Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Taxonomic and functional diversity of atrazine-degrading bacterial communities enriched from agrochemical factory soil

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Keywords
atrazine, *atz* genes, bacterial community, biodegradation, diversity, *trz* genes.

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2009/1138: received 24 June 2009, revised 8 January 2010 and accepted 29 January 2010

doi:10.1111/j.1365-2672.2010.04700.x

Abstract

Aims: To characterize atrazine-degrading potential of bacterial communities enriched from agrochemical factory soil by analysing diversity and organization of catabolic genes.

Methods and Results: The bacterial communities enriched from three different sites of varying atrazine contamination mineralized 65–80% of ^{14}C ring-labelled atrazine. The presence of *trzN-atzBC-trzD*, *trzN-atzABC-trzD* and *trzN-atzABC-DEF-trzD* gene combinations was determined by PCR. In all enriched communities, *trzN-atzBC* genes were located on a 165-kb plasmid, while *atzBC* or *atzC* genes were located on separated plasmids. Quantitative PCR revealed that catabolic genes were present in up to 4% of the community. Restriction analysis of 16S rDNA clone libraries of the three enrichments revealed marked differences in microbial community structure and diversity. Sequencing of selected clones identified members belonging to *Proteobacteria* (α-, *β*- and *γ*-subclasses), the *Actinobacteria*, *Bacteroidetes* and TM7 division. Several 16S rRNA gene sequences were closely related to atrazine-degrading community members previously isolated from the same contaminated site.

Conclusions: The enriched communities represent a complex and diverse bacterial associations displaying heterogeneity of catabolic genes and their functional redundancies at the first steps of the upper and lower atrazine-catabolic pathway. The presence of catabolic genes in small proportion suggests that only a subset of the community has the capacity to catabolize atrazine.

Significance and Impact of the Study: This study provides insights into the genetic specificity and the repertoire of catabolic genes within bacterial communities originating from soils exposed to long-term contamination by *s*-triazine compounds.

Introduction

Over the past 40 years, the *s*-triazine herbicide, atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine), has been widely used by agriculture to control grassy and broadleaf weeds. Agricultural activities and atrazine manufacturing plants are the primary atrazine point sources that lead to soil and water pollution. For example, atrazine can be present in the environment at very high concentrations (e.g. up to 2000 ppm), such as those typically found at spill sites or in manufacturing wastewaters, as well as at relatively low concentrations (200 ppb or lower), such as those occurring following minor spills or in run-off water (Mandelbaum and Wackett 1996). As microbial degradation of atrazine and other *s*-triazine compounds is one of the major modes of their removal from the environment, pure microbial cultures of atrazine degraders have been well characterized (Mandelbaum *et al.* 1995; Struthers *et al.*

1998; Topp *et al.* 2000a,b; Rousseaux *et al.* 2001; Strong *et al.* 2002).

Atrazine biodegradation usually begins *via* hydrolytic dechlorination, resulting in the production of hydroxyatrazine (Fig. 1). This first step is catalysed by a chlorohydrolase encoded by the *atzA* or *trzN* gene (De Souza *et al.* 1996; Topp *et al.* 2000a). Hydroxyatrazine is then converted to cyanuric acid by two amydohydrolases encoded by two other genes *atzB* and *atzC* of the upper degradation pathway (Boundy-Mills *et al.* 1997; Sadowsky *et al.* 1998). The lower atrazine-degradation pathway consists of three catabolic genes, *atzDEF* or *trzDEF*. These are essential for cleaving the cyanuric acid ring and for the hydrolysis of biuret and allophanate, respectively (Eaton and Karns 1991; Martinez *et al.* 2001; Cheng *et al.* 2005). Several studies revealed that atrazine-degrading genes are highly conserved, widespread and frequently associated with insertion sequences (IS) located on plasmids (De Souza *et al.* 1998b; Martinez *et al.* 2001; Rousseaux *et al.* 2002; Sajjaphan *et al.* 2004; Devers *et al.* 2007a). Consequently, the combination of IS-mediated rearrangement and plasmid transfer had been suggested to contribute to the assembly and dissemination of the atrazine-degrading capabilities in the environment (De Souza *et al.* 1998c; Devers *et al.* 2005, 2007b). This further suggests that atrazine-degrading communities are still actively evolving.

Although much research has been carried out in pure cultures, providing a substantial knowledge about the taxonomic diversity of atrazine-degrading microbes and individual metabolic pathways, limited knowledge is available about atrazine-degradation potential at the microbial community level. Degradation of atrazine is a multi-step process and although several bacteria are known to have a metabolic capacity to degrade atrazine to completion, such bacteria are thought to have evolved from a mixed atrazine-degrading microbial cultures (De Souza *et al.* 1998a). Therefore, investigating atrazine-catabolizing communities offers an advantage over studies of pure cultures. De Souza *et al.* (1998a) isolated from agricultural soil an atrazine-mineralizing community consisting of four or more bacterial species, but two members, a *Clavibacter* sp. and a *Pseudomonas* sp., collectively mineralized atrazine and carried out sequential steps in degradation pathway that involved the *atzABC* genes. By using a PCR-DGGE method, examination of another enrichment culture from the same soil sample revealed an 8-member atrazine-mineralizing community, including species of the genera *Agrobacterium*, *Caulobacter*, *Pseudomonas*, *Sphingomonas*, *Nocardia*, *Rhizobium*, *Flavobacterium* and *Variovax* (Smith *et al.* 2005). The atrazine-degrading genetic potential of this community included *trzN-atzBC-trzD* genes. The community contained multiple degradation pathways as well as considerable redundancy in

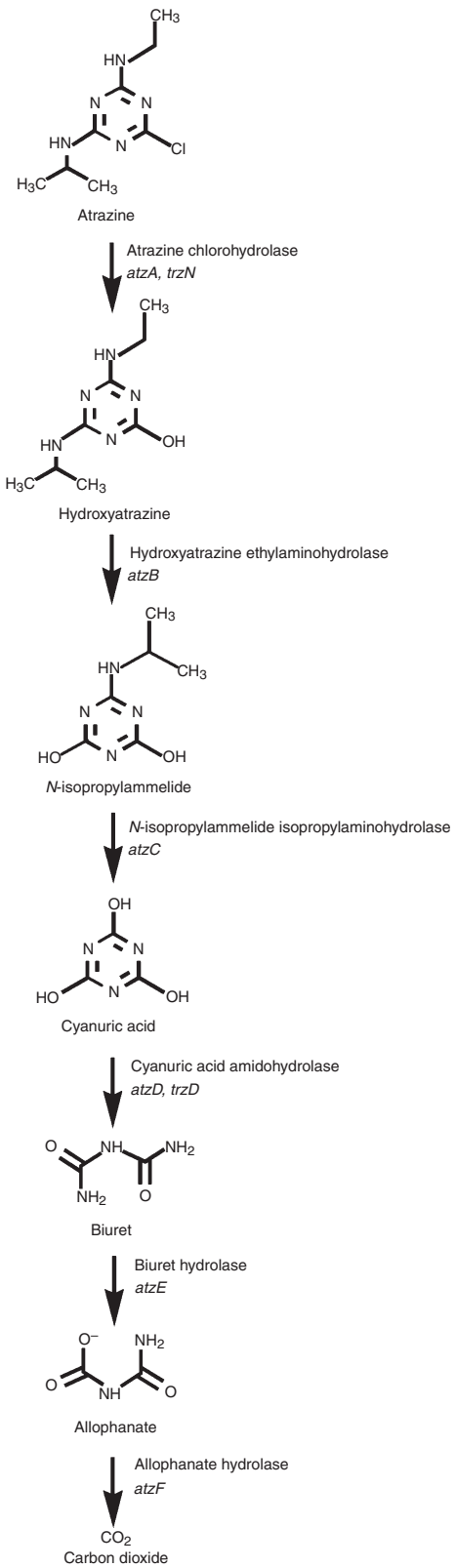

Figure 1 Complete atrazine-mineralizing pathway. Enzymes and genes involved in each step of atrazine mineralization are indicated.

catabolic genes. The *atzC* gene was redundant among all eight community members, whereas the *trzD* was present only in four members. The *atzB* and *trzN* genes were found in only one member each, *Rhizobium* sp. and *Nocardia* sp., respectively. In our recently published article (Kolić *et al.* 2007), we characterized an atrazine-mineralizing enrichment culture from soil at an agrochemical factory and determined by plate cultivation assays that *Arthrobacter* sp., *Ochrobactrum* sp. and *Pseudomonas* sp. are the key drivers for atrazine mineralization in that community.

Here, we use 16S rRNA gene cloning followed by restriction enzyme and sequence analysis to gain insights into the structure and diversity of three atrazine-mineralizing communities. We describe the catabolic genetic potential of these communities by exploring the prevalence of known atrazine-degrading genes, localization of these genes on catabolic plasmids and their abundance relative to the total community 16S rRNA gene pool using quantitative PCR (q-PCR).

Materials and methods

Chemicals

Technical atrazine (95·66%) was kindly donated by the herbicide factory Herbos, Sisak, Croatia.

Sampling sites

Upper soil layer samples (0–5 cm) were collected within the Herbos factory area from three different locations exposed to long-term contamination with atrazine and other *s*-triazine compounds (Table 1). One soil sample (S4) was taken from a site with a history of pesticide spills. Soils were homogenized and stored at 4°C prior to being used for enrichment.

Enrichment and growth conditions

For enrichment, 2 g of soil was added to 18 ml of mineral salts (MS) medium (Mandelbaum *et al.* 1993) with atrazine (100 mg l^{-1}) and supplemented with sodium citrate (1 g l^{-1}) and yeast extract (50 mg l^{-1}) as the source of vitamins and essential nutrients. Erlenmeyer flasks (100 ml) were shaken on a rotary shaker (150 rev min^{-1}) at 25°C. Every 2 weeks, 5% of enriched culture was transferred to fresh medium (40 ml) and incubated under the same conditions. After 2 months of enrichment (total of 4 culture transfers), biomass was centrifugated (10 000 ***g***, 5 min), resuspended in phosphate buffer (pH 7·5) and stored at −20°C under glycerol (16% v/v as the final concentration). For atrazine mineralization studies, frozen enrichments were grown in the MS-citrate medium containing 100 mg l^{-1} of atrazine on a rotary shaker at 25°C.

Atrazine mineralization kinetics

The atrazine-mineralizing capability of enriched communities (Z2, Z3, Z4) was determined by radiorespirometry over a 4-days incubation period as described previously (Kolić *et al.* 2007). Cells from enrichment cultures grown for 8 days were used as inoculum. The cells were harvested by centrifugation (6000 ***g***, 10 min), washed twice in MS medium and resuspended to an OD_{600} of 0·2 in MS-citrate medium supplemented with 30 mg l^{-1} of unlabelled atrazine and 52 Bq ml^{-1} of ^{14}C ring-labelled atrazine (Isotopchim, France, specific activity 910 MBq $mmol^{-1}$). The evolved $^{14}CO_2$ trapped in NaOH solution was measured by liquid scintillation counting.

The modified Gompertz model (Gompertz 1825; Zwietering *et al.* 1990) was fitted to mineralization data (Sigmaplot 4·0; Sigma). Three parameters were determined: A, the maximum percentage of atrazine mineralization; μ_m, the maximum mineralization rate; and λ, the lag time. A statistical analysis was performed by using single factor analysis of variance (ANOVA) followed by a Fisher procedure ($n = 3$, $P < 0.001$) (STATVIEW© 4.55 software; Abacus Concept, Inc., Los Angeles, CA).

DNA extraction and PCR amplification of *atz, trz* and 16S rRNA gene sequences

Genomic DNA from 7 ml of 8-day-old communities grown in MS-citrate medium containing 100 mg l^{-1} of

Table 1 Chemical properties of the contaminated soils within an agrochemical factory area, from which atrazine-degrading communities have been enriched

Sampling site	Enriched community	Organic matter (%)	Organic C (%)	Organic N (%)	Atr	Sim	Prop	Terb	Prom
					(mg kg^{-1} of dry soil)				
S2	Z2	5·56	2·65	0·33	2·22	0·08	0·08	0·04	ND*
S3	Z3	6·53	2·26	0·31	0·14	0·08	0·03	ND*	0·03
S4	Z4	5·50	1·61	0·27	416·8	761·1	1165·9	314·5	0·46

Atr, atrazine; Sim, simazine; Prop, propazine; Terb, terbuthylazine; Prom, prometryn.
*ND, not determined.

atrazine was extracted using Qiagen Blood and Cell culture Midi kit according to the manufacturer's instructions (Qiagen, France). Atrazine-catabolic genes (*atz* and *trz*) were detected by PCR amplification with primers specific for *atzA, B, C, D, E, F* and *trzD, N* genes. The annealing temperature for *atzA, B, D, E* genes was 60°C, for *atzC, F* genes was 57°C and for *trzD, N* genes was 55°C (Rousseaux *et al.* 2001; Mulbry *et al.* 2002; Devers *et al.* 2004). 16S rRNA genes were amplified with the universal 27f and 1492r primers (Gurtler and Stanisich 1996). Each reaction contained 0·2 mmol l^{-1} of each dNTP, 1·5 mmol l^{-1} $MgCl_2$, 1 μmol l^{-1} of each specific primer, 2·5 μl template DNA (25 ng), 1× PCR buffer and 1·25 U of *Taq* polymerase (QBiogene, France) and was carried out in a TGradient Thermocycler (Biometra, Germany) as follows: 5 min at 95°C, 35 cycles for 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.

Quantitative PCR assays

Quantitative PCR (q-PCR) was carried out in an ABI Prism 7900 (Applied Biosystems) by using SYBR Green® as the detection system in a reaction mixture of 20 μl containing: 12·5 μl of SYBR Green PCR master mix (QuantiTect™ SYBR® Green PCR Kit; Qiagen, France), 1 μmol l^{-1} of each primer and 5 μl of template DNA (10 ng). Thermal cycling conditions for the 16S rRNA, *atzA*, *B, C, D, E, F* and *trzD* genes were as previously described (Devers *et al.* 2004). The final step starting from 60 to 95°C (0·2°C s^{-1}) was added to obtain a specific dissociation curve. Purity of the PCR products was checked by the observation of a single melting peak and the presence of a unique band of the expected size in a 2% agarose gel. Three replicates were run for each gene target. Calibration curves of q-PCR relating to the log of the copy number of the target gene as function of the C_T (cycle threshold) were previously generated (Devers *et al.* 2004). For the *trzD* gene, calibration curve was as follows: $\log(trzD) = -0{\cdot}25 \times C_T + 10{\cdot}69$ ($r^2 = 0{\cdot}995$).

Plasmid profiling and hybridization

A modified Eckhardt plasmid extraction (Eckhardt 1978; Wheatcroft *et al.* 1990) was performed on bacterial pellets obtained by centrifuging (20 000 ***g***, 5 min) 2 ml of enrichment grown overnight in MS-citrate medium containing 30 mg l^{-1} of atrazine (OD_{600} 0·4). Plasmids were separated on a 0·75% agarose gel, vacuum-transferred onto Biodyne Plus membrane (Gelman Sciences, Merck Eurolab, France) and used for Southern hybridization with a Dig-labelled probe (*atzA*, *B, C, D, E, F; trzD*, *N*; IS*1071*) performed at high stringency conditions (Rousseaux *et al.* 2001). Plasmid size was estimated by calibration against the relative mobility of the plasmids used as standards: (i) pAT (543 kb, accession no. AE007872) and pTi (214 kb, accession no. AE007871) harboured by *Agrobacterium tumefaciens* C58, and (ii) a megaplasmid (>1500 kb), pRme41a::Tn7 (236 kb) and pRP4 (60 kb, accession no. L27758) harboured by a derivative of *Rhizobium meliloti* 41 (GMI328).

Clone library construction and amplified ribosomal DNA restriction analysis (ARDRA)

A 16S rRNA gene clone library was constructed for each of the three communities. 16S rRNA gene amplicons were cloned using pGEM-T Easy Vector System II according to the manufacturer's protocol (Promega). Approximately, 100 randomly selected clones from each library were checked for correct insert size by vector-targeted primers (T7, SP6). A total of 232 positive transformants were digested with the frequently cutting endonuclease *Alu*I (QBiogene). The digests were analysed on 3% high-resolution agarose gel (QBiogene). The ARDRA patterns obtained were compared and grouped into ARDRA families.

Rarefaction and diversity indices

Diversity coverage by the clone libraries was analysed with Analytic Rarefaction software (http://www.uga.edu/strata/software/). The Shannon–Weiner index of general diversity (H′) was calculated with the following equation: $H' = -\Sigma(p_i \ln p_i)$, where p_i was calculated as follows: $p_i = n_i/N$, where n_i is the number of clones in each phylotype, and N is the total number of clones. Using the same data, the Simpson index of dominance (D) was calculated by using the following function: $D = \Sigma(p_i^2)$.

Sequencing and phylogenetic analysis

One representative of each ARDRA family that contained at least two clones as well as selected individual clones were sequenced using the 926r primer (Cheneby *et al.* 2000) on a CEQ 8000 sequencer (Beckman Coulter®) following the manufacturer's instructions. All sequences were analysed using Blast against the GenBank 16S rRNA database (Altschul *et al.* 1990). Sequences were screened for chimeras using the Check_Chimera program (Maidak *et al.* 2001), and any potential candidates were excluded from the data set. The remaining sequences (a total of 47) were aligned to a global alignment using Greengenes NAST-aligner (DeSantis *et al.* 2006). Sequences were imported to the Greengenes May 2007

ARB database in the ARB software package (ver. 06.03.22) and inserted into the universal ARB dendrogram using parsimony function in ARB using a Lane mask filter (Ludwig *et al.* 2004). Primary taxonomic assignments were determined by the resulting ARB parsimony tree. Phylogenetic relationships of sequenced clones were additionally calculated by exporting aligned sequences as well as their closest relatives from the ARB database to the PAUP 4.01b software package (Sinauer Associates, Sunderland, MA, USA). A neighbour-joining tree and maximum-parsimony tree were constructed from the data set, and the bootstrap values (100 replicates) were obtained in PAUP (Swofford 1993).

Nucleotide sequence accession numbers

The 16S rDNA sequences retrieved in this study have been deposited in GenBank under accession numbers EU560927–EU560973.

Results

Enrichment and activity of bacterial communities from agrochemical factory soils

We obtained three microcosm enrichments from agrochemical factory soil samples of different *s*-triazine contamination levels (Table 1). Evidence of atrazine-degrading bacteria within the enrichment was observed by an increase in bacterial biomass and establishment of stable population densities within 2 months of cultivation.

Screening of atrazine-mineralizing activity within the enriched communities by using the radiorespirometric approach was performed under shaking conditions using atrazine labelled with ^{14}C on the *s*-triazine ring. The three enriched communities exhibited sigmoid mineralization curves (not shown) and were analysed using the modified Gompertz model. The results of the kinetic parameters presented in Table 2 showed that communities Z2 and Z4 exhibited similar and significantly higher A (maximum percentage of mineralization) values when compared to the community Z3. However, Z3 showed the shortest lag time (λ) of the three communities and a μ_m (mineralization rate) value similar to that of Z2.

Table 2 Parameters of atrazine mineralization kinetics after fitting the modified Gompertz model

Community	A (%)	μ_m (h^{-1})	λ (h)
Z2	75·6 (±0·7)[a]	3·6 (±0·4)[ab]	1·9 (±0·2)[a]
Z3	64·6 (±1·5)[b]	2·7 (±0·1)[a]	1·05 (±0·01)[b]
Z4	79·0 (±1·9)[a]	4·0 (±0·1)[b]	1·11 (±0·05)[c]

A, the maximum percentage of mineralization, μ_m, the maximum mineralization rate and λ, the lag time. Values are means ± SE (n = 3). Parameters were checked by applying a Fischer test, and values followed by the same letter do not differ significantly (n = 3, P < 0·001).

Atrazine-degrading gene composition and localization

Determination of the atrazine-degrading genetic composition of bacterial communities by PCR revealed various combinations of catabolic genes (Table 3). The most simple *trzN-atzBC-trzD* gene combination was observed in the community Z2, whereas the *trzN-atzBCDEF-trzD* and *trzN-atzABC-trzD* combinations were observed in the communities Z3 and Z4, respectively. These results indicate that community Z4 contained two different genes, *trzN* and *atzA*, encoding enzymes responsible for the first step of the upper degradation pathway, i.e. the transformation of atrazine to hydroxyatrazine (Fig. 1). Similarly, community Z3 contained two genes, *trzD* and *atzD*, coding for enzymes that catalyse the first step of the lower pathway, i.e. the transformation of cyanuric acid to biuret.

Plasmid profile analysis showed that community Z2 had six different-sized plasmids, whereas the communities Z3 and Z4 each had five (Table 3). The estimated sizes of recovered plasmids ranged from 40- to 295-kb.

Table 3 Prevalence and localization of atrazine-degrading genes and IS*1071* in enriched communities

Community	Atrazine-degrading gene composition (PCR)	IS*1071**	Chromosome (hybridization signal)	Number of plasmids†	Size of plasmids (kb) (hybridization signal)					
Z2	*trzN-atzBC-trzD*	+		6	295	165 (*trzN*, *atzBC*)	130 (*atzC*, *trzD*)	80	45	40
Z3	*trzN-atzBCDEF-trzD*	+‡	*trzD*	5	295	165 (*trzN*, *atzBC*)		80 (*atzBCDEF*)	45	40
Z4	*trzN-atzABC-trzD*	+		5	295	165 (*trzN*, *atzBC*)	125 (*atzC*, *trzD*)	55	45	

*The presence of sequences homologous to IS*1071* is indicated (+).
†Number of plasmids of different sizes detected in the bacterial community.
‡IS*1071* is not detected on 80-kb plasmid.

Interestingly, communities Z2 and Z3 carried plasmids of approximately the same sizes except a 130-kb plasmid that was absent from community Z3.

Southern blot analyses of plasmid profiles revealed that the 165-kb plasmid of all three communities produced hybridization signals with *trzN*, *atzB*, *atzC* and IS*1071* probes (Table 3). Nevertheless, in the community Z3, hybridization to a 80-kb plasmid band was seen with the *atzB, atzC, atzD, atzE* and *atzF* probes. However, we also detected a strong signal with the *trzD* probe on the chromosomal band in the community Z3 indicating localization of the *trzD* gene on chromosome. In communities Z2 and Z4, 130- and 125-kb plasmids also hybridized with *atzC, trzD* and IS*1071* probes (Table 3). Finally, although *atzA* was successfully amplified by PCR using Z4 community DNA as a template, the *atzA* probe did not hybridize with any of the plasmids.

Relative abundance of *atz* and *trz* sequences

The abundance of atrazine-degrading genes and 16S rRNA gene sequences was evaluated by q-PCR, and ratios of *atz* and *trz* sequences to 16S rRNA genes from the total community were calculated for *atzABCDEF* and *trzD,* showing similar PCR efficiencies.

Data on calculated ratios (Table 4) showed that the relative abundances of *atzB, C, D E* and *F* genes ranged from about 0·2–3·8%, while relative abundance of *trzD* ranged from about 0·01 to 0·09%. Compared to Z2 community, relative abundances of *atzB* and *atzC* genes in Z3 were about 10 times higher. The relative abundances of *atzD* and *atzF* genes within community Z3 were comparable to those of *atzB* and *atzC,* whereas the *atzE* was less represented. In contrast, community Z4 exhibited significantly higher relative abundance of *atzC* than that of the *atzB* gene, and this community had a higher *trzD*/16S rRNA gene ratio than the other two communities. In addition, the abundance of the *atzA* gene, detected only in the community Z4, was about 10 000-fold lower than the abundance of 16S rRNA genes. This observation is in accordance with the fact that *atzA* was detected by PCR but not by Southern blot hybridization.

Phylotype richness and distribution

16S rRNA gene clone libraries were constructed for each atrazine-degrading community. We analysed 60–90 clones from each library by ARDRA and grouped them by ARDRA patterns. A total of 26 (of 86 clones analysed), 33 (of 63) and 28 (of 82) different ARDRA patterns were detected within Z2, Z3 and Z4 clone libraries, respectively (Fig. 2). Comparison of the distribution of phylotypes from libraries Z2, Z3 and Z4 clearly exhibited significant differences (Fig. 2). The Z2 library was dominated by the phylotypes, in order of abundance, 5, 10, 1, 2 and 9. Together, these accounted for about 70% of this clone library, while within the Z3 library, ARDRA types 31, 30, 6 and 35 were the most abundant representing together 36% of the clone library. Further, the library from community Z4 was dominated by phylotypes 76, 66 and 61, which together accounted for 51% of the clone library. Calculation of the Simpson's index (D) revealed twofold higher values for Z2 and Z4 clone libraries (D = 0·11 and 0·10) than for the Z3 library (D = 0·05), indicating higher evenness in communities Z2 and Z4 when compared to Z3. We used rarefaction analysis to estimate how well the libraries were sampled. Rarefaction curves did not reach an asymptote, indicating that the diversity present within the libraries had not been sampled to saturation (Supporting information). Further, at the highest shared sample size (i.e. 63 clones), the Z2, Z3 and Z4 libraries had an estimated diversity of 22, 35 and 24 phylotypes, respectively, indicating the highest diversity in the Z3 library as well as lower and similar diversity in other two libraries. This was also confirmed by the calculation of the Shannon–Weiner index (H'), which was higher for the community Z3 (H' = 3·22) than for the other two communities (Z2, H' = 2·61; Z4, H' = 2·74).

Table 4 Relative abundance of atrazine-degrading (*atz* or *trz*) genes expressed as ratios of *atz* and *trz* gene copy numbers to 16S rRNA gene copy number determined by q-PCR

	Ratio*						
Community	*atzA*	*atzB*	*atzC*	*atzD*	*atzE*	*atzF*	*trzD*
Z2	–	0·22 ± 0·17	48 ± 0·32	–	–	–	0·032 ± 0·02
Z3	–	2·03 ± 0·21	2·94 ± 1·88	1·91 ± 0·88	0·69 ± 0·36	2·29 ± 1·20	0·006 ± 0·01
Z4	$3{\cdot}93 \times 10^{-4}$ $\pm 1{\cdot}04 \times 10^{-4}$	0·31 ± 0·07	3·84 ± 1·63	–	–	–	0·085 ± 0·01

Values are means ± SE (n = 3). – indicates the absence of gene.
*Ratio = [(*atz* or *trz* gene copy number/16S rRNA gene copy number) × 100].

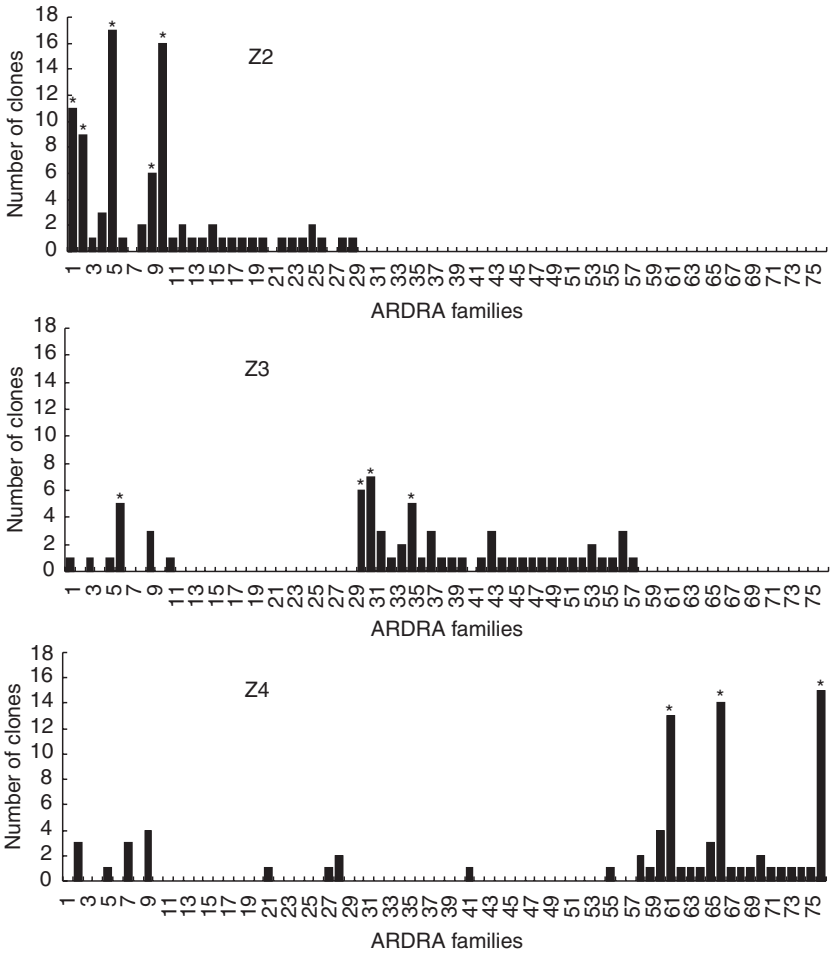

Figure 2 Distribution of the ARDRA families from the communities Z2, Z3 and Z4. Numerically predominant ARDRA families are indicated by an asterisk.

Phylogenetic diversity among ARDRA phylotypes

To evaluate the phylogenetic diversity represented by 68 ARDRA patterns obtained from the Z2, Z3 and Z4 clone libraries, partial 16S rRNA gene sequencing was performed, and phylogenetic trees were constructed using maximum-parsimony and neighbour-joining methods. As evident from the parsimony tree shown in Fig. 3, the clusters of the α- and β-subclasses of *Proteobacteria* as well as those of *Actinobacteria* were identified in all three libraries. Members of the γ-subclass of *Proteobacteria* were encountered in the Z2 and Z4 libraries, whereas the representatives of the *Bacteroidetes* phylum were found in the Z2 and Z3 libraries. The candidate division TM7 was represented only in the Z4 library.

The α-proteobacterial cluster was the largest and was represented mainly by the members of the families *Brucellaceae* and *Phyllobacteriaceae* (Fig. 3). Two phylotypes dominant in the Z2 (Z2_5) and Z3 libraries (Z3_35) displayed 99% similarity to the cyanuric acid degrading *Ochrobactrum* sp. CA1, isolated previously from an atrazine-degrading community originating from the current sampling location (Kolić *et al.* 2007).

The β-proteobacterial cluster represented the second largest cluster and comprised phylotypes clustering within the *Alcaligenes* and *Comamonas* groups (Fig. 3). *Alcaligenes*-related phylotypes, among which was the dominant phylotype of library Z2 (Z2_9, Fig. 2), were closely related to *Alcaligenes faecalis* ND1, isolated from atrazine-contaminated soil (Siripattanakul *et al.* 2009). A number of sequences that fell into the *Comamonas* group, among which were two predominant phylotypes from the Z3 library (Z3_30 and Z3_6, Fig. 2) displayed >98% similarity to *Hydrogenophaga palleronii* strain CCUG 20334.

The members of the γ-*Proteobacteria* belonged to the family *Pseudomonadaceae* (Fig. 3). One of the numerically dominant phylotypes in the Z2 library (Z2_2, Fig. 2) was highly similar to a cloned sequence from atrazine-catabolizing microbial association in the presence of methanol (KRA30 + 11). Additionally, several clones of the Z4 library were most similar (≥98%) to the cyanuric

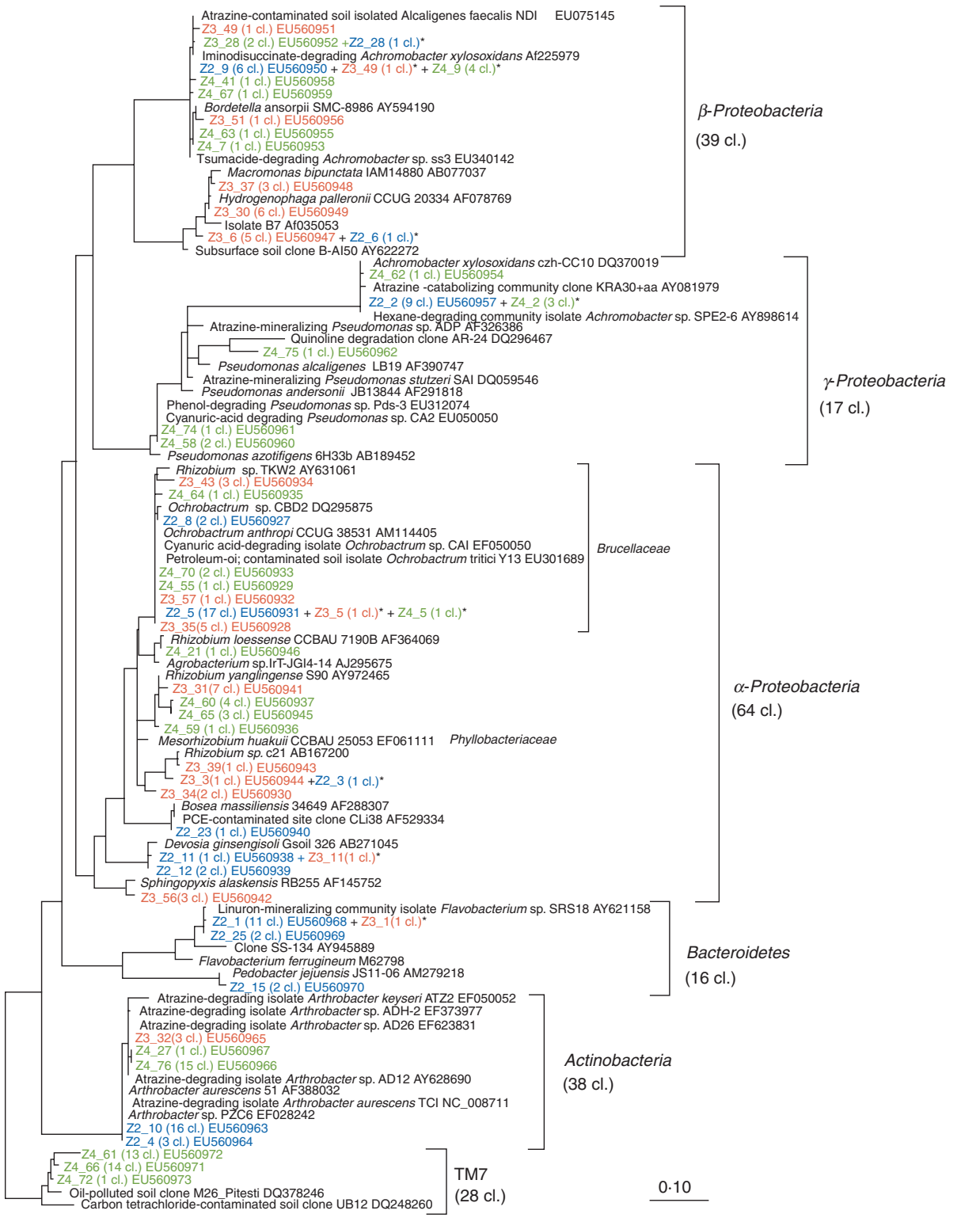

Figure 3 Parsimonious tree showing the affiliation of 16S rDNA clone sequences to selected reference sequences from various bacterial groups based on analysis of 374 bases of aligned sequences. The blue, red and green colours indicate the clones identified in libraries of communities Z2, Z3 and Z4, respectively. Number of clones is shown in brackets. The asterisk indicates the correlation based on ARDRA-typing.

acid degrading *Pseudomonas* sp. CA2 from an atrazine-degrading community isolated from the current sampling location (Kolić *et al.*2007).

The third numerically abundant cluster was a group of sequences branching within the *Actinobacteria* phylum (Fig. 3). The most abundant phylotypes of the Z2 and the Z4 libraries (Z2_10 and Z4_76) possessed high identity to *Arthrobacter keyseri* ATZ2, the atrazine-degrading member of bacterial community originating from the current sampling location (Kolić *et al.* 2007).

As evident from Fig. 3, clones of the Z2 library were representatives of the *Bacteroidetes* phylum, and the dominant phylotype Z2_1 was 99% similar to *Flavobacterium* sp. SRS18 recovered from a linuron-mineralizing bacterial community (Sorensen *et al.* 2005). Finally, two numerically abundant phylotypes (Z4_61 and Z4_66) from the Z4 library fell within the candidate division TM7 and matched the clone M26_Pitesti retrieved from oil-polluted soil collected in Romania and UB12 retrieved from carbon tetrachloride-polluted soil from USA.

Discussion

We obtained and compared three bacterial communities enriched following collection from agrochemical factory soils exposed to long-term contamination with atrazine and other related *s*-triazines. High degree of mineralization of ^{14}C ring-labelled atrazine by all enriched communities (65–80% within 4 days) confirmed the complete transformation of atrazine to carbon dioxide and ammonia.

Atrazine-degrading genetic potential

PCR analysis of atrazine-degrading genetic potential revealed that communities differed in their genetic capabilities to degrade atrazine; however, they all possessed hybrid *atz-trz* pathways, which extends the observation that such pathways are widespread among telluric atrazine-degrading communities (Smith *et al.* 2005; Kolić *et al.* 2007). The finding that all enriched communities contained a *trzN* gene for initiating atrazine metabolism supports the hypothesis that in addition to atrazine these communities might be efficient in the degradation of other *s*-triazines as well, because substrate specificity of TrzN is significantly broader than that of AtzA (Shapir *et al.* 2005). Based on our PCR results, it is evident that investigated communities possess different catabolic gene combinations in the degradation pathway of atrazine: *trzN-atzBC-trzD* (Z2), *trzN-atzBCDEF-trzD* (Z3) and *trzN-atzABC-trzD* (Z4), respectively. These results led us to assume that both *trzN* and *trzD* genes are more widespread among soil bacteria at the investigated location than the alternate *atzA* and *atzD* genes. Considering the catabolic gene composition harboured by communities Z2 and Z4 and their atrazine mineralization activities, it is likely that some other enzymes, TrzE and TrzF (Eaton and Karns 1991; Cheng *et al.* 2005) or unknown alternative enzymes (Kandil 2006) are involved in cyanuric acid metabolism initiated by TrzD. Furthermore, the presence of two different genes coding for the same function in Z4 (*atzA*, *trzN*; transformation of atrazine to hydroxyatrazine) and Z3 (*atzD*, *trzD*; transformation of cyanuric acid to biuret) suggests functional redundancies of key steps of the upper and lower atrazine-degradation pathway, which may help communities to survive when adverse environmental conditions prevent the functioning of the main metabolic pathway. As community Z4 originates from spill-site soil, the presence of two genes for initiating *s*-triazine metabolism, *atzA* and *trzN*, may constitute an effective metabolic system for overcoming the toxicity of highly concentrated *s*-triazine compounds (Accinelli *et al.* 2002).

In our communities, the atrazine-degradation pathways were encoded on plasmids as demonstrated by Southern blot analyses, and a 165-kb plasmid harbouring *trzN-atzBC* genes and IS*1071* sequences was common to all communities. This suggests either the dominance of bacterial populations harbouring this plasmid or its dispersion among atrazine-degrading bacteria within the communities. In addition, localization of *atzBC* genes to both a 165-kb plasmid and a 80-kb plasmid that did not harbour IS*1071* in the community Z3, suggests that conjugation could serve as a mechanism for dispersing catabolic genes within the community. Furthermore, the observed occurrence of *atzC* and IS*1071* on two plasmids differing in size in communities Z2 and Z4 allows the presumption that transposition may also contribute to the dissemination of *atzC* gene in these communities. All these hypotheses are corroborated by previous studies showing that both conjugation and IS-mediated genetic rearrangements have an important role in dispersing atrazine-degrading genes in the environment (De Souza *et al.* 1998b; Devers *et al.* 2005, 2007a,b). In addition, the increase in copy number of catabolic genes at different genomic locations may enhance catabolism via gene dosage effects (Devers *et al.* 2008). Redundancy of *atz* genes has previously been reported to be present in other environmental atrazine-degrading bacteria (Topp *et al.* 2000b; Smith *et al.* 2005; Devers *et al.* 2007a) suggesting that this could be a common feature and may help microbes adapt to changing environments.

To evaluate the abundance of atrazine degraders relative to total bacteria, percentages of *atzABCDEDF* and *trzD* genes in proportion to 16S rRNA genes were calculated. Absolute values of 16S rRNA gene copy numbers, however, cannot be accurately compared with cell

numbers because the number of 16S rRNA gene copies per genome is variable with a mean value generally estimated to 2–3 copies per microbial genome (Acinas *et al.* 2004). Regarding atrazine-degrading genes, only one to two copies per genome have been found thus far (Martinez *et al.* 2001; Devers *et al.* 2007a) with the exception of *trzN* gene, which can be present in up to six copies (Mongodin *et al.* 2006). Taking into account this information, the results of our study suggest that the targeted atrazine-degrading sequences can represent up to 0·5% of community Z2, up to 3% of Z3 and up to 4% of community Z4 (Table 4), indicating that only a subset of the community has the capacity to degrade atrazine. The present study also shows that in community Z3, cyanuric acid degraders having the *atzD* gene are about 300 times more abundant than those possessing the alternative *trzD* gene, which may indicate that the lower pathway initiated by the *atzD* gene is operative in this community. However, as the abundance of a functional gene does not need to be linked to enzyme expression, the operation of parallel lower pathways, initiated by *trzD* and *atzD*, cannot be excluded either.

Diversity and functional implications

Analysis of the clone libraries by ARDRA revealed considerable diversity within each community and differences in the structures of the enriched communities (Fig. 2). Given the studied communities were enrichments, the observed high biodiversity was unexpected. Rarefaction analysis further indicated that the actual diversity in the clone libraries was only partially sampled; however, the biases associated with the use of molecular techniques, which may underestimate or overestimate diversity cannot be excluded (Acinas *et al.* 2005). High phylotype richness, on the other hand, might reflect the bacterial community potential to respond to sudden changes in environmental conditions. Both rarefaction curves and diversity indices indicate a lower diversity in community Z4 originating from soil contaminated with high *s*-triazine content when compared to community Z3, which originated from the least polluted soil. Such diversity might be indicative of either initial lower diversity at high-polluted site or selection of efficient atrazine degraders in that community by specific enrichment conditions.

Further analysis of the distribution of clones and their 16S rDNA sequences indicated that the most abundant cloned phylotypes were related to genera *Ochrobactrum, Alcaligenes*, *Hydrogenophaga*, *Arthrobacter*, *Flavobacterium* and division TM7. Although clone frequency in the library is not an accurate quantitative measure of the bacterial abundance in the community, the dominant clones are expected to be more abundant and potentially more important in atrazine catabolism (Kisand and Wikner 2003). For some of the 16S rRNA gene clones, inference of likely catabolic functions is possible. In this context, we recently conducted a culture-based study to isolate and characterize metabolically active members of the atrazine-mineralizing community enriched from soil collected within the same agrochemical factory area (Kolić *et al.* 2007). This community consisted of four catabolic active members: two *Arthrobacter* sp. (strains ATZ1 and ATZ2) involved in the upper pathway, as well as *Ochrobactrum* sp. (CA1) and *Pseudomonas* sp. (CA2) involved in the lower pathway of atrazine degradation.

The dominance of clones almost identical to the 16S rDNA of *Arthrobacter* sp. ATZ2, with confirmed capability to transform atrazine to cyanuric acid in the clone libraries Z2 and Z4, suggests that these organisms are abundant and may also play important roles in atrazine catabolism within the studied communities. This further suggests that *Arthrobacter* strains, which are environmentally widespread, metabolically diverse and efficiently metabolize a variety of *s*-triazine compounds (Rousseaux *et al.* 2001; Strong *et al.* 2002; Cai *et al.* 2003; Aislabie *et al.* 2005; Kolić *et al.* 2007), may have a competitive advantage at the studied locations, particularly at the spill site, where those compounds are present at high concentrations. Furthermore, predominance of the clones most similar to cyanuric acid degrading isolate *Ochrobactrum* sp. CA1 in libraries Z2 and Z3 indicates that the bacteria represented by these sequences may play an important role in atrazine mineralization. However, this type of clone was not identified in the Z4 library, and low abundance clones affiliated with the cyanuric acid degrading *Pseudomonas* sp. CA2 were found, suggesting their potential role in the lower atrazine pathway. A specific feature of community Z4 was the predominance of clones clustering within the TM7 division, for which there are no reported cultivated representatives (Hugenholtz *et al.* 1998). This suggests that some, yet uncultivated, microorganisms may play a role in atrazine-degradation activity at this location.

In summary, our 16S rRNA gene clone library analyses showed considerable diversity in atrazine-mineralizing bacterial communities originating from contaminated soils of an agrochemical factory. The analyses of the organization of atrazine-degrading genes demonstrated their heterogeneity and their localization mainly on two catabolic plasmids as well as functional redundancies at the first steps of the upper and lower atrazine-catabolic pathway. The presence of catabolic genes in up to 4% in the community further suggests that only a subset of the enriched community has the capacity to catabolize atrazine.

Acknowledgements

This work was financially supported by a Croatian-French bilateral research project (COGITO, No. 09893YK) funded by the Croatian Ministry of Science, Education and Sports and by the French Ministry of Foreign Affairs. The authors are indebted to I. Smolčić from the agrochemical factory Herbos, Sisak, Croatia for his valuable engagement in collecting agrochemical soil samples and to N. Rouard from INRA, Laboratoire de Microbiologie du Sol et de l'Environnement, Dijon, France for her assistance in q-PCR experiments. Thanks are also extended to D. Bru from Service de Séquençage et de Génotypage (SSG, Dijon, France) for offering access to sequencing and q-PCR facilities.

References

Accinelli, C., Screpanti, C., Dinelli, G. and Vicari, A. (2002) Short-time effects of pure and formulated herbicides on soil microbial activity and biomass. *Int J Environ An Ch* **82**, 519–527.

Acinas, S.G., Marcelino, L.A., Klepac-Ceraj, V. and Polz, M.F. (2004) Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *J Bacteriol* **186**, 2629–2635.

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V. and Polz, M.F. (2005) PCR-induced sequences artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl Environ Microbiol* **71**, 8966–8969.

Aislabie, J., Bej, A.K., Ryburn, J., Lloyd, N. and Wilkins, A. (2005) Characterization of *Arthrobacter nicotinovorans* HIM, an atrazine-degrading bacterium, from agricultural soil New Zealand. *FEMS Microbiol Ecol* **52**, 279–286.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Boundy-Mills, K.L., De Souza, M.L., Mandelbaum, R.T., Wackett, L.P. and Sadowsky, M.J. (1997) The *atzB* gene of *Pseudomonas* sp. strain ADP encodes the second enzyme of a novel atrazine degradation pathway. *Appl Environ Microbiol* **63**, 916–923.

Cai, B., Han, Y., Liu, B., Ren, Y. and Jiang, S. (2003) Isolation and characterization of an atrazine-degrading bacterium from industrial wastewater in China. *Lett Appl Microbiol* **36**, 272–276.

Cheneby, D., Philippot, L., Hartmann, A., Henault, C. and Germon, J.C. (2000) 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. *FEMS Microbiol Ecol* **34**, 121–128.

Cheng, G., Shapir, N., Sadowsky, M.J. and Wackett, L.P. (2005) Allophanate hydrolase, not urease, functions in bacterial cyanuric acid metabolism. *Appl Environ Microbiol* **71**, 4437–4445.

De Souza, M.L., Sadowsky, M.J. and Wackett, L.P. (1996) Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. *J Bacteriol* **178**, 4894–4900.

De Souza, M.L., Newcombe, D., Alvey, S., Crowley, D.E., Hay, A., Sadowsky, M.J. and Wackett, L.P. (1998a) Molecular basis of a bacterial consortium: interspecies catabolism of atrazine. *Appl Environ Microbiol* **64**, 178–184.

De Souza, M.L., Seffernick, J., Martinez, B., Sadowsky, M.J. and Wackett, L.P. (1998b) The atrazine catabolism genes *atzABC* are widespread and highly conserved. *J Bacteriol* **180**, 1951–1954.

De Souza, M.L., Wackett, L.P. and Sadowsky, M.J. (1998c) The *atzABC* genes encoding atrazine catabolism are located on a self-transmissible plasmid in *Pseudomonas* sp. strain ADP. *Appl Environ Microbiol* **64**, 2323–2326.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D. *et al.* (2006) Greengenes, a chimera checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**, 5069–5072.

Devers, M., Soulas, G. and Martin-Laurent, F. (2004) Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. *J Microbiol Methods* **56**, 3–15.

Devers, M., Henry, H., Hartmann, A. and Martin-Laurent, F. (2005) Horizontal gene transfer of atrazine-degrading genes (*atz*) from *Agrobacterium tumefaciens* St96-4 pADP1::Tn5 to bacteria of maize-cultivated soil. *Pest Manag Sci* **61**, 870–880.

Devers, M., Azhari, N.E., Udikovic Kolic, N. and Martin-Laurent, F. (2007a) 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. *FEMS Microbiol Lett* **273**, 78–86.

Devers, M., Rouard, N. and Martin-Laurent, F. (2007b) Genetic rearrangement of the *atzAB* atrazine-degrading gene cassette from pADP1::Tn5 to the chromosome of *Variovorax* sp. MD1 and MD2. *Gene* **392**, 1–6.

Devers, M., Rouard, N. and Martin-Laurent, F. (2008) Fitness drift of an atrazine-degrading population under atrazine selection pressure. *Environ Microbiol* **10**, 676–684.

Eaton, R.W. and Karns, J.S. (1991) Cloning and analysis of *s*-triazine catabolic genes from *Pseudomonas* sp. strain NRRLB-12227. *J Bacteriol* **173**, 1215–1222.

Eckhardt, T. (1978) A rapid method for the identification of plasmid desoxyribonucleic acid bacteria. *Plasmid* **1**, 584–588.

Gompertz, B. (1825) On the nature and function expressive of the law of human mortality, and on a new mode of determining the value of life contingencies. *Philos Trans R Soc Lond* **115**, 513–585.

Gurtler, V. and Stanisich, V.A. (1996) New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **142**, 3–16.

Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**, 4765–4774.

Kandil, M.M. (2006) *Genetic diversity of soil bacteria degrading cyanuric acid: an intermediate in the atrazine biodegeneration pathway*. PhD Thesis, University of Minnesota, Minneapolis.

Kisand, V. and Wikner, J. (2003) Combining culture-dependent and -independent methodologies for estimation of richness of estuarine bacterioplankton consuming riverine dissolved organic matter. *Appl Environ Microbiol* **69**, 3607–3616.

Kolić, N.U., Hršak, D., Begonja Kolar, A., Petrić, I., Stipičević, S., Soulas, G. and Martin-Laurent, F. (2007) Combined metabolic activity within an atrazine-mineralizing community enriched from agrochemical factory soil. *Int Biodeter Biodegr* **60**, 299–307.

Ludwig, W., Strunk, O., Westram, R., Meier, H., Arno Buchner, Y., Lai, T., Steppi, S., Jobb, G. *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T.J., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J. *et al.* (2001) The RDP-II (Ribosomal database project). *Nucleic Acids Res* **29**, 173–174.

Mandelbaum, R.T. and Wackett, L.P. (1996) *Pseudomonas* strain for degradation of *s*-triazines in soil and water. *US patent* **5**, 508.

Mandelbaum, R.T., Wackett, L.P. and Allan, D.L. (1993) Mineralization of the *s*-triazine ring of atrazine by stable bacterial mixed cultures. *Appl Environ Microbiol* **59**, 1695–1701.

Mandelbaum, R.T., Allan, D.L. and Wackett, L.P. (1995) Isolation and characterization of a *Pseudomonas* sp. that mineralizes the *s*-triazine herbicide atrazine. *Appl Environ Microbiol* **61**, 1451–1457.

Martinez, B., Tomkins, J., Wacket, L.P., Wing, R. and Sadowsky, M.J. (2001) Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J Bacteriol* **183**, 5684–5697.

Mongodin, E.F., Shapir, N., Daugherty, S.C., Deboy, R.T., Emerson, J.B., Shvartzbeyn, A., Radune, D., Vamathevan, J. *et al.* (2006) Secrets of soil survival revealed by the genome sequence of *Arthrobacter aurescens* TC1. *PLoS Genet* **2**, 2094–2106.

Mulbry, W.W., Zhu, H., Nour, S.M. and Topp, E. (2002) The triazine hydrolase gene *trzN* from *Nocardioides* sp. strain C190: cloning and construction of gene-specific primers. *FEMS Microbiol Lett* **206**, 75–79.

Rousseaux, S., Hartmann, A. and Soulas, G. (2001) Isolation and characterisation of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. *FEMS Microbiol Ecol* **36**, 211–222.

Rousseaux, S., Soulas, G. and Hartmann, A. (2002) Plasmid localisation of atrazine-degrading genes in newly described *Chelatobacter* and *Arthrobacter* strains. *FEMS Microbiol Ecol* **41**, 69–75.

Sadowsky, M.J., Tong, Z.K., De Souza, M.L. and Wackett, L.P. (1998) AtzC is a new member of the amidohydrolase protein superfamily and is homologous to other atrazine-metabolizing enzymes. *J Bacteriol* **180**, 152–158.

Sajjaphan, K., Shapir, N., Wackett, L.P., Palmer, M., Blackmon, B., Tomkins, J. and Sadowsky, M.J. (2004) *Arthrobacter aurescens* TC1 atrazine catabolism genes *trzN*, *atzB*, and *atzC* are linked on a 160-kilobase region and are functional in *Escherichia coli*. *Appl Environ Microbiol* **70**, 4402–4407.

Shapir, N., Rosendahl, C., Johnson, G., Andreina, M., Sadowsky, M.J. and Wackett, L.P. (2005) Substrate specificity and colorimetric assay for recombinant TrzN derived from *Arthrobacter aurescens* TC1. *Appl Environ Microbiol* **71**, 2214–2220.

Siripattanakul, S., Wirojanagud, W., McEvoy, J., Limpiyacorn, T. and Khan, E. (2009) Atrazine degradation by stable mixed cultures enriched from agricultural soil and their characterization. *J Appl Microbiol* **106**, 986–992.

Smith, D., Alvey, S. and Crowley, D.E. (2005) Cooperative catabolic pathways within an atrazine-degrading enrichment culture isolated from soil. *FEMS Microbiol Ecol* **53**, 265–273.

Sorensen, S.R., Rasmussen, J., Jacobsen, C.S., Jacobsen, O.S., Juhler, R.K. and Aamand, J. (2005) Elucidating the key member of a linuron-mineralizing bacterial community by PCR and reverse transcription-PCR denaturing gradient gel electrophoresis 16S rRNA gene fingerprinting and cultivation. *Appl Environ Microbiol* **71**, 4144–4148.

Strong, L.C., Rosendahl, C., Johnson, G., Sadowsky, M.J. and Wackett, L.P. (2002) *Arthrobacter aurescens* TC1 metabolizes diverse *s*-triazine ring compounds. *Appl Environ Microbiol* **68**, 5973–5980.

Struthers, J.K., Jayachandran, K. and Moorman, T.B. (1998) Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. *Appl Environ Microbiol* **64**, 3368–3375.

Swofford, D.L. (1993) PAUP – a computer program for phylogenetic inference using maximum parsimony. *J Gen Physiol* **102**, A9–A9.

Topp, E., Mulbry, W.M., Zhu, H., Nour, S.M. and Cuppels, D. (2000a) Characterization of *s*-triazine herbicide metabolism by a *Nocardioides* sp. isolated from agricultural soils. *Appl Environ Microbiol* **66**, 3134–3141.

Topp, E., Zhu, H., Nour, S.M., Houot, S., Lewis, M. and Cuppels, D. (2000b) Characterization of an atrazine-degrading *Pseudaminobacter* sp. isolated from Canadian and French agricultural soils. *Appl Environ Microbiol* **66**, 2773–2782.

Wheatcroft, R., McRae, D.G. and Miller, R.W. (1990) Changes in the *Rhizobium meliloti* genome and the ability to detect

supercoiled plasmids during bacteroid development. *Mol Plant Microbe Interact* **3**, 9–17.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M. and van't Riet, K. (1990) Modeling of the bacterial growth curve. *Appl Environ Microbiol* **56**, 1875–1881.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Rarefaction curves of observed richness of ARDRA families in the communities Z2, Z3 and Z4. Error bars represent 95% confidence intervals calculated from the variance of the number of phylotypes.

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