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Biodegradation of *s*-triazine herbicide atrazine by *Enterobacter cloacae* and *Burkholderia cepacia sp.* from long-term treated sugarcane-cultivated soils in Kenya

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In this study soils from sugarcane-cultivated fields were screened for bacterial species capable of atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) degradation due to long exposure of the soils to this herbicide. To enrich for atrazine degraders, Minimal Salt Medium containing atrazine as the sole N source and glucose as the C source was inoculated with soils impacted with this herbicide and incubated. Bacterial growth was monitored by measuring optical density. The degradation of atrazine was followed by measuring residual atrazine in liquid cultures over a given time period by high performance liquid chromatography. Bacterial strains isolated from the enrichment cultures were characterized by biochemical tests and identified by 16S rRNA gene sequencing. Two bacterial strains coded ISL 8 and ISL 15 isolated from two different fields were shown to have 94 and 96% 16S rRNA gene sequence similarity to *Burkholderia cepacia* respectively. Another bacterial sp., ISL 14 was closely related to *Enterobacter cloacae* with a 96% 16S rRNA gene sequence similarity. There was not much difference between the extents of atrazine degradation by the enrichment cultures with communities (79–82% applied amount) from which pure strains were isolated and the pure strains themselves in liquid cultures that showed a degradation of 53–83% of applied amount. The study showed existence of bacterial strains in different sugarcane-cultivated fields which can use atrazine as a nitrogen source. The bacterial strains isolated can be used to enhance the degradation of atrazine in contaminated soils where atrazine is still considered to be recalcitrant.

Keywords: Atrazine, liquid culture, degradation, *Burkholderia cepacia*, *Enterobacter cloacae*, adaptation.

Introduction

S-triazine herbicides are extensively and frequently applied in sugarcane-cultivated soils. One such herbicide is atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), which has been used world-wide over the last 30 years for non-selective weed control in industrial and non-cropped land and for selective weed control in such crops as corn, sorghum, sugarcane, and pineapple.[1,2] Its widespread use has caused environmental concern due to the frequent detection of atrazine and its transformation products in surface and groundwater.[1] Atrazine can be degraded by both chemical and biological processes in soil environments, resulting in the formation of metabolites hydroxyl-atrazine (HA), deethyl-atrazine (DEA), and deisopropyl-atrazine (DIA).[3] Atrazine was considered to be poorly biodegradable during the 1960s to 1980s as soil indigenous microbes could not utilize atrazine as a source of C, N and energy.[4] However, in subsequent years, acclimation of the soil microflora to atrazine mineralization after repeated applications of this herbicide under field conditions was observed.[5]

The outcome of the acclimation of the soil microflora to atrazine mineralization has been enhanced degradation of atrazine, where in the past the chemical was recalcitrant in soils from geographically diverse locations.[6–8] From an agronomic perspective, residual weed control could be reduced in atrazine-adapted soils relative to non-adapted soils. From an environmental standpoint, enhanced degradation by adapted soil microflora could lead to reduced persistence of atrazine in soil thus reducing surface and groundwater pollution.[9] Shaner and Henry[10] reported that farmers in eastern Colorado in the United States had noticed that atrazine did not provide the length of weed control that they expected in fields that had received multiple applications of the herbicide. Laboratory degradation studies of atrazine in soils from eastern Colorado carried out by the same authors thereafter, showed that atrazine

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had an extremely short half-life. Other studies by Krutz et al., Jablonowski et al. and Shaner et al.[8,11,12] all showed that in adapted soils, the degradation of atrazine was enhanced thus reducing the ability of the chemical to control weeds.

In Kenyan soils under tropical conditions, atrazine has been used for more than 30 years to control weeds in sugarcane-cultivated fields. In a study to compare the extent of atrazine mineralization in adapted and non-adapted soils from the sugarcane-cultivated fields, atrazine was mineralized to CO_2 by 90% after 98 days of soil incubation in the laboratory. However, mineralization of atrazine in the non-adapted soil was negligible (0.16%) after 163 days of soil incubation.[13] Thereafter, from the same adapted soil atrazine degrading *Arthrobacter sp*. strain GZK-1 was isolated and characterized.[14] The objective of the present study was to screen soils from various sugarcane-cultivated fields for the presence of adapted atrazine-degrading bacteria. The utilization of atrazine for growth by the degraders in the soils was confirmed by measuring the $^{14}CO_2$ released from the mineralization of the uniformly ^{14}C-ring labeled atrazine in liquid culture.[14] Soils with adapted atrazine degraders could then be used as inoculants in liquid culture enrichments with atrazine as a substrate to isolate and characterize microorganisms responsible for atrazine degradation in the adapted soils.

Materials and methods

Soil

Soil samples were collected (0–20 cm) from three different sugarcane-growing areas, Nzoia (34° 34′ 00″ to 34° 51′ 30″ E and 0° 23′ 00″ to 0° 37′ 30″ N), Chemelil (34° 50′ 49″ E to 35° 35′ 41″ E and 0° 4′ 55″ to 0° 20′ 11″ N) and KESREF (34° 49′ 07″ to 34° 49′ 27″ E and 0° 02′ 10″ to 0° 02′ 12″ S) in Kenya where atrazine has been used for more than 25 years. The sugarcane farms were subdivided into different fields with different herbicide application practices. Soil samples were collected from a total of 16 different fields in the three growing areas. The fields were designated as D_8, F_{10C} and F_{26} (Chemelil area), F_9, F_6, F_1, F_{10} and 24D (KESREF area), 124, 7011, 312, 7011, 312, 7013, 314, 8100, 9090 and 1120 (Nzoia area). The soils from the fields were characterized for physico-chemical parameters (Table 1). The residual atrazine and its degradation metabolites were determined in the soils after sampling prior to laboratory degradation studies (Table 2).

Chemicals

Uniformly ^{14}C-ring-labeled atrazine (specific radioactivity of 9.5 mCi/mmol; radiopurity > 92% was purchased from Sigma, (St. Louis Missouri, USA). Analytical standards of atrazine, deethylatrazine, desisopropylatrazine, 2-hydroxyatrazine and deisopropyl-2-hydroxyatrazine, were purchased from Sigma Aldrich through Kobian Company (Nairobi, Kenya). High performance liquid chromatography (HPLC) grade solvents were purchased from Sigma Aldrich through Kobian Company (Nairobi, Kenya). Scintillation cocktails were obtained from Packard (Dreieich, Germany). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Soil analysis for residual atrazine and its metabolites

Atrazine and its metabolites were obtained from soil by soxhlet extraction method. The methanol extracts were cleaned and concentrated through triazine SPE cartridges before analysis by HPLC (LC-10AT VP Shimadzu, Japan). The HPLC was equipped with SPD-10A VP Shimadzu

Table 1. Characteristic of soils from different fields.

Sample fields no.	*pH*	*%N*	*%C*	*P mg/kg*	*S mg/kg*	*% Sand*	*% Clay*	*% Silt*
F_1	6.25	0.16	1.22	3.50	98.21	68	16	16
F_6	5.76	0.14	1.10	2.77	82.14	74	16	10
F_9	6.16	0.14	0.92	3.79	94.64	76	14	10
F_{10}	5.93	0.11	1.28	3.35	60.71	72	16	12
24D	6.07	0.28	1.96	2.48	47.32	40	38	22
D_8	5.43	0.17	1.66	2.19	320.54	52	32	26
F_{10C}	5.56	0.22	1.66	2.19	333.93	56	34	10
F_{26}	6.02	0.33	3.36	63.44	141.07	34	48	18
124	5.10	0.12	1.44	8.23	446.67	58	31	11
312	6.17	0.16	2.14	12.71	47.50	48	33	1
314	5.37	0.11	1.76	19.06	75.83	72	15	13
1120	5.01	0.15	1.68	3.90	236.67	64	25	11
7011	4.80	0.14	1.58	14.30	64.17	48	17	35
7013	4.60	0.15	1.50	2.89	19.17	40	33	27
8100	4.67	0.13	1.72	6.35	30.83	56	13	31
9090	5.08	0.05	0.80	8.23	78.33	72	13	15

Table 2. Concentration levels of atrazine and its metabolites residues in soils from various fields.

Sample field no.	*Atrazine* $\mu g\ g^{-1}$	*DEA* $\mu g\ g^{-1}$	*DIA* $\mu g\ g^{-1}$	*OH-DIA* $\mu g\ g^{-1}$	*HA* $\mu g\ g^{-1}$
F_1	2.81 ± 0.32	—	18.23 ± 0.53	0.76 ± 0.22	—
F_9	0.02 ± 0.01	—	—		—
F_{10}	—	—	7.61 ± 0.70	0.36 ± 0.12	—
24D	3.46 ± 0.26		—		—
D_8	—	—	—		23.45 ± 1.19
F_{10C}	0.15 ± 0.03	—	—		—
124	0.49 ± 0.01	—	—		—
314	0.73 ± 0.12	—	—		—
1120	0.6 ± 0.14	—	—		—
7011	—	—	—		10.07 ± 0.21
7013	0.77 ± 0.01	—	—		—
9090	0.85 ± 0.02	—	—		—

- denotes not detected; n = 3 ± standard deviation

UV-VIS detector and a reversed phase 110 C_{18} column 125 × 4 mm, 5 μm (Shimadzu). The measurements were conducted at the following conditions; Mobile phase (isocratic) acetonitrile: water (60:40) mixture at a flow rate of 1 mL min^{-1}, injection volume of 20 μL and the detector was set at 220 nm.

Screening soils for adapted degraders of atrazine

To screen soils for the presence of adapted atrazine degraders, atrazine mineral salt medium (MSM) was inoculated with soils from different sampling sites. Liquid culture medium for atrazine was prepared by taking 0.5 g of K_2HPO_4, $MgSO_4.7H_2O$ and 100 mgL^{-1} glucose as C source, dissolving together in 1litre of double distilled water and autoclaving at 121°C for one hour in a KT-30L autoclave (Alp Co. Ltd, Tokyo, Japan). The micronutrients consisting of 10 mg of $FeCl_3.H_2O$, 10 mg of $CaCl_2.H_2O$, 0.1 mg of $MnCl_2$ and 0.01 mg $ZnSO_4$ were dissolved together and sterile filtered through Millex GP filter units 0.22 μm. The micronutrients (50 μL aliquot from stock solutions) were then added to the macronutrients in a 500 mL-bottle to make a complete MSM.

Atrazine standard stock solution was prepared by dissolving 100 mg atrazine in 10 mL methanol to make a final concentration of 10 g/L. From the stock solution an aliquot of 110 μL of the solution was transferred into already autoclaved incubation flasks (100 mL). The methanol was allowed to evaporate in sterile hood. A 49 mL of the MSM solution was added to the atrazine crystals and the atrazine dissolved by vigorous shaking on the shaker for 12 hours. Soil samples (1 g) each were dissolved in 9 mL of 0.85% NaCl in double distilled sterilized water and vortexed for 2 minutes. The mixture was diluted to 10^{-3} in ten-fold dilution steps. After the third dilution, 1 mL of the solution was used for inoculating the 49 mL MSM with atrazine in 100 mL-Erlenmeyer flasks. The resulting inoculated medium contained an atrazine concentration of 22 mg/L in the MSM. The experiment was done in triplicate. The control that contained atrazine was uninoculated.

The liquid cultures were incubated at 25 ± 1°C in the dark with shaking at 100 rpm. A 3 mL aliquot was withdrawn periodically at three days interval for the first 30 days and five days thereafter to measure the optical density. Growth was determined by monitoring turbidity at optical density (OD_{600}) using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK). The same aliquot was centrifuged for 10 minutes at 5000 rpm and the supernatant was taken and analyzed for residual atrazine and metabolites by HPLC. A plot of optical density and degradation expressed as amount of atrazine remaining (mg/L) against time was obtained. Fields with soils in which high atrazine degradation (70–80%) was observed after 40 days of incubation were selected for further liquid culture experiments to enrich the adapted atrazine community degraders, and finally isolate and characterize the responsible organisms.

Mineralization of ^{14}C-ring labeled atrazine in liquid culture

The MSM with ^{14}C-atrazine as the sole N source and 100 mgL^{-1} glucose as C source was used for liquid culture experiments to confirm atrazine mineralization by adapted degraders in three soil samples from different fields. An aliquot of 55 μL of methanolic ^{14}C-atrazine solution (final concentration in liquid culture: 22 mgL^{-1}, 6.8×10^4 Bq L^{-1}) was applied in a sterile 100 mL-Erlenmeyer flask and methanol was left to evaporate. The resultant atrazine crystals were dissolved in 25 mL sterile MSM by shaking on an orbital shaker (GFL, Burgwedel, Germany) overnight. Thereafter, aliquots of 100 μL MSM were taken for radioactivity measurement to determine the initial ^{14}C-atrazine concentration. Subsequently, 5 g of soil (dry weight) were added to the medium and incubated on an orbital shaker at 100 rpm in the dark at 25±1°C. The incubation flasks were connected daily to the closed

laboratory system as described by Getenga et al.[14] and aerated for 1 h to trap the evolved $^{14}CO_2$ in order to determine the mineralization of atrazine by the atrazine degraders. Sterile filters (0.22 μm, Sartorius, Göttingen, Germany) were connected at the air inlet and outlet of the incubation flasks to ensure sterile conditions in the liquid cultures.

The mineralization of the ^{14}C-ring labeled atrazine in the liquid cultures was monitored by quantifying the $^{14}CO_2$ released. At regular time intervals (three times a week) the flasks from the rotary shaker were connected to the closed biodegradation systems filled with 10 mL 0.1 M NaOH to trap the accumulated $^{14}CO_2$ released to the headspace by flushing sterile $^{14}CO_2$-free air through the trapping system. A 2 mL aliquot of the absorption liquid, 1.0 M NaOH, was drawn and mixed with 3 mL of scintillation cocktail Ultima Flo AF (Packard, Netherlands). Radioactivity was detected by scintillation counting. [15]

Isolation of atrazine-degrading bacteria by enrichment techniques

Liquid cultures which showed enhanced degradation of atrazine from the screening process were subjected further to liquid culture enrichment experiments. Fresh atrazine liquid culture medium was prepared and sterilized. Aliquots of 49 mL were transferred to sterilized 100 mL flasks in a sterile hood and inoculated with 1 mL aliquots of the previous selected liquid cultures. A series of new liquid cultures were prepared from time to time to enrich the liquid cultures with atrazine degraders by using inoculums from the previous liquid culture. A fully enriched mixed liquid culture was attained after the 5th enrichment. After the 5th enrichment of the mixed liquid culture, an aliquot of 0.1 mL was taken and diluted to 1mL with a fresh MSM. Serial dilutions were made up to the 3th dilution in sterile Eppendorf tubes. MSM medium for atrazine, with the same composition as one used for liquid culture experiments, was prepared with 15 g of agar added to 1000 mL of the solution. The solution with agar was autoclaved and while it was hot atrazine and sterile micronutrients were added. Aliquots of the solution were poured on the agar plates and left to cool allowing the methanol in which atrazine was dissolved to evaporate.

From the 1st and 3rd dilutions, 0.1 mL aliquots were taken and spread on the agar plates in duplicates and the plates incubated at 30°C. The individual colonies were separately streaked on another set of agar plates and incubated at 30°C. The detailed procedure for isolation of the bacterial strains is as described by Getenga et al.[14] About 27 different single strains were isolated and transferred to liquid cultures to determine their capability to independently degrade and grow on atrazine substrate. Turbidity and concentration of atrazine and its metabolites were periodically measured. Six bacterial isolates coded as ISL 1A, ISL 2A, ISL 3A, ISL 4A, ISL 6A and ISL 7A capable of independently degrading and growing on atrazine in liquid culture were obtained. The isolates were then subjected to biochemical tests, and 16S rRNA gene analysis.

Characterization of isolates

Isolates were subjected to morphological and biochemical characterization[16] and also molecular characterization. The colonies were scrapped from agar plates and suspended in DNA extraction medium. Total bacterial DNA was extracted according to procedures described by Schmidt et al.,[17] purified and used as a template for amplification of 16S rRNA gene using bacterial primer pair 27F forward 5'-GAG TTT G(AC)T CCT GGC TCA G-3') and 1492R reverse, 5'-TAC GG(CT) TAC CTT GTT ACG ACT T-3' (Eurofins MWG GmbH). Amplification was performed using a model 9700 Fast Thermal Cycler from Applied Biosystems. PCR products were digested with MspI restriction enzyme (Promega Corporation Madison, USA). The PCR amplicons from the isolates were excised from the gel and purified using quickClean 5M gel Extraction kit (GenScript Corporation, 120 Centennial Ave, Piscataway, NJ 08854) according to the manufacturer's instructions. The sequencing of the 16S rRNA genes was done at BECA (Biosciences Eastern and Central Africa) at ILRI (International Livestock Research Institute) in Nairobi, Kenya. Sequencing was conducted with BigDye^{TM} Terminator technology. The sequencing products were run on ABI 3130 Genetic Analyzers. The forward and backward 16S rRNA gene sequences of the bacteria isolates were viewed and edited using Chromas software package (www.technelysium.com.au). They were then aligned using Bioedit sequence alignment editor software package[18] to provide full sequences of about 1500 nucleotide bases. The sequences were compared with sequences in the public databases with the BLAST search program on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rRNA gene sequences. The ARB database software package[19] was used to align and identify the closely related bacterial 16S rRNA gene sequences. The 16S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned and processed to produce Phylogenetic tree using MEGA software package (www.megasoftware.net).

Results and discussion

Soil characterization, atrazine and its metabolites residues in the soils

Soils from all the fields had pH values below 6.5, indicating that all the soils were acidic in nature. The concentration of residual atrazine and its metabolites in the soils varied from field to field depending on the herbicide application practices (Table 2). Hydroxyatrazine (HA) deisopropylatrazine (DIA) and deisopropyl-2-hydroxyatrazine (OH-DIA) were

detected as transformation products of atrazine in soils from only two different fields. DEA, which is known to be a product of microbial N-dealkylation of the ethyl side chain of atrazine,[3] was not detected in any of the soils. In soils from the two fields (D_8 and 7011) where HA was detected in concentrations of 23.45±1.19 and 10.07±0.2 μg g^{-1}, respectively, residual atrazine was not detected. Furthermore, the soils from the two fields were characterized with low pH values of 5.43 and 4.80, respectively.

Many pesticides are now present in surface and ground waters all over the world, especially in agricultural areas. The presence of HA metabolite in some of the soils is due to their low pH. Atrazine is known to be chemically transformed to HA at low pH conditions as the soils are likely to enhance the soil-catalyzed chemical process of atrazine dechlorination[20,21] while microbial N-dealkylation of the ethyl and isopropyl side chains is accomplished by nonspecific mono-oxygenases from indigenous soil microbes producing DEA and DIA.[20,22] DIA was detected in soils whereas there was no detection of DEA metabolite (Table 2). Dealkylation of atrazine initially proceeds through ethyl side chain removal resulting in DEA, later followed by the removal of isopropyl side chain.[1] DEA metabolite levels in soils may have been below detectable levels following the adopted extraction procedures. The residues in soil resulted from soils with atrazine applied a few months prior to sampling. Consequently DEA could have been transformed to other products which were not analyzed such as deethyl-2-hydroxyatrazine (OH-DEA), or would have been bound to soils leading to its detection being below the limits of detection. However, in the liquid culture, atrazine was freshly applied and the formation of DEA could easily be detected before being transformed to the next intermediate. There is no solid matrix to which newly formed DEA would have been bound leading to resistance of the method of extraction used. N-dealkylation of s-triazines is an important degradation pathway in many microorganisms, and Behki and Khan[23] found that the formation of deisopropylatrazine is favoured over the formation of deethylatrazine.

Atrazine mineralization in liquid cultures inoculated with soils from different fields

Out of the 16 fields from which soils were taken, 11 fields showed high values of both cumulative optical density and atrazine degradation in liquid cultures inoculated with the soils. Cumulative atrazine degradation after 40 days of incubation in the liquid cultures ranged from 58.3% to 82.9% in soils from fields with enhanced atrazine degradation (Table 3). The microbial degradation of atrazine by indigenous soil microbes is known to be poor unless the soil is known to have developed adapted microbes.[4,5] The high cumulative atrazine degradation observed in soils from the 11 fields could be due to the presence of adapted atrazine degraders. Field F_6 in KESREF currently uses atrazine but there was no residual atrazine at the time this study was conducted. This is a strong evidence that residual atrazine did not persist long to control weeds in the sugarcane-cultivated fields. Subsequently soils from six fields (F_9, 314, 1120, 8100, F_6 and F_{26}) with the highest atrazine degradation were used as inoculants in liquid culture enrichments with atrazine as a substrate to isolate key organisms responsible for enhanced atrazine degradation in the soil. The microbes' capability to mineralize atrazine in soils from fields F_9, F_1, and F_{10C} was determined by liquid culture experiments with ^{14}C-labeled atrazine. The microbes in these soils showed capability to mineralize atrazine with those in field F_1 mineralizing 24.1% of the applied amount after 35 days of incubation and a lag phase of 8 days. Those in field F_9 mineralized 21.7% of the applied amount of atrazine after 23 days incubation period and a lag phase of 9 days. Microbes in field F_{10C} mineralized 20.5% of the applied amount of atrazine after 35 days incubation period and a lag phase of 9 days (Fig. 1).

Table 3. Atrazine degradation in soil during screening in liquid cultures at 40 days incubation period.

Sample field no.	*Atrazine degradation (% of applied amount)*
F_1	58.9 ± 0.6
F_6	82.9 ± 1.2
F_9	70.8 ± 0.8
24D	59.1 ± 0.5
F_{10C}	66.8 ± 0.3
F_{26}	78.4 ± 1.7
312	66.1 ± 2.1
314	78.8 ± 0.6
1120	79.7 ± 0.6
8100	78.1 ± 0.5
9090	75.9 ± 1.1
Control	4.5 ± 1.1

n = 3 ± standard deviation

Atrazine degradation by liquid enrichment cultures

Growth as measured by increased optical density in the liquid enrichment cultures from different fields increased with increased atrazine degradation (Fig. 2). The growth curves for the different enrichment cultures from different fields exhibited different lag phases in the liquid cultures. The enrichment culture from soil in field F_6 had a lag phase of 15 days and reached its highest cell mass of 0.28 at OD_{600} on the 45th day. Up to 80% of the initial atrazine concentration of 22 mg L^{-1} in the liquid culture was degraded in 52 days at a degradation rate of 0.34 μg d^{-1}. Growth in enrichment cultures from soil in field 8100 had a shorter lag phase of 12 days and reached its highest cell mass of 0.24 at OD_{600} on the 40th day. Up to 79% of the initial atrazine concentration was degraded in 45 days at a degradation rate of 0.38 μg d^{-1}. Enrichment cultures from soil in field 1120 showed

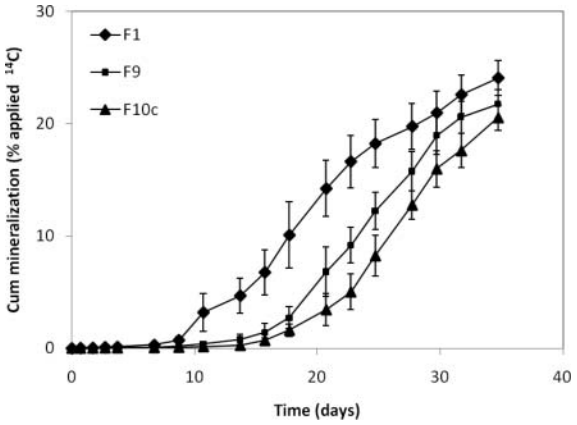

Fig. 1. ^{14}C-U-ring- atrazine mineralization by soils from fields F_1, F_9 and F_{10C}.

the longest lag phase of 17 days and reached its highest cell mass of 0.29 at OD $_{600}$ on the 45th day. The cultures degraded up to 82% of the atrazine applied in 54 days at a degradation rate of 0.33 μg d^{-1}.

Degradation of atrazine by pure isolates

Six different bacterial strains isolated from different fields independently mineralized atrazine ranging from 37.4% to 81.2% with optical densities of 0.063 to 0.105, respectively. Through biochemical tests, gel electrophoresis and profiling of DNA extracts, some of the isolates were found to be similar resulting in only three different isolates which were coded as ISL8, ISL14 and ISL15.

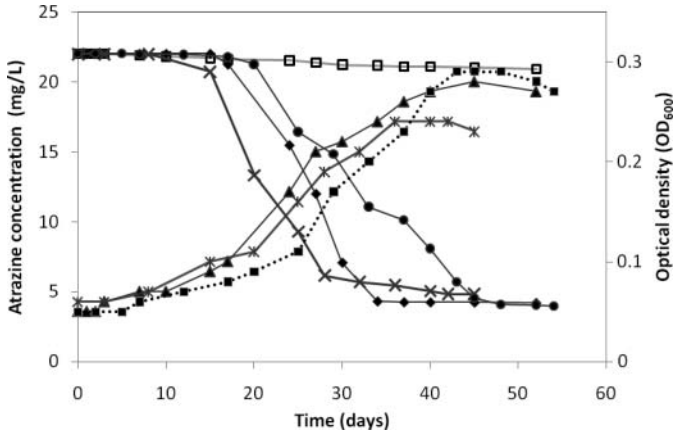

Fig. 2. Growth and atrazine degradation curves in liquid cultures inoculated with soils from different fields. Key: control, atrazine degradation and growth curve for enrichment cultures from sample F_6; atrazine degradation and growth curve for enrichment cultures from sample 8100; atrazine degradation and growth curve for enrichment cultures from sample 1120.

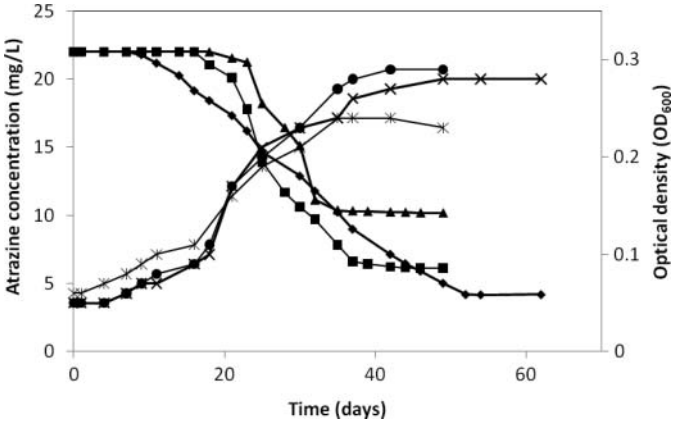

Fig. 3. Growth and atrazine degradation curves by pure isolates from different fields. Key: atrazine degradation and growth curve for ISL 8; atrazine degradation and growth curve for ISL 14; atrazine degradation and growth curve for ISL 15.

The isolates ISL8, ISL14 and ISL15 were isolated from soils in fields F_6 in KESREF, 8100 and 1120 in Nzoia, respectively. ISL8 degraded atrazine by 82.1% at the initial atrazine concentration of 22 mg L^{-1} in the liquid culture after 62 days of incubation with a lag phase of 10 days. Three metabolites were identified at the end of the incubation period as DIA (15%), OH-DIA (9%) and DEA (7%). Isolate ISL14 degraded atrazine by 72% after 49 days of incubation with a lag phase of 14 days. The degradation resulted to formation of DEA, DIA and OH-DIA metabolites constituting 5%, 13% and 8%, respectively. ISL15 degraded atrazine by 53.4% after 45 days of incubation with a lag phase of 23 days (Fig. 3). Three metabolites were present at the end of the incubation period; DEA, DIA and OH-DIA constituting 6, 10 and 7%, respectively. The degradation rates varied for each isolate reaching a maximum of 0.42, 0.35 and 0.34 μg d^{-1} for ISL 14, ISL 8 and ISL 15, respectively.

ISL 8 had degraded the highest amount of atrazine in liquid cultures. For this isolate, the intermediate metabolites DIA, DEA and OH-DIA formed at different sampling days were compared with the amount of degraded atrazine. There was a significant positive correlation between (P = 0.0046) between DIA and atrazine degradation. Similar significant correlations were established between DIA and atrazine degradation for ISL 14 and ISL 15. There was a significant correlation ($P = 0.0045$) between OH-DIA and atrazine degradation for ISL 8. Similar significant correlations were identified for ISL 14 and ISL 15. There was no significant correlation between ($P = > 0.10$) DEA and atrazine degradation established for the isolates.

In this study, it was observed that the degradation of atrazine by the isolates resulted to higher amounts of DIA and OH-DIA compared to DEA. Therefore, the significant positive correlation between DIA and degraded atrazine for the three isolates suggests the degradation pathway as

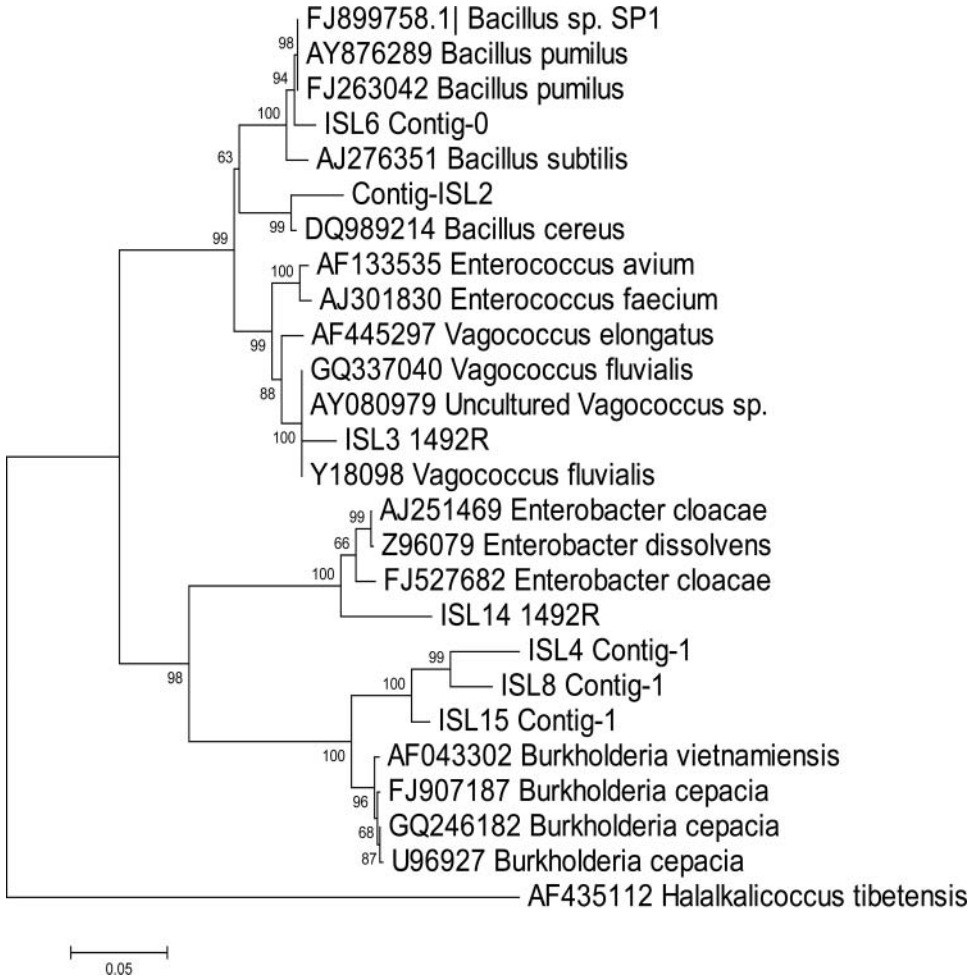

Fig. 4. Phylogenetic tree for bacterial isolates. The scale bar indicates approximately 5% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling. The 16S rRNA gene sequencing of *Halalkalicoccus tibetensis* AF435112 was used as an out group.

atrazine – DIA – OH-DIA. Dechlorination of DIA to the respective hydroxylated compound OH-DIA was demonstrated by Behki and Khan.[23] OH-DIA is an important intermediate product in atrazine mineralization since ring cleavage apparently occurs only after hydroxylation.[24]

Characterization of atrazine degrading strains

Phylogenic analysis showed that ISL8 and ISL15 clustered with the genus *Burkholderia,* supported by high bootstrap values of between 68 - 100% for the two isolates. ISL8 had 16S rRNA gene sequence similarity of 94% to *Burkholderia cepacia* FJ907187 while Isolate ISL15 had 16S rRNA gene sequence similarity of 96% to *Burkholderia cepacia* GQ246182. Phylogenic analysis of 16S rRNA gene sequences of isolate ISL14 showed that the isolate clustered with the genus *Enterobacter*. This clustering was supported by high bootstrap values of between 66–100% (Fig. 4). The isolate had 16S rDNA gene sequence similarity of 96% to *Enterobacter cloacae* FJ527682.

Isolates ISL8 and ISL 15 (*Burkholderia cepacia sp.*) were rod-shaped, free-living, motile gram- negative rods ranging from 1.6- 3.2 μm. The colonies of *Burkholderia cepacia* were circular, entire, sage green colonies with the medium changing from orange to bright pink. Biochemical tests showed that the strain as non-fermentative, oxidase-positive and utilized citrate. Acid was produced from fructose, glucose, arabinose, galactose, lactose, maltose, manitol, mamnose, and sucrose. Isolate 14 was rod-shaped, gram-negative, oxidase-negative and catalase-positive. Other biochemical tests showed that the strain was urease and citrate-positive and generated gas from glucose, fermented sucrose, arabinose and mannitol. It was both indole and H_2S-negative (Table 4 and 5).

Although numerous bacteria and a few fungi have been isolated from geographically diverse locations worldwide, most of them partially degrade atrazine and other s-triazines to cyanuric acid.[25–32] The genes trzN/atzA, atzB and atzC encode for the three hydrolases that catalyze the degradation of s-triazines to cyanuric acid.[33] However,

Table 4. Utilization of sugars by isolates.

	Isolates		
Metabolic versatility tests	*ISL 8*	*ISL 14*	*ISL 15*
Lactose	+	±	+
Sucrose	+	+	+
Glucose	+	+	+
Mannose	+	−	+
Arabinose	+	+	+
D-Manitol	+	+	+
D-Galactose	+	+	+
Maltose	−	+	−
Fructose	+	+	+
Dextrose	+	+	+

Symbols: + positive for substrate utilization, − negative for substrate utilization.

complete mineralization of atrazine has been reported for two *Pseudomonas sp*.[25,34] and a bacterial isolate from a pesticide mixing area.[1] Most recently from a sugarcane field in Kenya *Arthrobacter sp.* strain GZK- 1 which mineralized ^{14}C-ring-labeled atrazine by 88% to $^{14}CO_2$ and ^{14}C-ring-labeled terbuthylazine by 65% to $^{14}CO_2$ in a liquid culture within 14 days was isolated.[14] The enzymes necessary for the ring cleavage and the hydrolysis of cyanuric acid to CO_2 and NH_3 are encoded by the genes TrzD/AtzD, atzE and AtzF.[33] A number of original research papers and reviews have been cited[35,36] stating that cyanuric acid, an intermediate of atrazine metabolism, is metabolized in two steps via biuret to 2-nitrogen intermediate urea and then finally to CO_2 and NH_3. However, a study by Cheng et al.[36] showed that allophanate and not urea, was the 2-nitrogen intermediate in cyanuric acid metabolism in all the bacteria examined. Consequently, genes encoding the enzymes AtzE and AtzF, which produce and hydrolyze allophanate, respectively, were found in several cyanuric acid-metabolizing bacteria. Furthermore, s-triazine metabolism in a broad class of bacteria proceeded through allophanate using allophanate hydrolase, rather than through urea using urease. Shapir et al.[33] showed that allophanate hydrolase from *Enterobacter cloacae* strain 99 metabolized cyanuric acid, the intermediate in atrazine metabolism, to allophanate and then finally to CO_2 and NH_3.

Table 5. Biochemical characterization of the isolates from atrazine and hexazinone degradation.

	Isolates		
Biochemical tests	*ISL 8*	*ISL 14*	*ISL 15*
Cell type shape	rod	rod	Rod
Gram stain	−	−	−
Colony colour	cream white	cream white	cream white
Characteristics shape	round	circular entire margin	round
elevation	raised	raised	raised
surface	smooth	smooth	smooth
Citrate utilization	+	+	+
MR test	−	+	−
VP test	−	−	−
Urease test	+	−	+
Nitrate reduction	+	+	+
Motility at 37°C	+	+	+
Starch hydrolysis	−	−	−
H_2S production	−	−	−
Casein hydrolysis	−	−	−
Catalyse test	−	+	−
Oxidase	+	−	+
Indole	−	−	−

Symbols: + positive reaction,− negative reaction.

In the present study *Burkholderia* sp. was isolated from field F_6 where *Arthrobacter sp.* strain GZK- 1 had previously been isolated and from another field (1120) in Nzoia area. Both fields have a history of atrazine application with the latter having discontinued atrazine application 10 years before. Although *Burkholderia* species are widespread in soil, maize and sugarcane rhizospheres, animal species and hospital environments[37] no study has reported the isolation of *Burkholderia* sp. from soils impacted by atrazine application. However, Zhenmei et al.[38] reported the isolation of *Burkholderia cepacia* from soil collected from a pesticide-manufacturing plant for quinclorac (3,7-dichloro-8-quinone-carboxylic). The strain shares same physico-chemical characteristics with the *Burkholderia cepacia* sp. isolated in the present study. Many studies have reported the isolation of several *Burkholderia* strains from different sources degrading a variety of environmental pollutants. An example is the degradation of fenitrothion and 2,4-dinitrotoluene (2,4DNT) by *Burkholderia*[39,40] indicating their adaptation to different xenobiotics in the environment.

Enterobacter cloacae sp. was isolated from field 8100 in Nzoia area where atrazine had been discontinued 10 years before. However, Velpar 75DF (hexazinone) had been applied to the field in 2007 at the rate of 10 kg ha^{-1}. In a separate liquid culture enrichment experiment with hexazinone as a sole N-source, *Enterobacter cloacae* sp. was also isolated from soil sampled from field 7013 where hexazinone had been applied at the rate of 10 kg ha^{-1}. The strain degraded hexazinone by 26.3% after 35 days of incubation with a lag phase of 10 days in the liquid culture with an initial hexazinone concentration of 40 mg L^{-1}. Many studies have reported the isolation of *Enterobacter cloacae* and other *Enterobacter* strains from different sources degrading diverse pollutants.[41–44]

Conclusions

The present study shows that there could be many adapted atrazine degraders in tropical sugarcane-cultivated soils which have been exposed to repeated applications

of atrazine. This study identified *Enterobacter sp.* and *Burkholderia sp.* which degraded atrazine to different extents in liquid cultures. DIA was identified as an important intermediate in atrazine degradation as it was formed to a larger extent that DEA. In order to fully understand atrazine degradation in these soils, we suggest a complete elucidation of the degradation mechanisms involved.

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