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Isolation, characterization and identification of roundup degrading bacteria from the soil and gut of *Macrotermes michaelseni*

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Abstract

Roundup degrading bacteria were isolated from the soil and gut of fungus cultivating termites *Macrotermes michaelseni* using MM7 media supplemented with the pesticide as the sole source of carbon and energy. The isolates were gram negative rods the isolate from the soil was designated GS_1 while that from termite gut was designated GT_2. The 16S rRNA gene sequence of the isolates revealed that isolates Both isolates GS_1 and GT_2 are closely related to E*nterobacter sp* AY 0822447 with 99% sequence similarity. The results of this study highlight the potential of these bacterial isolates to be used in bioremediation.

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Introduction

The widespread use and disposal of pesticides by farmers, large plantations and the general public causes environmental contamination. Following release into the environment, pesticides create different problems. Pesticides which are sprayed can become airborne and may eventually end up in soil or water. Pesticides applied directly to the soil may be washed off the soil into water or may percolate through the soil to lower soil layers and groundwater. These chemical pesticides may have a variety of adverse ecological effects which include acute illness in human, fish and wildlife kills, reproductive failure in birds and forest decline. Ecological effects can be long-term or short-lived changes in the normal functioning of an ecosystem, resulting in economic, social, and aesthetic losses. Therefore there is need to device a way of removing the pesticides residues from the contaminated environment or of degrading the pesticides before it reaches underground water.

Interest in the biodegradation and environmental fate of xenobotics is prompted by their ubiquitous distribution and their potential deleterious effect on human health (Kanaly *et al.*, 2000). Understanding of the processes that affect the fate of organic contaminants including abiotic transformation and biotransformation is needed in order to make sound decisions about the hazards to the public and environment so that we can develop suitable mitigation techniques (Hardman, 1987; Atlas and Pramer, 1990).

Although bacteria have evolved mechanisms to degrade complex natural compounds like lignin and humic material, there are certain chemicals that were absent or rare in the environment before their manufacture and use as pesticide or industrial chemicals (Alexander, 1985). The introduction of these chemicals has lead to microbial adaptation to the new and unusual substrates as illustrated by various studies on the decomposition of xenobiotics (Eatton and Timmis, 1983; Stewart and Carlson, 1986; Trevor *et al.*, 1987; Schink *et al.*, 1992).

Isolation and characterization of organisms that can degrade xenobiotics could help in developing microbial strains that could attack persistent pollutants more aggressively. Since the genes encoding catabolic pathways are often clustered and plasmid borne (Chaudhry and Chapalamuguda, 1991), these clusters can be cloned and if they are readily expressed in the new host, then microorganisms with a broader substrate spectrum can be developed.

Termites are considered as ecosystem engineers because of their numerical and ecological significance (Brune and Fredrich, 2000). However most studies on termite gut microbiota have focused on wood feeders, anolagous studies on other feeding guilds, especially soil feeders and fungus cultivators remain few (Kane *et al.*, 2001). This is due to their typically remote habitat, delicate nature and difficulty in establishing permanent cultures (Rouland *et al.*, 1993). Species of the genus *macrotemes* that construct large epigeal nests and extensive underground gallery systems have major effect on the soil chemical and physical properties throughout the tropics and subtropics. An improved understanding of the termite gut microbial flora in the degradation of organic compounds especially xenobiotic in the tropical forests, savannah and wastelands is therefore absolutely essential (Brune *et al.,* 1995).

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 Roundup 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).

Roundup was added to the media (as a source of carbon) from a sterile stock solution to a final concentration of 1 mM. The cultures were incubated at 27° C with constant agitation (100 rpm) and monitored for loss of the pesticide supplement and turbidity increase due to bacterial growth. The culture were then plated in 1.5% agar incorporated with pesticide (1 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 Roundup 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 roundup from the media was monitored by High Performance Liquid Chromatography (HPLC) (Shimadzu Class-VP) on a reverse phase C-18 with a mobile phase of acetonitrile (80%) and 0.05% H_3PO_4 in water (20%). 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 commercial standards (Monsanto's round up 480g/l).

Growth curves

The bacterial growth curve for each isolate was determined using MM 7 broth culture with 1 mM of pesticide using the method described by Cappuccino and Sherman (2002).

A 500 ml conical flask containing 250 ml media supplemented with 1 mM of pesticide was inoculated with 5 ml of 24h inoculum. The initial $OD._{600}$ (at t_0) was recorded for the log phase culture and from these serial dilutions were made. Each culture was then placed in a shaker incubator (100 rpm) at 30°C. An aliquot (4 ml) of each culture was aseptically transferred to a cuvette and its OD determined after regular intervals of incubation.

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 assed 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 30ºC (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 carried out using soil and gut homogenate of fungus cultivating termite *Macrotermes michaelseni* using roundup as the only source of carbon and energy in each of the enrichment cultures.

Degradation of roundup was monitored using HPLC. Up to 87.60% of roundup was degraded in the soil enrichment culture while in the termite gut enrichment culture 86.26% of roundup was degraded after nine days of incubation (Fig. 1). Concentration of round up in the media remained the same after the ninth day. In the control experiment without inoculum roundup was not degraded.

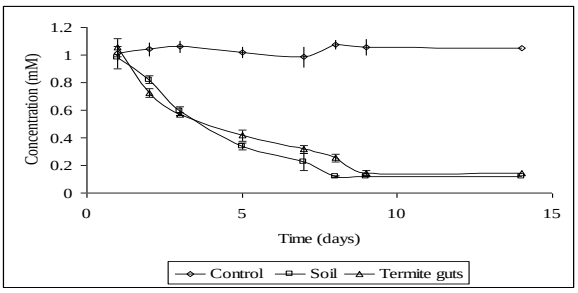

Fig.1. Degradation of Roundup in enrichment culture.

Isolation of pure cultures and degradation of Roundup by pure cultures

Four isolates were obtained from the enrichments with roundup; these isolates were named GS_1 and GS_2 from the soil and, GT_1, and GT_2 from the termites. Out of these four isolates only GS_1 and GT_2 were capable of degrading roundup, GS_1 could degrade 92.01% of roundup while GT2 could degrade 87.60% of roundup after 9 days of incubation (Fig. 2).

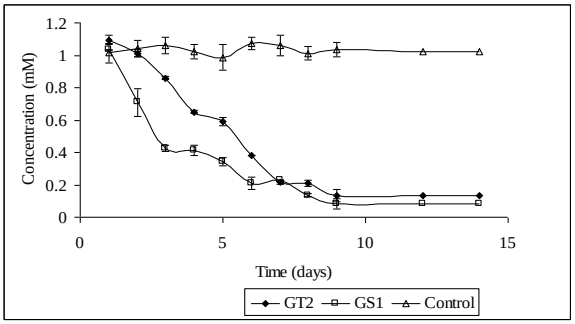

Fig. 2. Degradation of round up by isolates GT_2 and GS_1 in MM7 media with 1 mM roundup.

Growth of isolates on roundup

The two isolates GS_1 and GT_2 showed a slight difference in the rate of growth in roundup (Figure 3 and 4).

For isolate GS_1 maximum cell biomass (OD_{600}) was 0.317 and was obtained after 7 days as whereas for GT_2 maximum cell biomass (OD_{600}) was 0.305 and was obtained after 6 days, within the same time course and under the same conditions of growth (Fig. 3 and 4). In both the disappearance of Roundup corresponded with the increase in cell biomass. The doubling time for each isolate was determined using direct growth curve method. Both isolates GS_1 and GT_2 had a doubling time of approximately 3 days.

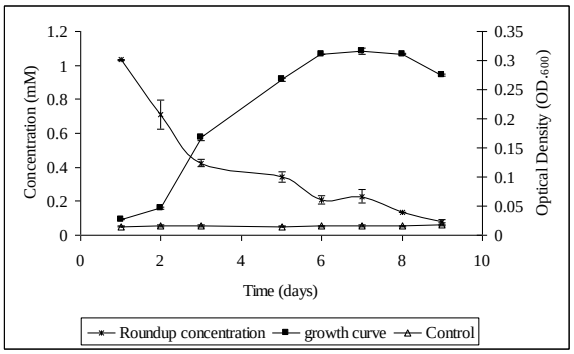

Fig. 3. Growth of isolate GS_1 on roundup. Growth was performed aerobically with 1 mM roundup in MM7 medium at 30°C.

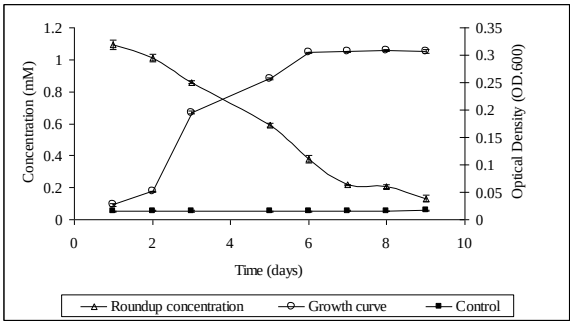

Fig. 4. Growth of isolate GT_2 on roundup. Growth was performed aerobically with 1 mM roundup in MM7 medium at 30°C.

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. The pH range for growth was pH 5 to pH 9 with optimum growth at pH 6

Table 1. Biochemical characteristics of isolates GS_1 and GT_2.

Characteristics	**Isolates**	
	GS_1	**GT_2**
Gram stain	Negative	Negative
Cell shape	Rod	Rod
Motility	Negative	Negative
Carbohydrate fermentation		
Lactose	Positive	Positive
Glucose	Positive	Positive
Sucrose	Positive	Positive
Catalase	Positive	Positive
Oxidase	Negative	Negative
Citrate	Positive	Positive
MR reaction	Positive	Positive
VP reaction	Negative	Negative
Indole	Negative	Negative
Nitrate reduction	Positive	Negative
Urease	Negative	Positive
Starch Hydrolysis	Negative	Negative
Gelatin liquefaction	Negative	Negative
H_2S production	Negative	Negative

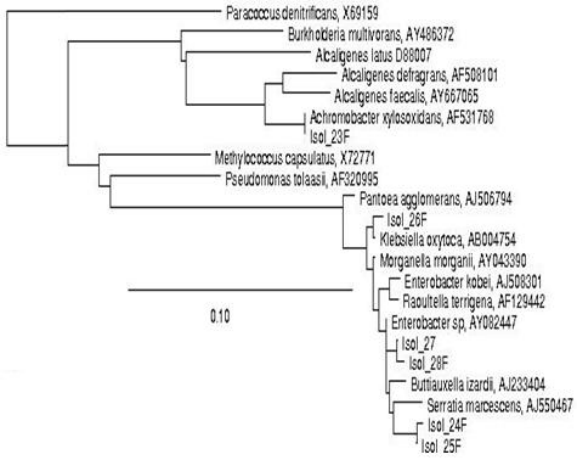

Fig. 5. Maximum likelihood phylogenetic tree showing position of the isolates, GT_2 (isol-27F) and GS_1 (isol-28). The gene bank accession number of the 16S r RNA sequence of each reference species is shown at the end of the species name. The bar indicates the estimated substitution per nucleotide position.

Metabolic versatility

The ability of the isolates to mineralize and transform a variety of aromatic and non-aromatic compounds under oxic conditions was tested. Both the isolates GS_1 and GT_2 could grow on resorcinol, maltose and fructose. The isolates did not grow g on phenol 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 Both isolates GS_1 and GT_2 are closely related to E*nterobacter sp* AY 0822447 with 99% sequence similarity (Figure 5).

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 GS_1 and GT_2 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 GS_1 and GT_2 displays the typical characteristics and biochemical properties of members of the genus *Enterobacter,* 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 it 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).

Previous Studies on degradation of roundup shows that roundup (glyphosate) is degraded in soil by bacteria which can use glyphosate as the sole nitrogen source. N-methyla minomethylphosphonic acid (AMPA) has been detected in some soils as the only significant metabolite (Lerbs, 1990). Complete degradation goes through glycine and sarcosine with an oxidation of the phosphonate to phosphate (Figure 13) (Kishore and Jacob, 1987). Some *Alcaligenes* spp stop with phosphate and sacrosine, using only the phosphorus and not the carbon if other carbon sources available. A *Flavobacterium* sp isolated (Lerbs, 1990) from a sewage treatment plant does the same even in the presence of inorganic phosphate. With other strains the presence of inorganic phosphate in the media may suppress the oxidation of glyphosate (Lerbs, 1990).

Conclusion

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 clean up 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.

References

Alexander M. 1985. Biodegradation of organic chemicals. Environmental Science and Technology **18,** 106-111.

Binks PR, French CE, Nicklin S, Bruce NC. 1996. Degradation of pentaerythritol by Enterobacter cloacae PB2, Applied and Environmental Microbiology **62**, 1214-1219.

Breznak JA, Switzer JM. 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Applied and Environmental Microbiology **52**, 623-630.

Brune A, Emersion D, Breznak A. 1995. The termite gut Microflora as an oxygen sink: microelectrode determination of oxygen and pH gradient in the guts of lower and higher termites. Applied Microbiology **61**, 2861-2687.

Brune A, Fredrich M. 2000. Micro ecology of termite gut: Structure and function on a micro scale. Curr.opin.Microbiol **3,** 263-269.

Cappucino JG, Sherman N. 2002. Microbiology A Laboratory Manual 6th Edition Pearson Education, Inc, Sanfransisco, C A.

Chaudhry RG, Chapalamaguda S. 1991. Biodegradation of halogenated organic compounds. America Society of Microbiology **55,** 59-79.

Diaz E, Ferrandez A, Prieto MA, **Garcia JL.** 2001. Biodegradation of aromatic compounds by *Escherichia coli.* Microbiology. Molecular biology annual review **65**, 523-569.

Eatton RW, **Timmis KN .**1983. Genetics of xenobiotic degradation. In Klug MJ, Reddy CA, (Ed.) Current perspectives in microbial ecology. American Society of Microbiology, Washington, DC. pp. 694-703.

Embley TM, Stackebrandt E. 1994. The molecular phylogeny and systematics of actinomycetes. Annual Review of Microbiology **48**, 257-289.

Farmer JT. 1995. Enterobactericeae: introduction and identification In: Manual of clinical microbiology (6th Edition) (Murray PR, Baron EJ, Pfaller MA, Tenover FC and Yolken RH Editors), American society of Microbiology, Washington DC. p. 435-450.

Felsenstein J. 1981. Evolutionary trees for DNA sequences: a maximum likelihood approach. Journal of Molecular Evolution **17,** 368-376.

Grant DJW. 1967. Kinetic aspects of growth of *Klebsiella aerogenes* with some benzoid carbon source. Journal of General Microbiology **46**, 213-224.

Hardman DJ. 1987. Microbial control of environmental pollution: the use of genetic techniques to engineer organisms with novel catabolic capabilities. pp. 295-317, In. Environmental biotechnology, Forste CF and Wase DAJ (Eds.) Ellis Hardwood, Chichester,Uk.

Kanaly RA, Bartha R, Watanabe K, **Harayama S.** 2000. Rapid mineralization of Benzo (a) pyrene by microbial consortium growing on diesel fuel. Applied Microbiology **66,** 4205-4211.

Kane DM, Dore J, Eggleton P, Bignell, Breznak A, Brauman A. 2001. Molecular phylogenetic profiling of prokaryotic communities in the gut of termites with different feeding habits. Federation of European Microbiology Society, Microbiology Ecology **35**, 27-36.

Lerbs W, Stock M, Parthier B. 1990. Physiological aspects of glyphosate degradation in *Alcaligenes* species strain GL. Archives of Microbiology **153**. p. 146-150.

Murray DW, Sowden LC, **Calvin RJ.** 1984. *Bacteroides cellusolven* sp. nov. a cellolytic species from sewage sludge. International Journal of Systematic bacteriology **34.** 2. 185-187.

Schink B, Brune A, Schenell S. 1992. Anaerobic degradation of aromatic compounds In Winkelman G (Ed) Microbial degradation of organic compounds VCH, Wienhein Germany.P. 220-242.

Stach EM, Maldonado AL, Masson GD, Ward CA, Goodfellow M, Bull TA. 2003. Statistical approaches for estimating actinobacterial diversity in marine sediment. Applied and Environmental Microbiology **69**, 6189-6200.

Trevor JT, Barkery T, Bourquin AV. 1987. Gene transfer among bacteria in soil and aquatic environments: a review. Canada Journal of Microbiology **33**, 191-198.

Yuste L, Corbella E, Turiegaro M, Karlso U, Puyet A, Rojo F. 2000. Characterization of bacterial strains able to grow on high molecular mass residue from the crude oil processing. Federation of European Microbiology Society Microbiology and Ecology **32**, 69- 75