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Molecular Microbiology (2006) **60**(2), 505–512
doi:10.1111/j.1365-2958.2006.05114.x
First published online 3 March 2006

Cloning, functional expression and characterization of *Mesorhizobium loti* arylamine *N*-acetyltransferases: rhizobial symbiosis supplies leguminous plants with the xenobiotic N-acetylation pathway

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Summary

Arylamine *N*-acetyltransferases (NATs) are xenobiotic-metabolizing enzymes involved in the detoxification of numerous aromatic chemicals. The NAT-dependent N-acetylation pathway has not previously been detected in plants. We demonstrate here the occurrence of the NAT-dependent pathway in leguminous plants, due to symbiosis with *Mesorhizobium loti*. We cloned two NAT enzymes from *M. loti* and showed that these two recombinant enzymes catalysed the N-acetylation of several known NAT substrates, including aniline-derived pesticide residues. We also demonstrate the existence of a functional NAT-dependent acetylation pathway in the root nodules of *Lotus japonicus* inoculated with *M. loti*. *M. loti* is the first non-eukaryotic organism shown to express two catalytically active NAT isoforms. This work also provides the first evidence for acquisition of a xenobiotic detoxification pathway by a plant through symbiosis with a soil microbe.

Introduction

Soils are complex ecosystems of many species, including plants and their associated microorganisms (Spaink, 2002). Human activities may result in high concentrations of xenobiotic compounds in the soil. In particular, soil contamination with pesticides, industrial chemicals and their derivatives is a major concern in industrialized countries (Harvey *et al*., 2002). Plants use several catabolic pathways to protect themselves against the potentially toxic effects of the natural products and xenobiotics present in their environment. These xenobiotic metabolism pathways involve the expression of endogenous xenobiotic-metabolizing enzymes (XME) such as cytochromes P450 (CYP450) (Morant *et al*., 2003) and glutathione-S-transferases (GST) (Pflugmacher *et al*., 2000). XME may also be involved in other metabolic processes in the plant, such as growth and development (Gong *et al*., 2005). XME are also expressed in prokaryotes and other eukaryotes, in which they play a well documented role in xenobiotic detoxification (Boelsterli, 2003).

Arylamine *N*-acetyltransferases (NAT) are XME catalysing the transfer of an acetyl group from acetylCoA (AcCoA) to the nitrogen or oxygen atom of arylamine-based xenobiotics and their hydroxylated metabolites (Pompeo *et al*., 2002). They play an important role in the detoxification of many therapeutic drugs and pollutants and have been shown to be involved in the biotransformation of several aniline derivatives, including chemical intermediates or by-products of additives, pharmaceuticals, dyes and pesticides (Hein *et al*., 2000; Westwood *et al*., 2005). Aniline-derived herbicides (such as diuron, linuron and propanil), and their by-products [such as 3,4-dichloroaniline (DCA)] are among the most toxic chemicals found in soil (Dearfield *et al*., 1999; Harvey *et al*., 2002). The N-acetylation of these chemicals has been shown to detoxify them (Tweedy *et al*., 1970; Tixier *et al*., 2002) and has been reported to occur in soil bacteria such as *Pseudomonas* sp. (Vol'nova *et al*., 1980; Westwood *et al*., 2005). NAT isoforms have been identified in several species from eubacteria to mammals (Pompeo *et al*., 2002), but screening of complete and incomplete genomes has identified no NAT enzymes in plants. We recently identified two putative NAT enzymes in the fully sequenced genome of *Mesorhizobium loti* (MAFF 303099 strain), a rhizobial nitrogen-fixing bacterium that lives in symbiosis with several species of the genus *Lotus*, including *Lotus corniculatus* (Rodrigues-Lima and

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Dupret, 2002) and *Lotus japonicus*. Rhizobial symbiosis provides the plant with metabolic and stress-related pathways, due to the production of rhizobial proteins in root nodules (Weidner *et al*., 2003; Djordjevic, 2004). These pathways enable host plants to survive in nitrogen-depleted soils and soils containing very low concentrations of chemically fixed nitrogen. However, rhizobia have never before been shown to provide plants with a xenobiotic-metabolizing pathway. We show here that symbiosis with a soil microorganism can provide leguminous plants with the NAT-dependent xenobiotic N-acetylation pathway. As many aromatic pollutants are detoxified by the NAT-dependent pathway, our results open up new possibilities in the field of rhizoremediation for organic soil pollutants.

Results and discussion

We previously reported the presence of two putative NAT genes (NAT1 and NAT2; GenBank ORF numbers: NP_105643 and NP_106410, respectively) on the single chromosome of the leguminous plant microsymbiont *M. loti* (MAFF 303099 strain, the only *Mesorhizobium* species for which the genome has been fully sequenced); these two genes are separated by 820 kb. The *M. loti* NAT1 and NAT2 genes, which are not located in a specific gene cluster, encode two polypeptide sequences, of 278 and 270 amino acids respectively (Rodrigues-Lima and Dupret, 2002). These polypeptides are of similar length to known NAT enzymes (Butcher *et al*., 2002). The two proteins have 27% identical amino-acid sequences and molecular masses of about 33 kDa. Alignment of the two *M. loti* NAT sequences with those of known bacterial and eukaryotic NATs showed that the *M. loti* NATs had amino-acid sequences 25–30% identical to those of other known NATs (data not shown). The *M. loti* NATs also contained all the known NAT-specific functional motifs, including the catalytic triad and the active-site loop (Fig. 1) (Sinclair *et al*., 2000; Rodrigues-Lima and Dupret, 2002). We cloned the cDNAs encoding *M. loti* NAT1 and NAT2, expressed them in *Escherichia coli* and purified the corresponding His-tagged recombinant proteins (Fig. 2). We obtained highly purified recombinant NAT1 and NAT2, with molecular masses consistent with predicted values. The recombinant *M. loti* NAT1 and NAT2 enzymes were readily detected with a polyclonal antibody against *Salmonella typhimurium* NAT. Finally, crystal structure determination (Holton *et al*., 2005) and homology modelling (data not shown) indicated that *M. loti* NAT1 and NAT2 adopted the NAT fold (which corresponds to a typical $\alpha+\beta$ protein consisting of three structural domains, i.e. a N-terminal α bundle, a central β-barrel and a C-terminal α/β lid), therefore confirming that these two proteins belong to the NAT enzyme family (Fig. 1) .

We further characterized the purified recombinant *M. loti* NAT1 and NAT2 enzymes by investigating whether they catalysed the AcCoA-dependent acetylation of several known NAT aromatic substrates. We tested a series of 20 NAT substrates, including drugs, industrial chemicals and pesticide residues. *M. loti* NAT1 acetylated 19 of the 20 substrates tested, but with large differences in acetylation rate (Table 1). For example, the rate of acetylation of hydralazine by NAT1 (2400 nmol min^{-1} mg^{-1} enzyme) was 480 times higher than the rate of procainamide acetylation. We observed no acetylation of 2,6-dichloro-4-nitroaniline. *M. loti* NAT1 seemed to be two to 400 times more active than NAT2 against the various substrates tested. Differences in substrate preference were also observed. The rate of hydralazine acetylation by NAT2 (36 nmol min^{-1} mg^{-1} enzyme) was 70 times higher than for the rate of 4-trifluoromethylaniline acetylation. No acetylation of 2,6-dichloro-4-nitroaniline was detected with NAT2, and this enzyme also displayed very low levels of activity with 3,4-dichloroaniline, which was acetylated by NAT1 (70 nmol min^{-1} mg^{-1} enzyme). Such differences in substrate preference and acetylation rate have been also reported for mammalian NAT paralogues

Table 1. N-acetylation of known aromatic NAT substrates by purified recombinant *M. loti* NAT1 and NAT2 enzymes.

Class/compound	Short name	Rate (nmol min^{-1} mg^{-1}) NAT1	NAT2
Arylamines and arylhydrazines			
5-Aminosalicylate	5-AS	616 ± 6	41.1 ± 4
4-Aminosalicylate	4-AS	1609 ± 48	3.7 ± 0.2
Sulphamethazine	SMZ	7 ± 0.8	0.7 ± 0.2
Procainamide	PRO	5 ± 0.6	2.3 ± 0.3
Phenylhydrazine	PHZ	17 ± 1	2.2 ± 0.1
Isoniazid	INH	31 ± 2	3.1 ± 0.2
4-Chlorobenzoic hydrazine	CBZ	225 ± 14	8.1 ± 0.4
Hydralazine	HDZ	2390 ± 17	36.2 ± 1
2-Aminofluorene[a]	2-AF	1540 ± 60	13.7 ± 0.6
Aniline derivatives			
4-Trifluoromethylaniline	TFMA	20 ± 4	0.5 ± 0.1
4-Bromoaniline	4-BA	35 ± 3	1.5 ± 0.2
4-Iodoaniline	4-IA	114 ± 8	3.6 ± 0.3
3,4-Dichloroaniline	DCA	68 ± 8	< 0.5
2,6-Dichloro 4-nitroaniline	DCNA	nd	nd
4-Phenoxyaniline	POA	306 ± 18	4.2 ± 0.3
4-Hexyloxyaniline[a]	HOA	1367 ± 44	12.4 ± 0.7
4-Methoxyaniline	4-MA	45 ± 4	1.6 ± 0.2
4-Ethoxyaniline	EOA	174 ± 9	6.7 ± 0.4
4-Butoxyaniline[a]	BOA	485 ± 15	7.3 ± 0.5
3,4-Dimethoxyaniline	DMA	51 ± 5	6.1 ± 0.6

The rate of hydrolysis of acetylCoA (AcCoA) (nmol min^{-1} mg^{-1} of NAT) was measured in the presence of purified NAT1 or NAT2, AcCoA (400 μM) and aromatic substrate (500 μM). The substrates tested are drugs (SMZ, PRO, 4-AS, 5-AS, INH, HDZ), industrial chemical intermediates (POA, HOA, 4-MA, BOA, EOA, CBZ, PHZ, DMA). TFMA, 4-BA, 4-IA, and DCA are pesticide residues. DCNA is a fungicide. 2-AF is an experimental carcinogen. All assays were carried out in quadruplicate. nd, no detectable activity.
a. Assay performed in the presence of 5% (v/v) DMSO.

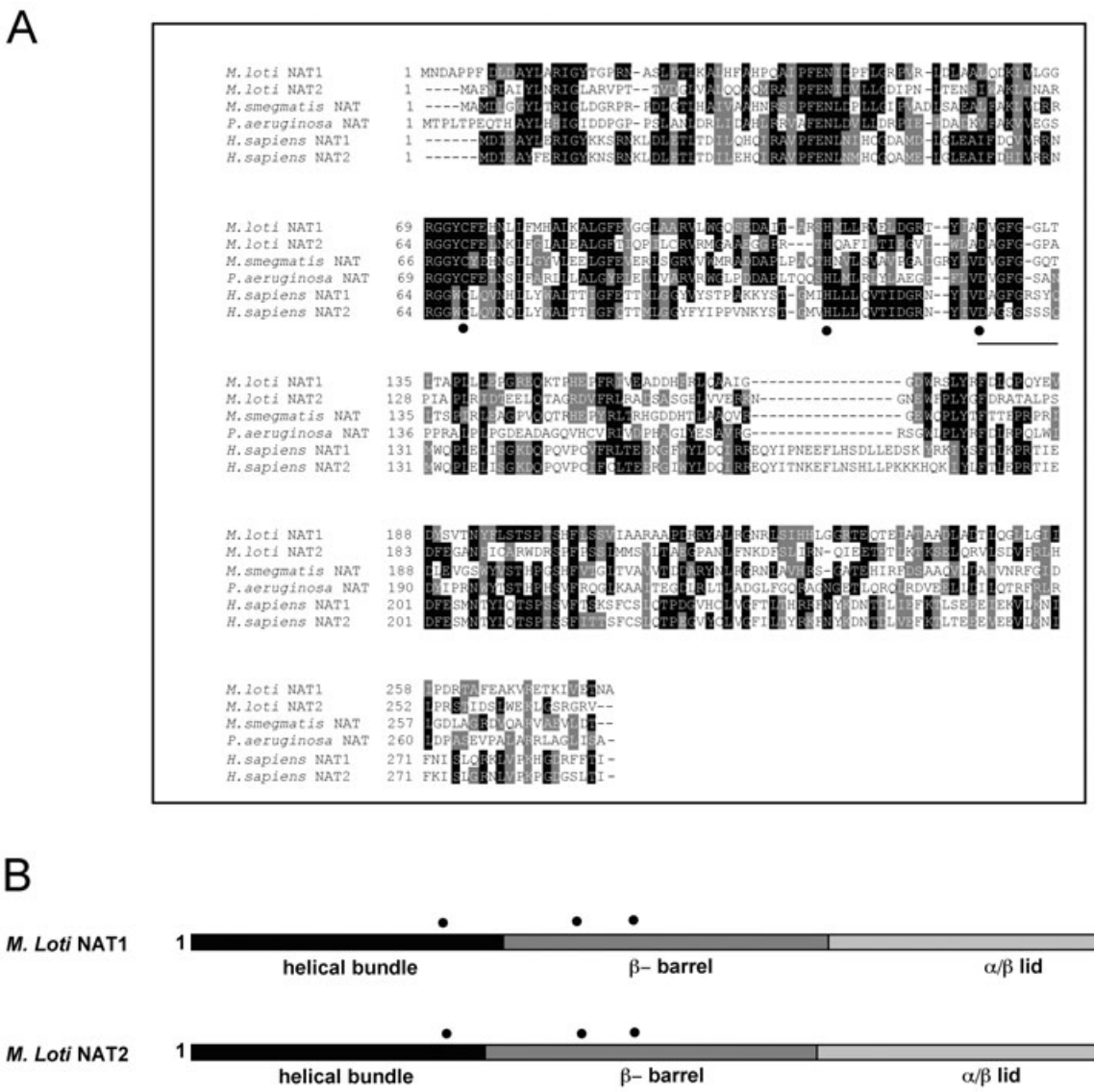

Fig. 1. Multiple alignment of *M. loti* NAT1 and NAT2 amino-acid sequences.
A. *M. loti* NAT protein sequences were aligned with well characterized NAT sequences from *Homo sapiens*, *Mycobacterium smegmatis* and *Pseudomonas aeruginosa*, using the Clustal W program. Catalytic residues are indicated by a dot. Active site loop residues are underlined.
B. Schematic representation of *M. loti* NAT1 and NAT2 structural domains.

(Butcher *et al*., 2002; Kawamura *et al*., 2005). Human NAT1 is more active against its known preferred substrates than is human NAT2 against its known preferred substrates. However, as observed for the *M. loti* NATs, certain substrates are acetylated by both human NAT1 and NAT2 (Kawamura *et al*., 2005). *M. loti* is the first non-eukaryotic organism shown to possess two catalytically active NAT enzymes. The presence of two NAT genes in *M. loti* may account for enzymatic specialization, as suggested for mammalian paralogues (Rodrigues-Lima and Dupret, 2002; Cornish *et al*., 2003; Sugamori *et al*., 2003).

We investigated whether the two *M. loti* NAT isoforms were present in both free-living bacteria and the root nodules of *Lotus* infected with the MAFF303099 strain of *M. loti*. We carried out reverse transcription polymerase chain reaction (RT-PCR) with specific primers and total RNA isolated from free-living *M. loti*. The mRNAs for *M. loti* NAT1 and NAT2 (837 and 813 bp, respectively) were both readily detected (Fig. 3, left panel). We carried out similar experiments with total RNA extracted from infected *Lotus* root nodules and uninfected roots. We detected mRNA for NAT1 and NAT2 in infected root nodules but not in uninfected plant roots (Fig. 3, right panel). Thus, the *NAT1* and *NAT2* genes are transcribed in free-living *M. loti* and in infected root nodules. We then carried out Western blotting to determine whether NAT proteins

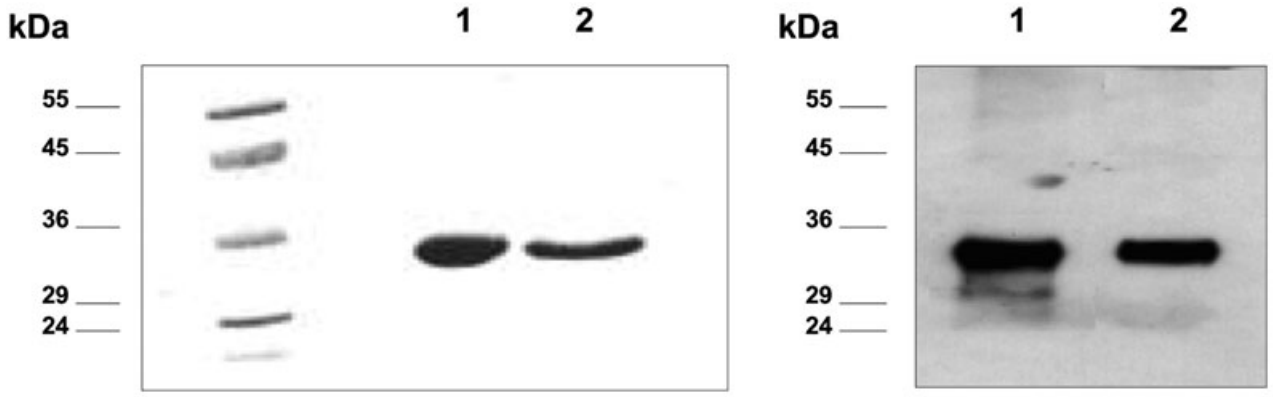

Fig. 2. Recombinant NAT from *Mesorhizobium loti*.
Left panel. Coomassie blue-stained SDS-PAGE gel showing the purified recombinant *M. loti* NAT enzymes (lane 1: NAT1; lane 2: NAT2).
Right panel. Specific detection of recombinant *M. loti* NAT1 (lane 1) and NAT2 (lane 2) by Western blotting with a polyclonal antibody against *Salmonella typhimurium* NAT (Westwood *et al*., 2005).

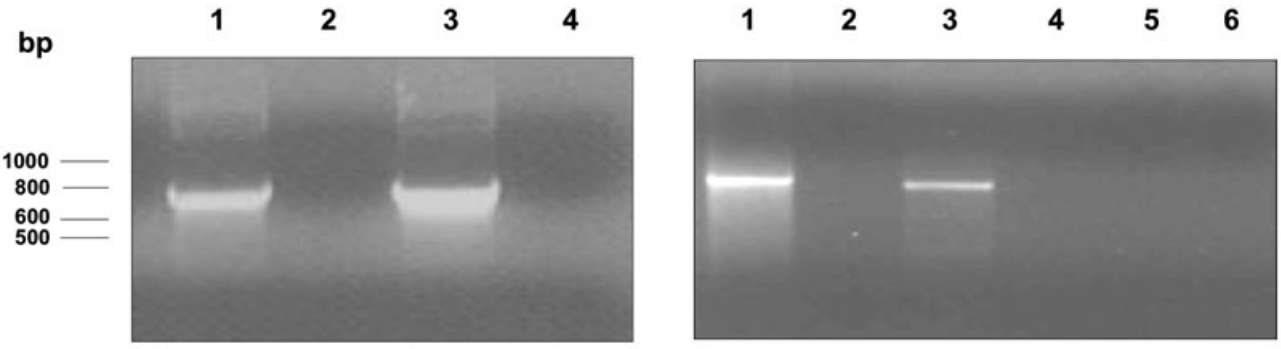

Fig. 3. Endogenous expression of NAT mRNA.
Left panel. Detection by RT-PCR of *M. loti* NAT1 (lane 1) and NAT2 (lane 3) mRNAs in free-living *M. loti*. The absence of genomic DNA contamination was confirmed with negative controls in which no reverse transcriptase was added for NAT1 (lane 2) and NAT2 (lane 4).
Right panel. Detection by RT-PCR of *M. loti* NAT1 (lane 1) and NAT2 (lane 3) mRNAs in infected root nodules. No NAT1 (lane 5) or NAT2 (lane 6) mRNA was detected in uninfected plant roots. The absence of genomic DNA contamination was confirmed with negative controls in which no reverse transcriptase was added for NAT1 (lane 2) and NAT2 (lane 4).

were present in total extracts of *M. loti* and infected *Lotus* root nodules. We detected a specific band at around 33 kDa in protein extracts from free-living *M. loti* and from infected *Lotus* root nodules (Fig. 4). No signal was obtained with uninfected roots. As the molecular masses of *M. loti* NAT1 and NAT2 are similar, we cannot tell which of the two enzymes is present in the protein extracts. However, as indicated by our RT-PCR results, both NAT enzymes are likely to be present in free-living *M. loti* and in root nodule extracts.

Finally, we investigated whether the corresponding total extracts catalysed the acetylation of NAT substrates previously shown to be acetylated by the recombinant *M. loti* NATs (Table 1), as a means of demonstrating the existence of a functional NAT-dependent detoxification pathway in infected root nodules and in free-living *M. loti*. AcCoA-dependent acetylation was found to occur in infected root nodule extracts (Table 2) and in extracts from

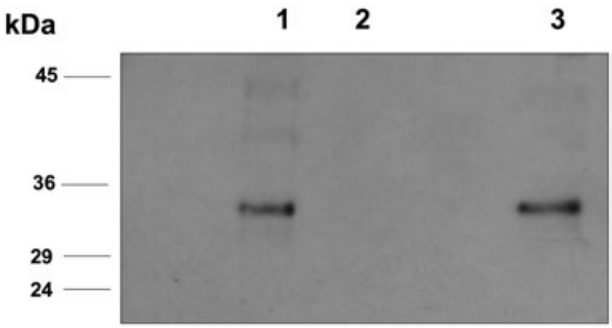

Fig. 4. Endogenous expression of NAT enzymes. Specific detection of endogenous *M. loti* NATs in infected root nodule extracts (lane 1) and in extracts from free-living *M. loti* (lane 3), by Western blotting with a polyclonal antibody against *Salmonella typhimurium* NAT (Westwood *et al*., 2005). No NAT protein was detected in uninfected plant roots (lane 2).

Table 2. N-acetylation of aromatic NAT substrates by root nodule extracts.

NAT substrates	NAT activity pmol min^{-1} mg^{-1}
4-Aminosalicylate (4-AS)	1568 ± 29
4-Hexyloxyaniline (HOA)	1001 ± 62
4-Butoxyaniline (BOA)	631 ± 43
4-Phenoxyaniline (POA)	335 ± 19
4-Bromoaniline (4-BA)	120 ± 9
4-Iodoaniline (4-IA)	47 ± 15
3,4-Dichloroaniline (DCA)	25 ± 5

The rate of acetylation of NAT substrates (pmol min^{-1} mg^{-1} of extract) was measured in the presence of root nodule extracts, AcCoA (800 μM) and aromatic substrate (200 μM). All assays were carried out in quadruplicate.

free-living *M. loti* (data not shown). By contrast, no acetylation was observed in uninfected plant roots. Although we cannot distinguish the specific contributions of *M. loti* NAT1 and NAT2 to the acetylation activity of the extracts, our results show that the NAT1 enzyme, at least, is functional, as 3,4-dichloroaniline (very poorly acetylated by NAT2) was readily acetylated by the extracts. Overall, our data clearly demonstrate that the NAT-dependent detoxification pathway occurs in *M. loti* and in the roots of leguminous plants infected with this microorganism.

Plants produce several XME, such as CYP450 and GST, allowing them to degrade a great variety of xenobiotics and mediating tolerance to pollutants (Coleman *et al*., 1997; Morrissey *et al*., 2004). However, the NAT-dependent detoxification pathway found in several bacteria and eukaryotes and NAT-related enzymes have never been found in plants. The data reported here clearly demonstrate that leguminous plants may acquire the NAT-dependent detoxification pathway through symbiosis with a soil microorganism.

This symbiosis-dependent expression of the NAT biotransformation pathway probably increases the detoxification capacity of infected plants, because this well known detoxification pathway is not present endogenously in plants. We assessed the contribution of the symbiosis-dependent NAT pathway to the detoxification capacity of infected plants, by carrying out a quantitative comparison of the NAT-dependent pathway and known endogenous plant detoxification pathways, such as the N-glucosylation and glutathione conjugation pathways. The persistent pollutant arylamine 3,4-dichloroaniline (DCA) is metabolized by N-glucosylation in *Arabidopsis thaliana* roots, at a rate of about 6–7 pmole min^{-1} mg^{-1} protein (Loutre *et al*., 2003). The GST-dependent biotransformation of chloroacetanilide xenobiotics (metolachlor, acetochlor), at rates of 180–1080 pmol min^{-1} mg^{-1} protein, has also been shown to contribute to the detoxification of these pollutants in *A. thaliana* seedlings (DeRidder *et al*., 2002). In *Lotus* root nodules, the rate of biotransformation of DCA (a relatively poor substrate) and of other aniline pollutants with NAT was in the same range (25–1000 pmol min^{-1} mg^{-1} protein) (Table 2) as those reported for N-glucosylation and glutathione conjugation. Moreover, estimated ratios of kinetic parameters (V_m/K_m) showed that the NAT-dependent pathway ($V_m/K_m = 5 \times 10^{-3}$ min^{-1}) and N-glucosylation ($V_m/K_m = 2 \times 10^{-3}$ min^{-1}) (Yang *et al*., 2005) were similarly efficient for the biotransformation of DCA. Overall, the level of activity and kinetics properties of the NAT pathway seem to be similar to those reported for other detoxification pathways in plants. Moreover, as many xenobiotics are preferentially detoxified via NAT-dependent pathways, the expression of the NAT pathway via symbiosis in a plant that would otherwise lack this pathway is likely to increase the capacity of the plant to detoxify a wider variety of substrates. Some substrates also seem to be acetylated more rapidly by NAT enzymes, which could be useful in screening for potential additional detoxification routes for new chemicals. Phytoremediation involves the phytoextraction, storage or phytodegradation of pollutants. XME are involved in the phytodegradation of soil pollutants. Thus, acquisition of the NAT-dependent biotransformation pathway by a plant will enhance its phytoremediation capacity.

Rhizobium-legume symbiosis is the most important known interaction between plants and bacteria because it leads to the transformation of atmospheric nitrogen into ammonia, a fixed form of nitrogen bioavailable to plants and microorganisms, which eventually becomes bioavailable to animals, following further transformations, in terrestrial ecosystems (Morrissey *et al*., 2004). The rhizobial proteins involved in biological nitrogen fixation are present in root nodules. Other nodule proteins have also been shown to be involved in vitamin synthesis pathways and in stress tolerance (heat shock, detoxification of reactive oxygen species) (Djordjevic, 2004). Thus, rhizobial symbiosis is not only important for leguminous plant growth, but also for stress tolerance, disease resistance and biodiversity. Rhizobium-plant symbiosis has also been shown to play a crucial role in the establishment of plants in polluted ecosystems (Morrissey *et al*., 2004). Tolerance to soil pollutants in plants is mediated primarily by XME, which catalyse the biotransformation of diverse phytotoxic compounds (Peuke and Rennenberg, 2005). We show here that functional NATs in the root nodules of *Lotus* plants inoculated with *M. loti* provide the host plant with access to the xenobiotic N-acetylation pathway. As several toxic soil pollutants, including pesticide-derived anilines, can be detoxified by N-acetylation (Tweedy *et al*., 1970; Tixier *et al*., 2002), the presence of NAT enzymes in root nodules may confer a wider spectrum of tolerance to these aromatic soil pollutants on plants infected with *M. loti*. This symbiosis-dependent NAT biotransformation pathway could potentially be transferred to other rhizobial

species via plasmids carrying the NAT genes described in this article, broadening the range of leguminous host plants that could be used for rhizoremediation. Leguminous plants grow in a wide variety of environments and some are able to tolerate extremes of salinity, temperature and drought. In addition, leguminous plants form extensive, branched roots systems penetrating from 50 cm (*Trifolium*, *Lotus*, *Vicia*, *Glycine soja*) to a few metres (*Acacia*, *Robinia*) into the soil and carrying newly formed nodules. Depending on the rhizobial-host plant combination, the host plant roots may carry as many as 100 nodules.

Our results open up new possibilities for the rhizoremediation of soils, providing a widely applicable means of dealing with aromatic pollutants. This new method of rhizomediation is based on the combined action of leguminous host plants and their associated microorganisms in the rhizosphere (Kuiper *et al*., 2004) and could be tailored to suit different environments. Leguminous plants that can be infected by rhizobia have been shown to be the most suitable plants for rhizoremediation. This plant–microbe association increases the degradation of soil pollutants by the root system, through the action of XME (Kuiper *et al*., 2004). Rhizoremediation could therefore be improved by the use of rhizobia that provide plants with an additional xenobiotic-metabolizing pathway, such as the NAT-dependent detoxification pathway described in this article.

Experimental procedures

Strains and culture conditions

Mesorhizobium loti strain MAFF303099 (*M. loti*) (also proposed as *Mesorhizobium huakuii* by Turner *et al*., 2002) was obtained from the MAFF Gene Bank (Ibaraki, Japan). Genomic DNA from *M. loti* MAFF303099 was kindly provided by Dr Kaneko (Kazuga DNA Research Institute, Chiba, Japan). Free-living *M. loti* cells were grown in YMB medium (Vincent, 1970) for 2 days at 28°C. Legume nodules were obtained as follows: *L. japonicus* ecotype Gifu seeds were scarified with sand paper, surface-sterilized by incubation for 20 min with 2% sodium hypochlorite and imbibed in sterile water overnight at room temperature. Seeds were germinated on solidified (0.5% agar) Jensen medium (Van Brussel *et al*., 1982) at 28°C in the dark for 2 days. Seedlings were then inoculated with *M. loti* strain MAFF303099. Inoculated seedlings were planted in tissue culture boxes (ECOLINE BVBA, Zottegem, Belgium), containing 2–2.5 mm sterile clay pellets (Hydro Jongkind Grond BV, Alsmeer, Holland) saturated with Jensen medium containing 1.5 mM NH_4NO_3. In parallel, we planted a set of *Lotus* seedlings that were not inoculated. Seedlings were grown in controlled conditions (16 h light/8 h dark cycle, 21°C-day/18°C-night temperature, 70% relative humidity). The three or four most developed nodules on each inoculated plant were detached from the roots 30–35 days after inoculation, immediately frozen in liquid nitrogen and stored at –80°C until use. Pieces of roots (1 cm in length) from non-inoculated plants were excised from equivalent sites, frozen in liquid nitrogen and stored at –80°C until use.

Molecular cloning and plasmid construction

The NAT1 and NAT2 cDNAs were cloned by PCR from *M. loti* genomic DNA, using the oligonucleotides 5′-AAGGATCCAT GAACGATGCTCCTCCC-3′ (sense) 5′-AACTCGAGTCAT GCGTTGGTCTCCAC-3′ (reverse) for NAT1 and 5′-CGG GATCCATGGCATTCAATATTGCG-3′ (sense) 5′-AAGCGGC CGCCTATACCCGTCCACGCGA-3′ (reverse) for NAT2. These amplified products were inserted into the BamHI/XhoI (NAT1) and BamHI/NotI (NAT2) sites of the expression vector pET-28 A (Invitrogen). The sequences of the inserts were checked by DNA sequencing.

Computer analysis of sequence data

DNA and protein sequences were retrieved from the NCBI GenBank and Genpept databases. Two sequence alignments were produced with BLAST (Tatusova and Madden, 1999) and multiple sequence alignments were generated with Clustal W (Higgins *et al*., 1996).

Protein production and purification

Escherichia coli BL21 (DE3) cells (Novagen) containing pET-28A-based plasmids encoding *M. loti* NAT1 or NAT2 cDNA were used for the production and purification of 6xHis-tagged recombinant proteins using nickel-agarose affinity chromatography as described in the pET System Manual (Novagen), and essentially as previously described (Westwood *et al*., 2005). Purified NAT1 and NAT2 enzymes were reduced with 10 mM di-thiothreitol (DTT) before dialysis against 25 mM Tris–HCl, pH 7.5, 1 mM EDTA. Purity of recombinant proteins were checked by Coomassie staining. In addition, mock-nickel agarose-purified fractions of *E. coli* BL21 (DE3) cells showed no NAT activity. Protein preparations were subjected to SDS-PAGE and Western blotting was carried out as described (Dairou *et al*., 2004).

Reverse transcription PCR experiments

Total RNA was extracted from free-living *M. loti*, infected root nodules and uninfected roots, using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The first-strand cDNA was synthesized from 2 μg of total RNA, using the *Carboxydothermus hydrogenoformans* RT kit from Roche and reverse primers for *M. loti* NAT1 and NAT2 (see above). PCR reactions were carried with the Roche polymerase and the NAT1 and NAT2 primers used for molecular cloning (see above). The absence of genomic DNA contamination was confirmed in controls to which no reverse transcriptase was added.

Total extract preparation

Total extracts were prepared by grinding pellets of free-living *M. loti*, infected root nodules or uninfected roots in Tris-HCl

buffer, pH 7.5 supplemented with 0.1% Triton X-100, 1 mM DTT and protease inhibitors [final concentrations were: leupeptin (20 μM), E64 (10 μM), pefabloc (500 μM) and EDTA (1 mM)]. The resulting suspensions were subjected to sonication and ultracentrifugation at 100 000 *g* for 1 h. The supernatants were removed and used for Western blotting and enzyme assays.

Enzyme assays

AcetylCoA-dependent acetylation of 20 known NAT aromatic substrates by recombinant enzymes and root extracts was carried out using the dithio-bis(2-nitrobenzoic acid) (DTNB) and 4-diethylaminobenzaldehyde (DMAB) assay methods, respectively, as described previously (Coroneos *et al*., 1991; Brooke *et al*., 2003). The rate of substrate acetylation by *M. loti* NAT1 and NAT2 was determined in 20 mM Tris-HCl, 1 mM EDTA, pH 7.5. Apparent kinetic parameters (V_m and K_m) for DCA were estimated as previously described (Brooke *et al*., 2003). For bi-bi ping-pong mechanisms (as for NAT enzymes), apparent V_m/apparent K_m ratios are equal to true kinetic parameter ratios (V_m/K_m). Apparent V_m/apparent K_m ratios were therefore used for comparing catalytic efficiencies. Controls were carried out in the absence of enzyme, AcCoA or aromatic substrates. Activity was measured in quadruplicate.

Acknowledgements

This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC). J.D. holds an Association Française contre les Myopathies (AFM) postdoctoral fellowship. E.S. is funded by the Wellcome Trust and E.S., J.D. and F.R.L. held a British Council/Alliance exchange award. M.C.R. held a Marie Curie UE fellowship. We thank Astrid Petzold (Erasmus student) for technical assistance.

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