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Metabolism of Atrazine in Liquid Cultures and Soil Microcosms by *Nocardioides* Strains Isolated from a Contaminated Nigerian Agricultural Soil

AYODELE E. OMOTAYO,[1] MATTHEW O. ILORI,[1] MARK RADOSEVICH,[2] AND OLUKAYODE O. AMUND[1]

[1]Department of Microbiology, University of Lagos, Lagos, Nigeria
[2]Biosystems Engineering & Soil Science, University of Tennessee, Knoxville, TN, USA

Atrazine-degrading microorganisms designated EAA-3 and EAA-4, belonging to the genus Nocardioides*, were obtained from an agricultural soil in Nigeria. The degradation kinetics of the two strains revealed total disappearance of 25 mg* l^{-1} *of atrazine in less than 72 h of incubation at the rate of 0.42 mg* l^{-1} h^{-1} *and 0.35 mg* l^{-1} h^{-1}*, respectively. Screening for atrazine catabolic genes in these organisms revealed the presence of* trzN, atzB, and atzC*. Other genes, specifically* atzA, atzD, *and* trzD, *were not detected. Potential intermediates of atrazine catabolic route such as hydroxyatrazine, desethylatrazine, and desisopropylatrazine were utilized as sources of carbon and energy, while desisopropyl desethyl-2-hydroxyatrazine and desisopropyl-2-hydroxyatrazine were attacked but in the presence of glucose. A soil microcosm study showed that degradation was faster in microcosms contaminated with 13 mg of atrazine per* g^{-1} *of soil compared with 480 mg* g^{-1} *of soil. In the former, degradation was 10% higher in the inoculated soil than the non-inoculated control (natural attenuation) over the 28-day study period. Corresponding value obtained for the latter was nearly 70% higher. This study has demonstrated that the bacterial strains isolated enhanced atrazine degradation and the catabolic activities of these strains were not affected with increasing soil atrazine concentration.*

Keywords BioSep beads, catabolic genes, biodegradation, soil, atrazine, nocardioides

Introduction

Atrazine {2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine} is a widely used herbicide and has been identified as a pollutant in the environment. Atrazine is persistent in water, mobile in soil, and is among the most frequently detected pesticides in groundwater (Shoseyov et al., 2006). High levels of atrazine concentrations >100 μg kg^{-1} in soil, 16 μg $liter^{-1}$ in surface water, and 1,500 μg $liter^{-1}$ in groundwater (Gannon, 1992; Kramer et al., 2001), which are well above the Environmental Protection Agency's maximum contaminant level of 3 μg $liter^{-1}$ for drinking water, have been detected in the environment (Kello, 1989). Due to its possible hazardous effect on human health and other

Address correspondence to Ayodele E. Omotayo, Department of Microbiology, University of Lagos, Lagos, Nigeria. E-mail: elizabethomotayo@yahoo.com

living things (Hayes et al., 2003; Mizota and Ueda, 2006), much attention has been paid to its fate in soil and to the chemical and biological processes involved in its degradation. Atrazine has been shown to be an endocrine-disrupting chemical in animal studies resulting in hermaphroditism in frogs (Hayes et al., 2003) and degranulation of mast cells in mice (Mizota and Ueda, 2006). Studies have shown that atrazine biodegradation can occur by dealkylation of the ethyl group (deethylation), removal of the isopropyl groups (deisopropylation), or by dechlorination after which the ring substituents are removed by amidohydrolases (Scribner et al., 2005). There are a number of reviews available on atrazine degradation pathways (Ellis et al., 2006; Govantes et al., 2009; Sene et al., 2010). The most characterized organism of atrazine degradation pathway is *Pseudomonas* sp. strain ADP (de Souza et al., 1998). The genes responsible for catabolism of atrazine are *atzA*, *atzB*, and *atzC*, which respectively encode the enzymes atrazine chlorohydrolase (AtzA), hydroxyatrazine ethylaminohydrolase (AtzB), and *N*-isopropylammelide isopropylaminohydrolase (AtzC), all of which sequentially convert atrazine to a central ring cleavage product, cyanuric acid. The genes of the lower pathway have also been documented and they include *atzD*, *atzE*, and *atzF*, which respectively code for cyanuric acid amidohydrolase, biuret hydrolase, and allophanate hydrolase (Martinez et al., 2001). The gene *trzN* also encodes the enzymes atrazine chlorohydrolase, which are present in the genera *Arthrobacter* and *Nocardioides* (Shapir et al., 2006). Generally, atrazine metabolic pathway could be encoded by gene cassettes *atzABCDEF* or *trzN-atzBCDEF*.

Previous reports on the degradation of atrazine by bacteria that are indigenous to tropical Africa are scarce, whereas the fate of the compound has been extensively studied in Europe and North America. Due to the wide geochemical and physical variations between the tropical and temperate environments, bacterial isolates of tropical origin may exhibit fascinating metabolic diversity and possess distinct catabolic properties for real-time degradation of atrazine. Therefore, this study set out to isolate from a soil with long history of atrazine contamination organisms with atrazine catabolic phenotypes. It also aimed to determine the existing catabolic pathways and to evaluate atrazine biodegradation potential of the isolates in soil ecosystems simulated in the laboratory.

Materials and Methods

Soil Sample and Bead Deployment

Soil samples were collected randomly at a depth of 0–10 cm (100 g) from an area covering 200 hectares of land, pooled, homogenized, and stored without drying at 4°C prior to use. In-situ enrichment for atrazine-degrading bacteria was conducted using atrazine-fortified BioSep beads as previously described by Ghosh et al. (2009). The highly adsorptive BioSep beads were amended by equilibration in aqueous atrazine solutions (48 h), such that the final concentrations attained were 20 and 200 mg atrazine kg^{-1} beads. Water-equilibrated beads (0 mg Kg^{-1}) served as control. The beads were incubated in 100 g of soil microcosms prepared in 250 ml conical flask and maintained at 80% of field capacity by the addition of sterile deionized water. These were left to equilibrate at room temperature (28°C) for approximately one month, thereby allowing time for bacteria to colonize the porous beads and metabolize the added atrazine.

Enrichment Protocol

Atrazine degraders indigenous to the baited beads were isolated by modified enrichment techniques described by Radosevich et al. (1995). Atrazine mineral salts (AMS) medium,

supplemented with 25 mg l^{-1} of atrazine as the sole sources of carbon, nitrogen, and energy, was inoculated with 25% w v^{-1} of beads and incubated aerobically with shaking at 28°C. A parallel enrichment medium but fortified with 2% glucose as a source of carbon (AMSG) was also set up. Uninoculated AMS and AMSG media served as controls. Pure cultures were isolated from the enrichment by spread plate technique on AMS and AMSG agar. Incubation was performed at room temperature (28°C). Random colonies appearing on the dilution plates were isolated, purified, and then screened for atrazine-utilization.

Degradation of Atrazine and its Potential Metabolites

Fresh cultures of atrazine-degrading bacteria were harvested at late exponential phase by centrifugation (Eppendorf 5415D, Hauppauge, NY, USA) at 10,000 rev min^{-1} for 5 min and washed twice with sterile AMS medium. Pelleted cells were re-suspended in the same medium and atrazine degradation was assessed using 10^4 cells ml^{-1} of the isolates. Degradation of atrazine (25 mg l^{-1}) was analyzed in 50 ml of AMS or AMSG medium inoculated with washed cells and incubated at 28°C on a rotary shaker (Innova 2100, Brunswick Scientific, Enfield, CT, USA) programmed at 150 rev min^{-1}. Culture samples were withdrawn at time intervals for the evaluation of residual substrate concentration. The samples were extracted with equal volume of acetonitrile and centrifuged at 12,000 rev min^{-1} for 5 min.

The ability of the isolates to utilize potential atrazine metabolites, including 2-hydroxyatrazine, desethylatrazine, desisopropylatrazine, desisopropyl-2-hydroxyatrazine, desisopropyl desethyl-2-hydroxyatrazine and cyanuric acid, was studied in a similar fashion in cultures grown for 7 d at 28°C in AMS and AMSG media containing 25 mg l^{-1} of respective compounds. Residual concentrations of the compounds were replicated.

Nucleic Acid Extraction and PCR Amplification

Total genomic DNA of the pure cultures was extracted using an ultra-clean-soil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA). The primers used for this study as well as the genes targeted are presented in Table 1. Identification of the organisms was carried out on the basis of 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified using the primers 27f (5′-AGR GTT TGA TCM TGG CTC AG-3′) as forward and 1492r (5′-GGY TAC CTT GTT ACG ACT T-3′) as reversed primer (Lane, 1991). PCR amplification and transformation were performed as described by Omotayo et al. (2011). The 16S rRNA gene was sequenced at the University of Tennessee, Knoxville, UTK Molecular Biology Resource Facility. The resulting 16S rDNA sequences were aligned and compared with entries in GenBank using the BLAST algorithm. Relevant atrazine catabolic genes existing in the isolates were screened by PCR amplification of the total genomic DNA according to the method of Smith et al. (2005). The PCR products were visualized in 1.0% agarose gel. All designed primers were validated against either *Pseudomonas* sp. ADP, *Arthrobacter aurescens* TC1, or *Ralstonia basilensis* M91–3.

Nucleotide Sequence Accession Numbers

The sequence data for the bacterial isolates have been deposited at GenBank under accession numbers FJ406513 and FJ406514. The sequence homology of the isolates was determined using ClustalW Multiple Sequence Alignment Program for DNA and Proteins.

Table 1
Primers for PCR amplification of catabolic genes

Target gene	Primer name	Nucleotide sequence (5′ direction 3′)	Source
atzA	AtzA-f	GGC GAT GAG ACC GGA GGA CG	This study
	AtzA-r	TGA AGC GTC CAC ATT ACC	This study
atzB	AtzB-f	CTT TGT CCG CAA TCT TGC C	This study
	AtzB-r	CTC CGC CAT CGG CAG GGT	This study
atzC	AtzC-f	AAT CCT TGG ATA TGG G	This study
	AtzC-r	AAA TCT CTG ATA TTG TCC GAA G	This study
atzD	AtzD-f	ACG CTC AGA TAA CGG AGA	This study
	AtzD-r	TGT CGG AGT CAC TTA GCA	This study
trzN	TrzN-f	GCG ACG GGA AGT TCG GTC	This study
	TrzN-r	CGA GCG TCA TCG ATG ACC T	This study
TrzD	TrzD-f	CAC TGC ACC ATC TTC ACC	This study
	TrzD-r	GTT ACG AAC CTC ACC GTC	This study

f- Forward primer; r- Reverse primer.

Soil Microcosm Experiment

The soil microcosm experiment was carried out as described by Morán et al. (2006). Prior to the microcosm set-up, the soil was sieved through a 2-mm sieve, air-dried, and analyzed for atrazine concentration. Microcosms were prepared for two different concentrations of atrazine; i.e., 13 mg g^{-1} and 480 mg g^{-1} of soil. The experiment was conducted in polyethylene foil-covered 50 ml Teflon tubes containing 5 g of treated soil at room temperature. The augmented soils were inoculated with 0.5 ml of 10^4 cells of the isolates g^{-1} of soil. An uninoculated microcosm set up to evaluate the contributions made by organisms indigenous to the soil served as control. Degradation was monitored by sampling (5 g) each system for growth (1 g) and disappearance of atrazine (4 g). Population densities were determined by plate count of colonies on nutrient agar at room temperature (28°C) incubated for 24–48 h.

Extraction of Atrazine from Soil Samples

The method of Morán et al. (2006) was adopted for extraction of atrazine from the microcosms. Briefly, 10 ml of methanol and water mixture (3:1) in a Teflon bottle was added to 4 g of soil. The mixture was agitated on a shaker overnight, after which it was left to stand for 6 h, and then agitated again for 30 min. Soil particles were allowed to settle for approximately 20 min and the entire content centrifuged at 4100 g min^{-1} for 30 min. $CaCl_2$ (10 mM, 200 μl) was added to precipitate humic acid in the solution. This was shaken for 5 min and allowed to stand for 20 min. The content was centrifuged. The supernatant (2 ml) was clarified by centrifugation at 15,000 rev min^{-1} for 10 min. Atrazine concentration in the extract was quantified via an HPLC.

HPLC Analysis of Atrazine and Other Compounds

Atrazine analysis was performed using a Shimadzu LC 10AD VP HPLC (System Columbia, MD, USA) equipped with an Alltech C-18 Econosphere column (5-μm pore size; length,

15 cm; diameter 4.6 mm; Grace Davison Discovery Sciences, Deerfield, IL, USA). Samples (50 μl) were eluted at 1.00 ml min^{-1} with an isocratic mobile phase of acetonitrile:water (3:2) and quantified with a UV-VIS detector set at 220 nm. Separation of potential atrazine metabolites, with the exception of cyanuric acid, was performed with a 3 mM phosphate buffer (pH 3.0) and acetonitrile (4:1) delivered over a linear gradient and flow rate of 1.40 ml min^{-1}. Cyanuric acid was eluted with an isocratic mobile phase of acetonitrile and water (1:1) at flow rate of 1.00 ml min^{-1}. Analytical standards of atrazine and metabolites were prepared in acetonitrile:water solutions (2:3) at concentrations ranging from 1 to 25 mg l^{-1}.

Statistical Analysis

Mean, standard deviation, Student t-test, analysis of variance (ANOVA), and other statistical tests were performed using the Prism computer software program version 5.00 (GraphPad Software, San Diego, CA). Significance limits were set at 95% confidence interval level.

Results

Bacterial Isolation and Identification

Two organisms exhibiting atrazine catabolic potentials from the 200 mg atrazine kg^{-1} bead AMSG with glucose supplement were isolated and purified. These isolates were designated strains EAA-3 and EAA-4. The partial nucleotide sequences of the 16S rRNA gene from the two strains showed more than 97% identity with those from the genus *Nocardioides*. The organisms were tentatively identified as *Nocardioides* sp. EAA-3 and *Nocardioides* sp. strains EAA-4. The sequence homology of the two isolates showed 97% sequence identity.

Atrazine Metabolism

Atrazine was readily degraded by both strains in AMS and AMSG media. At the onset of the study, strain EAA-3 showed no apparent response to the substrate (Figure 1A). However, between 36 and 48 h of incubation, atrazine was metabolized very rapidly at a degradation rate of 0.44 mg l^{-1} h^{-1}. Assuming the rate of utilization was constant, it would mean that the organisms degraded all the atrazine substrate at 0.42 mg l^{-1} h^{-1} in 60 h at 99% efficiency. A similar trend of metabolism was exhibited by EAA-4 (Figure 1A); however, atrazine was depleted at a slightly lower rate of 0.35 mg l^{-1} h^{-1}. With glucose supplementation, the overall degradation rates determined for EAA-3 and EAA-4 were 0.42 and 0.40 mg l^{-1} h^{-1}, respectively (Figure 1B), thus suggesting that glucose had no significant effect in enhancing the catabolic activity of the isolates. In general, the data obtained from the two incubations produced no significant differences in degradation kinetics when subjected to statistical analysis.

Amplification of Catabolic Genes and Utilization of Potential Products of the Pathway

The strains metabolized 2-Hydroxyatrazine, desethylatrazine and desisopropylatrazine in both AMS and AMSG media (Table 2). However, degradation of desisopropyl-2-hydroxyatrazine and desisopropyl desethyl-2-hydroxyatrazine was only observed when the medium was fortified with glucose. Cyanuric acid was not attacked in any of the medium used for cultivation. The catabolic genes *atzB, atzC*, and *trzN* were successfully amplified from the genome of the isolates. Gene *atzA, atzD,* and *trzD* were not detected.

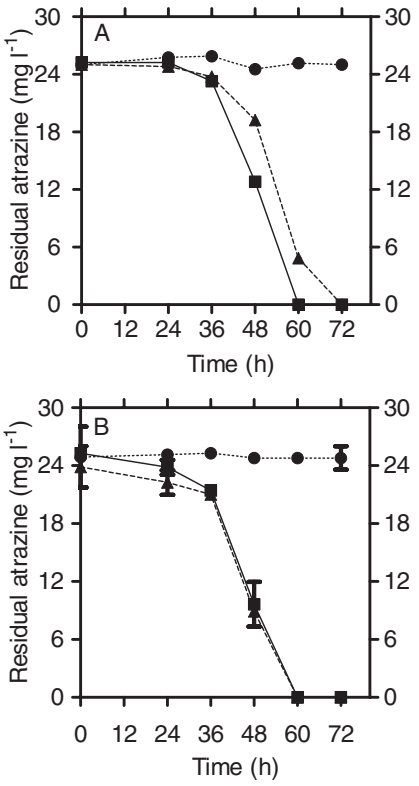

Figure 1. Degradation kinetics of atrazine at 25 mg l^{-1} by *Nocardioides* sp. strains EAA-3 and EAA-4 in atrazine mineral salts medium (AMS) (A) and atrazine mineral salts medium with glucose amendment (AMSG) (B). Control (•), EAA-3 residual concentration (■) and EAA-4 residual concentration (▲). Data presented are averages and standard deviations of three replicate determinations (n = 3). Error bars were too small to be seen in some cases.

Microcosm Studies

In the soil microcosm containing 13 mg g^{-1} atrazine (representing the concentration of atrazine found in soil polluted environment), over 10% atrazine disappearance in the uninoculated control system was observed (Figure 2AI). In the inoculated set-up, nearly 95% of the pollutant was degraded as early as the fourteenth day of incubation. The depletion rate was at 0.35 mg g^{-1} d^{-1} of atrazine (Figure 2AII and III). By the twenty-eighth day, virtually all the herbicide was consumed in the microcosms inoculated with either strain EAA-3 or EAA-4. In soil contaminated with 480 mg g^{-1} of the herbicide, a concentration that may be relevant for a hypothetical spill scenario, the indigenous microbial communities failed to effect any significant degradation (9%) (Figure 2BI). However, in the experimental soil, atrazine concentration was gradually depleted at a slower rate (Figure 2BII and III). As much as 290 mg g^{-1} of atrazine was recovered, yielding 39% substrate loss after 14 days of incubation. By day 28, about 89% and 82% degradation were obtained in microcosms augmented with strains EAA-3 and EAA-4, respectively, resulting in disappearance rates of 15.39 and 13.74 mg g^{-1} d^{-1}, respectively.

Table 2
Atrazine degrading-genes and substrate specificity test

	Isolate			
	EAA-3		EAA-4	
Parameter	AMS	AMSG	AMS	AMSG
Metabolite of atrazine				
2-Hydroxyatrazine	+	+	+	+
Desethylatrazine	+	+	+	+
Desisopropyl desethyl-2-hydroxyatrazine	−	+	−	+
Desisopropyl-2-Hydroxyatrazine	−	+	−	+
Desisopropylatrazine	+	+	+	+
Cyanuric acid	−	−	−	−
Atrazine Degrading-Genes				
atzA	−	−	−	−
atzB	+	+	+	+
atzC	+	+	+	+
atzD	−	−	−	−
trzN	+	+	+	+
trzD	−	−	−	−

AMS, Atrazine Mineral Salts Medium; AMSG, Atrazine Mineral Salts Medium with Glucose.

Between the two strains, there was no significant difference in the rate of disappearance of atrazine with time, neither was there a difference in the rate of degradation between the two concentrations tested. Changes in the Total Viable Count (TVC) presented in Figure 2A and B show that the bacterial population remained relatively stable with no significant increase over the investigation period. For instance, the population detected in the soils 28 days after contamination was similar to that in the soils at the commencement of the experiment, in spite of the inoculation with 10^4 cfu ml^{-1} of cells at onset of the study.

Discussion

In this work, atrazine-fortified BioSep beads were used for the isolation of atrazine-degrading bacteria to avoid culture bias inherent in laboratory enrichment protocols. Bacterial strains which are able to metabolize atrazine in liquid media and in soil systems simulated in the laboratory were isolated. The two isolates were identified as species of *Nocardioides* on the basis of the 16S rRNA gene sequences (GenBank accession numbers FJ406513 and FJ406514). *Nocardioides* species are widely distributed in agricultural soil and have been demonstrated by several investigators to degrade atrazine (Satsuma et al., 2006; Satsuma, 2009; Topp et al., 2000).

Degradation kinetics revealed total disappearance of atrazine from the culture media in less than 96 h of incubation. The addition of glucose as a carbon source to the culture media neither enhanced nor repressed atrazine utilization by the bacterial strains. Degradation of potential key atrazine metabolic products in the presence of glucose readily suggests a requirement of a carbon source as inducer of relevant enzymes of the pathways (Neumann et al., 2004). The addition of a carbon source is crucial for atrazine and other s-triazine

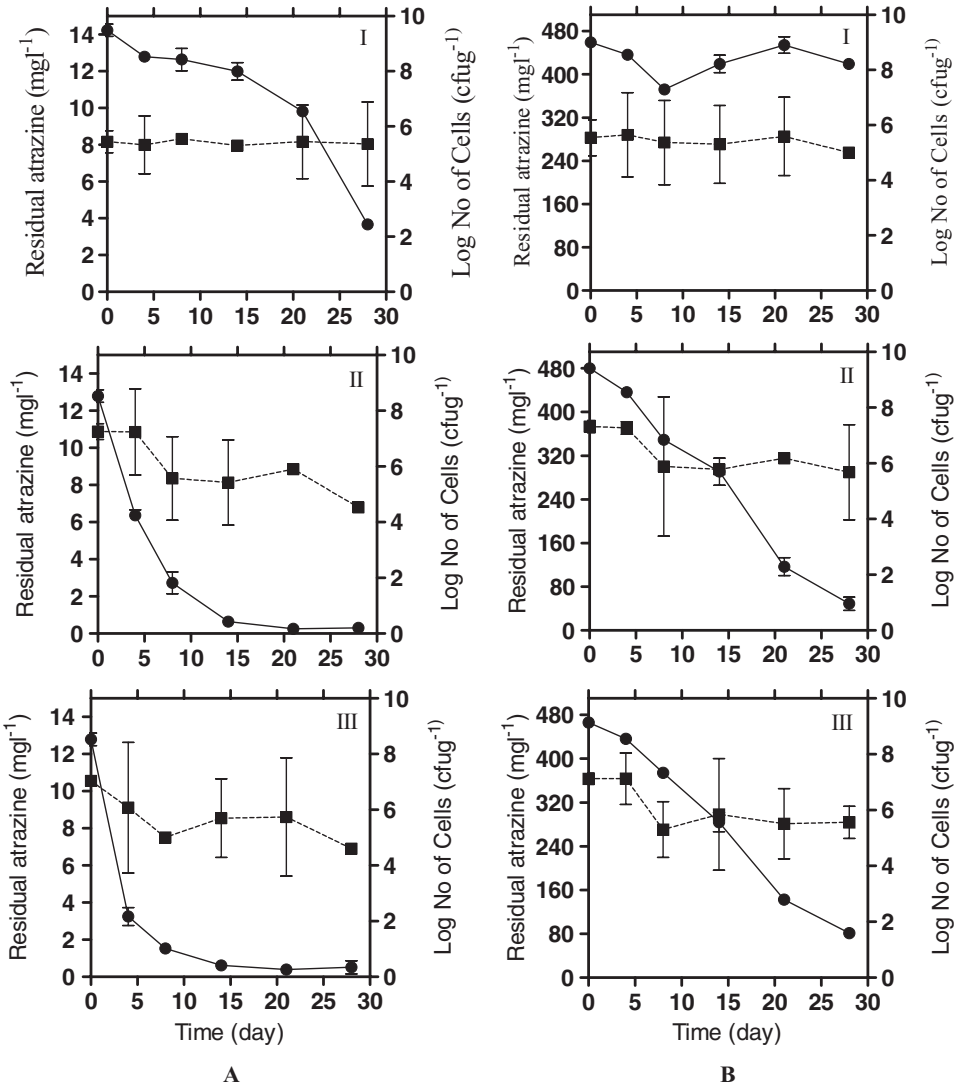

Figure 2. Degradation of atrazine in soil microcosms contaminated with 13 mg g^{-1} (A) and 480 mg g^{-1} (B) atrazine by indigenous communities alone (I), augmentation with strain EAA-3 (II), and strain EAA-4 (III). Logarithm number of cells (Log No of Cells), Microbial population (■) and concentration of atrazine recovered from the microcosms (•). Data presented are averages and standard deviations of three replicate determinations (n = 3). Error bars were too small to be seen in some cases.

degradation as more or less all known bacteria capable of degrading these substrates can only use them as nitrogen sources (Behki et al., 1993; Mandelbaum et al., 1995; Neumann et al., 2004; Strong et al., 2002). However, a few bacterial strains, like the isolates obtained in this study, are known to use atrazine as carbon and nitrogen sources (Struthers et al., 1998).

Cyanuric acid was regarded as a dead-end metabolite as it was not utilized by both strains with or without glucose supplementation. By implication, these organisms could

only transform and not mineralize atrazine. Mineralization of atrazine by pure cultures is a function restricted to few bacterial species. More importantly, atrazine mineralization is relatively unknown with *Nocardioides* species.

The growth of both *Nocardioides* sp. strains on desisopropylatrazine, desethylatarzine. and the other metabolites of atrazine suggests the synthesis of atrazine monooxygenase, an enzyme encoded by P450 cytochrome and s-triazine hydrolyses enzymes. The presence of *atzB* and *atzC* was unique to these isolates. Topp et al. (2000) reported a strain of *Nocardioides* that was barely able to metabolize atrazine to *N*-ethylammelide and isopropylamine as dead-end products. Likewise, Satsuma et al. (2006) showed the presence of *trz*N, *atz*B, and *atz*C in a *Nocardioides*-based consortium; however, the presence of the last two genes was not ascribed as originating from the *Nocardioides* strains. Strain AN4–4 isolated by Satsuma (2009) was shown to harbor *trzN* and *atzC* genes, but not *atzB*, even though the strain could grow on mineral salts agar containing ethylamine or isopropylamine as the only carbon and nitrogen sources.

Thus, the detection of this gene cassette in our strains allows us to reasonably assume that they could metabolize *N*-ethylammide and isopropylamine intermediates. The absence of *atzD* and *trzD* confirms that the enzymes of AtzD and the homologous, isofunctional enzyme of TrzD, which both have activity with cyanuric acid, were not synthesized. This is consistent with results obtained during screening on cyanuric acid as a growth substrate. Attempts to detect *atzA* in the strains were unsuccessful. This result was predictable and was not surprising (Satsuma, 2009), because *atzA* has mostly been found in Gram-negative strains (Shapir et al., 2006; Iwasaki et al., 2007). One exception is *Arthrobacter* sp. strain AD1, isolated from an industrial wastewater in China (Cai et al., 2003). Therefore, on the basis of evidence presented, we propose that atrazine is degraded by both dechlorination and dealkylation reactions to cyanuric acid by EAA-3 and EAA-4 strains.

Seeding of contaminated soil with strains EAA-3 and EAA-4 was successful in effecting real-time degradation of atrazine at the two different concentrations described, though it took a relatively longer time to achieve over 80% degradation of the pollutant at higher concentration. Accelerated degradation of atrazine in soils due to microbial seeding has also been reported previously (Lima et al., 2009; Struthers et al., 1998). Degradation of atrazine under natural attenuation conditions at 13 mg g^{-1} relatively compared with augmented soils, though it was higher in the latter. By interpretation, the attenuated soil harbors indigenous communities with active atrazine metabolic potentials. Earlier work by Omotayo et al. (2011) has shown the soil used in this study to be composed of active atrazine-degrading bacterial community. However, the situation contrasted sharply when the herbicide concentration was increased to 480 mg g^{-1}. Over the 28-day study period, the indigenous microflora were unable to exert significant degradation of the pollutant, as a result of which concentration of the atrazine recovered at the end of the study was 9% short of the initial dose. This result suggests that the heterotrophs, which are generally involved in the degradation of such pesticides, did not appear to decrease in number by their exposure to a high atrazine dose, and this is in contrast to the inability of the indigenous microflora to degrade atrazine. Therefore, in atrazine spill conditions, it may be unrealistic to rely on the natural metabolic functions of the microorganisms indigenous to the soil.

The enrichment culture from the contaminated soil utilized in this study consisted of at least two microbial species; i.e., strains EAA-3 and EAA-4. Both organisms were found to metabolize atrazine to cyanuric acid. Substrate spectra studies coupled with genetic analysis revealed that both strains utilized at least three pathways to bring about transformation of the herbicide to cyanuric acid as a dead-end metabolite. From growth and metabolic view points, it appears both strains share similar genetic and catabolic characteristics. This

inference is very consistent with 16S rRNA gene analysis of the genomic DNA and the sequence homology of the isolates. Until now, the presence of genes *atzB* and *atzC* in *Nocardioides* species has not been reported. One interesting attribute of these isolates is the fact that they were able to effectively enhance atrazine degradation in soil. It was unequivocally demonstrated that the catabolic activities of the organisms were not affected with increasing soil atrazine concentration, wherein the indigenous communities were very sensitive. The overall biodegradation results proved that the augmentation with degraders has high potential for soil decontamination. This study will be followed by an on-site, pilot-scale test for examining the effects of environmental stresses and competition with indigenous microorganisms.

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