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Impact of the maize rhizosphere on the genetic structure, the diversity and the atrazine-degrading gene composition of cultivable atrazine-degrading communities

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Abstract

Sixty-six atrazine-degrading bacterial communities utilizing atrazine as sole N source and citrate as principal C source were isolated from unplanted and maize planted soils treated with atrazine. Ribosomal intergenic spacer analysis (RISA) fingerprints revealed that the genetic structure of atrazine-degrading bacterial communities was modified in the maize rhizosphere. To assess the underlying microbial diversity, 16S rDNA sequences amplified from each bacterial community were cloned. Libraries containing 660 16S rDNA clones were screened by restriction fragment length polymorphism (RFLP) analysis. In all, 63 clone families were identified. Rarefaction curves did not reach a clear saturation, indicating that the analysis of a greater number of clones would have revealed further diversity. Recovered 16S rDNA sequences were related to *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*. The four dominant RFLP families were highly similar to *Variovorax paradoxus*, *Burkholderia cepacia*, *Arthrobacter* sp. and *Bosea* sp. The composition of most of the atrazine-degrading bacterial communities consisted of 2–7 different bacterial species. Various atrazine-degrading gene compositions were observed, two of these *atzABCDEF*, *trzND* and *atzBCDEF*, *trzN* being largely dominant. The first was more frequently detected in bacterial communities isolated from the maize rhizosphere whereas the second was more frequently detected in communities isolated from bulk soil. Monitoring of atrazine-degrading activity showed that 76% of the bacterial communities degraded up to 80% of the initially added atrazine within 15 days of culture. Altogether our results indicate that the maize rhizosphere has an impact on the genetic structure, the diversity and atrazine-degrading gene composition of the atrazine-degrading communities.

Introduction

The *s*-triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine), has been one of the world's most heavily used agricultural herbicides over the past 40 years and has been used to control a variety of broadleaf weeds competing with corn, sugarcane, sorghum, pineapple and other crops (Le Baron, 1982). In natural environments, the halflife of atrazine ranges from a few days to several months (Solomon et al., 1996). For

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almost 25 years, atrazine was degraded to deethylatrazine (DEA) and to deisopropylatrazine (DIA) by soil micro-organisms (Barriuso and Houot, 1996). As a result, atrazine and its main metabolites (DEA and DIA) are often detected in soil (Bowman, 1989), and in surface and ground water (Müller et al., 1997) at concentrations exceeding the European Union standard of 0.1 $\mu g\ l^{-1}$ (Pick et al., 1992; Spliid and Koppen, 1998). The contamination of soil, air and water resources with atrazine has led to its accumulation along the food chain (Kannan et al., 1994). As a result, the use of this herbicide has been forbidden in several countries in Europe including Austria, Denmark, France, Germany, Italy, Norway, and Sweden.

Numerous studies have described the fate of atrazine (adsorption, transfer, chemical and biological degradation) in the different compartments of the environment, and notably in soil which acts as a recharge zone for the aquifers (Mc Cormick, 1966). Until the nineties, atrazine biotransformation was reported to mainly occur *via N*-dealkylation reactions catalysed by a variety of soil bacterial and fungal monooxygenases, leading to the formation of metabolites (DEA and DIA) which accumulate in the environment (Erickson and Lee, 1989). Only relatively recently micro-organisms able to completely mineralize atrazine have been isolated from different soils (de Souza et al., 1998a; Mandelbaum et al., 1995; Rousseaux et al., 2001; Topp et al., 2000). The atrazine biodegradation pathway for *Pseudomonas* sp. ADP has been extensively described (Martinez et al., 2001). Atrazine is transformed to cyanuric acid by a chlorohydrolylase (*atzA* or *trzN*) and two amidohydrolases (*atzB* and *atzC*) (Boundy-Mills et al., 1997; de Souza et al., 1995; Sadowsky et al., 1998). Cyanuric acid is then transformed to CO_2 and NH_3 by three other amidohydrolases (*atzD*, *E* and *F*) (Cheng et al., 2005; Martinez et al., 2001). The *atz* genes coding these enzymes are located on a self-transmissible plasmid designated pADP1 (de Souza et al., 1998b). The *atzA*, *B*, *C* genes are highly conserved and widespread in the environment (de Souza et al., 1998b). Their presence has also been reported in different atrazine-degrading bacterial strains isolated from US, Canadian, French and Chinese soils (Piutti et al., 2003; Rousseaux et al., 2001; Strong et al., 2002; Topp et al., 2000b). Besides the *atz* genes, two *trz* genes involved in atrazine catabolism have also been described: *trzN* which transforms atrazine to hydroxyatrazine (Mulbry et al., 2002) and *trzD* which opens the *s*-triazine ring (Karns, 1999).

The mineralization of atrazine was shown to be stimulated by the addition of carbon sources to the soil (Yassir et al., 1998). It was also enhanced in the maize rhizosphere defined as the zone of influence of the root system which exudes up to 70% of the plant photosynthates into the soil thereby increasing carbon availability (Piutti et al., 2002a, 2002b). The maximal rate of atrazine mineralization in the maize rhizosphere was observed concomitantly to the maximal atrazine-degrading genetic potential which was estimated using *atzC* quantitative PCR performed on DNA directly extracted from the soil (Martin-Laurent et al., 2001, 2003; Piutti et al., 2002a). We therefore hypothesized that the maize rhizosphere is an ecological niche favouring the functioning of atrazine-degrading communities (Piutti et al., 2002b).

The aim of this study was to assess the impact of the maize rhizosphere on the structure of soil atrazine-degrading bacterial communities. Atrazine-degrading bacterial communities were isolated without microbial enrichment from unplanted and maize planted soils treated or not with atrazine. The genetic structure of each bacterial community was characterized using ribosomal intergenic spacer analysis (RISA). The bacterial diversity and bacterial composition of each bacterial community were determined by the cloning and sequencing of PCR amplifications of 16S rRNA gene fragments from genomic DNA. The capacity of each bacterial community to degrade atrazine was tested in liquid culture and the occurrence of atrazine-degrading genes (*atzA*, *B*, *C*, *D*, *E*, *F* and *trzD*, *N*) was detected by PCR and Southern blot analyses.

Materials and methods

Design of the experiment

The soil was collected from the first 15-cm top layer of an experimental field located at La Bouzule (Nancy, France). This is a redoxic

neoluvisol with the following physico-chemical characteristics: sand 15.4%, silt 51.3%, clay 33.3%, organic matter 26.4 g kg^{-1}, organic carbon 15.3 g kg^{-1}, pH 5.8 and cation exchange capacity 14.5 cmol kg^{-1}. The soil was sieved (5 mm), moistened to 80% of the water holding capacity (WHC) and placed in plastic pots (1.5 kg equivalent dry soil per pot) where it was subjected to four treatments: bulk soil (BS), bulk soil treated with atrazine (BSA), maize planted soil (MS) and maize planted soil treated with atrazine (MSA), replicated five times. The BSA and MSA soils were treated with atrazine (1.5 mg/kg). The MS and MSA soils were sown with four sterilized grains of maize (*Zea mays*, cultivar DEA). All the soil pots were kept in a greenhouse with the following controlled environmental conditions: day temperature 17 °C, night temperature 15 °C; day length 11 h, relative humidity 60% and cooling above 20 °C. Eight weeks after planting, the soils from the planted and unplanted plastic pots were recovered.

Isolation of atrazine-degrading bacterial communities

About 100 μl aliquots of serially diluted (from 10^{-2} to 10^{-4}) unplanted and maize-planted soils were plated on mineral salts medium (MSM) (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}, sodium citrate 1 g l^{-1}, thiamine–HCl 0.1 mg l^{-1}, biotin 40 μg l^{-1}, boric acid 2 mg l^{-1}, $MnSO_4 \cdot H_2O$ 1.8 mg l^{-1}, $ZnSO_4 \cdot$ 0.2 mg l^{-1}, $CuSO_4$ 0.1 mg l^{-1}, Na molybdate 0.25 mg l^{-1}, agar 16 g l^{-1}, pH 6.8–7) containing sodium citrate (10 mg l^{-1}) as sole carbon source and atrazine (500 mg l^{-1}) as principal nitrogen source. Plates were incubated at 28 °C for 3 weeks. Atrazine formed a whitish opaque veil on the surface of the MSM and bacterial colonies formed a clear halo of degraded atrazine. These were sub-cultivated once in liquid mineral salt medium supplemented with 30 mg l^{-1} of atrazine for 15 days at 28 °C under agitation (150 rpm). An aliquot of each microbial culture was collected, added with sterile glycerol (25% final concentration), frozen in liquid nitrogen and stored at −80 °C. HPLC and DNA based analyses were conducted directly on an aliquot of microbial culture.

Estimation of atrazine degrading activity of the bacterial communities

The atrazine-degrading activity of individual bacterial community was determined by quantifying the remaining atrazine by reverse phase HPLC using a 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 4.9 min.

Analysis of the genetic structure of the atrazine-degrading bacterial communities

The genetic structures of the atrazine-degrading bacterial communities were determined by RISA (Ribosomal Intergenic Spacer Analysis) which reveals the length polymorphism of the 16S–23S intergenic spacer (IGS) of the bacterial ribosomal operon. The 16S–23S IGS of the atrazine-degrading communities was amplified by PCR using a 2.5 μl aliquot of bacterial culture, 1 μM of universal primers 72f and 38r (Gurtler and Stanisich, 1996) and 2.5 U of *Taq* DNA polymerase (Qbiogene) in a 25 μl final volume. PCR reactions were performed in a PTC 200 Gradient ThermoCycler (MJ Research, Waltham, MA) under the following conditions: 95 °C for 4 min, 34 cycles of 94 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min, plus an additional 15 min cycle at 72 °C. PCR products (8 μl) were separated by electrophoresis on a 6% native polyacrylamide gel runs for 17 h at 8 mA. Gels were stained with SYBR green II (Molecular Probes), scanned with a Storm 960 (Molecular Dynamics) and analysed with the ImageQuant program (Molecular Dynamics). RISA fingerprints were analysed with the Bio-Profil V6.0 software (Vilber Lourmat). A similarity matrix was produced using the neighbor-joining analysis based on the test of Jaccard at 5% and represented as a tree (Bio-Profil V6.0 software, Vilber Lourmat).

Analysis of the atrazine-degrading gene composition of the atrazine-degrading bacterial communities

Atrazine-degrading genes (*atz* and *trz*) were detected in each bacterial community by PCR with gene-specific primers and by hybridization of gene-specific probes on bacterial communities directly dotted on to a nylon membrane. For each bacterial community *atzA*, *B*, *C*, *D*, *E*, *F* and *trzD*, *N* sequences were amplified from a 2.5 μl liquid culture aliquot using the specific primer pairs listed in Table 1. PCR reactions were conducted using the conditions described above with optimal annealing temperature (see Table 1 for details). The PCR products (5 μl) were separated by electrophoresis on 1.5% agarose gel (Type II, Medium, Sigma) and then capillary transferred to a nylon membrane (Pall biodyne® plus membrane, Merck Eurolab) as described previously (Martin-Laurent et al., 1995).

About 100 μl aliquots of the liquid culture of atrazine-degrading bacterial communities were dotted onto a nitrocellulose membrane (Pall biodyne® plus membrane, Merck Eurolab) using a Bio-DOT microtitration apparatus (Biorad). The bacteria were lyzed directly on the membrane and the bacterial DNA fixed to the membrane by UV exposure.

Digoxigenin (Dig)-labelled nucleic probes were prepared by PCR according to the manufacturer's instructions (Roche Biochemical). The *atz* probes were prepared from *Pseudomonas* sp. ADP (Mandelbaum et al., 1995), the *trzD* probe from *Chelatobacter heintzii* (Rousseaux et al., 2001) and the *trzN* probe from *Nocardioides* sp. SP12 (Piutti et al., 2003).

Membranes were hybridized with each of the *atz* and *trz* Dig-labelled probes under high stringency conditions as previously described (Rousseaux et al., 2001). The hybridized probe was detected using detection starter kit II (Roche Biochemical) as specified by the manufacturer.

Analysis of the bacterial composition of the atrazine-degrading bacterial communities

A 16S rDNA clone library was prepared for each of the 66 atrazine-degrading bacterial

Table 1. Nucleotide sequence of the primers used in this study

Gene target	Primers	Sequences	References
atzA	Ar	5′-TGA AGC GTC CAC ATT ACC-3′	de Souza et al. (1995)
	Af	5′-CCA TGT GAA CCA GAT CCT-3′	
atzB	Br	5′-CTC TCC CGC ATG GCA TCG GG-3′	de Souza et al. (1995)
	Bf	5′-TCA CCG GGG ATG TCG CGG GC-3′	
atzC	Cr	5′-GTA CCA TAT CAC CGT TGC CA-3′	de Souza et al. (1995)
	Cf	5′-GCT CAC ATG CAG GTA CTC CA-3′	
atzD	Df	5′-TCC CCA CCT GAC ATC ACA AAC-3′	Devers et al. (2004)
	Dr	5′-GGG TCT CGA GGT TTG ATT G-3′	
atzE	Ef	5′-GAG CCT CTG TCC GTA GAT CG-3′	Devers et al. (2004)
	Er	5′-GAT GGC GTG TAC CGT TTA CC-3′	
atzF	Ff	5′-ACC AGC CCT TGA ATC ATC AG-3′	Devers et al. (2004)
	Fr	5′-TAT TGT CCC GAT ACC CAA CG-3′	
trzD	trzDr	5′-TCG AAG GCA TAA CTG CAT TG-3′	Rousseaux et al. (2001)
	trzDf	5′-CCT CGC GTT CAA GGT CTA CT-3′	
trzN	trzNf	5′-CAC CAG CAC CTG TAC GAA GG-3′	Mulbry et al. (2002)
	trzNr	5′-GAT TCG AAC CAT CCA AAC G-3′	
16S rDNA	1492r	5′-TAC GGH TAC CTT GTT ACG ACT T-3′	Gurtler and Stanisich (1996)
	27f	5′-AGA GTT TGA TCM TGG CTC AG-3′	
16S rDNA	341f	5′-CCT ACG GGA GGC AGC AG-3′	Cheneby et al. (2000)
	907r	5′-CCG TCA ATT CMT TTR AGT TT-3′	
16S-23S rDNA	72f	5′-TGC GGC TGG ATC ACC TCC TT-3′	Gurtler and Stanisich (1996)
	38r	5′-CCG GGT TTC CCC ATT CGG-3′	

communities isolated. 16S rDNA sequences were amplified by PCR under the conditions described above and with the universal primers 341f and 907r (primer sequences shown in Table 1). The PCR products were separated by electrophoresis and extracted from the gel using the QIAEX extraction kit as recommended by the manufacturer (Qiagen). Purified PCR fragments (3 μl) were cloned using the pGEM®-T Easy II plasmid according to the producer's protocol (Promega). At least 10 recombinant clones isolated from each of the 66 libraries were screened for full-size inserts (approximately 570 bp) by transferring small aliquots of bacterial cells to PCR mixtures containing the vector primers SP6 and T7 and thermocycling. The PCR products were digested with the restriction endonuclease *Alu*I (Qbiogene). Restriction fragments were separated by electrophoresis in a 3% small fragment agarose gel (FMC). Clones showing identical patterns were grouped together into clone families. The nucleotide sequence of one member of each RFLP group was determined using the DTCS-1 kit (Beckman Coulter) and a CEQ2000-XL sequencer (Beckman Coulter) according to manufacturer's instructions. Vector primers SP6 and T7 were used for sequencing reactions. The resulting sequences were deposited in the GenBank sequence database under the accession numbers AY466719 to AY466781.

Phylogenetic analysis, rarefaction and diversity analysis

16S rDNA sequences were aligned using the ClustalX software version V.1.0.1 (Thompson et al., 1997). A phylogenetic tree based on 16S rDNA sequences alignment was constructed by inferring DNA sequence distances by the neighbor-joining method. Bootstrap analyses for nucleotide level (16S rDNA) phylogenetic analyses were performed using ClustalX. Each calculation was based on 1000 bootstrap resamplings. The diversity of the phylotypes in different samples was compared by rarefaction analysis using the Analytic Rarefaction software (http://www.uga.edu/strata/software/). The Simpson index of dominance concentration (D) was calculated by using the following relation: $D = \mathrm{T}P^2{}_i$ where P_i was calculated as follows: $P_i = n_i/N$, n_i is the number of clones in a single family, and N is the total number of clones (Simpson, 1949).

Results

Isolation of atrazine-degrading bacterial and communities

Atrazine-degrading bacterial communities were obtained directly from soil suspensions prepared from BS, MS, BSA and MSA samples without enrichment. The soil samples were serially diluted and aliquots plated on atrazine MSM plates. After three weeks of incubation at 20 °C, bacterial communities forming clear halos on atrazine MSM were isolated. A total of 66 atrazine-degrading communities were isolated i.e. 1 isolated from MS, 2 from BS, 30 from MSA and 33 from BSA (Table 2). Three out of 66 atrazine-degrading communities were isolated from soil samples not treated with atrazine. Similar numbers of atrazine-degrading communities were isolated from the MSA samples (i.e. 30 bacterial communities out of the 66 isolated) and from the BSA samples (i.e. 33 bacterial communities out of the 66 isolated).

Genetic structure of the atrazine-degrading bacterial communities

The genetic structures of the atrazine-degrading bacterial communities were assessed by applying ribosomal intergenic spacer analysis (RISA). RISA fingerprints were relatively complex and consisted of 15–25 major bands (data not shown). By comparing these different RISA fingerprints a tree was constructed representing the percentage similarity of each profile to each of the others (Figure 1). Based on this tree, the atrazine-degrading communities could be separated into three major groups (I, II and III) and a much smaller fourth group (IV) that represented only 2 of the 66 bacterial communities. Group I was composed of 13 atrazine-degrading bacterial communities 8 of which were isolated from MSA soil, 1 from MS soil and 4 from BSA soil. Group II was composed of 33 atrazine-degrading bacterial communities 20 of which were isolated from MSA soil, 2 from BS soil and 11 from BSA. Group III was composed of 18

Table 2. Molecular and physiological analyses of the atrazine-degrading bacterial communities isolated either from bulk soil (BS), maize planted soil (MS), bulk soil treated with atrazine (BSA) or maize planted soil treated with atrazine (MSA)

Treatment	Bacterial community	RISA group	Atrazine-degrading gene	Atrazine degradation (% remaining atrazine)
BS	71	II	abcdefn	0
	72	II	nd	0
BSA	3	III	abc	20
	4	III	bcdefn	0
	5	IV	bcdefn	0
	6	I	abcdeft	84
	7	II	abcdefnt	0
	8	II	abcdefnt	0
	10	I	bcdefn	89
	11	II	abcdefnt	60
	29	III	bcdefn	0
	30	III	bcdefn	0
	31	II	abcdefnt	0
	32	III	bcdefn	0
	33	III	nd	
	34	III	bn	0
	35	II	bcdefn	0
	36	III	bcdefn	0
	37	III	bcdef	0
	38	III	abcdefn	0
	39	III	abcdefn	0
	40	III	bcdef	0
	41	III	bcdefn	0
	42	III	bcn	0
	43	III	bcdefn	0
	59	II	abcdefnt	0
	60	III	abcdefn	0
	61	I	abcdefnt	0
	62	II	abcdefnt	0
	63	II	abcdefnt	0
	64	II	bcn	65
	65	II	abcdefnt	86
	66	I	abcdefnt	0
	67	II	abcdefnt	0
	72	II	abcn	0
MS	58	I	abcdefnt	0
MSA	12	I	abdeft	100
	14	I	abcdefnt	97
	15	I	abcdefnt	0
	16	I	abcdefnt	0
	18	I	abcdefnt	0
	21	II	abcdefnt	0
	22	II	abcdefnt	0
	26	IV	bcdefn	0
	45	II	bcdefn	0
	46	II	bcdefn	82
	47	II	abcdefnt	68

Table 2. Continued

Treatment	Bacterial community	RISA group	Atrazine-degrading gene	Atrazine degradation (% remaining atrazine)
	48	I	abcdefnt	82
	49	II	abcdefnt	72
	50	II	abct	76
	51	I	abcdefnt	0
	52	II	abcn	0
	53	II	acdefn	0
	55	II	bcdefn	0
	56	II	abcdefn	0
	68	II	abcdef	0
	69	II	abcdefnt	0
	70	II	abcdefnt	0
	75	I	abcdefnt	12
	76	II	abcdefnt	49
	78	II	abcdefnt	0
	79	II	abcdefnt	18
	80	III	abcdefnt	47
	82	II	abcdefnt	0
	83	II	abcdefnt	0
	84	II	abcdefnt	0

For each bacterial community the soil treatment, the bacterial community number, the RISA group, the atrazine-degrading gene composition and the atrazine-degrading activity is shown.
a: *atzA*, b: *atzB*, c: *atzC*, d: *atzD*, e: *atzE*, f: *atzF*, n: *trzN*, t: *trzD*, nd: not determined.

atrazine-degrading bacterial communities 17 of which were isolated from BSA but only 1 from maize rhizosphere soil. Most (70%) of the atrazine-degrading bacterial communities in group I had been isolated from maize-planted soil whereas most (94%) of those in group III had been isolated from bulk soil. In contrast, the atrazine-degrading bacterial communities of group II had been isolated from both maize-planted soil (60%) and bulk soil samples (33%).

Analysis of the bacterial composition of the atrazine-degrading bacterial communities RFLP analysis

The bacterial composition and the frequency of different microbial groups were determined for each atrazine-degrading bacterial community using a 16S rDNA-based approach. A 16S rDNA library was constructed for each of the 66 atrazine-degrading bacterial communities. A sample of at least 10 clones of the 566-bp 16S rDNA inserts, in each library, was screened by RFLP. Within the 660 16S rDNA inserts analysed, 63 different *Alu*I restriction banding patterns were detected with 30 represented by at least two clones. The distribution of the *Alu*I RFLP families in the four treatments is presented in Figure 2. Four major RFLP families were apparent with approximately 50% of the total number of clones belonging to the RFLP families AY466778, AY466728, AY466767 and AY466737. These four RFLP families were equally represented in both MSA and BSA atrazine-degrading bacterial communities. In addition, a similar proportion of the 38 RFLP families was detected specifically in either the BSA or MSA samples (i.e. 22 and 16 respectively). The diversity of the MSA and BSA libraries was estimated quantitatively by applying the Simpson's index (*D*). A Simpsons's index of 0.09 was obtained for the BSA library and of 0.07 for the MSA library with no significant difference between these values.

Rarefaction analysis

A rarefaction analysis relating the number of 16S rDNA clones analysed to the number of clone families sampled was performed. The rarefaction

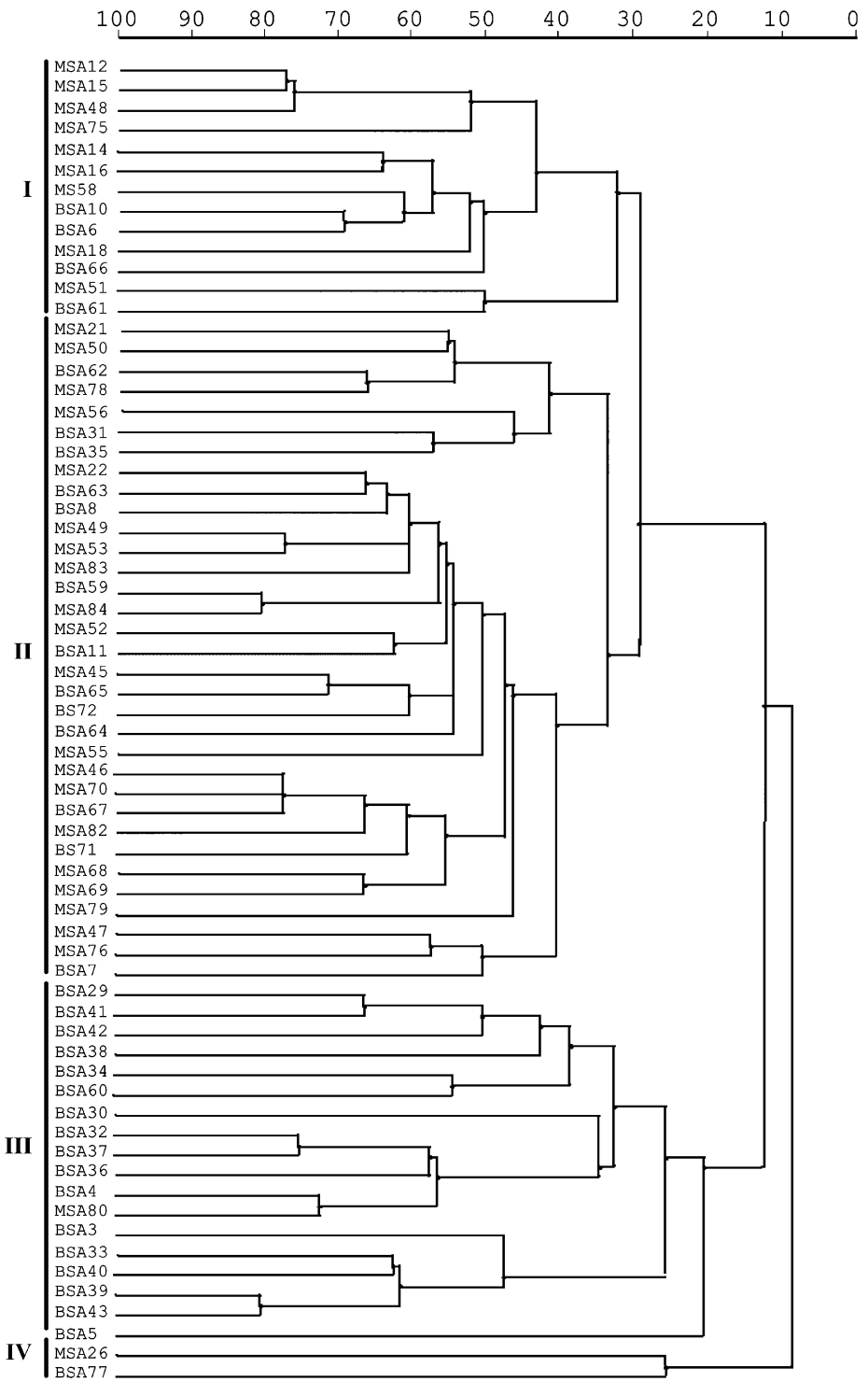


Figure 1. Phylogenetic relationship of the RISA patterns generated from PCR products amplified with 16S–23S rDNA universal primers (38r and 72f) from DNA extracted from atrazine-degrading bacterial communities. Phylogenetic distances were determined using the neighbour-joining analysis with the test of Jaccard at 5%.

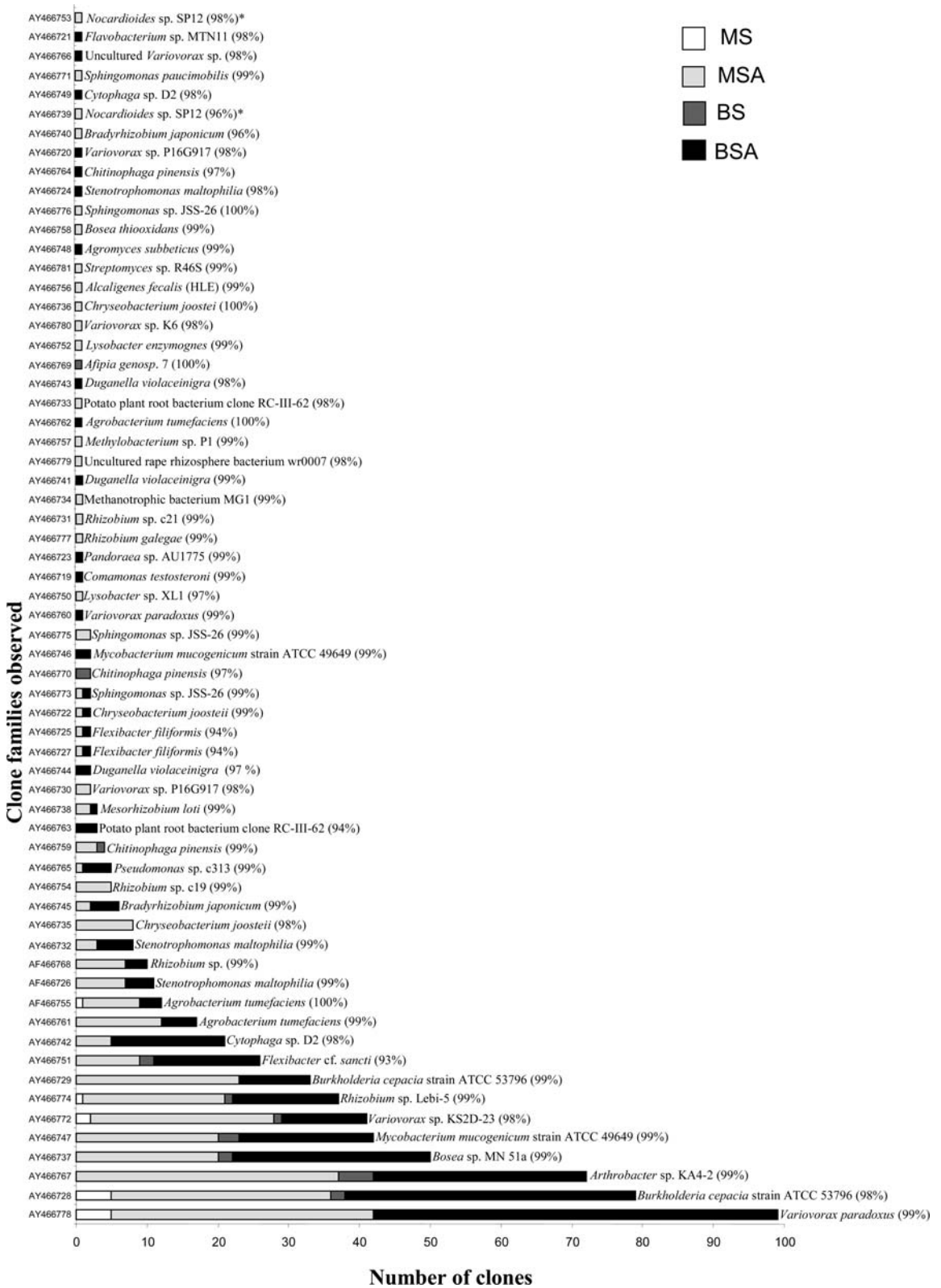

MS
MSA
BS
BSA
Clone families observed
AY466753 Nocardioides sp. SP12 (98%)*
AY466721 Flavobacterium sp. MTN11 (98%)
AY466766 Uncultured Variovorax sp. (98%)
AY466771 Sphingomonas paucimobilis (99%)
AY466749 Cytophaga sp. D2 (98%)
AY466739 Nocardioides sp. SP12 (96%)*
AY466740 Bradyrhizobium japonicum (96%)
AY466720 Variovorax sp. P16G917 (98%)
AY466764 Chitinophaga pinensis (97%)
AY466724 Stenotrophomonas maltophilia (98%)
AY466776 Sphingomonas sp. JSS-26 (100%)
AY466758 Bosea thiooxidans (99%)
AY466748 Agromyces subbeticus (99%)
AY466781 Streptomyces sp. R46S (99%)
AY466756 Alcaligenes fecalis (HLE) (99%)
AY466736 Chryseobacterium joostei (100%)
AY466780 Variovorax sp. K6 (98%)
AY466752 Lysobacter enzymognes (99%)
AY466769 Afipia genosp. 7 (100%)
AY466743 Duganella violaceinigra (98%)
AY466733 Potato plant root bacterium clone RC-III-62 (98%)
AY466762 Agrobacterium tumefaciens (100%)
AY466757 Methylobacterium sp. P1 (99%)
AY466779 Uncultured rape rhizosphere bacterium wr0007 (98%)
AY466741 Duganella violaceinigra (99%)
AY466734 Methanotrophic bacterium MG1 (99%)
AY466731 Rhizobium sp. c21 (99%)
AY466777 Rhizobium galegae (99%)
AY466723 Pandoraea sp. AU1775 (99%)
AY466719 Comamonas testosteroni (99%)
AY466750 Lysobacter sp. XL1 (97%)
AY466760 Variovorax paradoxus (99%)
AY466775 Sphingomonas sp. JSS-26 (99%)
AY466746 Mycobacterium mucogenicum strain ATCC 49649 (99%)
AY466770 Chitinophaga pinensis (97%)
AY466773 Sphingomonas sp. JSS-26 (99%)
AY466722 Chryseobacterium joosteii (99%)
AY466725 Flexibacter filiformis (94%)
AY466727 Flexibacter filiformis (94%)
AY466744 Duganella violaceinigra (97 %)
AY466730 Variovorax sp. P16G917 (98%)
AY466738 Mesorhizobium loti (99%)
AY466763 Potato plant root bacterium clone RC-III-62 (94%)
AY466759 Chitinophaga pinensis (99%)
AY466765 Pseudomonas sp. c313 (99%)
AY466754 Rhizobium sp. c19 (99%)
AY466745 Bradyrhizobium japonicum (99%)
AY466735 Chryseobacterium joosteii (98%)
AY466732 Stenotrophomonas maltophilia (99%)
AF466768 Rhizobium sp. (99%)
AF466726 Stenotrophomonas maltophilia (99%)
AF466755 Agrobacterium tumefaciens (100%)
AY466761 Agrobacterium tumefaciens (99%)
AY466742 Cytophaga sp. D2 (98%)
AY466751 Flexibacter cf. sancti (93%)
AY466729 Burkholderia cepacia strain ATCC 53796 (99%)
AY466774 Rhizobium sp. Lebi-5 (99%)
AY466772 Variovorax sp. KS2D-23 (98%)
AY466747 Mycobacterium mucogenicum strain ATCC 49649 (99%)
AY466737 Bosea sp. MN 51a (99%)
AY466767 Arthrobacter sp. KA4-2 (99%)
AY466728 Burkholderia cepacia strain ATCC 53796 (98%)
AY466778 Variovorax paradoxus (99%)
0
10
20
30
40
50
60
70
80
90
100
Number of clones

Figure 2. Distribution of the 16S rDNA OTUs from atrazine-degrading bacterial communities isolated from bulk soil (BS), maize cultivated soil (MS), bulk soil treated with atrazine (BSA) and maize planted soil treated with atrazine (MSA). The accession number and the highest percentage of similarity of each 16S rDNA sequence to known 16S rDNA sequences are indicated in italics. The Genbank accession numbers of the 16S rDNA sequences used here are: *Nocardioides* sp. SP12 (AF537327), *Flavobacterium* sp. MTN11 (AY162137), Uncultured *Variovorax* sp. (AY571833), *Sphingomonas paucimobilis* isolate 12H6 (AY367017), *Cytophaga* sp. D2 (AF250407), *Bradyrhizobium japonicum* (AF286361), *Variovorax* sp. P16G917 (AF214129), *Chitinophaga pinensis* (AF078775), *Stenotrophomonas maltophilia* (AF137357), *Sphingomonas* sp. JSS-26 (AF031240), *Bosea thiooxidans* (AF508803), *Agromyces subbeticus* (AY737778), *Streptomyces* sp. R46S (AY572485), *Alcaligenes fecalis* HLE (AY027506), *Chryseobacterium joostei* (AJ271010), *Variovorax* sp. K6 (AF532867), *Lysobacter enzymogenes* (AJ298291), *Afipia* genosp. 7 (U87773), *Duganella violaceingra* (AY376163), Potato plant root bacterium clone RC-III-62 (AJ252725), *Agrobacterium tumefaciens* (AY851692), *Methylobacterium* sp. P1 (AF148859), Uncultured rape rhizosphere WR007 (AJ295468), Methanotrophic bacterium MG1 (AF068118), *Rhizobium* sp. c21 (AB167200), *Rhizobium galegae* (AF025853), *Pandoreae* sp. AU1775 (AY043377), *Comamonas testosteroni* isolate Q10 (AF519533), *Lysobacter* sp. XL1 (AF472556), *Variovorax paradoxus* (AJ420329), *Mycobacterium mucogenicum* strain ATCC49649 (AF480585), *Flexibacter filiformis* (AB078049), *Mesorhizobium loti* (X67230), *Pseudomonas* sp. c313 (AB167182), *Bradyrhizobium japonicum* (X66024), *Rhizobium* sp. (Y10176), *Flexibacter* cf. *sancti* (AF181568), *Burkholderia cepacia* strain ATCC53796 (AY741355), *Rhizobium* sp. Lebi-5 (AY490119), *Variovorax* sp. KS2D-23 (AB196432), *Mycobacterium mucogenicum* strain ATCC 49649 (AF480585), *Bosea* sp. MN 51a (AJ313022), *Arthrobacter* sp. KA4-2 (AJ785761), *Burkhloderia cepacia* strain ATCC 53933 (AY741355).

curves obtained for atrazine-degrading bacterial communities isolated from the MS and BS soils are not shown since too few clones (i.e. 38) were analysed to permit a reliable analysis. However, the analysis performed on the atrazine-degrading bacterial communities isolated from MSA and BSA soils, for which 320 and 290 clones were screened respectively, is shown in Figure 3. At the highest shared sample size (i.e. 290 clones), 38 OTUs were observed in the BSA soil compared with 45 OTUs in the MSA (Figure 3), and the 95% confidential intervals (CIs) did not overlap.

Sequence analysis

A sequence analysis was carried out on one or two clones of each clone family. The 16S rDNA sequences determined were deposited in the GenBank sequence database and each RFLP family corresponded to a GenBank accession number (Figure 4). These results revealed that the 16S rDNA sequences obtained were affiliated with highly diverse bacterial groups such as the *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. Most of them were related to the *Proteobacteria* group which represented up to 66% of the RFLP groups identified. About 33.8% of the RFLP groups were related to the α-Proteobacteria subgroup, 22.6% to the β-Proteobacteria, 9.7% to the γ-Proteobacteria, 24.2% to the *Bacteroidetes* and 9.7% to the *Actinobacteria*. Although the α-Proteobacteria group was the largest of the RFLP groups, sequences related to the β-Proteobacteria were the most abundant representing up to 41% of the total number of sequences analysed whereas those related to the α-Proteobacteria represented only 25% of the total number of sequences analysed. In addition, although the β-Proteobacteria and *Bacteroidetes* groups were equivalently represented among the RFLP families, the percentage of 16S rDNA sequences related to the β-Proteobacteria was four times higher than that of CFB (only 11.8% of the total number of sequences). Similarly, the *γ-Proteobacteria* and the *Actinobacteria* subgroups were equivalently represented among the RFLP families, but the percentage of total 16S rDNA sequences related to the *Actinobacteria* (i.e. 18.17%) was four times higher than that of the γ-Proteobacteria (i.e. 4.19%). Based on partial 16S rDNA sequence analysis, the composition of most degrading bacterial communities consisted of 2–7 different bacterial species (data not shown).

Analysis of the atrazine-degrading genetic potential and of the activity of bacterial communities

The results of the analysis of the atrazine-degrading genetic potential of each bacterial community are presented in Table 2. Various combinations of atrazine-degrading genes were observed in the degrading bacterial community. Two gene combinations *atzABCDEF–strzND* and *atzBCDEF–trzN* were largely dominant and detected in 51% and 22% of the microbial communities, respectively. The *atzABCDEF–trzND* combination was

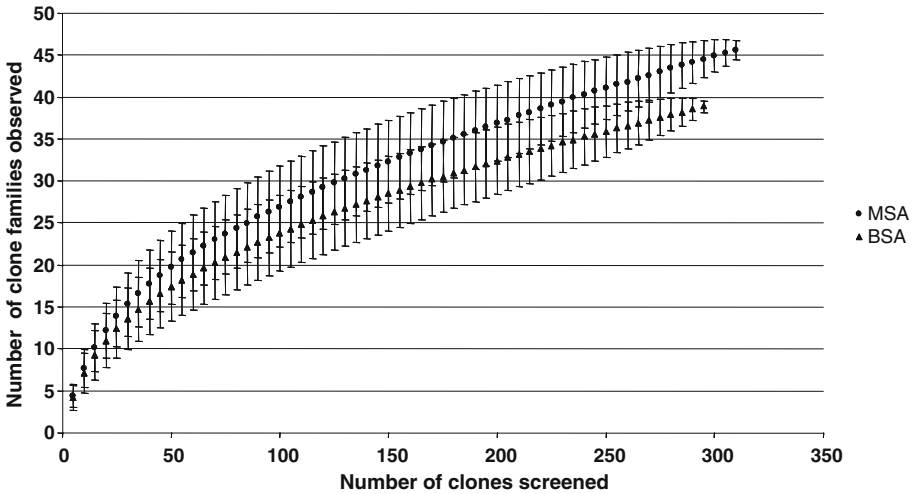


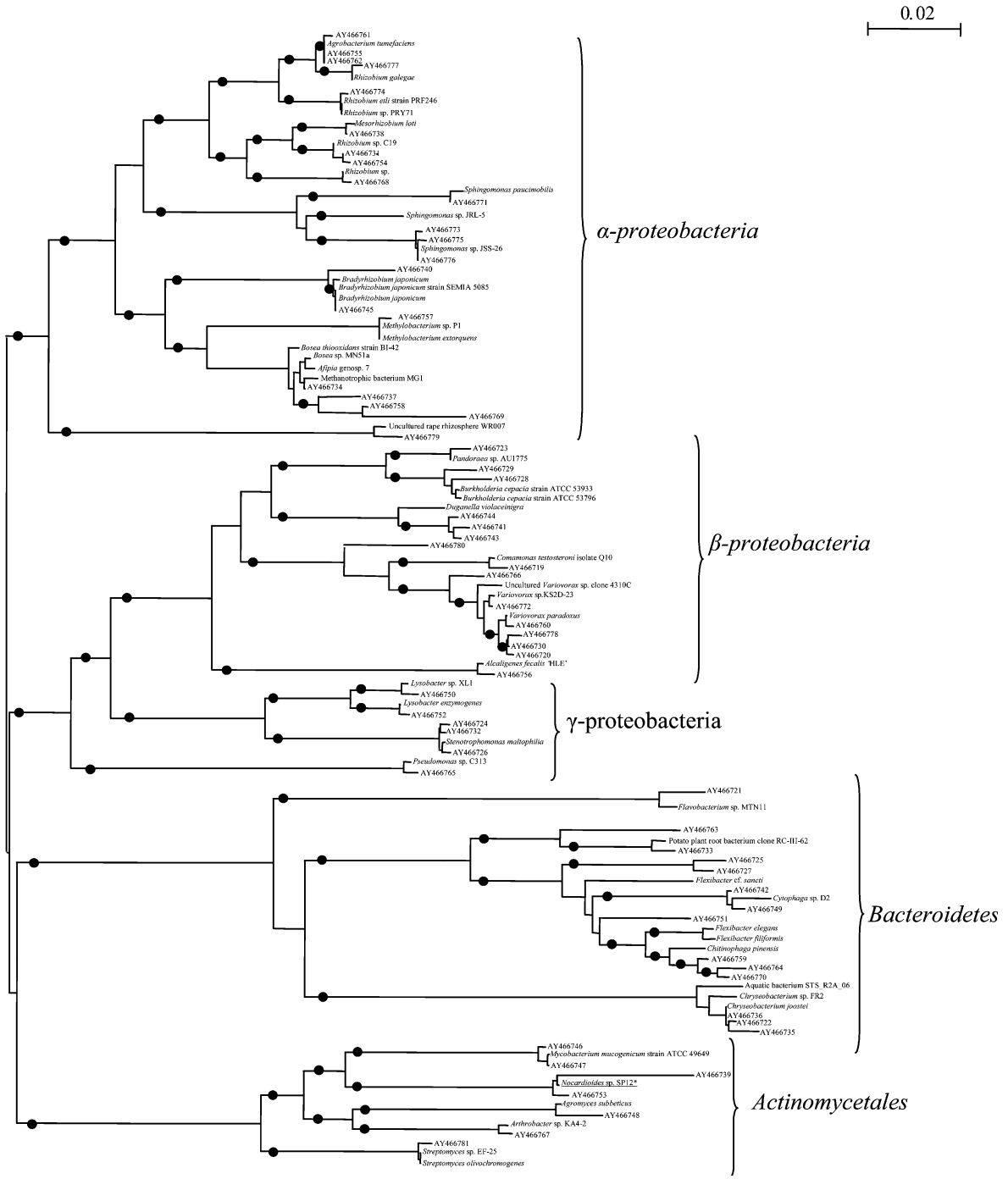
Figure 3. Rarefaction curves of observed richness of 16S rDNA OTU's in the atrazine-degrading bacterial communities isolated from bulk soil treated with atrazine (BSA) and maize planted soil treated with atrazine (MSA).

principally observed (62%) in bacterial communities isolated from maize rhizosphere soil whereas the *atzBCDEF–trzN* combination was mainly (64%) observed in bacterial communities isolated from bulk soil. Ten poorly represented gene combinations were also observed. Three of these (*atzABC–trzD, atzACDEF–trzN, atzABDEF–trzD*) were observed in a single bacterial community isolated from the maize rhizosphere. Five (*atzABC, atzB–trzN, atzBC–trzN, atzBCDEF, atzABCDEF–trzD*) were only observed in bacterial communities isolated from bulk soil and two others *(atzABC–trzN, atzABCDEF–trzN)* were observed in bacterial communities isolated either from bulk or maize rhizosphere soil.

Estimation of the atrazine-degrading activity of the bacterial communities revealed that within 15 days of culture, 76% of the communities had degraded up to 80% of the initially added atrazine (Table 2). However 20% of the bacterial communities poorly degraded atrazine (i.e. 47–89% of the initially added atrazine remained in the media) and only two communities MSA9 (abdeft gene combination) and MSA24 (bcdefn gene combination) did not degrade atrazine at all.

Discussion

Atrazine-degrading bacterial communities were isolated directly from soil suspensions prepared from bulk and maize rhizosphere soil samples without enrichment, contrary to the procedure in many previous studies (Mandelbaum et al., 1995; Topp et al., 2000a, 2000b). Enrichments were avoided on the premise that atrazine-degrading genes, frequently associated with transposons and located on a large plasmid, might be exchanged or rearranged under the selective pressure of enrichment, thereby leading to the development of microbial genotypes not encountered in soil (Rousseaux et al., 2001). Very few atrazine-degrading communities were isolated from soil samples not treated with atrazine. This result is in accordance with the fact that soils not treated with atrazine transform this herbicide very slowly (Piutti et al., 2002a). Similar numbers of atrazine-degrading communities were isolated from the MSA and BSA samples. This result indicated that although the maize rhizosphere had previously been reported to significantly increase the density and activity of atrazine-degrading communities (Piutti et al., 2002a), it did not modify the density of the cultivable atrazine-degrading communities. It should be noted that the uncultivability of many soil micro-organisms and the poor efficiency of the halo screening certainly biased the isolation of atrazine-degrading bacterial communities from soil. In addition, the obtaining of atrazine-degrading bacterial communities might have been biased by the fact that atrazine-degraders isolated were only those able to utilize citrate



as a carbon source. Moreover, it is most likely that the positive impact of atrazine, which exerted a selection pressure on atrazine-degrading communities, produced on atrazine-degrading communities may hide the impact of the maize rhizosphere on these communities.

To further asses the impact of the maize rhizosphere on atrazine degrading communities, ribosomal intergenic spacer analysis (RISA) which has previously been demonstrated to be relevant and sensitive enough to study bacterial communities from environmental samples

Figure 4. Phylogenetic analysis of the 16S rDNA sequences of atrazine-degrading bacterial communities. Phylogenetic distances were determined by neighbour-joining analysis. Nodes with more than 800 iterations (of 1000 bootstrap interations) are highlighted by a black circle. Asterisk indicates an atrazine-degrading bacterial strain. The Genbank accession numbers of the 16S rDNA sequences used here are: *Agrobacterium tumefaciens* (AY851692), *Rhizobium galegae* (AF025853), *Rhizobium* sp. SDW052 (AF345550), *Rhizobium etli* strain PRF246 (AY117661), *Rhizobium* sp. PRY71 (AF286362), *Mesorhizobium loti* (X67230), *Rhizobium* sp. C19 (AB167198), *Rhizobium* sp. (Y10176), *Sphingomonas paucimobilis* (AY367017), *Sphingomonas* sp. JRL-5 (AF181572), *Sphingomonas* sp. JSS-26 (AF131296), *Bradyrhizobium japonicum* (AF286361), *Bradyrhizobium japonicum* strain SEMIA 5080 (AF234889), *Methylobacterium* sp. P1 (AF148859), *Methylobacterium extorquens* (AF293375), *Bosea thiooxidans* strain BI-42 (AF508803), *Afipia* genosp. 7 (U87773), *Bosea* sp. MN 51a (AJ313022), Methanotrophic bacterium MG1 (AF068118), Uncultured rape rhizosphere bacterium WR007 (AJ295468), *Pandoreae* sp AU1775 (AY043377), *Burkholderia cepacia* strain ATCC 53933 (AY741357), *Burkholderia cepacia* strain ATCC 53796 (AY741355), *Duganella violaceingra* (AY376163), *Comamonas testosteroni* isolate Q10 (AF519533), Uncultured *Variovorax* sp. (AY571833), *Variovorax* sp. P9G781 (AF214127), *Variovorax* sp. K2SD-23 (AB196432), *Variovorax paradoxus* (AJ420329), *Alcaligenes fecalis* HLE (AY027506), *Lysobacter* sp. XL1 (AF472556), *Lysobacter enzymogenes* (AJ298291), *Stenotrophomonas maltophilia* (AF137357), *Pseudomonas* sp. C313 (AB167182), Rhizosphere soil bacterium clone RSC-II-76 (AJ252694), *Flavobacterium* sp. MTN11 (AY162137), Potato plant root bacterium clone RC-III-62 (AJ252725), *Flexibacter* cf. *sancti* (AF181568), *Cytophaga* sp. D2 (AF250407), *Flexibacter elegans* (M58782), *Flexibacter filiformis* (AB078049), *Chitinophaga pinensis* (AF078775), Aquatic bacterium STS_R2A_06 (AF125113), *Chryseobacterium* sp. FR2 (AF217562), *Chryseobacterium joostei* (AJ271010), *Mycobacterium mucogenicum* strain ATCC49649 (AF480585), *Nocardioides* sp. SP12 (AF537327), *Agromyces subbeticus* (AY737778), *Arthrobacter* sp. KA4-2 (AJ785761), *Streptomyces* sp. EF-25 (AF112170), *Streptomyces olivochromogenes* (AB122709).

(Borneman and Triplett, 1997; Robleto et al., 1998), was applied. RISA revealed that according to their genetic structure the 66 atrazine-degrading bacterial communities were separated in three major groups. The genetic structure of the atrazine-degrading bacterial communities in groups I and III depended on the soil compartment from which they had been isolated. Indeed, group I was essentially formed by bacterial communities isolated from maize-planted soil and the latter being in majority formed by communities isolated from bulk soil. Changes in diversity of the soil microbial community in the rhizosphere have been well documented (Kowalchuck et al., 2002; Kuske et al., 2002; Schmalenberger and Tebbe, 2003) although detailed studies of functional communities are scarce (Cheneby et al., 2003). Previous studies had shown that maize cultivation induced changes in the structure of soil bacterial communities resulting from the selective pressure exerted by root exudation, which stimulated the growth of microbial populations adapted to rhizospheric conditions (Curl and Truelove, 1986). We recently used a similar experimental procedure to show that the maize rhizosphere induced few changes in the structure of soil bacterial communities determined using RISA carried out on DNA directly extracted from soil (Philippot et al., 2002; Piutti et al., 2002a). It is most likely that RISA conducted on soil DNA extracts provides only a skewed view of the global structure of the bacterial community. This technique is probably not sensitive enough to detect the appearance of new phylotypes specific to the maize rhizosphere (Philippot et al., 2002) or to atrazine biodegradation (Piutti et al., 2002a). We demonstrated in our study of the atrazine-degrading microbial communities, which represent only a small part of the total microflora, that RISA was able to reveal the impact of the rhizosphere on the structure of these atrazine-degrading bacterial communities. Furthermore, these results are in accordance with recent reports where the structure of functional microbial communities, such as the nitrate-reducing community, was strongly modified in the maize rhizosphere although no effect was observed on the genetic structure of the microflora as a whole (Philippot et al., 2002).

To further characterize the bacterial populations forming atrazine-degrading bacterial communities a 16S rDNA library was elaborated for each bacterial community. The 16S rDNA library was screened by RFLP using *Alu*I restriction enzyme leading to the identification of 63 clone families. This analysis reveals a relatively high diversity of bacterial populations forming degrading communities. A rarefaction analysis relating the number of 16S rDNA clones analysed to the number of clone families sampled, was performed to see whether the number of clones sampled was sufficient to estimate the microbial

species richness of the atrazine-degrading communities isolated from MSA and BSA soils. The calculated rarefaction curves did not reach an asymptote, suggesting that the description of community richness (i.e. number of RFLP types observed) would have been further increased if a greater number of clones had been analysed. It also indicated the high diversity of the atrazine-degrading communities in both the bulk- and maize-planted soils. In addition, at the highest shared sample size, the number of OTUs observed in bulk soils was lower than that of maize planted soils. Although the statistical significance of this evidence cannot be tested, it suggests that the microbial richness of the atrazine-degrading bacterial communities isolated from the maize-planted soils was higher than that of the communities isolated from the bulk soils. However, the diversity of the MSA and BSA libraries estimated quantitatively by applying the Simpson's index (*D*), which accounts for the richness and the relative abundance of RFLP types from sampled biodiversity did not differ significantly.

Sequence analysis revealed that bacterial populations forming atrazine-degrading microbial communities were highly diverse being affiliated to bacterial groups such as *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. It also revealed the existence of four dominant RFLP families namely AY466779, AY466728, AY466767 and AY466737 which were highly similar to *Variovorax paradoxus* (99% similarity to AJ420329), *Burkholderia cepacia* strain ATCC53976 (98% similarity to AY741355), *Arthrobacter* sp. KA4-2 (99% similarity to AJ785761) and *Bosea* sp. MN51a (99% similarity to AJ313022), respectively. Of these, only *Arthrobacter* sp. have been isolated from different soils located in Europe, USA and China for their ability to mineralize atrazine (Cai et al., 2003; Rousseaux et al., 2001, 2002; Strong et al., 2002). *Variovorax* sp. and *Burkholderia* sp. are known to be well represented in the rhizosphere and able to degrade various organic pollutants such as 2,4-D (Vallaeys et al., 1999), linuron (Dejonghe et al., 2003), sulfolane (Greene et al., 2000) or toluene (Shields et al., 1995), polychlorinated phenols (PCPs) (Tomasi et al., 1995), and polyaromatic hydrocarbons (PAHs) (Kim et al., 2003), respectively. Up to now, most *Bosea* sp. isolates have been isolated from hospital environments and are known to oxidize thiosulfate but not to degrade organic compounds in soils.

Interestingly some poorly represented RFLP families showed high similarities to bacterial strains able to degrade pesticides or organic pollutants. Indeed, the RFLP families AY466726, AY466732 and AY466724 showed strong similarities with *Stenotrophomonas maltophilia* previously reported to mineralize atrazine (Rousseaux et al., 2001). The RFLP family AY466781 showed high similarity with *Streptomyces* sp., which is believed to degrade pesticide residues in contaminated soils (Shelton et al., 1996) and has been shown to degrade atrazine (Fadullon et al., 1998). The RFLP families AY466753 and AY466739 showed high similarities with *Nocardioides* sp. C157 and *Nocardioides* sp. SP12 which had previously been shown to degrade atrazine (Piutti et al., 2003; Topp et al., 2000a). It is noteworthy that *Nocardioides* sp. SP12 had already been isolated from the soil of La Bouzule (Nancy, France) (Piutti et al., 2003). Several other RFLP families showed homologies with *Rhizobium* sp. one isolate of which was shown to be able to dechlorinate atrazine (Bouquard et al., 1997). Sequence analysis also revealed that the composition of degrading communities in bacterial species was relatively complex being for most of them formed of two to seven different bacterial species. However, the strategy used in this study did not allow us to determine which species in the bacterial community possess atrazine-degrading genetic potential since the atrazine-degrading gene combination can only be determined for an individual community.

Indeed, the atrazine-degrading gene potential represented by the atrazine-degrading gene composition was studied for each atrazine-degrading bacterial community. Most of the bacterial communities harboured the *atz* genes. Various combinations of atrazine-degrading genes were observed in degrading bacterial communities suggesting that these genes are dispersed in the microflora of both bulk and maize-planted soils. This observation is in accordance with previous work showing that *atzA*, *B* and *C* genes are highly conserved and widely dispersed in the soil microflora (de Souza et al., 1998a) and also that *trzN* can be found in different Gram positive bacteria such as *Nocardioides* sp. SP12 and *Nocardioides* sp. C157 (Piutti et al., 2003; Topp

et al., 2000a). In addition, our results further indicate that the genes involved in the opening of the *s*-triazine ring of cyanuric acid (i.e. *atzD* and *trzD*), one of the key intermediary metabolites of atrazine are widespread among atrazine-degrading communities, as recently suggested (Fruchey et al., 2003). These authors reported that 10 bacteria that use cyanuric acid as a nitrogen source for growth were found to contain either *atzD* or *trzD*, but not both genes (Fruchey et al., 2003). Our results indicate that these two genes can be found in the same atrazine-degrading bacterial community. This result confirms that an atrazine catabolic pathway combining *trzN* with *atz* genes is widely distributed in atrazine-degrading communities as previously suggested (Piutti et al., 2003). Two dominant gene combinations *atzABCDEF*, *trzND* and *atzBCDEF*, *trzN* were observed; the first was preferentially observed in degrading bacterial communities isolated from maize-planted soils while the second was predominantly detected in degrading communities isolated from bulk soils. Interestingly, the gene combination observed in maize-planted soil was characterized by two functional redundancies on key steps of atrazine mineralisation. Indeed this gene combination harboured (i) *atzA* and *trzN* genes code for enzymes catalyzing the dechlorination of atrazine leading to the formation of hydroxyatrazine and (ii) *atzD* and *trzD* genes code for enzymes opening the *s*-triazine ring. These functional redundancies may contribute to a better accomplishment of the atrazine-degrading function. Although most atrazine-degrading gene combinations represented the completely atrazine catabolic pathway, some presented a truncated atrazine catabolic pathway and might therefore lead to a partial degradation of atrazine with accumulation of metabolites. Indeed, bacterial communities presenting the *atzABC*, *atzABC-trzN* or *atzBC–trzN* combinations might contribute to the accumulation of cyanuric acid and those presenting the *atzB–trzN* combination might accumulate *N*-isopropylammelide as previously shown (Piutti et al., 2003; Rousseaux et al., 2001). Together, all these results indicated that the atrazine-degrading genetic potential of the degrading bacterial communities was depending on the soil compartment from which they have been isolated.

The determination of atrazine-degrading activity of each bacterial community further indicated that most of them were able to successfully and rapidly degrade this herbicide. It showed that they were able to express their atrazine-degrading genetic potential at least under laboratory conditions. However, several bacterial communities poorly degraded atrazine and only two communities MSA9 (abdeft gene combination) and MSA24 (bcdefn gene combination) did not degrade atrazine at all. These results suggested that although these bacterial communities do possess an atrazine-degrading genetic potential, it was not expressed. This observation is in accordance with previous work showing that the expression of atrazine-degrading genetic potential is up-regulated by the substrate (atrazine) and depends on the host cell (Devers et al., 2004).

To conclude major factors regulating atrazine degradation can be modified in the maize-rhizosphere such as nitrate concentration (*via* N assimilation by plants) which may be decreased whereas C availability (*via* rhizodeposition) is generally increased. Since atrazine mainly provides a nitrogen source for the bacterial degrading community, the C-enriched maize rhizosphere may increase the capacity of the atrazine-degrading microbes since they are able to use this herbicide as N source. We previously showed that the maize rhizosphere contributes to an increase both in atrazine mineralization and the atrazine-degrading community (Piutti et al., 2002a). Our present work demonstrates that the maize rhizosphere also modifies the genetic structure, the diversity and the atrazine-degrading gene composition of these atrazine-degrading bacterial communities. It highlights the taxonomic and genetic complexity of atrazine-degrading communities and suggests that the atrazine-degrading genetic potential could be shared by several microbial strains associated within a degrading community in the soil environment. This hypothesis is in accord with recent work indicating the existence of cooperative catabolic pathways within an atrazine-degrading enrichment culture from soil (Smith et al., 2005). In addition, it is reinforced by our recent work reporting that *atz* degrading genes are horizontally transferred in soil and possibly subjected to gene rearrangement (Devers et al., 2005). Further work will aim to study the functioning of several atrazine-degrading bacterial communities isolated from the maize-rhizosphere differing in their taxonomic and genetic compositions.

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