PaNat2 genes encode two polypeptides, which, at 333 and 303 amino acids in length, are larger than any previously described NAT enzyme (20). The P. anserina NATs contain all the known NAT-specific
functional motifs (25). Sequence analyses (see Supplementary Fig. 2) showed the percentage identity between the P. anserina NAT isoforms (32%) to be lower than that between NAT isoforms in any other eukaryotic species (67-94%). This unusually low level of identity between two paralogous eukaryotic NAT enzymes may reflect functional divergence (26). The percentage of identity between the two P. anserina NAT and the other predicted fungal NAT proteins were found to range from 15% (with B. dandroboidis NAT) to 55% (with C. globosum NAT1 and NAT2) (supplementary Fig. 2). PaNAT1 and PaNAT2 were found to share around 30% identity with a newly characterized NAT isoform (called FDB2) from Fusarium verticilloides which is involved in benzoxazolinone metabolism (27). When compared with characterized mammalian NAT enzymes such as human NAT1 and NAT2, identities were found to be around 25-30% (data not shown). Protein sequence identities between PaNAT1 and PaNAT2 and known bacterial NAT enzymes such as the Mycobacterium smegmatis or Salmonella typhimurium NAT isoforms were around 15-20% (data not shown). Predicted fungal NAT enzymes and the mammalian and bacterial isoforms also differ in their protein sequence lengths (supplementary Fig. 2). Almost all mammalian and bacterial NAT enzymes identified so far are less than 295 amino acids long (28). On the contrary, all fungal NAT identified in this study (including PaNAT1 and PaNAT2) range between 303 and 387 amino acids (supplementary Fig. 2). So far, no NAT enzyme has been identified in plants.

We purified recombinant P. anserina NAT isoforms and showed that they were readily detected with an anti-NAT antibody (supplementary Fig. 3). We further characterized the purified P. anserina NATs, by investigating whether they catalyzed the AcCoA-dependent acetylation of several AA (Fig. 1a and Table 1). We tested a series of different substrates, including a carcinogen (2-AF), drugs (SMX, SMZ, 4-AS, 5-AS, INH, HDZ), industrial chemical intermediates (4-BOA, 4-EOA, 4-PD, 4-ANS, 4-AMV) and pesticide residues (4-BA, 4-IA, 3,4-DCA). Kinetic parameters (Vm, kcat and Km) were estimated for aromatic NAT substrates (14) (Fig. 1b-c). P. anserina NAT enzymes were highly active against most of the AA substrates tested, with PaNat2 systematically more active than PaNat1 (see Table 1 and Fig. 1b-c). The catalytic efficiency (kcat/Km) of PaNat2 was up to 80 times higher than that of PaNat1 (Fig. 1c). The catalytic efficiency of PaNat1 with 3,4-DCA was 2.5 times higher and that of PaNat2 was five times higher than that of the Pseudomonas aeruginosa NAT, which is currently considered to be the most efficient NAT enzyme ever described, particularly with 3,4-DCA (29). When compared with the two NAT isoforms from the plant symbiotic bacterium Mesorhizobium loti, the catalytic efficiency towards 3,4-DCA by PaNat2 (kcat/Km=17400 M⁻¹.s⁻¹) was 220 and 120 times higher than that of M. loti NAT1 and M. loti NAT2, respectively (14).

Effects of the targeted disruption of NAT enzymes on the tolerance of P. anserina to toxic AA- We investigated the functions of the proteins encoded by the PaNat1 and PaNat2 genes in P. anserina, focusing particularly on their contribution to AA tolerance, by carrying out targeted gene disruption (see Methods and supplementary Fig. 1). The phenotypes of mutants lacking either one (ΔPaNat1 and ΔPaNat2) or both (ΔPaNat1/2) NAT genes were compared with that of the WT strain (11). No obvious differences in key biological features, including growth, differentiation, defense against competitors, aging, sexual reproduction and ascospore germination, were observed (data not shown). We then evaluated the AA tolerance of P. anserina WT and mutant strains. We first assessed AA tolerance in a minimal medium to which selected aromatic compounds (3,4-DCA, 2-AF, 4-BOA) were added. All the fungal isolates (WT and mutants) were screened by assessing radial growth for three days. The growth of strains lacking PaNat2 was strongly impaired in the presence of 2-AF, 3,4-DCA or 4-BOA, whereas strains lacking PaNat1 were less affected and grew similarly to the WT (Fig. 2a). Sensitivity to AA cosegregated in crosses with the phleomycin resistance marker gene used to inactivate PaNat2, and the introduction of a PaNat2 gene by cotransformation with a hygromycin B resistance marker restored wild-type (WT) levels of growth in the ΔPaNat2 mutants. Hypersensitivity to AA therefore resulted from PaNat2 deletion. The WT and mutant strains grew similarly in the presence of chemically synthesized N-acetylated forms.
of 2-AF, 3,4-DCA and 4-BOA (Fig. 2a). The N-acetylation of these three AA by PaNat2 therefore seems to be a key mechanism underlying tolerance to these toxic aromatic chemicals. Measurements of N-acetylation activity in various fungal extracts confirmed that 3,4-DCA, 2-AF and 4-BOA were N-acetylated principally by PaNat2 (Fig. 2b). Low level of AA acetylation was detected with the ΔPaNat1/2 extracts (Fig. 2b and Fig. 3a). This is likely due to background acetylation by non-NAT acetyltransferases present in P. anserina.

**Detoxification of the highly toxic pesticide residue 3,4-DCA and bioremediation applications using experimentally polluted soils**- We characterized the role of PaNat2 in tolerance to AA in more detail, focusing on 3,4-DCA, a highly toxic pesticide-derived AA persistent in soil, surface water and groundwater. This compound is the major breakdown product of the phenylamide herbicides diuron, linuron and propanil (30). The N-acetylated form of 3,4-DCA has been shown to be much less toxic than the parental compound (19). We therefore investigated whether 3,4-DCA was acetylated in vivo by WT and mutant P. anserina strains. Similar amounts of the N-acetylated form of 3,4-DCA were detected by HPLC in the liquid medium of WT and ΔPaNat1 strains grown in the presence of a toxic dose of 3,4-DCA (250 μM; Fig. 3a). By contrast, very low levels of acetyl-3,4-DCA were found in the media of strains lacking PaNat2, mainly due to the very poor growth of these strains in the presence of 3,4-DCA. Acetyl-3,4-DCA generation was time-dependent. After three days of incubation, 45% of the 3,4-DCA had been biotransformed into its acetylated product, 3,4-dichloroacetanilide, (3,4-DCA) and bioremediation medium previously incubated with the ΔPaNat1/2 strain. Conversely, seeds were able to germinate and grow in contaminated medium previously incubated with WT P. anserina. Thus, PaNat2 is sufficient for the detoxification of 3,4-DCA in vivo. These data also suggest that no toxic (at least for the seeds) fungal compound was released by P. anserina strains.

*P. anserina* is found on herbivore dung in nature, but can grow in soil in the presence of plant debris. This species reproduces by sexual means only, with mating occurring only between partners of opposite mating types (32). The spread of this non pathogenic fungus is therefore easy to control, making it an attractive candidate for safe bioremediation. As proof of principle, we assessed the capacity of the NAT-dependent acetylation pathway of *P. anserina* to N-acylate 3,4-DCA present in soil samples. For this purpose, we inoculated soils highly contaminated with 3,4-DCA (final concentration 25 mg/kg soil) with WT or ΔPaNat1/2 P. anserina strains and incubated the mixtures at 25°C for two days. We then extracted 3,4-DCA and its acetylated metabolites for detection by HPLC (Fig. 4a). Acetylated 3,4-DCA was readily detected in soil samples incubated with WT *P. anserina*. The amount of acetyl-3,4-DCA was found to depend on the amount of fungus used for soil inoculation (Fig. 4a, black and dotted lines). We found that 40% of the 3,4-DCA present could be N-acetylated within 48 hours (Fig. 4b). In the same conditions, no acetylation of 3,4 DCA was detected with ΔPaNat1/2 P. anserina (Fig. 4a, dashed line; Fig. 4b). We analyzed *L. sativa* seed germination and growth in 3,4-DCA-contaminated (80 mg/kg soil) soils after incubation with WT or ΔPaNat1/2 P. anserina strains for 72 hours (Fig. 4c). *L. sativa* seed germination and early growth were completely abolished in soils contaminated with 3,4-DCA. No seed germination or growth was observed with 3,4-DCA-contaminated soil inoculated with the ΔPaNat1/2 strain (Fig. 4c). Conversely, in contaminated soil treated with WT *P. anserina*, seed germination and growth were restored to the levels observed with soil treated with chemically-acetylated 3,4-DCA (Fig. 4c). Thus, the inoculation of a 3,4-DCA contaminated soil with a *P. anserina* strain harboring a functional NAT-dependent

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**Fig. 3a** shows the levels of 3,4-DCA and its acetylated metabolites in media of WT and ΔPaNat1 strains incubated with 3,4-DCA-contaminated soil. The levels of 3,4-DCA and its acetylated metabolites were significantly lower in media of ΔPaNat1 strains than in media of WT strains. **Fig. 3b** shows the levels of 3,4-DCA and its acetylated metabolites in media of WT and ΔPaNat1 strains incubated with 3,4-DCA-contaminated soil. The levels of 3,4-DCA and its acetylated metabolites were significantly lower in media of ΔPaNat1 strains than in media of WT strains. **Fig. 3c** shows the levels of 3,4-DCA and its acetylated metabolites in media of WT and ΔPaNat1 strains incubated with 3,4-DCA-contaminated soil. The levels of 3,4-DCA and its acetylated metabolites were significantly lower in media of ΔPaNat1 strains than in media of WT strains. **Fig. 3d** shows the levels of 3,4-DCA and its acetylated metabolites in media of WT and ΔPaNat1 strains incubated with 3,4-DCA-contaminated soil. The levels of 3,4-DCA and its acetylated metabolites were significantly lower in media of ΔPaNat1 strains than in media of WT strains. **Fig. 3e** shows the levels of 3,4-DCA and its acetylated metabolites in media of WT and ΔPaNat1 strains incubated with 3,4-DCA-contaminated soil. The levels of 3,4-DCA and its acetylated metabolites were significantly lower in media of ΔPaNat1 strains than in media of WT strains. **Fig. 3f** shows the levels of 3,4-DCA and its acetylated metabolites in media of WT and ΔPaNat1 strains incubated with 3,4-DCA-contaminated soil. The levels of 3,4-DCA and its acetylated metabolites were significantly lower in media of ΔPaNat1 strains than in media of WT strains.
acetylation pathways leads, in situ, to significant detoxification of this toxic compound, making possible the germination of L. sativa seeds and the early growth of the seedlings. Concentrations around 100 µg/kg of 3,4-DCA have been reported in contaminated soils (33). Our study shows that even at higher 3,4-DCA concentrations (25 mg/kg), P. anserina mediates efficient bioremediation of this compound in soil.

These results pave the way for use of the fungal NAT metabolic pathway in the bioremediation of AA pollution in soils. The potential of fungi metabolic pathways for the bioremediation of AA pollution, particularly in soils contaminated with aniline pesticide residues such as 3,4-DCA, has been little studied. Certain fungi and bacteria have nonetheless been shown to biotransform 3,4-DCA to its acetylated form (19,34). In the bacterium Mesorhizobium loti, a NAT isoform has been shown to acetylate 3,4-DCA (14). In plants, glucosylation of 3,4-DCA was described but this pathway was considered as non effective at detoxifying this AA (35). Our results should facilitate prospective studies of AA bioremediation and the rationalization of future strategies based on the fungal NAT pathway for AA detoxification. In addition, our findings further emphasize that certain well characterized fungi may constitute a more efficient alternative model, with a reasonable cost and a low environmental impact.

Studies on model fungi have provided much of our understanding of biological processes. Such models also help to understand the general mechanisms by which living organisms protect themselves against potentially toxic effects of natural products or xenobiotics present in their environment (7,8). The NAT-dependent xenobiotic biotransformation pathway is found in many organisms ranging from bacteria to humans (26). However, the relevance of this pathway to AA tolerance in living organisms has remained unclear. So far, studies done on KO NAT mouse models have not demonstrated a role for NAT enzymes in preventing AA toxicity (21). Our P. anserina model provides the first clear molecular and functional evidence indicating that the NAT-dependent xenobiotic-biotransformation pathway can afford complete tolerance towards toxic AA in a eukaryotic organism. In addition to the existing KO mouse model, our P. anserina model should be helpful to uncover the potential endogenous functions of NAT enzymes. Overall, our data underline the role of certain XME in the adaptation of organisms to their chemical environment and emphasize the potential biotechnological applications of such enzymatic pathways.

**FOOTNOTE**

NAT, arylamine N-acetyltransferase; XME, xenobiotic-metabolizing enzyme; AA, aromatic amine; 3,4-DCA, 3,4-dichloroaniline; 2-AF, 2-aminofluorene

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**REFERENCES**

FIGURE LEGENDS

Figure 1: Functional characterization of PaNat1 and PaNat2; a, Schematic representation of the NAT-dependent acetylation of three aromatic amines: 3,4-DCA, 4-BOA and 2-AF. b, Detailed Michaelis-Menten kinetic characterization of PaNat1 (black lines) and PaNat2 (dotted lines) with 3,4-DCA, 4-BOA, 2-AF, 5-AS and HDZ. All assays were performed in quadruplicate. c, Comparison of catalytic efficiencies, as estimated from ratios of kinetic parameters (kcat/Km), expressed in M⁻¹.sec⁻¹.

Figure 2: Contribution of PaNAT enzymes to tolerance to the toxic aromatic amines 2-AF, 3,4-DCA and 4-BOA in P. anserina. a, 8 cm Petri dish containing the indicated strains grown on M2-agar medium with 2-AF, 3,4-DCA and 4-BOA or their acetylated forms at the indicated final concentrations. Photographs were taken after 3 days of growth at 27°C. DMSO (0.25% final concentration) in solidified M2 medium was used as a control and had no effect on growth (data not shown). The data presented are representative of three independent experiments. b, Rate of acetylation of 3,4-DCA, 2-AF and 4-BOA (pmol.mg⁻¹.min⁻¹) in fungal extracts. All assays were carried out in quadruplicate. *p<0.01 versus WT and ΔPaNat1 strains. The data presented are representative of three independent experiments.

Figure 3: In vivo acetylation of 3,4-DCA by P. anserina. a, The WT, ΔPaNat1, ΔPaNat2 and ΔPaNat1/2 strains (0.25 g of fungal dry mass) were grown in M2 medium in the presence of 250 µM 3,4-DCA. At different time points, acetyl-3,4-DCA and 3,4-DCA were detected in the growth medium (absorbance at 254 nm) and quantified by HPLC (data were normalized to take into account the molar absorbance at 254 nm of acetyl-3,4-DCA being 2.6 times higher than that of 3,4-DCA). b, WT, ΔPaNat1, ΔPaNat2 and ΔPaNat1/2 strains (1.5 g of fungal dry mass) were grown in M2 medium in the presence of 400 µM 3,4-DCA for 2 days at 27°C. M2 media were filtered and used for the preparation of agar plates (50% v/v conditioned and normal M2 medium). Sterilized Lactuca sativa seeds (n=10) were allowed to germinate and grow for 7 days at room temperature. The data shown are representative of three independent experiments.

Figure 4: Remediation of soils contaminated with 3,4-DCA. a, WT or ΔPaNat1/2 (0.3 g and 0.9 g of fungal dry mass) was used to inoculate soils (3 g) contaminated with 3,4-DCA (25 mg/kg soil), which were then incubated for 2 days at 25 °C. At various time points, 0.5 g of soil was sampled and 3,4-DCA and acetyl-3,4-DCA were extracted with absolute ethanol and quantified by HPLC. The dashed line corresponds to ethanol extractions from soil incubated with ΔPaNat1/2 (0.9 g); the solid and dotted lines correspond to ethanol extractions from soil incubated with 0.3 g and 0.9 g of WT P. anserina, respectively. b, Time-course of the disappearance of 3,4-DCA (conversion to its acetylated form) from contaminated soil treated with ΔPaNat1/2 (0.9 g) (triangles) or WT P. anserina (0.9 g) (squares). c, 3,4-DCA-contaminated soils (20 g per pot, 80 mg 3,4-DCA /kg soil) were inoculated every 24 h (over a period of 72 h) with 0.5 g of WT or ΔPaNat1/2 strains and incubated for 72 h at 25 °C. Lactuca sativa seeds (20 per pot) were sown and allowed to germinate and grow for 8 days at 25°C. Controls were set up with acetyl-3,4-DCA (80 mg/kg soil) and H2O.
Table 1: N-Acetylation of known aromatic NAT substrates by purified recombinant *Podospora anserina* enzymes (PaNAT1 and PaNAT2). The rate of hydrolysis of AcetylCoA (AcCoA) (nmol.min⁻¹.mg⁻¹ of NAT) was measured in the presence of purified PaNAT1 or PaNAT2, AcCoA (400 µM) and aromatic substrate (500 µM).

<table>
<thead>
<tr>
<th>Class/compound</th>
<th>Short name</th>
<th>Rate (nmol.min⁻¹.mg⁻¹)</th>
<th>PaNAT1</th>
<th>PaNAT2</th>
</tr>
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<tbody>
<tr>
<td><strong>Arylamines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>SMX</td>
<td>43 ± 1</td>
<td>41 ± 2</td>
<td></td>
</tr>
<tr>
<td>Sulphamethazine</td>
<td>SMZ</td>
<td>75 ± 3</td>
<td>62 ± 3</td>
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<tr>
<td>4-Aminobenzoic acid</td>
<td>4-ABA</td>
<td>134 ± 7</td>
<td>303 ± 15</td>
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<tr>
<td>Procainamide</td>
<td>PRO</td>
<td>250 ± 8</td>
<td>1 540 ± 184</td>
<td></td>
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<tr>
<td>2-Phenylenediamine</td>
<td>2-PD</td>
<td>5 270 ± 421</td>
<td>6 130 ± 490</td>
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<tr>
<td>3-Phenylenediamine</td>
<td>3-PD</td>
<td>3 280 ± 197</td>
<td>6 470 ± 776</td>
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<tr>
<td>4-Aminosalicylate</td>
<td>4-AS</td>
<td>750 ± 52</td>
<td>9 580 ± 287</td>
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<tr>
<td>4-Aminophenol</td>
<td>4-AMP</td>
<td>9 680 ± 871</td>
<td>11 700 ± 129</td>
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<tr>
<td>2-Aminofluorene</td>
<td>2-AF</td>
<td>2 790 ± 279</td>
<td>14 300 ± 143</td>
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<td>5 150 ± 309</td>
<td>16 800 ± 673</td>
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<td>20 300 ± 244</td>
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<td><strong>Aniline derivatives</strong></td>
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<td>4-Iodoaniline</td>
<td>4-IA</td>
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<td>4-Aniside</td>
<td>4-ANS</td>
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<td>7 980 ± 851</td>
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<td>4-Butoxyaniline</td>
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<td>3 920 ± 900</td>
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<td>7 990 ± 639</td>
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<td>3,4-Dichloroaniline</td>
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<td>18 500 ± 166</td>
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<td>85 ± 6</td>
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<td>Hydralazine</td>
<td>HDZ</td>
<td>2 930 ± 235</td>
<td>12 200 ± 731</td>
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</tbody>
</table>
Martins et al Figure 1

(a)

3,4-DCA
\[
\begin{align*}
3,4-\text{DCA} & \quad \text{Acetyl 3,4-DCA} \\
4-\text{BOA} & \quad \text{Acetyl 4-BOA} \\
2-\text{AF} & \quad \text{Acetyl 2-AF}
\end{align*}
\]

(b)

\begin{align*}
K_m &= 197 \pm 30 \mu M \\
K_m &= 211 \pm 7 \mu M \\
K_m &= 137 \pm 9 \mu M \\
K_m &= 172 \pm 12 \mu M \\
K_m &= 70 \pm 4 \mu M \\
K_m &= 529 \pm 50 \mu M \\
K_m &= 545 \pm 50 \mu M \\
K_m &= 639 \pm 101 \mu M \\
K_m &= 1100 \pm 313 \mu M
\end{align*}

(c)

\[ k_{cat} \frac{\text{M}^{-1} \cdot \text{s}^{-1}}{K_m} \]
Martins et al Figure 2

a

WT  ΔPaNat1  ΔPaNat2  ΔPaNat1/2

2-AF (100 µM)  3,4-DCA (250 µM)  4-BOA (250 µM)

Acetyl 2-AF (100 µM)  Acetyl 3,4-DCA (250 µM)  Acetyl 4-BOA (250 µM)

b

Acetylation (pmol.mg\(^{-1}\).min\(^{-1}\))

Wild type  ΔPaNAT1  ΔPaNAT2  ΔPaNAT1/2

3,4-DCA  2-AF  4-BOA

* *
Martins et al Figure 3

**a**

<table>
<thead>
<tr>
<th></th>
<th>Acetyl 3,4-DCA</th>
<th>Acetyl 3,4-DCA</th>
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</table>

Wild Type  | ∆PaNat1        | ∆PaNat2        | ∆PaNat1/2      |

**b**

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<tr>
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<th>3,4-DCA</th>
<th>DMSO</th>
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<tr>
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<td>3,4-DCA + WT</td>
<td>3,4-DCA + ∆PaNat1/2</td>
</tr>
</tbody>
</table>
Martins et al Figure 4

**a**

Absorbance (254 nm) vs Time (min)

- 0.9g ΔPaNat1/2
- 0.3g Wild type
- 0.9g Wild type

**b**

3,4-DCA (%) vs Time (hours)

- ΔPaNat1/2
- Wild type

**c**

Control

<table>
<thead>
<tr>
<th>H2O</th>
<th>3,4-DCA</th>
<th>Acetyl 3,4-DCA</th>
</tr>
</thead>
</table>

WT

<table>
<thead>
<tr>
<th>H2O</th>
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</table>

ΔPaNAT1/2

<table>
<thead>
<tr>
<th>H2O</th>
<th>3,4-DCA</th>
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An acetyltransferase conferring tolerance to toxic aromatic amine chemicals: molecular and functional studies
Marta Martins, Fernando Rodrigues-Lima, Julien Dairou, Aazdine Lamouri, Fabienne Malagnac, Philippe Silar and Jean-Marie Dupret

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