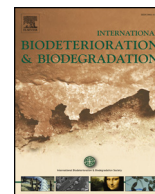




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Degradation of atrazine by *Pseudomonas* sp. and *Achromobacter* sp. isolated from Brazilian agricultural soil

Ana Flavia Tonelli Fernandes^a, Vânia Santos Braz^a, Anelize Bauermeister^b,
Jonas Augusto Rizzato Paschoal^c, Norberto Peporine Lopes^b, Eliana Guedes Stehling^{a,*}

^a Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil

^b Núcleo de Pesquisa em Produtos Naturais e Sintéticos (NPPNS), Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil

^c Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil

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ABSTRACT

Atrazine is a herbicide used worldwide to control weeds in maize, sorghum and sugarcane crops. This study presents two bacteria isolated from Brazilian soil samples with atrazine application history. The isolates, identified as *Pseudomonas* sp. and *Achromobacter* sp., showed potential to degrade atrazine in solid medium. *Pseudomonas* sp. exhibited all the six *atx* genes that encode enzymes of the degradation process. This isolate was capable of degrading 99% of atrazine *in vitro* after 24 h of incubation. *Achromobacter* sp. presented only *atxA*, *atxB* and *atxC* genes, which are responsible for the first steps of the degradation pathway, and showed a different degradation profile. The three initial metabolites formed by atrazine degradation were detected in samples containing both *Pseudomonas* sp. and *Achromobacter* sp., suggesting atrazine transformation. By Northern Blot assay, *atxA*, *atxB*, *atxC* and *atxD* genes were differentially expressed in the presence of atrazine in *Pseudomonas* sp., however, this difference was not observed in *Achromobacter* sp. This study confirms the worldwide dispersion of *atx* genes.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is an s-triazine herbicide used worldwide to control grassy and broadleaf weeds in corn, sorghum and sugarcane crops. It has been applied alone or in combination with other pesticides since the 1950's (Fan and Song, 2014). Atrazine is classified as a moderately persistent herbicide due to the presence of chloride and N-alkyl groups and is frequently detected in surface and groundwater at high concentration levels (Radosevich et al., 1995; Dutta and Singh, 2013) due to its long half-life and low absorption in soil (Fan and Song, 2014).

United States Environmental Protection Agency (USEPA, 2006) has classified atrazine as a class C chemical (possible human carcinogen) and according to Sanderson et al. (2000), atrazine presents hormonal disrupting and tumor-promoting properties. Atrazine is slowly degraded in the environment and its half-life may vary between 13 and 260 days (USEPA, 2006). The primary mechanism for atrazine dissipation is a biological degradation (Radosevich et al., 1995), starting with dechlorination and formation of hydroxyatrazine, which is transformed in N-isopropylammelide by a dealkylation, with posterior yielding of cyanuric acid, the substrate for the ring cleavage (Cook

et al., 1985; Dutta and Singh, 2013). Pure and mixed cultures are capable of utilizing atrazine as a nitrogen source and have been isolated from contaminated sites (Mandelbaum et al., 1993).

The metabolism of the s-triazine compounds by pure bacterial cultures has been studied and genes encoding enzymes involved in atrazine degradation under aerobic conditions have been well characterized (Vibber et al., 2007). *Pseudomonas* sp. ADP (Atrazine Degrading *Pseudomonas*) is the most well-characterized atrazine-degrading microorganism and its metabolic pathway is completely established (Mandelbaum et al., 1995; De Souza et al., 1998a). This bacterium possesses a self-transmissible plasmid pADP-1, where *atx* genes are located, which are responsible for encoding enzymes for atrazine degradation (Martinez et al., 2001). Atrazine mineralization by *Pseudomonas* sp. ADP involves six enzyme steps encoded by *atxA*, *atxB* and *atxC* genes and *atxDEF* operon. Products of *atxA*, *atxB* and *atxC* genes are responsible for the chlorine, isopropylamine and ethylamine removal, producing hydroxyatrazine, n-isopropylammelide and cyanuric acid, respectively (De Souza et al., 1996; Boundy-Mills et al., 1997; Sadowsky et al., 1998; García-González et al., 2005). To complete atrazine degradation cycle, a LysR-type regulator *atxR*, in response to cyanuric acid and nitrogen limitation, is responsible for the activation of the *atxDEF*

* Corresponding author. Av do Café S/N, Monte Alegre, 14040-903, Ribeirão Preto, SP, Brazil.
E-mail address: elianags@usp.br (E.G. Stehling).

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operon that encoded enzymes that convert cyanuric acid in ammonium and carbon dioxide (Shapir et al., 2005; Porrúa et al., 2010).

Atrazine degradation genes are widespread and highly conserved (De Souza et al., 1998a) and have been detected in Canada, United States of America, Chile, France, Croatia, China, Brazil, India and Egypt with similarity above 97% (Vaishampayan et al., 2007; Hernández et al., 2008; Sene et al., 2010; El Sebai et al., 2011; Fernandes et al., 2014). Gene organization appears to be variable since *atz* genes may be located in different plasmids and chromosomal DNA (Rousseaux et al., 2002; Vaishampayan et al., 2007).

Atrazine biodegradation is not only performed by *Pseudomonas* sp. Beyond mineralization by pure cultures, bacterial consortia containing up to eight different species capable of degrading atrazine have been reported. Studies demonstrate that *atz* genes have been detected in *Caulobacter*, *Variovorax*, *Rhizobium*, *Sphingomonas*, *Burkholderia*, *Nocardia*, *Flavobacterium*, *Achromobacter*, *Arthrobacter*, *Ensifer* and others genera (Smith et al., 2005; Vaishampayan et al., 2007; Udiković-Kolić et al., 2010; El Sebai et al., 2011; Limin et al., 2017).

Despite having been banned in several countries, atrazine is a herbicide widely used in Brazil. Therefore, studies related to atrazine degradation are essential for a better understanding of atrazine-degrading bacteria. The aim of this study was to verify if Brazilian soil has potential to biodegrade atrazine naturally through the analysis of atrazine degradation ability by two new isolates from Brazilian soil as pure colonies and as a bacterial consortium.

2. Materials and methods

2.1. Soil sampling and reagents

Contaminated soil samples were obtained from cornfields with historical use of atrazine. Collection sites are located at “Experimental Farm” in Ribeirão Preto, São Paulo, Brazil. Atrazine was spread on the fields one month prior to samples collection. The top 5 cm and 10 cm layer were collected from seven sites in the field and were transported to the laboratory to be processed immediately.

Commercial atrazine (Nortox, Brazil) used for enrichment cultivation was a courtesy from a local corn producer. Atrazine (99% pure) used for degradation assays was purchased from Sigma-Aldrich (Germany). Atrazine medium (ATZ-R) used for bacterial isolation and degradation assay contains (L^{-1}): 67 mL 1 M KH_2PO_4 (pH 6.8), 2 g sodium citrate, 5 mL of R-Salt (16 g $MgSO_4 \cdot 7H_2O$, 0.4 g $FeSO_4 \cdot 7H_2O$, 0.8 mL HCl), 0.2 mL 1 M $CaCl_2$ and 10 mL 20% glucose (Mandelbaum et al., 1993). For solid medium preparation, it was added 1.5% of bacteriological agar.

2.2. Microbial isolation and identification

A total of 50 g of each soil sample was added to 200 mL of atrazine liquid medium (ATZ-R) containing $1000 \text{ mg } L^{-1}$ of atrazine as the single nitrogen source. Samples were incubated at $30^\circ C$ for up to 4 weeks under agitation (150 rpm). After 7 days of incubation, 200 μL were spread on atrazine solid medium (ATZ-R). Next, 100 mL of atrazine liquid medium were added to the initial sample. This procedure was performed once a week during 4-weeks incubation and bacterial growth was monitored through bacterial growth in ATZ-R agar plates. Plates were incubated at $30^\circ C$ up to 5 days and isolates were selected according to the formation of clear zones around the colonies, suggesting atrazine degradation (adapted from Gargouri et al., 2014).

Isolates were identified by 16S rRNA gene sequencing using primers 63F (5'-CAGGCTAACACATGCAAGTC-3') e 1387R (5'-GGGCGGWTGTACAAGGC-3') (Marchesi et al., 1998).

2.3. Detection of atrazine catabolic genes and sequencing

Genomic and plasmid DNA from isolates were extracted to be used

Table 1

Primers used in this work.

Target gene	Primer Sequence (from 5' to 3')	Annealing Temperature ($^\circ C$)	Product Size (bp)
<i>atzA</i>	ACGGGCGTCAATTCTATGAC CACCCACCTCACCATAGACC	58	200
<i>atzB</i>	TCA CCG GGG ATG TCG CGG GC CTC TCC CGC ATG GCA TCG GG	58	500
<i>atzC</i>	GCT CAC ATG CAG GTA CTC CA GTA CCA TAT CAC CGT TTG CCA	50	600
<i>atzD</i>	GGA GAC ATC ATA TGT ATC ACATCG ACG TTT TC CCA ATA AGC TTA GCG CGG GCAATG ACT GCA	60	1100
<i>atzE</i>	TAC GCG GTA AAG AAT CTG TT GGA GAC CGG CTG AGT GAG A	52	1000
<i>atzF</i>	CGA TCG GAA AAA CGA ACC TC CGA TCG CCC CAT CTT CGA AC	52	900

$^\circ C$: degrees Celsius; bp: base pairs.

as template in PCR (Polymerase Chain Reaction). Genomic DNA was extracted using QIAmp DNA mini kit (QIAGEN, Germany) and plasmid DNA was extracted using *Plasmid Midi Kit* (QIAGEN), according to the manufacturer's recommendations.

Atrazine degradation genes were amplified according to Zhang et al. (2011). The reaction conditions were as follow: denaturation for 5 min at $95^\circ C$, followed by 30 cycles of $95^\circ C$ for 1 min, annealing temperatures specific for each primer (Table 1) and a final extension of $72^\circ C$ for 10 min. All PCR products obtained in the above reactions were separated on 1% agarose gel. PCR products were sequenced in ABI 3130 Genetic Analyser (Applied Biosystems, EUA) and analyzed in GenBank using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.4. RNA extraction

Total RNA from bacterial isolates was extracted to be used as a template in Northern Blot assays for analysis of the expression of *atzA*, *atzB*, *atzC* and *atzD* genes before and after atrazine induction ($100 \text{ mg } L^{-1}$). RNA extraction was performed using the Reasy RNA Extraction kit (QIAGEN) according to the manufacturer's recommendations, combined with the Trizol reagent (Life Technologies). Isolates without atrazine induction were incubated in BHI liquid medium at $30^\circ C$ until bacterial growth reached the exponential phase (OD_{600nm} 1.0) and RNA extraction was followed.

For the atrazine induction assay, isolates were incubated in ATZ-R liquid medium with addition of atrazine ($100 \text{ mg } L^{-1}$) at $30^\circ C$ and 150 rpm for 48 h. Then, 30 μL of the bacterial growth was transferred to BHI liquid medium. Samples were incubated at $30^\circ C$ until bacterial growth reached the exponential phase (OD_{600nm} 1.0). After this period, samples were centrifuged and the supernatant was discarded. Cells were resuspended in 2 mL of ATZ-R liquid medium with addition of $100 \text{ mg } L^{-1}$ of atrazine and then incubated for 2 h at $30^\circ C$ and 150 rpm (adapted from Devers et al., 2004). Finally, the total of each sample (2 mL) was centrifuged and RNA extraction was followed. RNA integrity was evaluated by agarose-formaldehyde gel electrophoresis. An aliquot of each extracted RNA preparation was quantified in NanoDrop 2000 Spectrophotometer (Thermo Scientific), using wavelengths (λ) of 260 and 280 nm.

2.5. Northern Blot

Northern Blot assay was performed to verify *atzA*, *atzB*, *atzC* and *atzD* gene expression before and after atrazine induction. To obtain the probes used for mRNA detection it was required to isolate the fragments through PCR reactions containing the internal regions of the genes: 200 bp fragment for *atzA*; 500 bp fragment for *atzB*; 600 bp fragment for

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