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Plasmid Mediated Degradation of Diazinon by Three Bacterial Strains *Pseudomonas* sp., *Flavobacterium* sp. and *Agrobacterium* sp.

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Three microorganisms, which utilize diazinon as a sole source of carbon, phosphorus and energy for growth, were isolated from an enrichment culture. The microorganisms were identified as *Pseudomonas* sp., *Agrobacterium* sp. and *Flavobacterium* sp. The bacterial strains were found to harbour three endogenous plasmids. Two of the plasmids were lost when the microorganisms were treated with ethidium. Loss of the plasmids in the organisms was correlated with the loss of the ability to metabolize diazinon. Phenotypic testing of the wild type and cured strains revealed that the gene(s) responsible for diazinon degradation may reside upon the plasmids.

Key Words: Plasmid, Diazinon degradation, *Pseudomonas* sp., *Agrobacterium* sp., *Flavobacterium* sp.

INTRODUCTION

In a non-polluted environment, bacteria, fungi, protists and other microorganisms are constantly at work breaking down organic matter. It is a matter of research to investigate. What would happen if an organic pollutant such as dye, pesticide, plasticizer, crude oil or aromatic compound contaminated this environment. Some of the microorganisms would die. The behaviour of pesticides in soils has already been reported[1, 2].

While some organisms are capable to degrade pollutant compounds, soil and water bacteria in general and members of the genus *Pseudomonas* in particular, able to degrade and use as sources of carbon and energy[3, 4]. A wide range of organic compounds including some that are quite noxious and otherwise, biocide (*e.g.* pesticides) and these exotic properties are not found in commensal and parasitic bacteria, such as *Escherichia coli*[5].

The degradative plasmids endow on their hosts the ability to utilize rather uncommon organic compounds as their carbon and energy sources[6–11]. Because many such compounds are toxic to the microorganisms, the presence and expression of such plasmids allow the host cells to quickly reduce the toxic concentration of these compounds[12].

Ecologically, plasmid-encoded pathways are advantageous because they provide genetically flexible systems and can be maintained in the population and transferred between bacteria species[13]. There are many papers dealing with pesticides (including insecticides) degradation in soil and metabolism by microorganisms[14–19].

Direct insecticide additions to water for purposes of pest control and discharges as industrial wastes are in most cases carefully regulated; however, in our geographical area, two types of operations, rice farming and mosquito control, make it likely that insecticides will reach surface waters in repeated doses during relatively long periods of time each year. In mass applications of insecticide, as in mosquito control, the chemical may reach surface water directly or adsorb on particulate matter from which it may move to surface waters in agricultural or urban run-off[3, 4, 20–22].

Organophosphours insecticides are widely used in agriculture, despite their biodegradation nature, some are highly toxic and their residues are found in the environment there are some paper dealing with degradation of methyl parathion by and some insecticide bacteria[7, 16, 18–21].

EXPERIMENTAL

Isolation of diazinon degrading microorganisms from enrichment culture, soil (0.5 g), river water (1 mL) and sewage (1 mL) were added to individual 250 mL Erlenmeyer flasks each containing 100 mL of sterile mineral salt (MS) with the following ingredients: KNO_3 (2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), NaCl (0.1 g), $FeCl_3 \cdot 6H_2O$ (0.01 g) and 1 mL of trace elements, $MnCl_2 \cdot 4H_2O$ (100 mg), $CoCl_2$ (20 mg), $CuSO_4$ (10 mg), $Na_2MoO_4 \cdot 2H_2O$ (10 mg), $ZnCl_2$ (20 mg), LiCl (5 mg), $SnCl_2 \cdot 2H_2O$ (5 mg), H_3BO_3 (10 mg), KBr (20 mg), $BaCl_2$ (5 mg) and EDTA-Na-Fe^{3+} (8 mg)[23]. The pH was adjusted to 7.4 with NaOH and standard diazinon (75 mg L^{-1}) was added. Control flasks without an inoculum were also prepared to take account of any abiotic disapearance of diazinon. This primary enrichment culture was incubated several days at 30°C in a shaker incubator at 150 rpm. Numerous attempts have been made to obtain single colonies of diazinon degrading bacteria. Microorganisms capable of degrading diazinon isolated from enrichment culture were streaked on to agar solidified medium containing mineral salt and diazinon concentration (75 mg L^{-1}), when the plates were incubated at 30°C, small pure culture of diazinon degrading bacteria was achieved by repeated transfer from agar plate to agar plate and agar to liquid cultures media both containing diazinon (75 mg L^{-1}).

Identification of diazinon degrading microorganisms: For the identification of diazinon degrading microorganisms, microscopic observation and growth characteristics as well as biochemical tests and fermentative characteristics were studied. The microorganisms were identified according to Bergey's Manual of Systematic Bacteriology[24].

Plasmid extraction: The plasmid DNA of the microorganisms was isolated according to Maniatis *et al.*[25]

Plasmid curing: Methods were taken from the review on plasmid curing by Trevors[26]. Curing agents used were ethidium bromide, acriflavin and sodium dodecyl sulfate (SDS). In addition, repeated subculture in nutrient broth in the absence of appropriate carbon source was used as a curing strategy.

RESULTS AND DISCUSSION

Isolation of the diazinon degrading bacteria: By selective enrichment culture for growth on diazinon as the only source of carbon, phosphorus and energy, three bacterial strains were isolated.

Identification of the bacterial strains: Microscopic observation and growth characteristics as well as biochemical tests and fermentative characteristics of the isolated bacterial strains 1, 2 and 3 were studied and are represented in Tables 1(a), 1(b), 2(a), 2(b) and 3(a), 3(b) respectively. From the results of the different tests[24] shown in Tables 1(a) and 1(b), 2(a) and 2(b), 3(a) and 3(b), the isolated bacteria seem to be *Pseudomonas* sp., *Flavobacterium* sp. and *Agrobacterium* sp. respectively.

TABLE-1(a)
MICROSCOPIC OBSERVATION AND GROWTH CHARACTERISTICS OF THE ISOLATED BACTERIAL STRAIN 1

	Tests	Obeservations
Microscopic examination	I. Simple staining	Rod shaped
	II. Gram's staining	Gram-negative
	III. Flagella staining	Lophotrichous
Growth characteristics	I. Agar colony	Abundant, moist, creamy
	II. Agar slant	Luxuriant, moist, creamy, spreading growth
	III. Nutrient Broth	Turbid, becomes greenish
	IV. Growth on semi-solid medium	Motile
	V. Growth at 4°C	Growth

TABLE-1(b)
BIOCHEMICAL AND FERMENTATIVE TESTS OF THE ISOLATED BACTERIA STRAIN 1

Test	Result
Catalase	Positive
Gelatin liquefaction	Positive
Test of nitrate	Positive
Test of ammonia production	Positive
Hydrolysis of starch	Starch hydrolysis weak
Fermentation test	No acid from glucose, starch, lactose,sucrose, maltose, glycerol or mannitol
Malonate	Positive
O.F. (oxidation-fermentation) test	Oxidation
Poly β-hydroxy butyric acid (P.H.B.)	Positive

Plasmid content of wild type and cured bacterial strains: To determine the possible role of plasmid DNA in the metabolism of diazinon, the plasmid content of wild type and cured strains was examined. The wild type strains of the three bacteria contained three DNA bands of plasmid. The cured strains of the

three bacteria were found to have lost two bands of plasmid DNA. The loss of the two bands in the cured strain proved that the bands found on the gel belong to the DNA carrying the gene(s) responsible for diazinon degradation. Thus, the loss of two bands with the treatment of ethidium bromide (200 mg mL^{-1}) suggested that there was strong correlation between the inability to metabolize diazinon with the lost DNA bands. This result strongly suggests that the gene(s) responsible for the ability to metabolize diazinon might be the plasmid DNA.

TABLE-2(a)
MICROSCOPIC OBSERVATION AND GROWTH CHARACTERISTICS OF THE ISOLATED BACTERIAL STRAINS 2

	Tests	Obesrvation
Microscopic examination	I. Simple staining	Rod-shaped
	II. Gram's staining	Gram-negative
	III. Flagella staining	No flagella
Growth characteristics	I. Agar colony	Spherical, colony with smooth margin, shiny
	II. Pigment on nutrient agar	Negative
	III. Nutrient Broth	Turbid
	IV. Growth on semisolid medium	Nonmotile
	V. Fluorescent pigment	Negative
	VI. Growth on MacConkey agar	Positive
	VII. Growth at 4°C	No growth

TABLE-2(b)
BIOCHEMICAL AND FERMENTATIVE TESTS OF THE ISOLATED BACTERIA STRAIN 2

Tests	Result
Oxidase	Positive
Catalase	Positive
Phosphatase	Positive
Citrate	Negative
Hydrolysis of starch	Negative
Test of nitrate	Negative
Gelatin liquefaction	Positive
Urease	Positive
Lipase	Positive
Casein	Positive
Cellulase	Positive
DNAse	Positive
Fermentation test	No acid from glucose, lactose, fructose, inositol, sorbitol, adonitol, dulcitol
Oxidation-Fermentation test (O.F.)	Oxidative
Poly, β-hydroxbutyric acid (P.H.B)	Negative

TABLE-3(a)
MICROSCOPIC OBSERVATION AND GROWTH CHARACTERISTICS OF THE ISOLATED BACTERIAL STRAINS 3

Tests		Observation
Microscopic examination	I. Simple staining	Rod-shaped
	II. Gram's staining	Gram-negative
	III. Flagella staining	Peritrichous
Growth characteristics	I. Growth on nutrient agar	Smooth, convex colony
	II. Pigmentation on nutrient agar	Colourless
	III. Growth on semi-solid medium	Motile
	IV. Growth at 4°C	Negative

TABLE-3(b)
BIOCHMICAL AND FERMENTATIVE TESTS OF THE ISOLATED BACTERIA STRAIN 3

Tests	Result
Oxidases	Positive
Catalase	Positive
Citrate	Negative
Hydrolysis of starch	Negative
Gelatin liquefaction	Negative
Hydrolysis of casein	Negative
Cellulase	Negative
Hydrogen sulfide (T.S.I .A)	Negative
Urease	Negative
Acid production from	D-glucose, D-fructose, L-arabionose, D-xylose, Adonitol, Lactose, Maltose, Sucrose
Gas from glucose	Negative

In this study, microorganisms capable of using diazinon as their sole source of carbon, energy and phosphorus were isolated from the enrichment cultures. Pure culture of diazinon degrading bacteria was isolated by plating out on mineral salt agar medium containing diazinon. When the morpholoical, physiological and biochemical tests were done, three microorganisms were found; all of them were Gram-negative, rod-shaped, identified as *Pseudomonas* sp., *Flavobacterium* sp. and *Agrobacterium* sp.

There are a number of papers dealing with degradation of organophosphorus insecticides by soil microorganisms; they are able to utilize them as the sole source of carbon, phosphorus and energy was isolated from soil surface water contamination with pesticides and from activated sludge[16, 19, 27–35]. Rosenberg and Alexander[18] used enrichment cultures from soil and sewage to obtain bacteria able to utilize aspon, azodrin, dasanit, diazinon, malathion, orthene, parathion, trithion, dimethoate, dylox, methyl-parathion and vapona.

Ramanathan and Lalithakumari[21] through enrichment technique from soil also

isolated *Pseudomonas* sp. that was able to complete the mineralization of methyl-parathion. Sheela and Pai[4] also used enrichment culture from soil to obtain bacteria capable of degrading fensulfothion, organophosphorus pesticide used to control the golden nematode *Heterode rostochiensis*. Two of the microbial isolates were *Pseudomonas alcaligenese* and *Alcaligenes* sp. In the present study, we further tried to find out whether the ability to degrade diazinon by these three bacteria species was encoded by chromosomal DNA or extra-chromosomal DNA. The three bacterial strains, after the plasmid curing experiment, by using ethidium bromide, lost the ability to grow in medium containing diazinon suggest the involvement of plasmids. A number of soil bacteria capable of degrading atrazine and related s-triazine compounds as pesticides were isolated from diverse geographic locations. The result of these studies indicated that different size plasmids were found to habour atrazine catabolism genes in *Pseudomonas* sp. and *Agrobacterium* sp.[36]. Also there is an evidence that the biodegration of nylon oligomers by *Flavobacterium* sp. and *Pseudomonas* sp. is dependent on degradative plasmids. Electron microscopy and restriction endonuclease analyses revealed that the *Flavobacterium* sp. K172 possessed three kinds of plasmids, $pOAD_1$ [39 kilo-base-pair (kb)], $pOAD_2$ (44 kb) and $pOAD_3$ (56 kb). Curing readily eliminated $pOAD_2$ with loss of the 6-aminohexanoatecyclic-dimer hydrolase (EI), 6-aminohexanoate-dimer hydrolase (EII) enzymes activity that were responsible for degradation of the oligomers. These results demonstrated that the EI and EII enzymes were encoded on plasmid[37–40].

The genes of the catabolic pathways of many compounds are coded in plasmids[12, 41] including those for the degradation of salicylates[41], camphore[42], octane[43], naphthalene[44], toluene and xylene[45, 46], carbofuran[47], 2,4-dichlorophenoxy acetate[48], chlorinated aromatic compounds[49] and plasmid mediated degradation of phenol[50].

Agarose gel electrophoresis showed that the wild type organisms carries three bands of plasmid DNA (Fig. 1) whereas the cured one carries only one (Fig. 2) since the cured strains were found unable to grow in the medium containing diazinon which was the sole source of carbon and phosphorus and energy; the loss of the two

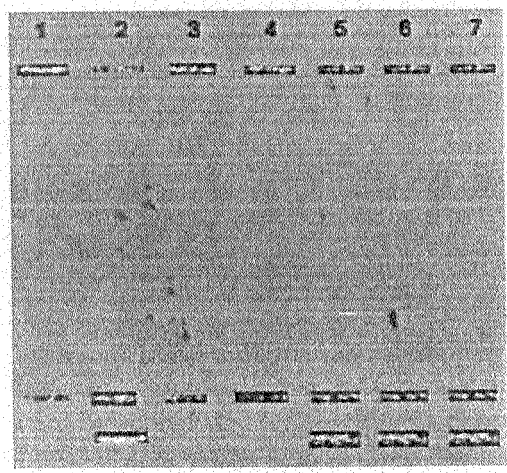

Fig. 1. Plasmid DNA of wild type *Pseudomonas* sp. (lanes 1, 2 and 3), *Flavobacterium* sp. (lanes 4 and 5) and *Agrobacterium* sp. (lanes 6 and 7). The DNA was resolved on 1% agarose gel and stained with ethidium bromide 10 μg /mL.

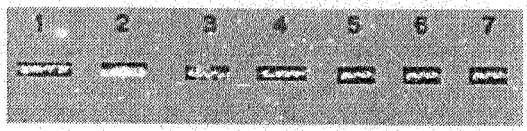

Fig. 2. Cured strain of *Pseudomonas* sp. (lanes 1, 2 and 3), cured strains of *Flavobacterium* sp. (lanes 4 and 5) and cured strain of *Agrobacterium* sp. (lanes 6 and 7); in the three organisms (2 bands of) plasmid DNA were lost when treated with ethidium bromide.

bands of plasmid DNA in the cured strains proved that the DNA carrying the gene(s) is responsible for diazinon degradation. It is also evident from earlier studies that there are a significant number of pathways for the catabolism of aromatic compounds. Timmis *et al.*[5] analyzed the plasmid encoded pathways for the catabolism of aromatic compounds. Shingler *et al.*[51] also demonstrated the growth of *Pseudomonas* strain CF 600 on phenol and 3,4-dimethyl phenol, as sole carbon and energy sources by virtue of plasmid encoded phenol hydroxylase and involves a meta-cleavage pathway.

The role of plasmids in the degradation of diazinon has provided a lucrative ground for examining the potential for and mechanisms of bacterial evolution in nature and practical consequences in terms of pollution control. More research should be performed on the genetic construction of bacteria for expanded catabolic activity, which may involve more than simple combining genes from different catabolic pathways; in addition, gene expression of the enzymes must be properly regulated and unproductive enzyme pathways deactivated in order to form man-made bacterial colonies that will clean up a factory's waste before it leaves the plant to minimize contamination and reduced or eliminate the environmental hazard.

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