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# Horizontal gene transfer of atrazine-degrading genes (*atz)* from *Agrobacterium tumefaciens* St96-4 pADP1::Tn5 to bacteria of maize-cultivated soil†

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**Abstract: The plasmid pADP1::Tn5 derived from pADP1[Atr$^+$] carrying a Tn5 transposon conferring kanamycin and streptomycin resistances was constructed and introduced in *Agrobacterium tumefaciens* St96-4. This genetically modified strain was inoculated ($\sim 10^8$ cfu g$^{-1}$) in potted soils planted with maize and treated or not with atrazine (1.5 mg kg$^{-1}$). Bulk and maize rhizosphere soils were sampled 39 days after planting to look for soil indigenous bacteria that had acquired pADP1::Tn5. Four transconjugants were isolated from four different soil samples. The estimated transfer frequency of pADP1::Tn5 was $10^{-4}$ per donor. Maize rhizosphere and atrazine treatment had no obvious effect on pADP1::Tn5 transfer frequency. The sequencing of the 16S rDNA sequences of the transconjugants revealed that they were almost identical and highly similar to that of *Variovorax* spp (97%). In addition, their characterization suggested that the *atzA* and *atzB* genes had been transferred from pADP1::Tn5 to the bacterial chromosome in two of the four transconjugants. These data suggest that the *atz* degrading genes are horizontally transferred in soil and possibly subjected to gene rearrangement.**
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**Keywords:** horizontal gene transfer; atrazine; biodegradation; *atz* genes

## 1 INTRODUCTION

During the past forty years, atrazine (6-chloro-$N^2$-ethyl-$N^4$-isopropyl-1,3,5-triazine-2,4-diamine) has been widely used to control a variety of broadleaf weeds in corn, sugarcane, sorghum, pineapple and other crops. As for many other xenobiotics, the enzymatic arsenal required to completely metabolize atrazine is not naturally available in the soil microflora. As a result, for almost thirty years, atrazine was only converted to desethylatrazine (DEA) and desisopropylatrazine (DIA) by non-specific monooxygenases,[1] and small amounts were degraded into hydroxyatrazine by chemical processes.[2] Consequently atrazine was relatively persistent in soil, with a half-life ranging from a few days to several months.[3] In addition, atrazine was frequently detected in surface water, tile drainage and ground water at concentrations exceeding the European Union standard of 0.1 μg litre$^{-1}$.[4]

However, over the past ten years, enhanced degradation of atrazine has been observed in many soils repeatedly treated with this herbicide. In these soils, the bacterial microflora mineralizes up to 80% of the initially applied atrazine within a week.[5–8] Numerous atrazine-degrading strains belonging to diverse bacterial genera have been isolated from geographically separated sites exposed to atrazine.[9–17] Descriptions of the metabolic pathway and genetic determinants of atrazine mineralization were obtained from investigations in *Pseudomonas* sp ADP.[18] Briefly, atrazine is converted into cyanuric acid through three amidohydrolases encoded by *atzA, atzB* and *atzC* genes.[18–21] Cyanuric acid is then completely degraded to $CO_2$ and $NH_3$ by three other hydrolases encoded by the *atzD, atzE,* and *atzF* genes. These latter three genes are organized in an operon-like structure.[22] In *Pseudomonas* sp ADP, the *atz* genes are located on pADP1, a self-transmissible plasmid that has been entirely sequenced.[22,23] The study of several atrazine-degrading strains revealed that the a*tzA, B* and *C* genes are widely dispersed and highly conserved.[24–26] In addition, they have always been located on large plasmids[16,23,27] except in *Arthrobacter* sp AD1 in which the *atzA* gene was located on the bacterial chromosome.[24] Finally, for some strains, and notably *Pseudomonas* sp ADP, the *atzA, B* and *C* genes were associated with IS1071-like sequences.[16,22,27]

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These data suggest that soil microflora adaptation to atrazine mineralization may rely on horizontal gene transfer (HGT) but its involvement has not yet been evidenced in soil. The aim of our study was to demonstrate that *atz* genes can be transferred by conjugation from a donor strain to members of the indigenous soil microflora. The selection pressures exerted by the pesticide and the rhizosphere had been reported to enhance HGT[28] and promote atrazine mineralization in soil,[29] so these two parameters were investigated. *Agrobacterium tumefaciens* pADP1::Tn5 was inoculated into potted soils planted with maize and treated or not with atrazine. Bulk and maize rhizosphere soils were sampled 39 days after inoculation to (1) enumerate the total culturable bacteria and the donor bacteria, (2) isolate transconjugants and (3) characterize them physiologically, genetically and taxonomically.

## 2 MATERIALS AND METHODS

### 2.1 Soil

The soil was collected from the surface horizon of the soil at La Bouzule (Nancy, France) and sieved (5 mm). This is a redoxic neoluvisol : clay (<2 µm) 33.3% w/w, sand (2–200 µm) 51.3% w/w, silt (200–2000 µm) 15.4% w/w, humidity (equivalent at 1 kg) 33.0%, organic matter 26.4 $g\,kg^{-1}$, organic carbon 15.3 $g\,kg^{-1}$, organic nitrogen 1.7 $g\,kg^{-1}$, C/N yield 9.2 and pH 5.8 (in water).

### 2.2 Bacterial strains and growth conditions

The bacterial strains used in this study are shown in Table 1. *Escherichia coli* strains were grown at 37 °C on Luria-Bertani medium (LB) [bactotryptone (10 g $litre^{-1}$), yeast extract (5 g $litre^{-1}$), NaCl (5 g $litre^{-1}$)]. The other strains were grown at 28 °C on TY medium [bactotryptone (5 g $litre^{-1}$), yeast extract (3 g $litre^{-1}$), $CaCl_2$ (0.6 mM)] or on mineral salt medium (MSA) [$K_2HPO_4$ (1.6 g $litre^{-1}$), $KH_2PO_4$ (0.4 g $litre^{-1}$), NaCl (0.1 g $litre^{-1}$), $MgSO_4{\cdot}7H_2O$ (0.2 g $litre^{-1}$), $CaCl_2$ (0.02 g $litre^{-1}$), $FeSO_4{\cdot}6H_2O$ (5 mg $litre^{-1}$), borate (2 mg $litre^{-1}$), $MnSO_4{\cdot}H_2O$ (1.8 mg $litre^{-1}$), $ZnSO_4$ (0.2 mg $litre^{-1}$), $CuSO_4$ (0.1 mg $litre^{-1}$), $Na_2MoO_4$ (0.25 mg $litre^{-1}$), biotin (0.1 mg $litre^{-1}$), thiamine (0.04 mg $litre^{-1}$)], containing citrate (1 g $litre^{-1}$) as carbon source and atrazine as sole nitrogen source (500 mg $litre^{-1}$; chemical purity of 99%; Syngenta, Switzerland). When needed, the media were supplemented with antibiotics: kanamycin (50 mg $litre^{-1}$), rifampicin (100 mg $litre^{-1}$), streptomycin (50 mg $litre^{-1}$), and/or cycloheximide (100 mg $litre^{-1}$).

### 2.3 Filter-mating experiments

Overnight cultures of donor or recipient cells were centrifuged at 6000 rpm for 5 min then washed twice in 5 ml of TY medium. They were resuspended in this medium at a final bacterial concentration of one OD unit (600 nm). One millilitre of each bacterial suspension was filtered through a 0.45 µm filter (HA sterile filter, Millipore, Ireland) and placed on a TY agar plate overnight at 28 °C. The cells were then washed off the filter with 9 ml of ultra-pure water and serially diluted tenfold. Dilutions of $10^0$ to $10^{-2}$ were plated on selective agar medium, and incubated at optimal temperature for transconjugant growth (28 or 37 °C). Controls, consisting of filtering the donor and recipient through separate filters, were included.

### 2.4 Construction of a recombinant strain containing a plasmid bearing antibiotic markers

Antibiotic resistance was introduced in pADP1 using the Tn5 [$Km^R$; $Sm^R$] transposon. *Agrobacterium tumefaciens* GMI9023 [$Rif^R$] pADP1 was conjugated with *E coli* WA803 containing pGS9::Tn5, a suicide plasmid which cannot be replicated in the recipient.[30] *Agrobacterium* cells that had integrated

**Table 1.** Bacterial strains

| Strain | Relevant characteristics[a] | Reference |
|---|---|---|
| *Escherichia coli* WA803 pGS9 | pGS9 is a Tn5 [$Km^R$, $Sm^R$] suicide vehicle | 30 |
| *Agrobacterium tumefaciens* GMI9023 pADP1 | [$Rif^R$, $Sm^R$]<br>Cured of pTi and pAT plasmids<br>pADP1 [$Atr^+$] | 27 |
| *Agrobacterium tumefaciens* GMI9023 pADP1::Tn5 | [$Rif^R$, $Sm^R$]<br>Cured of pTi and pAT plasmids<br>pADP1::Tn5 [$Atr^+$, $Km^R$, $Sm^R$] | This study |
| *Escherichia coli* JM107 | Overnight growth at 37 °C | 47 |
| *Escherichia coli* JM107 pADP1::Tn5 | Overnight growth at 37 °C<br>pADP1::Tn5 [$Atr^+$, $Km^R$, $Sm^R$] | This study |
| *Agrobacterium tumefaciens* St-96-4 | C58 C9 [$Rf^R$, $Sm^R$, $Ade^-$, $Thr^-$, $Ilv^-$]<br>Cured of pTi plasmid | 48 |
| *Agrobacterium tumefaciens* St-96-4 pADP1::Tn5 | C58 C9 [$Rf^R$, $Sm^R$, $Ade^-$, $Thr^-$, $Ilv^-$]<br>Cured of pTi plasmid<br>pADP1::Tn5 [$Atr^+$, $Km^R$, $Sm^R$] | This study |

[a] $Km^R$, $Sm^R$, $Rf^R$: resistance to kanamycin, streptomycin, and rifampicin, respectively; $Ade^-$, $Thr^-$, $Ilv^-$: inability to synthesize adenine, threonine, isoleucine and valine, respectively; $Atr^+$: ability to mineralize atrazine.

Tn5 in their DNA were screened on a TY agar plate supplemented with rifampicin (100 mg $litre^{-1}$) and kanamycin (50 mg $litre^{-1}$). Plasmid profiles were carried out on the screened cells and hybridized to Tn5 probes. *Agrobacterium tumefaciens* GMI9023 cells showing a hybridization signal on their plasmid were selected. The ADP1::Tn5 plasmid was then transferred from *A tumefaciens* GMI9023 pADP1::Tn5 to *E coli* JM107 by filter mating. *Escherichia coli* JM107 pADP1::Tn5 cells were screened on LB medium containing kanamycin after 24 h of incubation at 37 °C. The ADP1::Tn5 plasmid was then transferred from *E coli* JM107 pADP1::Tn5 to *A tumefaciens* St96-4 by filter mating. *Agrobacterium tumefaciens* St96-4 cells containing pADP1::Tn5 were selected on TY medium containing rifampicin and kanamycin. The *A tumefaciens* St96-4 pADP1::Tn5 cells were checked for (1) atrazine mineralization, (2) the presence of *atzA*, *B*, *C*, *D*, *E*, *F* genes by PCR, and (3) the presence of a 108-kb plasmid hybridizing with the *atzA* probe.

### 2.5 Soil treatment and sampling procedure

*Agrobacterium tumefaciens* St96-4 pADP1::Tn5 was grown on TY liquid medium supplemented with kanamycin, washed twice and suspended in a mineral salt buffer [$K_2HPO_4$ (1 g $litre^{-1}$), $KH_2PO_4$ (1 g $litre^{-1}$), $MgSO_4 \cdot 7H_2O$ (40 mg $litre^{-1}$), $FeCl_3$ (4 mg $litre^{-1}$); pH 6.6]. Soil was inoculated or not with a theoretical density of $10^8$ cfu of the donor strain per gram. The soil was homogenized and placed in plastic pots (1.5 kg equivalent-dry weight). The surface of the soil was spread with either an aqueous atrazine solution (1.5 mg $kg^{-1}$ soil) or water. The soil moisture was adjusted to 80% of the water holding capacity. Three sterilized seeds of maize (*Zea mays* L, cv DEA) were sown per pot and were grown in a greenhouse. The pots were watered daily with sterile water to keep the soil moisture at 80% of the water holding capacity (weight/volume). After 15 days, they were also watered weekly with nutrient solution [$KNO_3$ (0.002 M), $Ca(NO_3)_2$ (0.003 M), $K_2HPO_4$ (0.0008 M), $MgSO_4$ (0.0012 M), NaCl (0.001 M), $NH_4NO_3$ (0.002 M)]. Each set of culture conditions [not treated and not inoculated (NT-NI), treated and not inoculated (T-NI), not treated and inoculated (NT-I), and treated and inoculated (T-I)] was replicated five times.

Maize plants were harvested after 39 days and the root systems shaken to remove excess soil. Rhizosphere soil was recovered by scraping off the soil particles still associated with roots. Soil remaining in the pot was considered as bulk soil. Soil samples were sieved to 2 mm and stored at 4 °C for less than 5 days before experiments. Remaining soil was placed in another pot and subjected to a second cropping cycle following the procedure described above.

### 2.6 Enumeration of bacteria from soil and search for transconjugants

10 g of soil sample (equivalent-dry weight) were mixed with 90 ml of sterile water for 1 min using a Waring blender (New Hartford, Conn, USA). The resulting soil suspensions were serially diluted tenfold in sterile water, and 100 µl aliquots of appropriate dilutions were spread on specific agar plates containing cycloheximide (100 mg $litre^{-1}$), benlate (10 mg $litre^{-1}$) and *para*-nitrochlorobenzene (PNCB, 5 mg $litre^{-1}$) as antifungal agents. Each dilution was duplicated. Total culturable heterotrophic bacteria were enumerated on 1/10 nutrient agar medium (Difco, France) after two weeks of incubation at 20 °C. The donor population was enumerated on TY medium supplemented with rifampicin (100 mg $litre^{-1}$) and kanamycin (50 mg $litre^{-1}$) after 5 days of incubation at 28 °C. Transconjugants were screened on MSA medium containing atrazine (500 mg $litre^{-1}$), kanamycin (50 mg $litre^{-1}$) and streptomycin (50 mg $litre^{-1}$). Bacterial colonies forming a clear zone corresponding to atrazine degradation[18] were collected and used for further analysis.

### 2.7 DNA extraction

DNA was extracted from 1 ml of bacterial suspension cells (1 OD unit at 600 nm) grown overnight in TY medium using the High pure PCR template preparation kit according to the manufacturer's instructions (Roche, Germany).

### 2.8 PCR analysis

PCR analyses were conducted in a final volume of 25 µl by using 0.2 µM of dNTPs, 1 µM of specific primers (for primer sequences see Table 2) and 1.25 U of Taq DNA polymerase (Q-biogene). DNA amplifications were carried out in a PTC 200 gradient cycler (MJ Research, Waltham, MA, USA) as follows: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at the optimal temperature for primers annealing and 2 min at 72 °C, plus an additional 10-min cycle at 72 °C. PCR products were then separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide (100 µg $litre^{-1}$) (Gibco-BRL, Life Technologies, CA, USA) and photographed with a CCD camera (Sony, France).

### 2.9 Repetitive extragenic palendromic (REP) PCR

REP PCR was carried out in a 25 µl mixture using 50 ng of extracted DNA as template. The PCR conditions were as follows: 5 µl of 5× Giescher buffer [83 mM $(NH_4)_2SO_4$, 335 mM Tris HCl, 150 mM $\beta$-mercaptoethanol, 33.5 µM EDTA, pH 8.8], 6.5 mM $MgCl_2$, 0.17 mg $ml^{-1}$ BSA, 1.25 mM of each of dNTP, 0.1 µl $ml^{-1}$ DMSO, 2 µM of each of the two primers (REPIR-1 and REP2-2, Table 2) and 1 U of Taq polymerase (Q-biogene). Amplifications were carried out in a PTC 200 gradient cycler as follows: 1 cycle at 95 °C for 6 min, 30 cycles at 94 °C for 1 min, 40 °C

**Table 2.** Primers

| Target sequence | Primer | Nucleotide sequence [a] (5′ → 3′) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|
| *atzA* | A1f | ACG GGC GTC AAT TCT ATG AC | 60 | 25 |
| | A1r | CAC CCA CCT CAC CAT AGA CC | | |
| *atzB* | B1f | AGG GTG TTg AGG TGG TGA AC | 60 | 25 |
| | B1r | CAC CAC TGT GCT GTG GTA GA | | |
| *atzC* | Cf | GCT CAC ATG CAG GTA CTC CA | 57 | 25 |
| | C1r | TCC CCC AAC TAA ATC ACA GC | | |
| *atzD* | Df | GGG TCT CGA GGa TTT GAT TG | 60 | 49 |
| | Dr | TCC CAC CTG ACA TCA CAA AC | | |
| *atzE* | Ef | GAG CCT CTG TCC GTA GAT CG | 60 | 49 |
| | Er | GAT GGC GTG TAC CGT TTA CC | | |
| *atzF* | Ff | ACC AGC CCT TGA ATC ATC AG | 60 | 49 |
| | Fr | ACT TAC AAA CGC ACC GAA CC | | |
| Tn5 | Tn5Kmu | TGG ACC CCT TGG CGT CAT CAA | 60 | This study |
| | Tn5Kml | TTG TCG GCA GCC TGG TTC ATC | | |
| *AtzA* upstream sequence | ORF35f | GCT TGA CCT TCA GCT CCT TG | 57 | 49 |
| | AtzA1771r | GAC GAT CCG TCC ATC CTG | | |
| *AtzB* upstream sequence | ORF35f | GCT TGA CCT TCA GCT CCT TG | 57 | 49 |
| | AtzB2203r | CCC CGT CTC TCA CCA GAA TA | | |
| IGS of the donor strain | C58f | ATT CGC AAG AAT TGA TGC CTG T | 56 | This study |
| | C58r | AGC GAT CCT TCC TAC CTC CAA C | | |
| 16S | 27f | AGA GTT TGA TC(A/C) TGG CTC AG | 55 | 50 |
| | 1492r | TAC GG(A/C/T) TAC CTT GTT ACG ACT T | | |
| IGS | 72f | TGC GGC TGG ATC ACC TCC TT | 55 | 50 |
| | 38r | CCG GGT TTC CCC ATT CGG | | |
| REP | REPIR-1 | III ICG ICG ICA TCI GGC | 40 | 51 |
| | REP2-1 | ICG ICT TAT CIG GCC TAC | | |

[a] The letter I corresponds to inosine.

for 1 min and 65 °C for 4 min, plus an extension cycle at 65 °C for 16 min. Amplified DNA fragments were separated by electrophoresis on 1% agarose gel.

### 2.10 Plasmid profile analysis, blotting and hybridization

Plasmids were separated from total DNA using the Eckhardt method[31] as modified by Wheatcroft *et al.*[32] Plasmid bands were observed under UV illumination after staining with ethidium bromide. DNA was then transferred to a Biodyne Plus membrane (Gelman Sciences) under vacuum using a Vacu Gene apparatus (Pharmacia). The membranes were washed in 5 × SSC [NaCl (0.75 M), tri-sodium citrate (0.075 M)]. DNA was cross-linked to the membrane by UV irradiation for 3 min and by heating for 1 h at 80 °C.

Non-radioactive Dig labeled *atz*A, *atz*B, *atz*C, *atzD*, *atz*E, *atzF* and Tn5 Probes were produced from *Agrobacterium tumefaciens* St-96-4 pADP1::Tn5 by PCR. PCR conditions were as described above except that the dNTP concentrations were as follows: dATP, dCTP and dGTP 200 mmol litre$^{-1}$ each, dTTP 26 mmol litre$^{-1}$, and Dig 11-dUTP 13 mmol litre$^{-1}$ (Roche Biochemical). Hybridization of the membrane was carried out overnight at 68 °C in a solution containing 5 × SSC 0.1% blocking agent, 0.1% *N*-laurylsarcosinate, 0.02% SDS and 5 µl of probe targeting *atzA*, atz*B*, atz*C*, atz*D*, atz*E*, atz*F* or Tn5. Post-hybridization washes were performed twice in 2 × SSC and 0.1% SDS solution for 5 min at room temperature and twice in 0.1 × SSC and 0.1% SDS solution for 15 min at 68 °C. Hybridized probes were detected using the Dig Luminescent Detection Kit and CDP Star as a chemiluminescent substrate according to the manufacturer's instructions (Roche Biochemical).

### 2.11 Restriction length polymorphism analysis

16S rDNA and *atz* upstream sequences were amplified as described above and 5 µl of PCR products were digested either with *Rsa*I, *Hha*I or *Hae*III (Appligene-Oncor, France) overnight at 37 °C. Digested PCR products were separated by electrophoresis on 3% Small Fragment agarose gel (Appligene-Oncor, France) in 1× TBE (Interchim, France), stained with ethidium bromide and visualized under UV illumination.

### 2.12 16S rDNA sequencing

16S rDNA PCR products were cloned into the pGemT-EasyII vector according to manufacturer's recommendations (Promega, Madison, WI, USA) and sequenced with the DTCS-1 kit (Beckman Coulter, France). Sequences were compared with those from the GenBank database using BlastN. Multiple alignments were carried out with the ClustalX software and phylogenetic analyses were done with NJplot software. The 16S rDNA sequences of strains MD1, MD2 and MD3 were deposited

into GenBank under accession numbers AY738 707, AY738 708, AY738 709, respectively.

### 2.13 Determination of growth parameters

Growth parameters of strains were determined by culturing cells overnight at 28 °C in TY medium supplemented with kanamycin, washing twice in ultra-pure water and resuspending in ultra-pure water to obtain an optical density of 0.6 OD (600 nm) corresponding to $5.1 \times 10^8 \pm 1.8\,\text{cfu ml}^{-1}$ of each strain. Cellular suspensions were serially diluted in TY medium and then incubated in a Bioscreen apparatus (Labsystem, Finland) which enabled (1) incubation at a precise temperature, (2) agitation of cultures, and (3) measurement of optical density at 600 nm every 30 min. Dilutions were replicated three times.

A modified Gompertz model[33] was fitted to the bacterial growth curves using SigmaPlot 4.0 software. The model equation was as follows:

$$y = A \cdot \exp\{-\exp[1 + \mu m.\exp(1).(\lambda - t)/A]\}$$

where $y$ is the OD value at 600 nm (AU), $t$ the time (h), $\mu m$ is the maximum specific growth rate ($h^{-1}$), $A$ is the maximum OD value attained (AU) and $\lambda$ is the lag time (h). The parameters determined were validated by a Student $t$ test ($P < 0.005$, $n = 3$).

### 2.14 Determination of atrazine mineralization kinetics of the strains

The capacity of bacterial isolates to mineralize atrazine was tested by radiorespirometry in liquid culture. Isolates were grown overnight at 28 °C in TY medium containing kanamycin (50 mg litre$^{-1}$), and then centrifuged at 6000 rpm for 5 min. Pellets were washed twice in ultra-pure water and resuspended to 0.2 OD unit (600 nm) in MSA medium containing 30 mg litre$^{-1}$ of atrazine and 170 Bq ml$^{-1}$ of $^{14}C$ ring-labelled atrazine. Bacterial suspensions were incubated at 28 °C in a sterile jar and shaken (150 rpm). Each jar contained a $CO_2$ trap (5 ml of 0.2 M NaOH in a scintillation vial) to determine $^{14}CO_2$ production. Radioactivity in the NaOH solution was measured periodically by liquid scintillation counting (Packard) using 10 ml of ACSII scintillation fluid (Amersham). Cultures were triplicated.

The atrazine-mineralization parameters of the bacterial strains were determined using the Gompertz model. The model equation was as follows:

$$y = a \cdot \exp(-\exp(-k(t - ti)))$$

where $y$ is the rate of atrazine mineralization (%), a is the maximum rate of atrazine mineralization (%), $k$ is the constant rate of atrazine mineralization (day$^{-1}$), $t$ is the time (day), and $ti$ is the abscissa of the inflection point (day). These analyses were performed with the SigmaPlot 4.0 software and the determined parameters were validated by a Student $t$ test ($P < 0.005$, $n = 3$).

### 2.15 Statistical analysis

Transconjugants were compared with regard to atrazine mineralization and bacterial growth by subjecting the model parameters to a single factor analysis of variance (ANOVA) followed by a Fisher procedure ($n = 3, P < 0.05$) (Statview© 4.55 software, Abacus Concept, Inc).

## 3 RESULTS

### 3.1 Enumeration of total culturable bacteria and of the donor strain in soil

Total culturable soil bacteria were enumerated to determine the impact of (1) *A tumefaciens* St96-4 pADP1::Tn5 inoculation, (2) the maize rhizosphere and (3) the atrazine treatment on the soil microflora (Table 3). Initially, $1.2 \times 10^8$ cfu were enumerated per gram of soil. Thirty-nine days after inoculation, $0.93–2.00 \times 10^8\,\text{cfu g}^{-1}$ of culturable bacteria were enumerated. Analysis of variance (ANOVA) revealed that the number of culturable soil bacteria was significantly higher in the rhizosphere than in bulk soil ($n = 5$, $P < 0.05$). In addition, the number of culturable soil bacteria was lower in the inoculated soil than in the uninoculated soil. Atrazine treatment did not significantly affect the size of the microflora. No interaction between the three analyzed parameters was observed.

The donor strain was enumerated in soil samples using a rich medium supplemented with rifampicin

**Table 3.** Soil bacteria and donor cell enumeration initially (T0) and after 39 days of soil cultivation. Treatments were as follows: soil treated or not with atrazine, bulk or rhizosphere soil, and soil inoculated or not (I or NI)[a]

| | | Not treated soil | | | | Atrazine-treated soil | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Bulk | | Rhizosphere | | Bulk | | Rhizosphere | |
| cfu g$^{-1}$ | T0 | NI | I | NI | I | NI | I | NI | I |
| Soil bacteria $\times 10^8$ | 1.2 (±0.16) | 1.1 (±0.9) | 0.93 (±0.19) | 2.0 (±1.1) | 1.2 (±0.15) | 1.2 (±0.24) | 0.95 (±0.11) | 1.5 (±0.41) | 1.5 (±0.59) |
| Donor strain $\times 10^5$ | 505 (±58) | — | 2.2 (±0.38) | — | 2.3 (±0.41) | — | 1.8 (±0.44) | — | 2.5 (±0.67) |

[a] Values are means ± standard error ($n = 5$).

(100 mg litre$^{-1}$) and kanamycin (50 mg litre$^{-1}$) allowing the selection of donor bacteria [Rif$^R$] possessing pADP1::Tn5 [Km$^R$] (Table 3). At the beginning of the experiment, $5.05 \times 10^7$ cfu were enumerated per gram of soil. Whatever the atrazine treatment and soil compartment, the number of culturable donor bacteria decreased to approximately $2.0 \times 10^5$ cfu g$^{-1}$ 39 days after inoculation. Neither atrazine treatment nor rhizosphere had any significant effect on the size of the culturable donor population in soil ($n = 5$, $P < 0.05$). At the end of the second cropping cycle, the quantity of donor bacteria in soil was below the detection limit of $10^3$ cfu g$^{-1}$ (data not shown).

### 3.2 Transconjugant isolation

After 39 days of maize cultivation, the presumptive transconjugants were isolated on MSA plates containing kanamycin and streptomycin. Presumptive transconjugants resistant to these antibiotics and forming clear zones corresponding to the degradation of atrazine crystals were selected. The estimated detection limit of the transconjugant was approximately $10^4$ cfu g$^{-1}$ due to the growth of fungi and bacteria on the selective media despite the presence of two antibiotics and three antifungal agents. No presumptive transconjugant could have been isolated from soils not inoculated with the donor strain. Four presumptive transconjugants were recovered from the inoculated soils. One was isolated from untreated inoculated bulk soil (NT-I-B), one from treated inoculated bulk soil (T-I-B) and the other two from two different treated inoculated rhizosphere soils (T-I-R). Presumptive transconjugants were further purified by successive plating on selective medium yielding four isolates designated as MD1, MD2, MD3 and MD4. PCR amplifications of the *atzA* gene and Tn5 transposon performed on these isolates yielded PCR products of the expected size (data not shown). Plasmid profile analysis revealed the presence of a plasmid similar in size to pADP1::Tn5 in isolates MD3 and MD4 (Fig 1A). Surprisingly, a plasmid slightly smaller than pADP1::Tn5 was observed in isolates MD1 and MD2.

Results of plasmid hybridization with *atzA, atzB, atzC, atzD, atzE, atzF* and Tn5 probes are presented in Fig 1B. The plasmid of isolate MD3 produced a hybridization signal with all probes. The plasmid of isolate MD4 also hybridized with all probes but produced only a low signal due to a poor plasmid extraction as shown by the plasmid profile (Fig 1A). The plasmids of isolates MD1 and MD2 produced a hybridization signal only with *atzC, atzD, atzE, atzF* and Tn5 probes. For these isolates, the *atzA* and *atzB* probes produced a hybridization signal on the bacterial chromosome.

To test whether the chromosomal *atzA* and *atzB* genes of isolates MD1 and MD2 originated from pADP1::Tn5, the sequences upstream from these genes were analyzed by PCR targeted on the *atzA* region between −897 to +102 bp and on the *atzB* region between −897 to +97 bp as described on

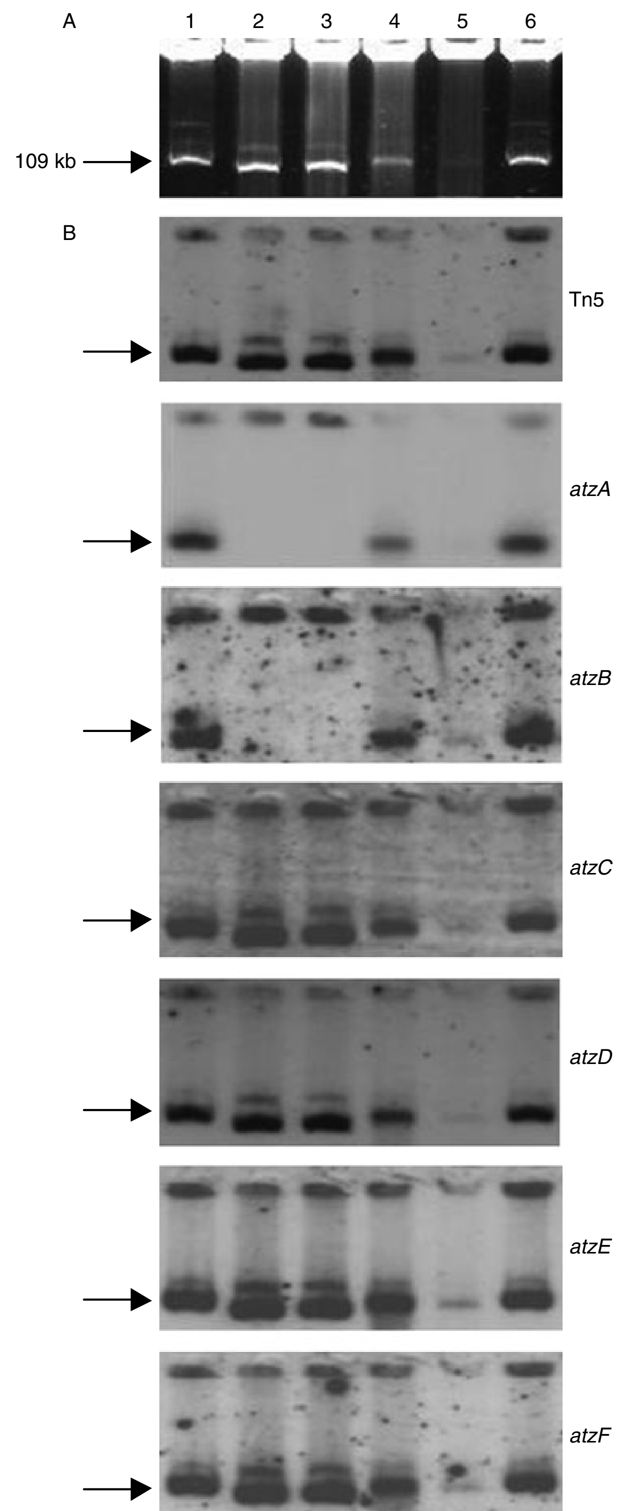


**Figure 1.** Plasmid profiles of donor and transconjugant strains (panel A), and corresponding hybridizations with Tn5, *atz*A, *atz*B, *atz*C, *atzD, atzE,* or *atzF* probes (panel B). Lanes 1 and 6: donor strain; lane 2 to 5: isolates MD1, MD2, MD3 and MD4, respectively. The arrows indicate pADP1::Tn5 size.

pADP1 (Fig 2). PCR fragments of the expected size were obtained for both genes from the four isolates and the donor (data not shown). In addition, for each gene, the digestion of these PCR products with *Hha*I and *Hae*III produced similar patterns for the isolates MD1, MD2 and the donor strain (Fig 3).

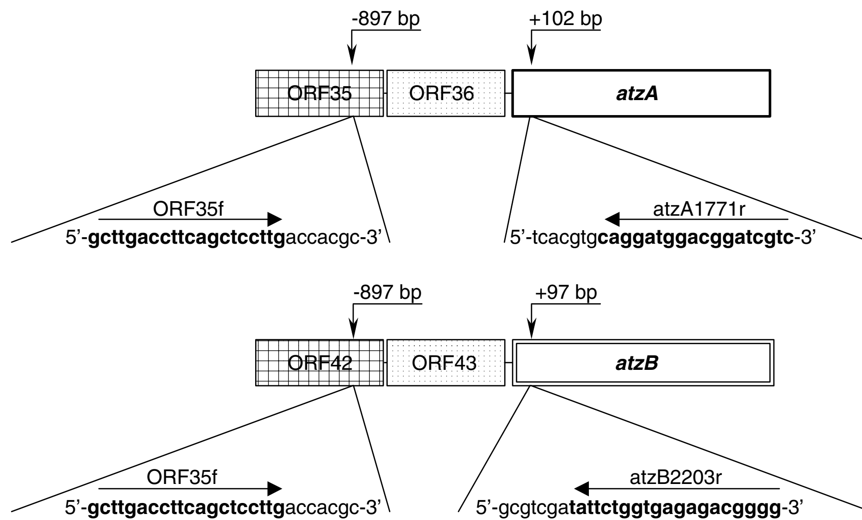


**Figure 2.** Map of the upstream regions of *atzA* and *atzB* genes on pADP1. An arrow indicates the sequences targeted by the primers used to amplify the upstream regions of both genes. The positions given in base pairs are related to the first transcripted nucleotide (+1 bp) of *atzA* or *atzB* genes.

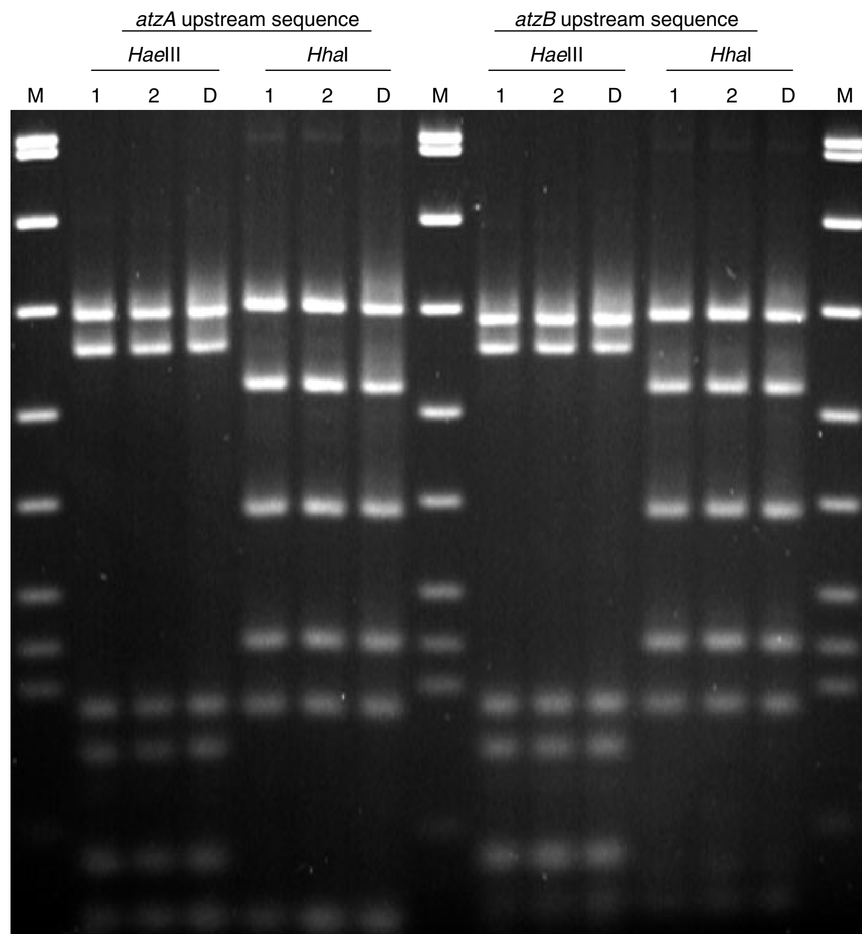


**Figure 3.** RFLP analysis of the 5′-upstream sequence of *atzA* and *atzB* from the isolates MD1 and MD2 and the donor. The upstream sequences of *atzA* and *atzB* genes were amplified by PCR and digested with the restriction enzymes *Hha*I, *Hae*III. Lanes 1, 2, D correspond to the isolates MD1, MD2, and the donor, respectively. BVIII molecular weight marker is shown in lane M (band sizes in bp: 501, 489, 404, 320, 242, 190, 147, 124, 110 and 67).

### 3.3 Transconjugant characterization

All isolates exhibited the same colony morphology on MSA medium. They were yellow, circular (1 mm diameter), with an entire margin and opaque with mucoid aspect. The four isolates presented identical ARDRA profiles with *Hae*III, *Hha*I, and *Rsa*I restriction enzymes (data not shown). The amplification of the 16S-23S rDNA intergenic spacer (IGS) yielded a unique 770-bp PCR fragment for all the transconjugants (data not shown). Repetitive

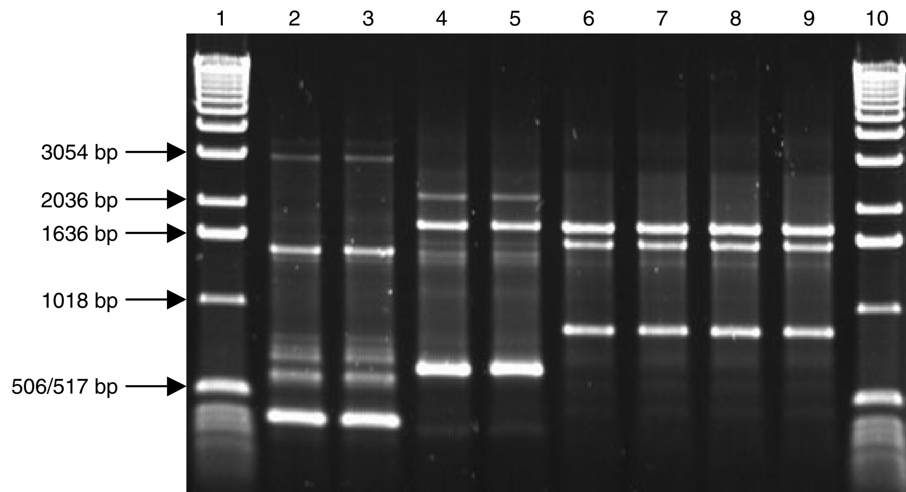


**Figure 4.** REP-PCR analysis of the transconjugants. REP-PCR analyses were performed in duplicate for each transconjugant. Lanes 1 and 10: 1-kb ladder (molecular weight is indicated in bp); lanes 2 and 3: isolate MD1; lanes 4 and 5: isolate MD2; lanes 6 and 7: isolate MD3; lanes 8 and 9: isolate MD4.

extragenic palendromic (REP) PCR fingerprints were different for isolates MD1 and MD2 but identical for isolates MD3 and MD4 (Fig 4). Comparison of 16S rDNA sequences of isolates MD1, MD2 and MD3 with those available in the databases showed the highest level of similarity (97%) to *Variovorax* spp. Moreover, multiple alignments of the 16S rDNA sequences also revealed a close phylogenetic relationship with several *Variovorax* spp (Fig 5). As a result, they were classified as Bacteria, phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, family Comamonadaceae and close to the genus *Variovorax*.

Growth of the transconjugants was monitored by cultivation on rich medium. Strains MD3 and MD4 had no significantly different growth parameters ($n = 3$, $P < 0.05$). They showed a maximum absorbance value of ~0.7 AU, a maximum specific growth rate of $\sim 0.08\,h^{-1}$, and a lag time of ~27 h ($n = 3$, $P < 0.05$) (Table 4). Strains MD1 and MD2 were characterized by a longer lag time than MD3 and MD4 (~30 h). In addition, strain MD1 had a lower maximum absorbance value (0.59 AU) and a higher maximum specific growth rate ($0.089\,h^{-1}$) than strain MD2 (0.71 AU, $0.075\,h^{-1}$).

Mineralization activities of the four transconjugants were measured by radiorespirometric analysis using $^{14}C$-ring-labelled atrazine. The mineralization results obtained for strains MD1 and MD2 were consistent and permitted determination of the atrazine mineralization parameters *a*, *k* and *ti*. These two strains showed similar constant rates of atrazine mineralization (Table 4). Nevertheless strain MD2 needed only 15.2 h to reach its maximum mineralization rate (*ti*) whereas strain MD1 required 29.5 h. In addition, strain MD1 showed a higher maximal percentage of mineralized atrazine (ie 63.6%) than strain MD2 (ie 47.3%) ($n = 3$, $P < 0.005$).

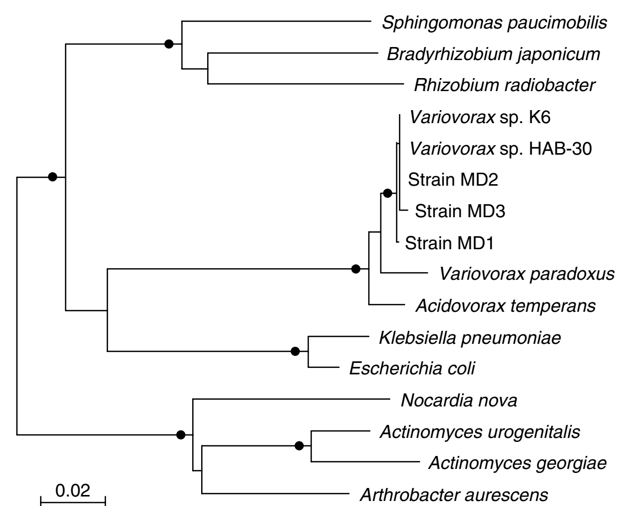


**Figure 5.** 16S rDNA phylogenetic (NJ) analysis of the isolated transconjugants with related 16S rDNA, sequences found in the GenBank database. Only bootstrap values up to 9000 of 10 000 iterations are highlighted by a black dot. The strains used for phylogenetic analysis and the GenBank accession numbers of their 16S rDNA sequences are: *Sphingomonas paucimobilis* (D16144), *Bradyrhizobium japonicum* (X87 272), *Rhizobium radiobacter* (M11223), *Variovorax* sp K6 (AF532867), *Variovorax* sp HAB-30 (AB051691), *Variovorax paradoxus* (AF250030), *Acidovorax temperans* (AF078766), *Klebsiella pneumoniae* (Y17 656), *Escherichia coli* (X80 725), *Nocardia nova* (Z36 930), *Actinomyces urogenitalis* (AJ243791), *Actinomyces georgiae* (X80 413), *Arthrobacter aurescens* (X83 405).

## 4 DISCUSSION AND CONCLUSIONS

Repeated application of atrazine leads to the establishment of atrazine-accelerated biodegradation relying on the acquisition by soil microflora of *atz* genes encoding catabolic enzymes responsible for atrazine degradation.[5,29] Since (1) *atzA*, *B* and *C* genes are highly conserved and plasmid-borne[25] and (2) the maize rhizosphere is assumed to be a hotspot for HGT,[28] our study aimed to reveal the existence of horizontal *atz* gene transfer in both bulk and maize rhizosphere soils treated or not with this herbicide.

**Table 4.** Growth and atrazine mineralization parameters for transconjugants. Bacterial growth and atrazine mineralization parameters were determined after modelling growth and mineralization curves using the Gompertz model as modified or not by Zwietering *et al.*[33] *a* is either the maximum OD value (AU) or the maximum mineralization rate (%), $\mu_m$ is the maximum specific growth rate ($h^{-1}$), $\lambda$ is the lag time (h), *k* is the constant rate of atrazine mineralization ($h^{-1}$), and *ti* is the abscissa of the inflection point (h). Values are means ± standard error. For each parameter, values followed by the same letter do not differ significantly ($n = 3$, $P < 0.05$)

| | Growth | | | Atrazine mineralization | | |
|---|---|---|---|---|---|---|
| | *a* (AU) | $\mu_m$ ($h^{-1}$) | $\lambda$ (h) | *a* (%) | *k* ($h^{-1}$) | *ti* (h) |
| Strain MD1 | 0.59 (±0.01)[a] | 0.089 (±0.002)[a] | 29.8 (±0.2)[a] | 63.6 (±1.5)[a] | 0.048 (±0.004)[a] | 29.5 (±0.7)[a] |
| Strain MD2 | 0.71 (±0.03)[b] | 0.075 (±0.003)[b] | 29.9 (±0.3)[a] | 47.3 (±3.5)[b] | 0.059 (±0.017)[a] | 15.2 (±2.6)[b] |
| Strain MD3 | 0.65 (±0.03)[c] | 0.081 (±0.006)[abc] | 27.1 (±0.2)[b] | nd | nd | nd |
| Strain MD4 | 0.68 (±0.01)[bc] | 0.086 (±0.005)[ac] | 26.9 (±0.2)[b] | nd | nd | nd |

nd: not determined.

The number of culturable bacteria was higher in the maize rhizosphere than in bulk soil, confirming that the growth of microorganisms is promoted in the rhizosphere.[29] It further suggests that the higher density of bacteria in the maize rhizosphere could favour contact between bacterial cells and thus conjugation.[28] The number of donor bacteria decreased from $5 \times 10^7$ cfu $g^{-1}$ at the beginning of the experiment to $10^5$ cfu $g^{-1}$ of soil at the end of the first culture cycle and was not detectable (ie below $10^3$ cfu $g^{-1}$) after the second cycle. In addition, atrazine treatment did not visibly favour the maintenance of the donor strain. These results suggest that the donor population was not efficient enough to colonize ecological niches already occupied by indigenous soil bacteria. Furthermore, this observation underlines the interest of horizontal *atz* gene transfer for bioremediation purposes when inoculating poorly competitive atrazine-degrading bacteria to soil. Indeed, even if the inoculated atrazine-degrading bacteria did not survive in the soil, their atrazine-degrading genetic potential might be disseminated within the indigenous soil microflora.

Horizontal *atz* gene transfer was investigated using a polyauxotrophic donor strain ($Ile^-$, $Ade^-$, $Ilv^-$) which was counter-selectable on the minimal medium used to select transconjugants in order to avoid plate mating bias. However, the donor strain grew on this medium probably because of the presence of amino acid traces in the diluted soil suspensions. This observation highlights a limitation to the counter-selection method based on the use of auxotrophic donor bacteria which has not been stated in previous reviews.[34,35] Plate mating greatly depends on the density of donor and recipient cells spread on the selective plates. Previous work showed that this phenomenon did not occur when less than $10^4$ cfu of donors and recipients were spread on the plate.[36] In our study, transconjugants were isolated 39 days after inoculation when a maximum of $2 \times 10^5$ cfu of donor bacteria were enumerated per gram of soil, corresponding to a maximum of 20 cfu of donor bacteria on the selective plates. Under these conditions the probability of plate mating was almost nil.

Four bacterial isolates that had integrated the plasmid pADP1::Tn5 were isolated from soil 39 days after inoculation with *Agrobacterium tumefaciens* St96-4 pADP1::Tn5. By convention they were designated as transconjugants even though transformation or generalized transduction cannot be excluded. The estimated frequency of pADP1::Tn5 transfer was approximately $10^{-4}$ per donor cell. Nevertheless, the conjugation frequency depends on several parameters such as the nature of the donor population or the selection pressure exerted on soil.[37] Therefore, although we demonstrated that transfer of ADP1::Tn5 atrazine-degrading plasmid occurred under our experimental conditions, the transfer frequency of pADP1::Tn5 might not reflect HGT frequency in natural soil conditions.

The low number of isolated transconjugants did not allow us to draw conclusions about atrazine treatment and rhizosphere effects on the transfer frequency of pADP1::Tn5. However, it has already been shown for other catabolic genes that (1) the selective pressure exerted by the xenobiotic can enhance the survival and growth of transconjugants in soil,[38,39] and that (2) the rhizosphere can act as a hotspot for plasmid transfer.[40–44] In addition, no effects of either atrazine treatment or rhizosphere were observed on the nature of the isolated transconjugants. Indeed, whatever the soil sample from which they were isolated, they were all closely related to the *Variovorax* genus. Species of this genus are frequently isolated from natural environments for their ability to degrade xenobiotics.[39,45,46] However nothing can be concluded since this could result from experimental bias such as (1) better culturability of the transconjugants on the selective medium, and (2) their high density in soil.

In addition to horizontal *atz* gene transfer, we obtained good evidence of the recombination of *atzA* and *atzB* genes. The observed plasmid in two of the four transconjugants (MD1 and MD2), was smaller than pADP1::Tn5 and did not contain the *atzA* and *atzB* genes. However, both these genes were detected in MD1 and MD2. In addition, the restriction patterns of the 5′-upstream sequence of these two genes were identical for the donor and both these strains. The

results suggest that *atzA* and *atzB* genes moved from the ADP1::Tn5 plasmid to the chromosome. As the *atzA* and *atzB* genes are flanked by IS1071 insertion sequences, a possible rearrangement could be the transposition of a catabolic transposon made of two copies of IS1071 and containing *atzA* and/or *atzB* genes. This is supported by the fact that IS1071 has been associated with *atz* genes in strains of *Pseudaminobacter* C147,[16] *Arthrobacter aurescens*,[26] *Chelatobacter heintzii, Arthrobacter crystallopoietes* and *Aminobacter aminovorans*.[27] Further work will aim at evaluating the involvement of IS1071 in *atz* gene rearrangement in several atrazine-degrading strains.

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