Detection and organization of atrazine-degrading genetic potential of seventeen bacterial isolates belonging to divergent taxa indicate a recent common origin of their catabolic functions

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Abstract

A collection of 17 atrazine-degrading bacteria isolated from soils was studied to determine the composition of the atrazine-degrading genetic potential (i.e. *trzN*, *trzD* and *atz*) and the presence of IS*1071*. The characterization of seven new atrazine-degrading bacteria revealed for the first time the *trzN-atzBC* gene composition in Gram-negative bacteria such as *Sinorhizobium* sp. or *Polaromonas* sp. Three main atrazine-degrading gene combinations (i) *trzN–atzBC*, (ii) *atzABC–trzD* and (iii) *atzABCDEF* were observed. The *atz* and *trz* genes were often located on plasmids, suggesting that plasmid conjugation could play an important role in their dispersion. In addition, the observation of these genes (i) on the chromosome, (ii) on the same DNA fragment but on different plasmids and (iii) on DNA fragments also hybridizing with IS*1071* suggests that transposition may also contribute to disperse the atrazine-degrading genes.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is a herbicide widely used to control a variety of broadleaf weeds mainly in corn and sugarcane crops. Its extensive use over the last 40 years has led to the contamination of water resources by the parent compound and its main metabolites (deethylatrazine and deisopropylatrazine).

However, an enhancement of atrazine biodegradation has been evident in several soils frequently treated with this herbicide (Barriuso & Houot, 1996). This phenomenon results from the evolution of soil bacterial communities genetically able to use atrazine as a nutrient source. The characterization of atrazine-degrading bacterial strains revealed the existence of *atzA*, *B*, *C*, *D*, *E* and *F* genes encoding enzymes responsible for the mineralization of this herbicide (De Souza *et al.*, 1996; Boundy-Mills *et al.*, 1997; Sadowsky *et al.*, 1998; Martinez *et al.*, 2001). Two other atrazine-degrading genes have also been characterized: *trzN* coding an atrazine chlorohydrolase (equivalent to *atzA*) and *trzD* coding a cyanuric acid hydrolase (equivalent to *atzD*) (Karns, 1999; Mulbry *et al.*, 2002). Further studies revealed that *atzA*, *atzB* and *atzC* are worldly widespread and highly conserved in different microbial genera arguing for a recent unique evolutionary origin and a global dispersion (De Souza *et al.*, 1998a, b; Rousseaux *et al.*, 2001).

Horizontal gene transfer and notably plasmid conjugation has a central role in dispersing the atrazine-degrading genes in the environment. Indeed, except for *Arthrobacter* sp. AD1, in which *atzA* has been located on the chromosome (Cai *et al.*, 2003), the *atz* and *trz* genes have always been located on plasmids, which is a prerequisite for conjugation. In addition, experiments conducted in the laboratory or in soil microcosms with bacteria harbouring the *atz* genes on a catabolic plasmid demonstrated that these genes could be transferred to indigenous bacteria by plasmid conjugation (De Souza *et al.*, 1998a, b; Devers *et al.*, 2005).

However, one can hypothesize that plasmid conjugation might not be the only mechanism responsible for dispersion of the atrazine-degrading genes. Actually, *atz* and *trz* genes have been observed at different genomic locations (i.e. plasmid and/or chromosome) within a bacterium (Rousseaux *et al.*, 2002) suggesting their involvement in genetic rearrangements. Furthermore, the *atz* genes are often associated with one or more copies of insertion sequences related to IS*1071* (Topp *et al.*, 2000a, b; Martinez *et al.*,

2001; Sajjaphan *et al.*, 2004). It has therefore been hypothesized that the observed occurrence at different genomic locations might result from the transposition of a composite transposon consisting of two copies of IS*1071* flanking an atrazine-degrading gene cassette (Devers *et al.*, 2005).

In this context, the aim of this work was to describe the genomic organization of the atrazine-degrading genes (i.e. *atzA*, *B*, *C*, *D*, *E*, *F*, *trzN* and *trzD*) and of IS*1071* in 17 atrazine-degrading bacterial strains. This bacterial collection was analysed by (1) hybridizing their plasmid profiles with *atz*, *trz* and IS*1071* probes in order to determine their genomic location (plasmid vs. chromosome) and by (2) Southern blot analyses to determine whether the atrazine-degrading genes were located in genomic DNA fragments containing IS*1071*.

Materials and methods

Strains and media

The strains used in this study are presented in Table 1. They were grown at 28 °C on mineral salt medium (MSA) [K_2HPO_4 (1.6 g L^{-1}), KH_2PO_4 (0.4 g L^{-1}), NaCl (0.1 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}), $FeSO_4 \cdot 6H_2O$ (5 mg L^{-1}), borate (2 mg L^{-1}), $MnSO_4 \cdot H_2O$ (1.8 mg L^{-1}), $ZnSO_4$ (0.2 mg L^{-1}), $CuSO_4$ (0.1 mg L^{-1}), NaMo (0.25 mg L^{-1}), biotin (0.1 mg L^{-1}), thiamine (0.04 mg L^{-1})], containing atrazine as sole nitrogen source (99% chemical purity; Syngenta, Switzerland) and citrate as carbon source (1 g L^{-1}) (Devers *et al.*, 2005). The concentration of atrazine in the liquid and solid MSA media was 30 and 500 mg L^{-1}, respectively.

Isolation of atrazine-degrading bacteria from soil

Two soils were used one was collected from an agricultural field in Vannecourt (Moselle, North-east France) and the other from a location within an atrazine-producing factory (Sisak, Northern Croatia). The soils were collected from the first 10 cm, sieved (2 mm), moistened (70% water-holding capacity) and treated with 1.5 mg of atrazine per kilogram of soil. They were incubated for 2 weeks in the dark at 22 °C and 60% relative humidity. The soils were then serially diluted in water and 100 μL aliquots of dilutions (from 10^{-2} to 10^{-4}) were plated on MSA medium. The plates were incubated at 28 °C for 3 weeks. Atrazine formed a whitish opaque veil on the surface of the MSA plates. Any bacterial colonies that produced clear halos corresponding to atrazine degradation were further purified by repeated streaking on MSA medium. To avoid any losses of atrazine-degrading genes, the purified strains were kept on solid MSA medium. To store pure strains on the long term, they were cultivated in liquid MSA and an aliquot of the culture was added with 50% glycerol (v/v) and kept at − 80 °C until use. Microbial biomass was obtained by cultivating the strains in liquid MSA medium.

Table 1. Bacterial strains used in this study together with strain names, isolation location, PCR detection of atrazine-degrading genes and atrazine-degradation abilities

Strain	Location of isolation*	PCR detection of atrazine-degrading genes	Atrazine degradation (% remaining atrazine)	Atrazine degradation	Reference
Chelatobacter heintzii Cit1	Citeaux, France	*atzABC*, *trzD*	ND	Mineralization	Rousseaux *et al.* (2001)
Chelatobacter heintzii Sal1-3	Salinis, France	*atzABC*, *trzD*	ND	Mineralization	Rousseaux *et al.* (2001)
Chelatobacter heintzii LR3-3	Le Rheu, France	*atzABC*, *trzD*	ND	Mineralization	Rousseaux *et al.* (2001)
Chelatobacter heintzii LRA	Le Rheu, France	*atzABC*, *trzD*	ND	Mineralization	Rousseaux *et al.* (2001)
Chelatobacter heintzii SalB	Salinis, France	*atzABC*, *trzD*	ND	Mineralization	Rousseaux *et al.* (2001)
Chelatobacter heintzii Lous2-3	Loustalet, France	*atzA*	44	Hydroxyatrazine	Rousseaux *et al.* (2001)
Chelatobacter heintzii Sal2	Salinis, France	*atzA*	ND	Hydroxyatrazine	Rousseaux *et al.* (2001)
Pseudomonas sp. ADP	USA	*atzABCDEF*	ND	Mineralization	Mandelbaum *et al.* (1995)
Agrobacterium sp. NEA-D	Vannecourt, France	*atzABCDEF*	ND	Mineralization	This study
Nocardioides sp. NEA-A	Vannecourt, France	*trzN*, *atzBC*	ND	Cyanuric acid	This study
Arthrobacter cristallopoietes Cit2	Citeaux, France	*trzN*, *atzBC*	ND	Cyanuric acid	Rousseaux *et al.* (2001)
Sinorhizobium sp. NEA-B	Vannecourt, France	*trzN*, *atzBC*	ND	Cyanuric acid	This study
Polaromonas sp. NEA-C	Vannecourt, France	*trzN*, *atzBC*	ND	Cyanuric acid	This study
Nocardioides sp. SP12	La Bouzule, France	*trzN*, *atzBC*	ND	Cyanuric acid	Piutti *et al.* (2003)
Nocardioides sp. 1D	Croatia	*trzN*	ND	Hydroxyatrazine	This study
Arthrobacter sp. 2B	Croatia	*trzN*	ND	Hydroxyatrazine	This study
Arthrobacter sp. 3A	Croatia	*trzN*	ND	Hydroxyatrazine	This study

*For the French soils the sample locations were indicated.
ND, not detected.

Estimation of atrazine-degrading activity of the bacterial strains

The strains were grown on liquid MSA medium for 15 days with shaking at 150 r.p.m. The atrazine-degrading activity of each strain was determined by quantifying the remaining atrazine by reverse-phase HPLC using an LC Star System® (Varian) equipped with a Microsorb-MV C18 column (length 25 cm, internal diameter 4.6 mm, Varian). The solvent system consisted of methanol/ultra pure water (75/25, v/v) delivered at a flow rate of 1 mL min^{-1}. Atrazine was detected at 220 nm and showed a retention time of 6.9 min.

PCR detection of the *atz*, *trz* genes and of IS*1071* in the bacterial isolates

DNA extracts were prepared from the bacterial isolates using a proteinase K procedure as described elsewhere (Cheneby *et al.*, 2004). PCR reactions were conducted in a final volume of 25 μL using 2.5 μL of DNA template, 0.2 μM of dNTPs, 1 μM of each specific primer and 1.25 U of Taq DNA polymerase (Q-biogene, France). DNA amplifications were carried out in a PTC 200 gradient cycler (MJ Research, Waltham, MA) as follows: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at the optimal temperature for primer annealing and 2 min at 72 °C, plus an additional 10-min cycle at 72 °C. The PCR products were then separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide (100 μg L^{-1}) and photographed with a CCD camera. The primer pairs used were A1f/A1r for *atzA*, B1f/B1r for *atzB*, Cf/C1r for *atzC*, Df/Dr for *atzD*, Ef/Er for *atzE*, Ff/Fr for *atzF* (Devers *et al.*, 2005), trzDf1/trzDr1 for *trzD* (Rousseaux *et al.*, 2001), C190-10/C190-11 for *trzN* (Mulbry *et al.*, 2002). For the IS*1071* sequence, a primer set [IS*1071*f1(5′-TAT GAG GCT GGC GTG CAA-3′)/IS*1071*r1(5′-GCG CAA TCT TCC CGA TCA GT-3′)] was designed based on a multiple alignment of IS*1071* sequences (data not shown). The specificity of this premier pair for IS*1071* sequences was checked *in silico* using the IS finder programme (http://www-is.biotoul.fr/).

16S rRNA gene sequencing

16S rRNAs gene from the seven newly isolated atrazine-degrading bacteria were amplified by PCR using the 27f (5′-AGAGTTTGATCMTGGCTCAG-3′), positions 8–27 of *Escherichia coli* 16S rRNA gene) and 1492r (5′-TACGGHTACCTTGTTACGACTT-3′), positions 1492–1513 of *E. coli* 16S rRNA gene universal primers and cloned in pGEM-T as described previously (Devers *et al.*, 2005). Partial sequencing of cloned 16S rRNA gene sequences was performed using the 341f (5′-CCT ACG GGA GGC AGC AG-3′) and 926r (5′-CCG TCA ATT CMT TTR AGT TT-3′ primers) using a CEQ8000 sequencer (Beckman Coulter) according to the manufacturer's instructions. Partial 16S rRNA gene sequences of strains NEA-A, NEA-B, NEA-C, NEA-D, 1D, 2B and 3A were deposited in GenBank under the Accession Numbers DQ485404, DQ485406, DQ485410, DQ485405, DQ485407, DQ485408 and DQ485409, respectively.

Genomic DNA extraction – restriction enzymes digestion

The strains were grown in liquid MSA for 1 week at 28 °C with shaking at 150 r.p.m. Genomic DNA was extracted from 75 mL of bacterial suspension using the QIAGEN Blood and Cell culture DNA Midi Kit according to the manufacturer's instructions (QIAGEN, France). Digestions were performed in 40 μL containing 200 ng of extracted DNA, 20 U of NruI or SmaI restriction enzymes incubated overnight at 37 °C. Twenty microliters of the digested DNA were separated by electrophoresis in a 0.9% agarose gel overnight at 40 V in the presence of the digoxigenin-labelled DNA molecular weight marker II (Roche Applied Science, France).

Plasmid profiles

Bacterial cells were grown in liquid MSA medium as described above. A volume of 1 mL of culture adjusted to an OD of 0.2 at 600 nm was pelleted by centrifugation (15 000 ***g*** for 3 min). Plasmid profiles were obtained from the bacterial pellets using Eckhardt's method (Eckhardt, 1978) as modified by Wheatcroft *et al.* (1990). Plasmids were separated on an 0.75% (w/v) TBE agarose gel by electrophoresis at 40 V for 30 min followed by 100 V for 3 h. Plasmid sizes were determined by calibrating towards the relative mobility of the plasmids used as standards: (i) pAT (543 kb, accession number AE007872) and pTi (214 kb, accession number AE007871) harboured by *Agrobacterium tumefaciens* C58, and (ii) a megaplasmid (>1500 kb), pRme41a::Tn7 (236 kb) and pRP4 (60 kb, accession number L27758), harboured by a derivative of *Rhizobium meliloti* 41 (GMI328).

Hybridization analysis

Plasmid profiles and digested genomic DNAs were vacuum transferred to a Biodyne Plus membrane (Gelman Sciences, Merck Eurolab, France) and hybridized with each of the following probes: *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, *trzD*, *trzN* and IS*1071*. Dig-labelled probes were produced by PCR using specific primer pairs as described above. PCR was performed on the DNA of *Pseudomonas* sp. ADP (*atzABCDEF*, IS*1071*), *Chelatobacter heintzii* Cit1 (*trzD*) or *Nocardioides* sp. SP12 (*trzN*). DNA hybrids were detected under high-stringency conditions as described previously (Rousseaux *et al.*, 2001).

Results

Characterization of seven new atrazine-degrading isolates

The descriptions of seven new atrazine-degrading bacterial isolates are reported. Four strains (i.e. NEA-A, NEA-B, NEA-C and NEA-D) were isolated from a French agricultural soil repeatedly cropped with maize. Three (i.e. 1D, 2B and 3A) were isolated from a Croatian agrochemical factory soil (Table 1). Their atrazine-degrading activity was estimated by HPLC (Fig. 1). Partial sequencing of the 16S rRNAs gene revealed that strain NEA-A was taxonomically close to *Nocardioides* sp. (100% similarity over 568 bp), strain NEA-B close to *Sinorhizobium* sp. (99% over 901 bp), strain NEA-C close to *Polaromonas* sp. (99% over 723 bp), strain NEA-D close to *Agrobacterium* sp. (99% over 858 bp), strain 1D close to *Nocardioides* sp. (99% over 496 bp) and strains 2B and 3A close to *Arthrobacter* sp. (99% over 508 and 841 bp, respectively). Determination of the atrazine-degrading genetic compositions revealed that three of the French isolates (i.e. NEA-A, NEA-B, NEA-C) harboured the *trzN–atzBC* gene composition whereas NEA-D harboured the *atzABC-DEF* gene composition. The three strains isolated from the Croatian soil contained only the *trzN* gene (Table 1).

Plasmid location of the atrazine-degrading genes and IS*1071*

The genomic location of the atrazine-degrading genes and the insertion sequence IS*1071* was determined by hybridizing the plasmid profiles of each atrazine-degrading strain with *atz*, *trz* and IS*1071* probes (Fig. 2, Table 2). No plasmid could be recovered from the three Croatian strains (i.e. 1D, 2B, 3A). This suggests either that these strains did not possess plasmids or that the method used to extract and separate plasmids was not successful with these strains. One to nine plasmids were recovered from each of the other strains, with estimated sizes ranging from 23 to 583 kb.

In all nine atrazine-degrading strains harbouring *atzA*, the *atzA* probe hybridized on a plasmid. More precisely, an *atzA* signal was observed on a 77 kb plasmid in strains Cit1, SalB, LR3-3, LRA, Lous2-3 and Sal2. These plasmids did not contain any copy of IS*1071*. The *atzA* hybridization signal in strain Sal1-3 was on a 73-kb plasmid, which also produced a hybridization signal with the IS*1071* probe. Finally, the *atzA* hybridization signal in strains NEA-D and ADP was observed on their single plasmids of 137 and 110 kb, respectively. Both plasmids also hybridized with the IS*1071* probe.

In the eight atrazine-degrading strains harbouring the *trzN* gene, plasmids were only recovered from strains Cit2, SP12, NEA-A, NEA-B and NEA-C. In the Cit2 and SP12 strains, *trzN* was detected on a plasmid of 200 kb that also hybridized with the IS*1071* probe. No *trzN* hybridization was observed on the 200-kb plasmid of the three other strains (i.e. NEA-A, NEA-B and NEA-C).

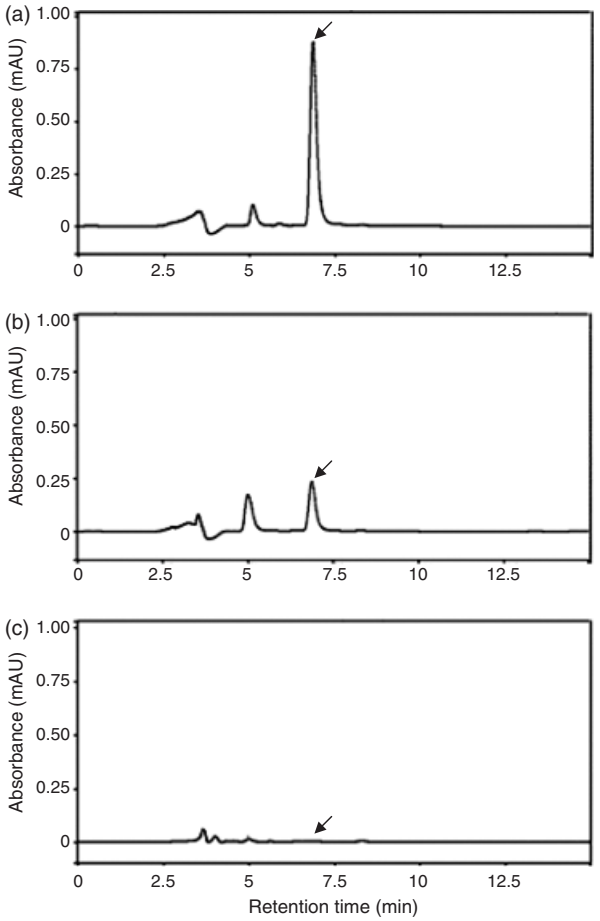

Fig. 1. HPLC analysis of either control atrazine mineral media. (a), *Chelatobacter heintzii* Lous 2–3 inoculated atrazine mineral media (b) or *Nocardioides* sp. 1D inoculated atrazine mineral media (c). The peak corresponding to atrazine is indicated by an arrow.

Nine of the 12 strains presenting *atzB* and *atzC* genes harboured at least one copy of these genes on plasmids. Depending on the strains, *atzB* hybridization signals were detected on plasmids ranging from 110 to 325 kb in size and also producing an IS*1071* hybridization signal. An *atzC* hybridization signal was always observed on the plasmids producing a hybridization signal with the *atzB* probe. Nevertheless, a second plasmid hybridizing with the *atzC* probe was observed in strains LR3-3, LRA and Sal1-3. This plasmid also hybridized with either (i) IS*1071* in strains LR3-3 and LRA or (ii) with *atzA*, and IS*1071* probes in strain Sal1-3. The bacterial strains containing the *trzD* gene produced a *trzD* hybridization signal with plasmids that also hybridized with the *atzB*, *atzC* and IS*1071* probes. Finally, only two atrazine-degrading bacteria (i.e. NEA-D and ADP)

Table 2. Localization of the atrazine-degrading genes and IS*1071* on the plasmids of the 17 atrazine-degrading bacterial strains

Strain	Plasmid number	Plasmids with atrazine degrading-genes (kb)			Other plasmids (kb)						
Cit1	7	325 (*atzBC*, *trzD*, IS*1071*)		77 (*atzA*)	533	186	137	99	49		
SalB	9	199 (*atzBC*, *trzD*, IS*1071*)		77 (*atzA*)	533	338	137	116	48	30	23
Sal1-3	7	130 (*atzBC*, *trzD*, IS*1071*)	73 (*atzAC*, IS*1071*)		488	301	186	64	23		
LR3-3	6	199 (*atzBC*, *trzD*, IS*1071*)	137 (*atzC*, IS*1071*)	77 (*atzA*)	583	380	49				
LRA	8	186 (*atzBC*, *trzD*, IS*1071*)	130 (*atzC*, IS*1071*)	77 (*atzA*)	510	351	56	45	24		
Lous2-3	7			77 (*atzA*)	533	325	186	130	45	23	
Sal2	7			77 (*atzA*)	557	351	199	137	49	23	
Cit2	1	200 (*trzN*, *atzBC*)									
SP12	1	200 (*trzN*, *atzBC*)									
NEA-D	1	137 (*atzABCDEF*, IS*1071*)									
ADP	1	110 (*atzABCDEF*, IS*1071*)									
NEA-A	1				200						
NEA-B	1				200						
NEA-C	1				200						
1D	0										
2B	0										
3A	0										

The estimated sizes of the plasmids are given and those carrying atrazine-degrading genes and IS*1071* are indicated in parentheses.

produced hybridization signals with *atzD*, *atzE* and *atzF* probes on their unique plasmid.

Genomic organization of the atrazine-degrading genes and of IS*1071*

The relative genomic location of the *trzN*, *trzD*, *atz* genes and of IS*1071* was investigated by performing Southern blot analyses on genomic DNA extracts from each strain. These genomic DNA extracts were digested with either NruI or SmaI, two restriction enzymes that theoretically do not recognize any restriction site into the *atz*, *trz* or IS*1071* available sequences. Only the results obtained with SmaI are presented (Fig. 3, Table 3). Southern blots performed with the IS*1071* probes revealed complex IS*1071*-hybridization patterns consisting of 0–9 hybridization signals.

Nine atrazine-degrading bacterial strains possessed the *atzA* gene. In seven strains, the *atzA* probe hybridized with a 2.6-kb band. In strains NEA-D and ADP, the *atzA* probe hybridized with a 19.7-kb band that also hybridized with the IS*1071* probe.

The eight strains that did not have the *atzA* gene possessed the *trzN* gene. Strains 1D, 2B and 3A presented the same *trzN* hybridization signal on a unique 8.1-kb band. Strain SP12 presented two *trzN* hybridization signals on DNA fragments of 2.9 and 14.9 kb. Similarly, a *trzN* hybridization signal was observed on a DNA fragment of 2.9 kb for strain Cit2, and of 14.9 kb for strains NEA-A, NEA-B and NEA-C. It has to be noticed that the 14.9-kb DNA fragment hybridizing with the *trzN* probe also hybridized with the IS*1071* probe, whatever the strain.

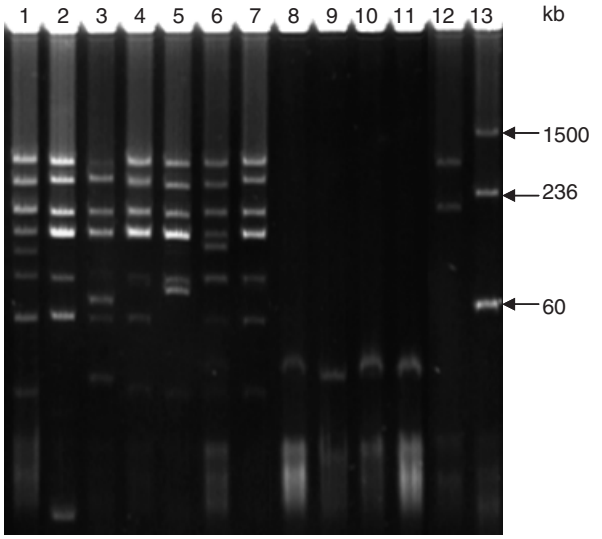

Fig. 2. Plasmid profiles carried out on 11 atrazine-degrading strains. Line 1, *Chelatobacter heintzii* Cit1; line 2, *Chelatobacter heintzii* LR3-3.; line 3, *Chelatobacter heintzii* LRA; line 4, *Chelatobacter heintzii* Lous2-3; line 5, *Chelatobacter heintzii* Sal1-3; line 6, *Chelatobacter heintzii* SalB: line 7, *Chelatobacter heintzii* Sal2; line 8, *Nocardioides* sp. SP12; line 9, *Nocardioides* sp. NEA-A; line 10, *Sinorhizobium* sp. NEA-B; line 11, *Polaromonas* sp. NEA-C; line 12, *Agrobacterium tumefaciens* C58; line 13, *Rhizobium meliloti* 41.

Twelve atrazine-degrading bacterial strains produced hybridization signals with the *atzB* probe. In strains Cit1, LR3-3, LRA, Sal1-3 and SalB, a band of 7.5 kb hybridized with the *atzB* probe. Only LR3-3 also presented an IS*1071* hybridization signal on this band. Strain SP12 presented two *atzB*

Table 3. Localization of the atrazine-degrading genes on SmaI-restriction fragments (in kb) of the 17 atrazine-degrading bacterial strains

	Number of IS*1071*	*trzN*	*atzA*	*atzB*	*atzC*	*trzD*	*atzD*	*atzE*	*atzF*
Cit1	9	–	2.6	7.5	19.8	7.5	–	–	–
LR3-3	9	–	2.6	7.5	24	7.5	–	–	–
LRA	8	–	2.6	7.5	24	7.5	–	–	–
Sal1-3	6	–	2.6	7.5	24.1	7.5	–	–	–
Lous2-3	0	–	2.6	–	–	–	–	–	–
SalB	7	–	2.6	7.5	21.3	–	–	–	–
Sal2	6	–	2.6	–	–	–	–	–	–
Cit2	5	2.9	–	8.7	8.7	–	–	–	–
Sp12	6	2.9	–	8.7	8.7	–	–	–	–
		14.9		14.9	14.9				
NEA-A	6	14.9	–	14.9	14.9	–	–	–	–
NEA-B	7	14.9	–	14.9	14.9	–	–	–	–
NEA-C	8	14.9	–	14.9	14.9	–	–	–	–
1D	0	8.1	–	–	–	–	–	–	–
2B	0	8.1	–	–	–	–	–	–	–
3A	0	8.1	–	–	–	–	–	–	–
NEA-D	3	–	19.7	19.7	10.7	–	23.8	23.8	23.8
				10.7					
ADP	2	–	19.7	19.7	10.7	–	23.8	23.8	23.8

The fragment sizes that are underlined correspond to DNA fragments that also hybridized with IS*1071* probes.

hybridization signals on 8.7- and 14.9-kb bands. Similarly, strains NEA-A, NEA-B and NEA-C hybridized with *atzB* on a 14.9-kb band, while strain Cit2 hybridized with *atzB* on an 8.7-kb band. Finally, strains NEA-D and ADP presented an *atzB* hybridization signal on a 19.7-kb band. Strain NEA-D also presented a second *atzB* hybridization signal on a 10.7-kb band. However, only one hybridization signal on a 23.7-kb band was observed when the NruI restriction enzyme was used (data not shown). Finally, it should be noted that seven of the 12 strains hybridizing with *atzB* harboured a DNA fragment that hybridized with both *atzB* and IS*1071* probe.

Twelve atrazine-degrading strains produced hybridization signals with the *atzC* probe. The *atzC* hybridization signals in strains Cit2, SP12, NEA-A, NEA-B and NEA-C were identical to those observed for *atzB*. In strains NEA-D and ADP, the *atzC* probe hybridized on a 10.7-kb band. Contrary to strain ADP, the DNA fragment of NEA-D hybridizing with *atzC* also hybridized with *atzB* and IS*1071* probes. In strains Cit1, LR3-3 and LRA, Sal1-3 and SalB, the *atzC* probe hybridized on DNA fragments ranging from 19.8 to 24.1 kb. Strains LR3-3 and LRA presented identical *atzC* hybridization profiles with a single hybridization signal on a band of 24 kb.

Five atrazine-degrading bacteria (i.e. strains Cit1, LR3-3, LRA, Sal1-3 and SalB) produced a hybridization signal with the *trzD* probe. In these strains, the *trzD* probe hybridized on a 7.5-kb band also hybridizing with the *atzB* probe. Only strain LR3-3 also harboured an IS*1071* signal on this band.

Finally, only strains NEA-D and ADP hybridized with the *atzD*, *atzE* and *atzF* probes (data not shown). They presented identical hybridization signals: (i) with the SmaI restriction enzyme, all three probes hybridized with a same band of 23.8 kb, and (ii) with the NruI enzyme, the *atzD* and *atzE* probes hybridized on a 4.4 kb-band whereas the *atzF* probe hybridized on a 3.5-kb band.

Discussion

This report provides an analysis of the atrazine-degrading genetic potential of seventeen cultivable atrazine-degrading bacteria isolated from geographically different soils and includes seven newly described strains.

Six of the seven newly isolated strains possess the *trzN* gene. This high proportion of atrazine-degrading bacteria possessing the *trzN* gene, which encodes an atrazine chlorohydrolase (Mulbry *et al.*, 2002), suggests its widespread occurrence throughout the atrazine-degrading bacterial

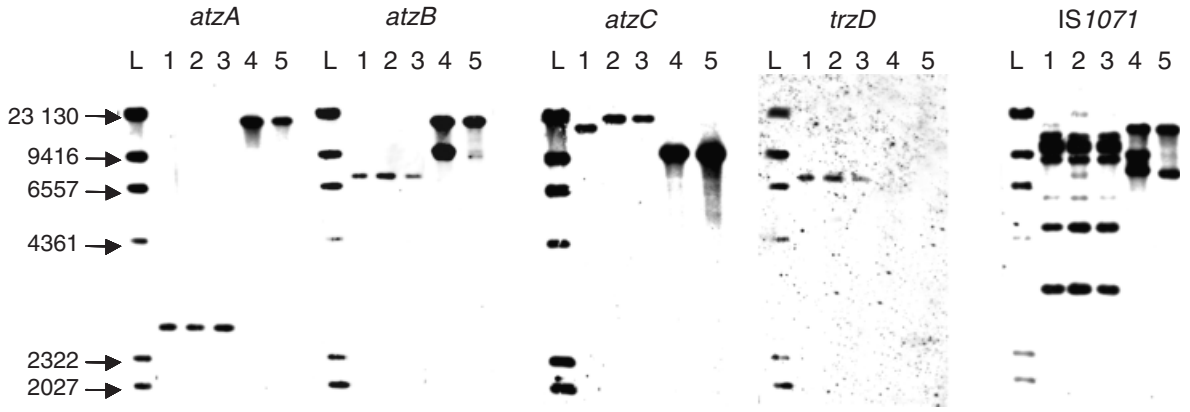

Fig. 3. Southern blot analysis carried out on bacterial genomic DNA digested with SmaI restriction enzyme and hybridized with *atzA*, *atzB*, *atzC*, *trzD*, and IS*1071* probes. The sizes of the DNA ladder (L) are given in kb. Line L, Dig-labelled DNA ladder; line 1, *Chelatobacter heintzii* Cit1; line 2, *Chelatobacter heintzii* LR3-3.; line 3, *Chelatobacter heintzii* LRA; line 4, *Agrobacterium* sp. NEA-D; line5: *Pseudomonas* sp. ADP.

community as it has previously been reported (Piutti *et al.*, 2003). Up to now, the *trzN* gene has been found in several Gram-positive bacteria (i.e. *Arthrobacter aurescens*, *Nocardioides* C190, *Nocardioides* sp. 12 and *Nocardia* sp.) (Topp *et al.*, 2000a, b; Piutti *et al.*, 2003; Sajjaphan *et al.*, 2004; Smith *et al.*, 2005). This is the first report describing the presence of this gene in Gram-negative bacteria such as *Sinorhizobium* sp. and *Polaromonas* sp.

Five strains over the seventeen harboured the *trzN–atzBC* gene combination. This atrazine-degrading gene composition, which leads to the transformation of atrazine to cyanuric acid (Piutti *et al.*, 2003), has been reported only in *Arthrobacter aurescens* TC1 and *Nocardioides* sp. SP12 (Piutti *et al.*, 2003; Sajjaphan *et al.*, 2004). The results therefore suggest that the *trzN–atzBC* gene composition may be widespread among Gram-positive and Gram-negative atrazine-degrading strains. It has to be highlighted that *Nocardioides* sp. SP12 contained at least two copies of the *trzN, atzB* and *atzC* genes. Although the functionality of the different copies of these genes has not been evaluated, their presence suggests a redundancy of the atrazine-degrading function. Up to now, such a redundancy has been observed only in atrazine-degrading consortia establishing a cooperative catabolic pathway (Smith *et al.*, 2005).

Interestingly, in contrast with *Nocardioides* sp. SP12 and *Arthrobacter crystallopoietes* Cit2 in which the *trzN*, *atzB* and *atzC* genes were located on a plasmid, *Nocardioides* sp. NEA-A, *Sinorhizobium* sp. NEA-B and *Polaromonas* sp. NEA-C harboured these genes on the chromosome. Although *atzA* and *atzB* genes have already been located on the chromosome of *Arthrobacter* sp. AD1 (Cai *et al.*, 2003) and *Variovorax* sp. MD1 and MD2 (Devers *et al.*, 2005), this is the first report showing the presence of *atzC* and *trzN* genes on the bacterial chromosome. In addition, Southern blot analyses suggested that the *trzN, atzB* and *atzC* genes were located on a single DNA fragment of 14.9 kb in strains NEA-A, NEA-B and NEA-C and in *Nocardioides* sp. SP12. Because these strains belong to different genera but harbour a similar *trzN–atzBC* gene cassette, one could hypothesize that this fragment might be part of a mobile genetic element responsible for its insertion at different genomic locations (chromosome vs. plasmid) in these strains.

The *atzABC–trzD* gene combination was found in most of the *Chelatobacter heintzii* strains and the *atzA* gene was usually located on a 77-kb plasmid. Although the plasmid sequence was unknown and no other known plasmid marker was available, this observation led us to hypothesize that the conjugation of this 77-kb plasmid could be one of the processes responsible for the spread of *atzA* gene between these strains. Although this gene combination has been reported only in strains isolated from different French soils (Rousseaux *et al.*, 2001), *trzD* has also been observed in several atrazine-degrading consortia isolated from Swiss (Smith *et al.*, 2005), American (Eaton & Karns, 1991), French (Martin-Laurent *et al.*, 2006) and Croatian soils (N. Udikovic-Kolic, pers. commun.). Data of this study therefore confirm that *trzD*, which codes for an enzyme responsible for the cleavage of the *s*-triazine ring, is widespread and often associated with *atzABC* genes in different atrazine-degrading strains (Karns, 1999; Fruchey *et al.*, 2003). In several strains harbouring this gene combination (i.e. *Chelatobacter heintzii* Cit1, LRA, Sal1-3 and SalB), the *atzB* and *trzD* genes were detected on a 7.5-kb DNA fragment but located on plasmids of different sizes. Similarly, the *atzC* gene was observed on a single DNA band but located on two different plasmids in strains Sal1-3, LRA, LR3-3. These observations suggest that all three genes could have been recruited by means of conserved mobile genetic elements.

Finally, the *atzABCDEF* gene composition was only found in *Pseudomonas* sp. ADP and *Agrobacterium* sp. NEA-D. All the atrazine-degrading genes in these two bacterial strains, as well as IS*1071*, were located on a unique plasmid of 110 kb for ADP and 137 kb for NEA-D. The *atzAB* genes and IS*1071* seems to be located on a DNA fragment of *c.* 19.7 kb in both strains. Thus, one could hypothesize that in *Agrobacterium* sp. NEA-D, these genes may be flanked by IS*1071* and organized as a composite transposon-like structure as observed in the plasmid ADP1 of *Pseudomonas* sp. ADP. In addition, the observation of two copies of *atzB* on a single NruI-DNA fragment in NEA-D suggests a duplication of this gene on its plasmid and reveals the genomic plasticity of the atrazine-degrading genetic potential. The versatility of the atrazine-degrading genes has already been shown by Topp *et al.* who observed that *atzB* was easily lost by *Aminobacter ciceronei* strain C147 formerly *Pseudaminobacter* sp. in the absence of atrazine selection pressure (Topp *et al.*, 2000a, b; McDonald *et al.*, 2005).

Interestingly, this study revealed that the *atzDEF* genes, initially described in *Pseudomonas* sp. ADP, were also found in *Agrobacterium* sp. NEA-D. It further confirms that such genes are widespread (Fruchey *et al.*, 2003). Southern blot analyses revealed that the *atzDEF* genes were similarly organized in *Agrobacterium* sp. NEA-D and in *Pseudomonas* sp. ADP where they are structured as an operon located on pADP1 (Martinez *et al.*, 2001; Garcia-Gonzalez *et al.*, 2005). From these data it can be hypothesized either that (i) the plasmids in the ADP and NEA-D strains derived from the same ancestor or alternatively (ii) that the catabolic plasmids have recruited conserved DNA cassettes containing *atzDEF*.

Finally, it was shown that the insertion sequences IS*1071* were located near *atz* and *trz* genes or at least on atrazine-degrading catabolic plasmids in most of the strains studied here (i.e. 13/17). IS*1071* is already known to be associated with other genes that encode enzymes responsible for the biodegradation of pollutants such as 2,4-D (Clément *et al.*,

2001; Vedler *et al*., 2004) or chlorobenzoate (Nakatsu *et al*., 1991; Di Gioia *et al*., 1998). The association of IS*1071* with *atz* genes has already been observed in three strains of atrazine-degrading bacteria (Topp *et al*., 2000a, b; Martinez *et al*., 2001; Sajjaphan *et al*., 2004). Results of this study suggest that the association of the atrazine-degrading genes with IS*1071* could be a common feature and may play a crucial role in atrazine-degrading gene dispersion.

Later studies will aim to sequence the DNA fragment containing *atz* and IS*1071* sequences in these strains and verify the involvement of IS*1071* in atrazine-degrading gene dispersal.

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