Atrazine-Degrading Bacteria Isolated from a Mississippi Delta Soil Expressing the Potential for Accelerated Atrazine Degradation

Robert M. Zablotowicz, Cesare Accinelli, L. Jason Krutz and Mark A. Weaver

USDA Agricultural Research Service, SWSRU, Stoneville, Mississippi, 38776 USA
University of Bologna, Dept of Agro-Environmental Science, Bologna, Italy

Introduction

Accelerated atrazine degradation has been reported in many Mississippi soils following exposure to even a single atrazine application (Zablotowicz et al., 2006, 2007). The general pathway for atrazine degradation is initiated by either or two atrazine chlorohydrolases *atzA* or *trzN* (Fig 1). Initial studies were unable to detect *atzA* in Mississippi soils, however recent studies detected the *atzA* gene in one Mississippi soil using PCR (Krutz et al. 2008). Enrichment techniques to isolate atrazine-degraders using the herbicide as sole nitrogen or sole carbon and nitrogen source have been highly successful as reviewed by Shapir et al. (2007).

The objectives of this study were to isolate and characterize atrazine-degraders from a Dundee silt loam soil with six years of atrazine history (Zablotowicz et al., 2007, Krutz et al., 2007). The *s*-triazine substrate range (atrazine, simazine and cyanazine), and the presence of atrazine degrading genes (*atzA*, *atzB*, *atzC* and *trzN*) was determined, the *atzA*, *trzN* genes were sequenced, and species identified based on 16s RNA.

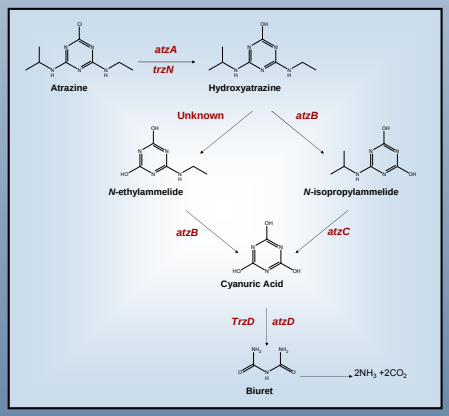

Figure 1. Atrazine degradation pathway

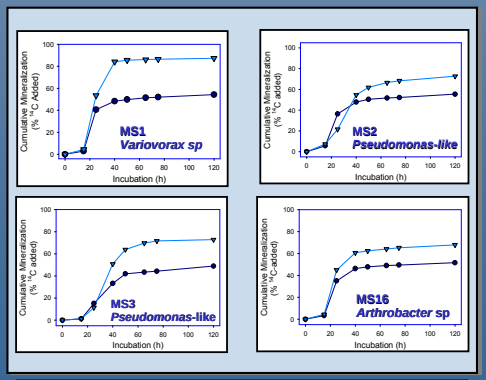

Figure 2. Atrazine (circles) and simazine (triangles) mineralization by four bacterial isolates

Materials & Methods

Bacteria capable of growing on atrazine as a sole carbon and nitrogen source were isolated from a Dundee silt loam using techniques described elsewhere (Zablotowicz et al., 2007). Following four sequential enrichments on mineral salts media with atrazine (66 mg / L) single colony isolates were obtained with a atrazine colony clearing assay (Mandelbaum et al., 1996). Atrazine dissipation was monitored by HPLC (Krutz et al., 2008).

Mineralization of ^{14}C-labeled atrazine and simazine was assessed in biometer flasks containing 20 mL mineral salts with 33 mg / L herbicide (300 Bq / mL) as sole carbon and nitrogen. Mineralization was monitored daily for 120 h and residual herbicide or metabolites monitored at termination by RAD-HPLC. Cyanazine metabolism was determined using unlabelled herbicide (66 mg / mL) and analyzed by HPLC after 120 h.

The presence of four atrazine degrading genes (*atzA*, *atzB*, *atzC*, and *trzN*) were assessed using PCR with primers described in Table 1. Bacterial DNA was isolated using Mo-Bio kits and DNA extracts were purified with Wizard DNA Clean-Up System (Promega). The PCR was performed with RedTaq ReadyMix (Sigma), 0·5 µM of each primer, 5–10 ng template DNA using a MJ Research PTC-100 thermal cycler. PCR products were separated on a 1% agarose gel and stained with SYBR Green. Bacterial 16s RNA genes were amplified using PRBA338d (ACTCCTACGGGAGGCACAG) and PRUN518r (ATTACCGCGGCTGCTGG). These primers were also used for sequencing analysis. After clean-up with ExoSAP-IT (USB, Cleveland, OH), PCR products were sequenced using the ABI Dye Terminator Cycle Sequencing Ready Reaction Kit and analyzed using an ABI 310 automated sequencer (The Perkin-Elmer). Sequences were aligned using DNAMAN software (Lynnon Co, Quebec, Canada).

Table 1 Primers used for detection of atrazine-degrading genes

***atzA* -F**	**5'CCATGTGAACCAGATCCT3'**	**Shapir et al., 2000**
***atzA*-R**	**5' TGAAGCGTCCACAT TACC3'**	**527 bp**
***atzB*-F**	**5'CATGGCGGCGGCAGGTGTG3**	**Shapir et al., 2000**
***atzB*-R**	**5'CCC GGGCCAGCTCCATT TCA3'**	**406 bp**
***atzC*-F**	**5'AGTCAGCGAAGGGCGTAGGTATCA3'**	**Shapir et al., 2000**
***atzC*-R**	**5'GACAAATCCGGGAGACACAAGGTT3'**	**423 bp**
***trzN*10-F**	**5'CACCAGCACCTGTACGAAGG3'**	**Mulbry et al., 2002**
***trzN*11-R**	**5'GATTCGAACCATTCCAAACG3'**	**420 bp**

Table 2. Triazine metabolism and detection of atrazine-degrading genes in Mississippi bacterial isolates.

Isolate	Atrazine*	Simazine*	Cyanuric acid**	Cyanazine remaining	*atzA*	*trzN*	*atzB*	*atzC*
MS1	54	87	ND	ND	-	+	+	+
MS2	55	73	ND	ND	-	+	+	+
MS3	49	76	ND	ND	-	+	+	+
MS7	53	64	ND	ND	-	+	+	+
MS8	< 1	< 1	+	NT	-	+	+	+
MS10	52	68	ND	+	-	+	+	+
MS11	50	32	ND	ND	-	+	+	+
MS14	< 1	< 1	+	ND	-	+	+	+
MS16	52	68	ND	ND	+	+	+	+
MS18	12	29	+	+	-	+	+	+
MW14	< 1	< 1	+	NT	+	-	+	+

*** = % Mineralized** **** = Residual from atrazine and simazine**
ND = None Detected, NT =Not Tested

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Results & Discussion

After four enrichments, 20 single colony isolates were obtained using the colony clearing assay. Based on HPLC, only eleven isolates were capable of atrazine metabolism with no hydroxyatrazine accumulation. Although eleven strains used atrazine as sole carbon and nitrogen source, only eight of the isolates mineralized the ring of atrazine and simazine, with a greater mineralization of atrazine compared to simazine (Table 2, Figure 2). Non-mineralizing isolates accumulated a metabolite that eluted with the solvent peak corresponding with cyanuric acid. All eleven isolates metabolized cyanazine but metabolite identification was not pursued.

Only two isolates (MS16 and MW14) produced PCR products using *atzA* primers described in Table 1, however, all atrazine-degrading isolates possessed *atzB* and *atzC* genes (Table 2). The fragments of *atzA* and *atzB* were of appropriate size, however DNA amplified with *atzC* was 100 bp greater than predicted (Figure 3). All strains except MW14 produced amplified DNA fragments of the appropriate size with *trzN* primers (420 bp). PCR producrs with the trzN primers displayed > 99% homology to *Nocardiodes* sp. strain C190 and *Arthrobacter aurescens* strain TC1. All Mississippi 10 isolates had unique base substitutions in the *trzN* gene sequence. The atzA PCR products from isolale MS16 had high homology to both *Arthrobacter* sp. AD1 and *Pseudomonas* sp ADP.

The taxonomic classification of several atrazine-degrading isolates was identified using 16s RNA sequences. Only six of the eleven strains were identified. MS1 and MS14 had sequence homology to *Variovarax* spp, MS3 and MW14 similar to several *Pseudomonas*-like organisms, and MS16 and MS18 similar to *Arthrobacter* spp. All three species have been previously described to possess the potential for degrading atrazine and related triazines. However the *Arthrobacter* isolate MS16 may be the first degrader to possess both *atzA* and *trzN* encoding for two distinct chlorohydrolases.

Mississippi soils had a long-term history of exposure to cyanazine as this herbicide was used in cotton production. The evolution of a s-trizaine degrading population based on *trzN* instead of *atzA* as the initial dechlorinating gene product is logical considering the cyanazine history and the wider substrate range of *trzN* chlorohydrolase.

Figure 3. PCR analysis of Isolates

MS16 (1=*atzA*, 2=*atzB*, 3=*atzC*, 4= *trzN*)
MS3 (5=*atzA*, 6=*atzB*, 7=*atzC*)
8= 100 kb ladder