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Isolation, characterization and identification of Diazinon degrading bacteria from the soil and gut of macrotermes

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Abstract

Diazinon degrading bacteria were isolated from the soil and gut of fungus cultivating termites Macrotermes michaelseni using MM7 media supplemented with pesticide as the sole source of carbon and energy. The isolates were gram negative rods the isolates from the soil were designated DS2, DS3 and DS5 while from termite gut was designated DT2. 16S rRNA gene sequence analysis showed that diazinon degrading isolate DS2 (from the soil) is closely related to Achromobacter xylosoxidan AF508101 (100%), isolate DT2 from termite gut is closely related to Klebsiella oxytoca AB004754 (99.7%). Whereas isolates DS3 and DS5 , both from the soil are closely related to p., AY082447 (99%). The ability of the isolates to degrade diazinon highlights their potential to be used in bioremediation.

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Introduction

Pesticides are widely used in Kenya and the rest of Africa in agriculture, livestock development, and disease vectors control (Wandiga, 2001). Over 1000 pesticides have been marketed worldwide for chemical pest control (Charles and Barrie, 1983). This widespread use of pesticides in agriculture and public health has led to fears over the persistence of these toxic chemicals in the environment, bioaccumulation in the food chains and risks to non-target organisms (Atterby *et al.*, 2002). The pesticides enter the environment via losses that arise during production, transport and storage. Losses can also occur in the use of product, improper waste disposal and accidental spillage (Hallberg, 1989). Environmental contamination by pesticides can either be low level contamination from continued use of pesticides and remnant of persistent pesticides used in the past or heavy pollution of soil and surface or ground water in defined areas due to disposal or accidental releases of concentrated pesticide formulations (Attar by *et al.*, 2002).

Bioremediation is a pollution control technology that uses biological systems to catalyze the degradation and transformation of various toxic chemicals to less harmful forms. Microorganisms are utilized in many forms of bioremediation (Atlas and Unterman, 1999). In bioremediation process, heterotrophic microorganisms break down hazardous compounds to obtain chemical energy, hence hazardous compounds can serve as carbon, energy, and nutrient source for microbial growth (Tariq *et al.*, 2003).

Microorganisms, especially bacteria, have been shown to degrade a large number of xenobiotics. These are compounds that are foreign to biological systems. Such microorganisms have developed an impressive adaptive capacity for degrading synthetic compounds, even those that show little resemblance to preexisting templates among natural compounds (Alexander, 1985; Schink *et al.*, 1992; 2000). Over the past decades major advances in the understanding of the range and rates at which aromatic compound of varying degrees of complexity can be degraded in natural environments have been reported (Gibson and Harwood, 2002). A number of bacterial strains that degrade aromatic compounds in pure cultures have recently been isolated, thus opening the door for more detailed physiological, biochemical and molecular genetic studies (Gibson and Harwood, 2002).

The ability to mineralize chemical compounds has been demonstrated in termites belonging to the lower and higher groups (Brune *et al.*, 1995). The termite hindgut harbors symbiotic bacteria and protozoa, which have been implicated in this degradative process (Neuhauser *et al.*, 1978; Gibson and Subramanian, 1984). Moreover studies with a variety of soil invertebrates' show evidence those members of the gut microbiota are responsible for aromatic-ring mineralization (Neuhauser and Hartenstein, 1976; Neuhauser *et al.*, 1978). In tropical and sub-tropical regions termites play an important role in degradation of organic matter, where they are abundant termites have a remarkable effect on soil structure, nutrient cycle and growth of vegetation through movement of soil to build runways and nest, these activities translate into efficient conversion of large quantities of vegetation into fecal residues and termite tissues (Wood and Johnson, 1986). The termites possess specialized hindgut biota (Breznak, 1982; Boga, 2000), which enable them to degrade lignin (Slaytor1992). Due to resemblance of lignin to some xenobiotic compounds some of which persist in the environment such as catechol, dioxin, resorcinol and polycyclic aromatic hydrocarbon (Lijinsky, 1991), a question arises whether termites could be used to clean soils contaminated with xenobiotic compounds and pesticides. In addition Biodegradation has been observed to progress much faster in soils occupied by termites than soils without termites because of the litter harvesting and fungus cultivation by *Macrotermes* (Collins, 1981). Despite these apparent and significant roles played by soil feeding and fungus cultivating termite species, their contribution in degradation of xenobiotics compounds has received little attention. Therefore, this study is aimed at isolating characterizing and identifying bacterial

isolates capable of degrading pesticides from intestinal tract of fungus cultivating termites.

Materials and methods

Sample collection

Soil samples and termites were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) farm. Worker caste of fungus cultivating termite *Macrotermes michaelseni* was used. The termites were freshly collected from their mound and used for the homogenate preparation.

Enrichment and isolation of diazinon degrading bacteria from the soil

Soil sample (1g) was homogenized then inoculated in 25ml of MM7 culture broth containing the following compounds per liter NaCl (1.7g), KCl (6.5g), $Mgcl_2.6H_2O$ (0.5g), $CaCl_2.2H_2O$ (0.1g), NH_4Cl (5..6g), Na_2SO_4 (1.0g) and KH_2PO_4 (1.0g). Diazinon was added to the media (as a source of carbon) from a sterile stock solution to a final concentration of 0.5 mM. The cultures were incubated at 27^o C with constant agitation (100 rpm) and monitored for loss of the pesticide supplement and turbidity increase due to bacterial growth. The culture was then plated in 1.5% agar incorporated with pesticide (0.5 mM) in order to ascertain the different colonies of bacteria present. Individual colonies were then inoculated in fresh MM 7 media to ascertain their ability to degrade the pesticides (Yuste *et al.*, 2000).

Enrichment and isolation of diazinon degrading bacteria from the termite gut

Worker caste fungus cultivating termites were dissected using sterile fine-tipped forceps. Homogenate of 10 gut sections were made in sterile buffered salt solution (BSS) (Breznak and Switzer, 1986) using a glass tissue homogenizer and serially diluted (1:10). BSS contained the following compounds per liter; K_2HPO_4 (2.0 g), KH_2PO_4 (1.0 g), KCl (1.5 g) and NaCl (1.5 g). The homogenate was then inoculated in MM 7 supplemented with pesticide using the same procedure as the soil sample.

Analytical methods

The loss of diazinon from the media was monitored by high performance liquid chromatography (HPLC) (Shimadzu Class-VP) on a reverse phase C-18 column (250 by 4.6 mm) fitted with C-18 silica reverse phase guard column and equipped with a UV detector. A mobile phase of acetonitrile (75%) in demineralized water (25%) was used. Chromatography was carried out at an ambient temperature at a flow rate of 1.0 ml/min wavelength of 206 nm and run time of 10 minutes. The concentration of pesticide was calculated using standards.

Characterization of the isolates

Cell morphology of the isolates was determined using classical gram staining method (Bartholomew, 1992) and 3.0% (w/v) potassium hydroxide (KOH) test. Motility was passed by direct microscopic observation during growth in SIMS agar (pH 7.3) and by testing the ability of the isolates to migrate from the point of inoculation through semi solid (0.3%) agar plates (Ball *et al.,* 1996). Temperature and pH ranges and optima were determined in nutrient broth. The cultures were incubated at different temperature regimes and growth was measured after 48 hours with a Shimadzu model UV240 Spectrophotometer at 600 nm in curettes with 1-cm light path. Biochemical characterization of the isolate was carried out using the procedures of Cappuccino and Sherman (2002) and Atlas (1995).

Metabolic versatility

The ability of the isolates to utilize various selected substrates was tested using MM7 in screw cap tubes, at concentrations of 1% (w/v) for non-aromatic compounds and 1mM concentration for aromatic compounds. The compounds tested were, Ninhydrin, Fructose, Phenol, 2-4-Dimethylamino-benzadehyde, Salicylic acid, Mannose, 2, 6-dimethylphenol, Maltose and Resorcinol. The test media were inoculated with 0.05 ml of 48- hour old culture grown in nutrient broth and incubated at 30^oC (Murray *et al.*, 1984) in a shaker (100 rpm) for up to two weeks .

Phylogenetic analysis

The genomic DNA was extracted from exponential growth phase cells of the isolates grown aerobically in nutrient broth. Total DNA was extracted using Ultra-Clean Microbial DNA Isolation kit (Mo Bio Laboratories, Solana Beach, Calif) according to the manufacturer's instructions based on the method of Stach *et al.,* 2003. Purified total DNA from each isolate was used as a template for amplification of the16S rDNA genes. This was done using the HotStar Taq Master Mix Kit (Qiagen, USA) according to the manufacturer's instructions. Nearly full-length 16S rRNA gene sequences were PCR-amplified using primers 27F (forward 5'-TAG AGT TTG ATC CTG GCT CAG-3') and 1392R (reverse, 5'-GAC GGG CGG TGT GTA CA-3') (Sigma) according to the position in relation to *Escherichia coli* gene sequence (Embley and Stackebrandt, 1994). PCR (35 cycles) was performed with a model PTC-100 thermal cycler (MJ research inc., USA).

The sequences were compared to sequences in the public database with Blast search program on the National Center for Biotechnology Information (NCBI) website (http:/www.ncbi.nlm.nih.gov) to find closely related bacterial 16S rRNA sequences. The ARB software package (Ludwig and Struck, 1996) was used to align the sequences. Alignment were checked and corrected manually where necessary. Highly variable regions of the 16s rRNA gene sequences and sequence position with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences. Phylogenetic trees were calculated according to the neighbour joining method (Saitou and Nei, 1987) and maximum-likelihood (Felsenstein, 1981), and visualized using tree view of the same software. Sequence similarity matrices were calculated.

Results

Enrichment experiments with Termite guts and soil

Two independent enrichment procedures were performed using soil and gut homogenate of fungus cultivating termites *Macrotermes michaelseni* using diazinon as the only source of carbon and energy in each of the enrichment cultures. Transformation of diazinon in the cultures was then assessed using HPLC. Diazinon was degraded in the soil enrichment culture (71.77%) while in the termite gut enrichment culture 75.80% of diazinon was degraded after nine days of incubation (Fig.1.). After nine days the concentration of diazinon remained constant. In the control experiment without inoculum diazinon was not degraded (Fig.1.) and remained constant during the fourteen days of incubation.

Enrichment and isolation of diazinon degrading bacteria from the termite gut

There were nine bacterial isolates were obtained from the enrichment cultures in MM 7 media with diazinon as the sole source of carbon and energy out of these five were from the soil and were designated DS_1, DS_2, DS_3, DS_4 and DS_5, while from termite guts there were four isolates, which were designated DT_1, DT_2, DT_3 and DT_4. However out of the nine isolates only four were able to degrade diazinon. The percentage of diazinon degraded by DS_2, DS_3, DS_5 and DT_2 was 73.89% 78.85% 91.48% and 79.54% respectively after 9 days of incubation (Fig.2.). After nine days the concentration of diazinon remained constant (Fig.2.). After 9 days of incubation the concentration of Diazinon in the media remained constant.

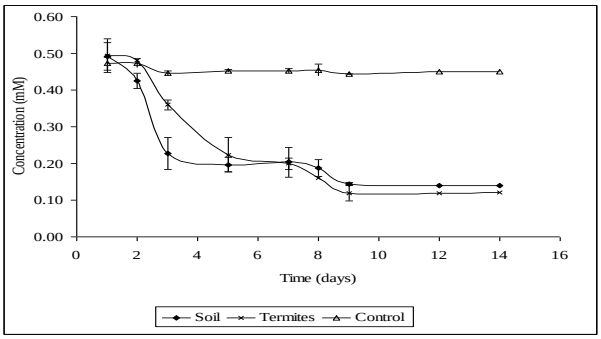

Fig. 1. Degradation of Diazinon in enrichment culture.

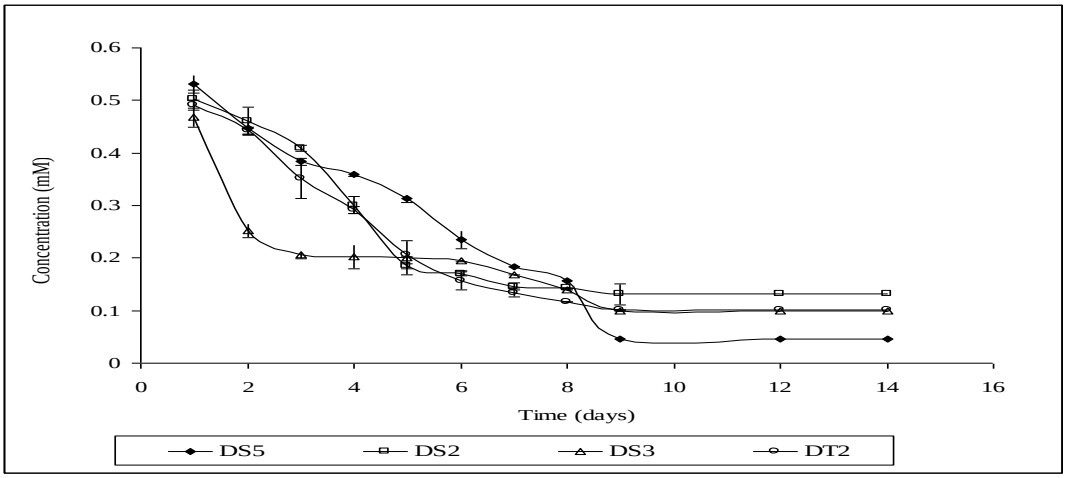

Fig.2. Degradation of Diazinon by isolates DS_2, DS_3, DS_5 and DT_2 in MM7 media with 0.5 mM Diazinon.

Characterization of the isolates

The isolates were characterized using morphological and phenotypic characteristics (Table 1.). The colonies formed on nutrient agar were cream colored opaque slightly raised with smooth margins. Gram stain and 3% KOH test revealed that the cells were gram negative and rod shaped. The isolate grew slowly on Enriched media, visible growth was observed after 3 days. Growth on nutrient agar or nutrient broth takes 36- 48 hours. The isolates grew at temperature range of 10°C to 40 °C with the optimum growth at 30 °C for DS_3 and DT_2 and 37 °C for DS_5 and DS_2. The pH range for growth was pH 5 to pH 9 with DS_2 while DS_3, DS_5 and DT_2 had optimum growth at pH 6

Table 1. Biochemical characteristics of isolates DS_2, DS_3, DS_5 and DT_2 .

Characteristics	**Isolate**			
	DT_2	**DS_2,**	**DS_3,**	**DS_5**
Gram stain	Negative	Negative	Negative	Negative
Cell shape	Rod	Rod	Rod	Rod
Motility	Negative	Negative	Negative	Negative
Carbohydrate fermentation				
Lactose	Positive	Negative	Positive	Positive
Glucose	Positive	Negative	Positive	Positive
Sucrose	Positive	Negative	Positive	Positive
Catalase	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Negative
Citrate	Positive	Positive	Positive	Positive
MR reaction	Positive	Negative	Positive	Positive
VP reaction	Positive	Negative	Negative	Negative
Indole	Positive	Negative	Negative	Negative
Nitrate reduction	Positive	Positive	Positive	Positive

Urease	Positive	Negative	Positive	Positive
Starch Hydrolysis	Negative	Negative	Negative	Negative
Gelatin liquefaction	Negative	Negative	Negative	Negative
H_2S production	Positive	Positive	Negative	Negative

Metabolic versatility

The ability of the isolates to mineralize and transform a variety of aromatic and non- aromatic compounds under oxic conditions was tested. all the isolates could grow on resorcinol and fructose whereas only isolate DT_2 grew on phenol and only DS_2 did not grow on maltose. No isolate grew on ninhydrin 2, 4-Dimethylamino-benzadehyde, Salicylic acid, Mannose and 2,6-dimethylphenol.

Phylogenetic analysis

The 16S rRNA gene sequence of the isolates revealed that isolates DT_2 is closely related to *Klebsiella oxytoca* AB 504754 with 99% sequence similarity, isolates DS_3 and DS5, clustered with E*nterobacter sp* AY 0822447 with 99% sequence similarity. Isolate DS_2 clustered with *Achromobacter xylosidans* AF 531768 with 100% s sequence similarity (Fig. 3.)

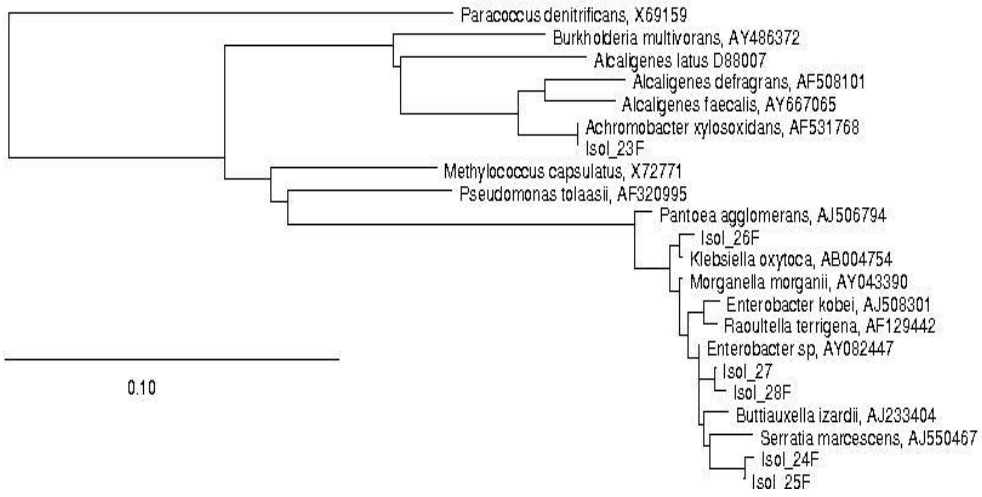

Fig. 3. Maximum likelihood phylogenetic tree showing position of the isolates, DS_2 (isol-23F), DS_3 (isol-24F), DS_5 (isol-25F) and DT_2 (isol-26F), The gene bank accession number of the 16S r RNA sequence of each reference species is shown at the end of the species name. The bar indicates the estimated substitution per nucleotide position.

Discussion

The microbial world is characterized by an incredible metabolic and physiological versatility that permits microorganisms to inhabit hostile ecological niches and to exploit, as carbon and energy sources, compounds unpalatable for higher organisms (Timmis *et al.,* 1994). A number of studies have demonstrated bacterial utilization of aromatic compounds that comprise xenobiotics. To this point however, there have been few studies involving utilization of aromatic compounds by individual bacteria species isolated from gut of fungus cultivating termites (Brune *et al.*, 1995). In this study bacteria that are able to degrade diazinon were isolated from the soil and intestinal tract of fungus cultivating termites.

The phylogenetic analysis of the isolate DS_3, and DS_5, shows that the isolates are closely related to members of the genus *Enterobacter* with 99% 16S rRNA sequence similarity. *Enterobacters* are described as gram negative, citrate positive, indole negative and TSI positive with gas. The species of the genus *Enterobacter* are ubiquitous in nature occurring in fresh water, soil, sewage, plants, vegetables and animals and human feaces. Several species have including *E.aerogenes*, *E. cloacae*, *E. sakazaki pantoea* (formerly *Enterobacter*) *agglomerans* and *E.gergoviae,* are notable opportunistic pathogens (Farmer, 1995). Isolates DS_3 and DS_5, displays the typical characteristics and biochemical properties of members of the genus *Enterobacte,* they were able to utilize wide range of carbohydrate substrates. This characteristic is shared by nearly all members of the family *Enterobacteriaceae* (Holt *et al.*, 1994), and is consistent with their wide distribution in most environmental compartments.

The metabolic capacity of the members of the genus *Enterobacter* has been explored, *E. cloacae* strain PB2 (Binks *et al.*, 1996) was isolated from a munitions plant in UK by its ability to utilize nitrate esters explosives as a sole nitrogen source in growth. There is also wealth of information on aromatic compounds degradation by *Escherichia coli* as reviewed by Diaz *et al.,* (2001). These investigations highlight various aromatic compounds utilized by *E. coli* such as hydroxicinnamic acid, phenyl acetate, styrene, benzoate and aromatic hydrocarbons. Other members of *Enterobactericeae* like *Enterobacter* (formerly *Klebsiella) aerogenes* have also been reported to degrade aromatic compounds (Grant 1967).

Klebsiella can be described as gram-negative, non-sporing, oxidase-negative, rod-shaped bacteria, capable of aerobic and facultative anaerobic growth in the presence of bile salts of other surface active agents with similar growth-inhibiting properties. They are able to ferment lactose, with the production of acid and gas within 48 hours at 35–37°C. (Grimond *et al.*, 1991). Isolate DT_2 adhere to all the signature phenotypic characteristics of *Klebsiella oxytoca* including the fact that it is a facultative gram negative anaerobe, ferment lactose with production of acid, Indole positive, oxidase negative and grows in laboratory media at temperatures between 15°C-40°C.

Bacteria of the genus *Klebsiella* are widely distributed in nature, in the soil and in water. They are also part of the normal flora of the intestinal tract, but usually in low numbers compared with E. coli. *Klebsiella* are opportunistic pathogens that can cause pneumonia, urinary tract infections, and bacteremia. A number of studies have shown that *K. oxytoca* is involved in degradation of hydrocarbons. For instance 2,4-dichlorophenol-degrading strain, *Klebsiella oxytoca*, was isolated from phenol-activated sludge in Tianjin (Qiang *et al.*, 2005). The cyanide-degrading *Klebsiella oxytoca* was isolated from the waste water of a metal-plating plant. The *K. oxytoca* was capable of utilizing tetracyanonickelate as its sole nitrogen source (Lin, 2001).

Phylogenetic position of isolate DS_2 showed that it is closely related with *Achromobacter xylosoxidans* with 100% sequence similarity. *A. xylosoxidans* is an aerobic, catalase and oxidase positive, motile, non-lactose-fermenting gram-negative rod that oxidizes xylose and glucose. This organism was briefly classified as genus *Alcaligenes* but was recently reclassified as *Achromobacter* (Yabuuchi *et al* 1998). The organism exists in a water environment and may be confused with *Pseudomonas* species. Unlike *Pseudomonas, Achromobacter* has peritrichous flagella (Igra-Siegman *et al.*, 1980). *Achromobacter xylosoxidans* is also multi-drug resistant (Bizet, 1995). Physical and biochemical characteristics of isolate DS_2 shows that it is related to *A. xylosoxidans*.

Members of the genus *Achromobacter* that are ubiquitous in soil are capable of degrading a wide variety of naturally occurring aromatic and aliphatic compounds. Several studies have shown that a variety of synthetic compounds such as pesticides are

degraded by members of the genus *Achromobacter* (Karns *et al.,* 1986). *Achromobacter* have also been shown to degrade aromatic compound for instance *Achromobacter* strain WM 111 can degrade carbofuran (Topp *et al.*, 1993). The *Achromobacter* strain (DT_2) isolated in this study was able to degrade up to 79.53% of Diazinon in the media. The strain was isolated from termite gut an indication that the fungus cultivating termite harbours diazinon degrading organisms' termite.

The cleavage of the phosphorus ester bond, leading directly, or via diazoxon, to the pyrimidyl metabolite plays the major role in the metabolism of diazinon. Sethunathan and Yoshida, (1973) Isolated a *Flavobacterium* sp. ATCC 27551 from paddy water samples that had been previously treated with diazinon, *Flavobacterium* showed an exceptional capacity to hydrolyse diazinon and then metabolize its hydrolysis product, 2-isopropyl-4-methyl-6-hydroxypyrimidine to CO_2. Balthazor and Hallas, (1986) also isolated Diazinon-degrading *Flavobacterium* sp. and *Pseudomona aeruginosa.*

In Conclusion the isolation of pesticide degrading bacteria from the gut of fungus cultivating termites and the soil shows that they harbor pesticide degrading bacteria which are potentially useful in industrial and environmental biotechnology. The results of this study highlight the potential of these bacteria to be used in the cleanup of pesticide contaminated environment. Use of bacteria from soil and termite guts in bioremediation will provide an environmentally safe and affordable means for rapidly and effectively eliminating a variety of hydrocarbon contaminants from the environment.

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