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ORIGINAL ARTICLE

Isolation and characterization of atrazine-degrading *Arthrobacter* sp. strains from Argentine agricultural soils

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Abstract Three bacterial strains capable of degrading atrazine were isolated from Manfredi soils (Argentine) using enrichment culture techniques. These soils were used to grow corn and were treated with atrazine for weed control during 3 years. The strains were nonmotile Gram-positive bacilli which formed cleared zones on atrazine solid medium, and the 16S rDNA sequences indicated that they were *Arthrobacter* sp. strains. The atrazine-degrading activity of the isolates was characterized by the ability to grow with atrazine as the sole nitrogen source, the concomitant herbicide disappearance, and the chloride release. The atrazine-degrader strain *Pseudomonas* sp. ADP was used for comparative purposes. According to the results, all of the isolates used atrazine as sole source of nitrogen, and sucrose and sodium citrate as the carbon sources for growth. HPLC analyses confirmed herbicide clearance. PCR analysis revealed the presence of the atrazine catabolic genes *trzN*, *atzB*, and *atzC*. The results of this work lead to a better understanding of microbial degradation activity in order to consider the potential application of the isolated strains in bioremediation of atrazine-polluted agricultural soils in Argentina.

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Introduction

The intensive use of herbicides in agricultural practices has received particular consideration not only because of their immediate pollution potential but also due to their persistence in the environment. In this regard, atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) is important because it has been found in much higher concentrations than permitted, many years after its application (Houot et al. 2000; Singh et al. 2004). Atrazine is a selective systemic herbicide that blocks photosynthesis, and it is the predominant member of a broad class of triazine herbicides used for pre- and post-emergence control of annual grass and broad-leaved weeds, mainly for maize and sorghum cropping (Roberts 1998). This herbicide may damage future crops, may reduce other organism populations, and may contaminate water and food (Delmonte et al. 1997; Popov et al. 2005). It has been reported that atrazine is an endocrine disrupting chemical, interrupting regular hormone function and causing birth defects, reproductive tumors, and weight loss in amphibians as well as in humans. An association has been proposed between atrazine pollution and an increased incidence of various types of cancer (breast, ovarian, and uterine tumors), as well as of leukemia and lymphoma (Singh et al. 2004).

A variety of atrazine-degrading fungi and bacteria belonging to different genera have been isolated from agricultural soils (Piutti et al. 2003; Smith et al. 2005; Topp et al 2000a, b; Vibber et al. 2007; Marecik et al. 2008). Hydrolytic dechlorination, dealkylation, and *s*-triazine ring

cleavage are the key reactions involved in atrazine biodegradation (Mandelbaum et al. 1993). Given the large-scale utilization and the extreme toxicity of the molecule, there is a need to find new organisms capable of degrading atrazine not only at the laboratory level but also in the environment. For this purpose, the bacteria best suited would be those that are already members of the atrazine-polluted soil community.

Therefore, the aims of this work were to isolate atrazine-degrading bacteria from agricultural soils and to characterize them with respect to their activity and mechanism of degradation. In addition, molecular analyses were performed to identify isolates and to detect atrazine degrading genes.

Materials and methods

Soils

Samples were taken from the surface soil layer (0–10 cm) of five agricultural sites located in Argentina's flatlands (Pampean region). Three fields were placed in Pergamino department (33°52′59.6″S, 60°35′00.3″W) in the northern area of Buenos Aires, and were coded as follows: Per 1, Per 2, and Per 3. The other sites were located in Manfredi (3025′47.2″S, 64º21′04.5″W) in the north-central region of Córdoba, and were coded: Man 1 and Man 2. The physicochemical properties of the sampled soils have been reported elsewhere (Fernández et al. 2008). Man 1 soil had been used to grow corn and had been treated with atrazine for weed control according to normal farming practice for 3 years. The rest of the studied soils had irregular applications of the herbicide.

Bacteria and growth media

Pseudomonas sp. strain ADP (Mandelbaum et al. 1995) was provided by Dr. Lawrence Wackett (University of Minnesota, St. Paul, USA). The atrazine liquid growth medium (ALM; Mandelbaum et al. 1993) contained 0.1 % (w/v) sodium citrate and sucrose as the carbon sources, and atrazine as the only nitrogen source (from 50 to 125 mg/l). The atrazine solid medium (ASM) used to detect atrazine degradation contained the same mineral salts as the ALM, 1.5 % (w/v) agar (Britania, Argentina) and atrazine at a final concentration of 250 ppm.

Chemicals

Atrazine (98 %) was used to maintain and characterize atrazine-degrading bacteria and was kindly provided by INRA-CMSE, Dijon Cedex, France. The atrazine used for enrichment techniques was an aqueous formulation at 90 % (w/v) active ingredient, commercially distributed as Gesaprim® 90 WDG (Syngenta, Argentina), and it was diluted in water at 0.05 % (w/v), which is the equivalent of 0.5 kg ha^{-1} (on the basis of 1,000 t soil ha^{-1}). Atrazine, hydroxyatrazine, deethylatrazine, and desisopropylatrazine for HPLC analysis were purchased as analytical standards with 99 % purity from Accustandard (USA).

Enrichment techniques

Two enrichment techniques were carried out in this study to obtain indigenous microbial cultures capable of degrading atrazine, following the methods of Focht (1994) and Forlani et al. (1999).

Enrichment 1 Plastic containers (21×30 cm) were filled with 1 kg of soil and were kept during 1 year at 28 °C. Once a month, an atrazine commercial solution was applied to soils using a hand-sprayer. At the end of the atrazine loading period, 5 g of soil were transferred into flasks with 15 ml of sterile 0.85 % (w/v) NaCl and were shaken during 30 min at 400 rpm.

Enrichment 2 A second experiment which examined the influence of corn plants on isolation of atrazine degrading consortia was done. Briefly, maize seeds were surface-disinfected with 2.5 % (w/v) sodium hypochlorite followed by six rinses in sterile distilled water, and then sown in pots (18 × 16 cm) filled with 2 kg of soil. Plants were grown in a growth chamber and were periodically watered as required with sterile distilled water. One month after planting, 10 ml of an atrazine commercial solution was applied to the soil surface of the pot using a hand-sprayer. The treatment was repeated every 2 weeks during 2 months. After 3 months, roots were thoroughly washed with sterile distilled water for 10 min to remove all the loosely adhering soil particles. Fragments of 5 cm were transferred into flasks with 15 ml of sterile 0.85 % (w/v) NaCl and were shaken during 30 min at 400 rpm.

Isolation, maintenance and characterization of atrazine-degrading bacteria

Aliquots of 5 ml from the suspensions prepared from both enrichment techniques were inoculated into 125-ml flasks with 50 ml of ALM containing 50 mg/l of atrazine and 0.01 % (w/v) cycloheximide. The enrichment cultures were incubated aerobically with shaking at 120 rpm and 28 °C. Every two weeks, 5 ml of culture were transferred to 50 ml

of freshly ALM medium in which the atrazine concentration was increased stepwise in 25 mg/l. After the fourth subculture, atrazine-degrading bacteria were isolated by plating 100 μl of culture onto ASM. Because atrazine forms an opaque precipitate in the plates, atrazine-metabolizing isolated were identified by the formation of cleared zones around colonies in the atrazine layer (Aislabie et al. 2004). Bacteria were routinely maintained on ASM with atrazine (98 %) and were stored frozen in 15 % glycerol at −20 °C.

Atrazine-degrading activity

Bacteria were grown in 25 ml of a chloride free ALM containing 100 mg/l atrazine (98 %) in 125-ml Erlenmeyers flasks. Cultures were incubated aerobically with shaking at 120 rpm and 28 °C during 60 h. Three replicates per isolate were done. Optical density at 610 nm (OD_{610}) was determined periodically at 12, 24, 30, 36 and 48 h, using a Metrolab 330 UV Vis spectrophotometer. At the end of the incubation period, chloride release and protein concentration were determined. Chloride release was determined according to Fotch (1994). For estimation of protein content, cells were digested with with 4.6 mM NaOH, and the cleared lysate was analyzed by the Bradford method (Bradford 1976) using bovine serum albumin as standard. The disappearance of atrazine was analyzed by diluting culture samples 1:2 with distilled water and determining their ultraviolet absorption spectra on a Shimadzu 2100 UV Vis spectrophotometer. Herbicide utilization was associated with complete disappearance of the 220-nm atrazine absorption peak. Finally, culture samples were centrifuged at 13,000 rpm for 10 min and the supernatants were collected for HPLC analysis.

Characterization of atrazine metabolites

The atrazine catabolic products present in culture supernatants were identified on the basis of their retention time and quantified by reverse phase HPLC using a Hewlett-Packard 1100 automatic injection device (Agilent Technologies, Waldbronn, Germany) equipped with a Phenomenex C18 column (length 15 cm, internal diameter 4.60 mm). The solvent system consisted of ACN/ water (50/50, v/v) delivered at a flow rate of 1 ml/min. The products were analyzed with a diode array detector (Agilent Series).

Molecular analysis

Bacterial DNA was extracted according to standard protocol (Sambrook et al 1989). To approach the taxonomic identity of the organisms, a gene region of 1,300 bp encoding 16S rDNA was amplified by PCR using universal 16S rDNA primers P0 and P6 (Picard et al. 2000). PCR reactions (20 μl) contained 1 U Taq DNA polymerase (PB-L, Argentina), P0 and P6 primers at 1 μM, 1× Taq DNA polymerase buffer (PB-L, Argentina), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and the corresponding DNA template (approx. 100 ng). The thermocycling reactions were performed in a DNA thermal cycler (Biometra, USA), and consisted of an initial denaturation step of 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and a final elongation step of 72 °C for 10 min. The PCR reactions were checked in 1 % agarose gels (Ultrapure™ Agarose, Invitrogen), run at 2 V/cm for 40 min. A 100 bp-ladder (PB-L, Argentina) was used as a size standards. The 16S rDNA amplicons were sequenced bidirectionally with primers P0 and P6 at Macrogen Inc. (Korea), and the obtained sequences were used to query the Seqmatch tool of the Ribosomal database project II (Cole et al. 2009).

PCR amplification of *atzB*, *atzC*, and *trzN* genes was carried out using gene-specific oligonucleotide primers (Devers et al. 2004; De Souza et al. 1998; Piutti et al. 2003). All primers were a gift from Fabrice Martin-Laurent (INRA-CMSE). Genomic DNA was subjected to PCR amplification in reactions containing 1.25 U Taq DNA polymerase (Promega, WI, USA), 1 μM of each primer, 1× green buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and the corresponding DNA template (approx. 100 ng). The cycling protocol consisted in: 3 min at 95 °C; 5 cycles at 94 °C for 45 s, starting with an annealing temperature of 60 °C for 45 s, which decreased by 1 °C every cycle, 72 °C for 45 s; 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C; 1 cycle of 10 min at 72 °C. PCR reactions were carried out in a DNA thermal cycler (BIOER Technology, China). The amplicons were fractionated in 1.8 % agarose gels (Seakem) and their size was estimated using the Fast Ruler Low Range (Fermentas, Life Science) as a reference. Images were analysed with the Kodak 1D 3.0 software (Kodak, New Haven, CT, USA).

Results

Isolation and identification of atrazine-degrading bacteria from Argentine agricultural soils

Three morphologically distinct colonies which formed clear zones on ASM plates were isolated and characterized. All of them were obtained from one of the soils sampled in Manfredi (Man 1) that had been treated with atrazine for 3 years. No atrazine-degrading isolates could be obtained from the other sampled soils, which had no records of continuous atrazine application. The three isolates were nonmotile Gram-positive bacilli. The comparative analysis of the 16S rDNA sequence revealed that the isolates are closely related to the actinomycete strain *Arthrobacter* sp. AD7, an

Table 1 Phylogenetic assignment of atrazine-degrading isolates SD, MD and FD

Isolate	Origin	Closest relative based on 16S rDNA / Accession number	Organism / Accession Number	Similarity (%)
FD	Man 1	*Arthrobacter* sp. AD7 / JF775581	*Arthrobacter* sp. FD / JN601428	100
MD	Man 1	*Arthrobacter sp.* AD7 / JF775581	*Arthrobacter* sp. MD / JN601430	100
SD	Man 1	*Arthrobacter* sp. AD7 / JF775581	*Arthrobacter* sp. SD / JN601429	100

atrazine-degrader isolate from wastewater in China (Acc. Nr. JF775581; Table 1). The *Arthrobacter* sp. isolates were denoted as SD, FD, and MD. SD was isolated using the enrichment procedure 1 (from soil treated with atrazine), whereas FD and MD were obtained by enrichment 2 (from the rhizosphere of maize plants grown in soil treated with atrazine).

Atrazine-degrading activity of the *Arthrobacter* sp. isolates

The atrazine catabolic efficiency of the isolates after 60 h of incubation at 28 °C, compared with that of *Pseudomonas* sp. ADP, is shown in Fig. 1. Typical growth curves were observed in the studied bacteria as well as in the control strain *Pseudomonas* sp. ADP, indicating that, a priori, atrazine served as a nitrogen source. A plateau of absorbance was observed for both the isolate MD and the strain *Pseudomonas* sp. ADP between 30 and 36 h after inoculation, suggesting that the atrazine metabolic process was finished. For isolates SD and FD, the absorbance continued to rise, indicating a lower atrazine degrading capacity.

The three isolates tested positive for chloride release. Isolate SD released approximately 0.26 mM Cl^- from the initial 0.46 mM concentration of atrazine (98 %) in solution, resulting in a 57 % yield. MD and FD released 0.33 mM Cl^-, resulting in a 72 % yield. Finally, 0.28 mM Cl^- was detected in the liquid culture of *Pseudomonas* sp. ADP, which corresponds to a 62 % yield. The protein content of the cultures was comparable among the three isolates, ranging from 111 to 127 μg/ml, whereas the protein accumulation of the *Pseudomonas* sp. ADP culture was lower (82 μg/ml).

Atrazine clearance was monitored using a rapid and simple spectrophotometric method (Vibber et al. 2007). The characteristic single peak at 220 nm was observed in the ultraviolet absorption spectra of the culture with atrazine (98 %). Spectra of the strains and of *Pseudomonas* sp. ADP were similar: there were no peaks at 220 nm which means that herbicide content was reduced below the detection level (data not shown).

Atrazine degradation was confirmed in all isolates using HPLC. The presence of atrazine in the growth medium before bacterial growth was detected as a single peak with a retention time of 4.8 min, and no additional peaks were detected during the 6-min elution period (Fig. 2a). Culture supernatants were sampled from exponential through stationary growth phase of our isolates and of *Pseudomonas* sp. ADP. The concentration of atrazine was observed to decrease throughout the experiment. However, at the end of the experiment in the strain of reference, herbicide concentration was higher (Fig. 2b). This result indicated us that our degrading strains were more efficient (Fig. 2c). Metabolites of atrazine, hydroxyatrazine, deethylatrazine and desisopropylatrazine, from the first step in the different characterized degradation pathways, could not be detected by HPLC analysis of culture supernatants (detection limit: 0.5 mg/l) (Fig. 2b, c). Many other unidentified metabolites eluted between 1.6 and 2.2 min.

PCR detection of atrazine-degrading genes

To determine if the isolates contain genes involved in atrazine degradation that are similar to those found in *Pseudomonas* sp. ADP, PCR analysis with primers specific for internal regions of the *atzB* and *atzC* were performed. The DNA of *Pseudomonas* sp. ADP gave an *atzB* amplicon of 400 bp (Fig. 3). In contrast, the DNA of the *Arthrobacter* sp. isolates described in this work produced shorter amplicons (200 bp). For *atzC*, all DNA samples (from the reference strain ADP and the three isolates) generated amplicons of about 600 bp (Fig. 3). The presence of another gene involved in atrazine catabolism, *trzN*, was studied by PCR. The three *Arthrobacter* sp. isolates SD, FD and MD were positive for the *trzN* PCR reaction (Fig. 4). Although no strain of reference was available for comparison, the size of the *trzN* amplicons from the isolates was 400 bp and matched the size of what have been published for this gene.

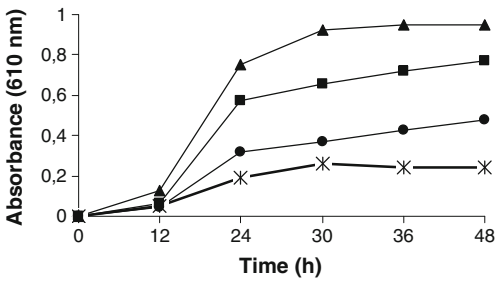

Fig. 1 Growth curves of SD, MD, and FD atrazine-degrading isolates and of *Pseudomonas* sp. ADP in aerobic batch cultures (28 °C) in liquid growth medium containing 100 mg/l of atrazine (98 %). (●), isolate FD; (■), isolate SD; (▲), isolate MD; (*), *Pseudomonas* sp. ADP

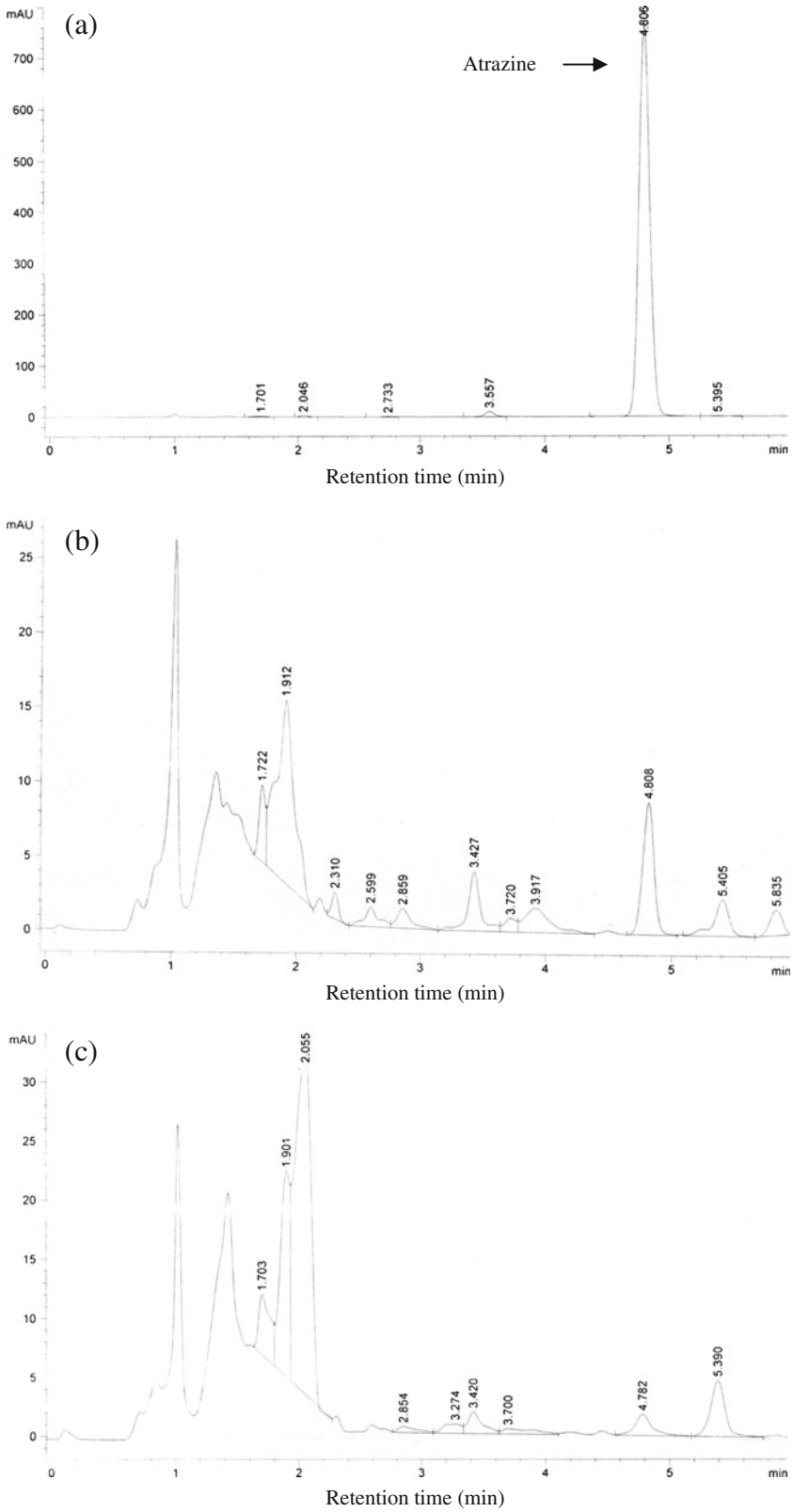

Fig. 2 Chromatograms of atrazine metabolic products obtained from: **a** growth medium with atrazine (98 %), before inoculation, **b** culture extracts of *Pseudomonas* sp. ADP, **c** culture extracts of MD isolate which is representative of the other isolates (FD and SD), incubated 60 h at 28 °C in the presence of the herbicide

Discussion

Several studies have described increased atrazine degradation in agricultural soil in correlation with increased numbers of atrazine-degrading microorganisms (Anderson et al. 1993; Wenk et al. 1998). It is well known that exposure of soil to atrazine enhances the abundance and activity of atrazine-degrading bacteria (Topp et al. 2000b; Aislabie et

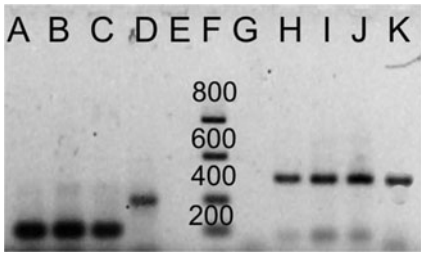

Fig. 3 Detection of *atzB* and *atzC* genes in atrazine-degrading isolates. PCR amplification of total genomic DNA from MD, FD, and SD isolates with primers targeting the *atzB* (*lanes A–E*) and *atzC* (*lanes G–K*) genes from *Pseudomonas* sp. strain ADP. *Lanes A, B, and C* genomic DNA from MD, FD, and SD isolates, respectively; *D* positive control (genomic DNA from *Pseudomonas* sp. strain ADP); *E* negative control (no template); *F* DNA size marker; *G* negative control (no template); *H, I, and J* genomic DNA from MD, FD, and SD isolates, respectively; *K* positive control (genomic DNA from *Pseudomonas* sp. strain ADP)

al. 2004; Yassir et al. 1999; Barriuso and Houot 1996). In this work, all isolates were obtained from a sampled soil that had been treated with the herbicide for 3 years. It is likely that any enrichment technique is useful to isolate atrazine-degrading microorganisms. However, Alvey and Crowley (1996) suggested that plant rhizosphere may be particularly important in promoting biological degradation of xenobiotics and would facilitate the isolation of pollutant degraders. Moreover, Martin-Laurent et al. (2004) showed that as a result of repeated atrazine application and of maize cultivation, atrazine-degrading bacterial communities were stimulated.

Most of studies done in our country have evaluated the persistence of atrazine through biossays (Delmonte et al. 1997; Fuscaldo et al. 1999) and its mobility in the soil (Bedmar et al. 2004; Hang et al. 2003, 2005). To our knowledge, this is the first report of isolation of atrazine-degrading strains belonging to the *Arthrobacter* genus from Argentine soils. This bacterial genus is indeed associated with atrazine degradation in different soils around the world. Rousseaux et al. (2001) isolated 25 bacterial strains able to degrade atrazine by an enrichment method from 10 different French soils. Three

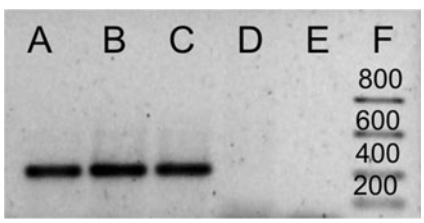

Fig. 4 Detection of *trzN* gene in atrazine-degrading isolates. PCR amplification of total genomic DNA from MD, FD and SD isolates with primers targeting *trzN*. *Lanes: A, B, and C* genomic DNA from MD, FD, and SD isolates, respectively; *D* negative control (genomic DNA from *Bradyrhizobium* sp.); *E* negative control (no template); *F* DNA size marker (0.1 Kb FastRuler DNA Ladder, Low Range, Fermentas)

isolates were identified as *A. crystallopoietes*. In a subsequent study, Rousseaux et al. (2002) localized the atrazine-degrading genes in two or three plasmids with variable molecular masses. Aislabie et al. (2005) isolated *Arthrobacter nicotinovorans* HIM from an agricultural soil in New Zealand. Getenga et al. (2009) isolated the atrazine-degrading strain *Arthrobacter* sp. strain GZK-1 from a tropical soil of Kenya, while Sajjaphan et al. (2010) isolated *Arthrobacter histidinolovorans* strain KU001 from a Thailandese soil. Both fields were cultivated with sugarcane. On the other hand, not just soil has been a source of atrazine-degrader isolates belonging to the *Arthrobacter* genus; highly efficient atrazine-degrader *Arthrobacter* strains have been isolated from industrial wastewater samples (Cai et al. 2003; Li et al. (2008).

Other genera of Gram-positive bacteria have been cited as atrazine degraders. For instance, different species of actinomycetes other than *Arthrobacter* (Topp et al. 2000b; Piutti et al. 2003; Satsuma 2006; De Schrijber and De Mot 1999), *Bacillus licheniformis* and *B. megaterium*, have been isolated from the rhizosphere of sweet flag (*Acorus calamus* L.) (Marecik et al. 2008), and *Rhodococcus* sp. strains (Behki et al. 1993).

Isolates FD, MD, and SD were able to utilize atrazine as a sole nitrogen source, and sucrose and sodium citrate as the carbon sources for growth. The growth curves on defined medium containing 100 mg of atrazine per liter revealed different atrazine degradation activity between the isolates (Fig. 1). HPLC analyses demonstrated a significant reduction in the atrazine content of the growth medium and the absence of intermediate metabolites (Fig. 2). As reported elsewhere for other *Arthrobacter* isolates (Strong et al. 2002; Sajjaphan et al. 2004; Zhu et al. 2009), our strains most probably degraded the herbicide via cyanuric acid to CO_2 and ammonia.

We evaluated the presence of atrazine degradation genes, *atzB*, *atzC*, and *trzN*, in the isolated strains FD, MD, and SD. The PCR analysis showed that all our *Arthrobacter* sp. isolates produced bands using targeting to *atzC* and *trzN* genes, with the expected size according to De Souza et al. (1998) and Piutti et al. (2003) (Figs. 3 and 4). However, the size of the *atzB* amplicon from the three isolates was lower than the expected one (Devers et al. 2004) (Fig. 3). It would be interesting to evaluate the nucleotide sequence of the detected genes in our strains, in order to study their relatedness to those from other *Arthrobacter* sp.

The enzymatic basis of atrazine mineralization has been extensively studied in *Pseudomonas* sp. strain ADP (Mandelbaum et al. 1995; De Souza et al. 1995). In this strain, displacement of the three substituents on the *s*-triazine ring is mediated by three enzymatic steps encoded by the genes *atzA*, *atzB*, and *atzC*. The first enzyme, AtzA, catalyzes the hydrolytic dechlorination of atrazine to yield hydroxyatrazine. AtzB subsequently catalyzes hydroxyatrazine deamidation, yielding *N*-isopropylammelide, and the third enzyme, AtzC,

stoichiometrically metabolizes *N*-isopropylammelide to cyanuric acid and *N*-isopropylamine. Cyanuric acid is subsequently metabolized to CO_2 and NH_3 by the products of the *atzD*, *atzE*, and *atzF* genes (Martínez et al. 2001). In contrast, most species from *Arthrobacter* genera and other Gram-positive bacteria, combine *trzN* and *atzB* and *atzC* gene products in their catabolic pathway and degrade the herbicide to cyanuric acid without mineralizing the *s*-triazine ring to CO_2 and NH_3 (Vibber et al. 2007; Zhu et al. 2009; Arbeli and Fuentes 2010). *trzN* encodes an atrazine amidohydrolase originally discovered in *Nocardioides* strain C190 (Topp et al. 2000b). Next, *atz*B catalyzes hydroxiatrazine deamination yielding *N*-isopropylammelide, and the third enzyme, *atz*C, metabolizes *N*-isopropylammelide to cyanuric acid and *N*-isopropylamine (Strong et al. 2002). These observations are in agreement with our results from detection of atrazine-degrading genes (Figs. 3 and 4), indicating that our strains effectively degraded the herbicide via the *trzN-atzB-atzC* pathway. Nonetheless, as suggested recently (Ralebitso et al. 2002), there might be species and novel catabolic mechanisms which await identification and elucidation.

In conclusion, three bacterial strains capable of degrading atrazine were isolated from Argentine agricultural soils using enrichment culture. 16S rRNA gene sequencing positioned FD, MD, and SD strains close to *Arthrobacter* sp. strain DP7. The three isolates used atrazine as sole source of nitrogen with sucrose and sodium citrate as the carbon source for growth, and exhibited different efficiency of atrazine degradation activity. HPLC analyses of culture supernatants confirmed herbicide clearance. PCR experiments demonstrated the presence of *atzB*, *atzC*, and *trzN* atrazine-degrading genes in the strains. Further studies of these atrazine-degrading bacteria are required to analyze their potential application in bioremediation of argentine agricultural soils.

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