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SPLIT ROLE OF PLASMID GENES IN THE DEGRADATION OF CHLORPYRIFOS BY INDIGENOUSLY ISOLATED *PSEUDOMONAS PUTIDA* MAS-1

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Abstract

A chlorpyrifos degrading bacterium *Pseudomonas putida* MAS-1 was isolated from the cotton grown soil of NIAB, Faisalabad, Pakistan. Genetic studies based on plasmid curing and electroporation mediated transformation were performed on this bacterium. The bacterium lost the property to grow on the nutrient agar containing 10mg/mL chlorpyrifos after acridine orange mediated curing. The plasmid (bearing chlorpyrifos degrading determinants / genes) was isolated and transferred into *E. coli* DH5α. The transformants however, could not resist and grow in the chlorpyrifos containing medium. It may be concluded that chlorpyrifos degradation *Pseudomonas putida* MAS-1 is accomplished by the combined action of plasmid and chromosomal genes.

Introduction

Chlorpyrifos [0, 0-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is being used globally as an agriculture based pesticide (Cho *et al*., 2002). This pesticide has been extensively used for the control of foliar insects in cotton and paddy fields and on vegetable crops. The environmental fate of chlorpyrifos has been extensively studied. Its half life in soil varies from 10 to 120 days. 3,5,6-trichloro-2-pyridinol (TCP) has been reported to be the major degradation product (Racke *et al*., 1988). Studies have been conducted on the microbial degradation of chlorpyrifos (Singh *et al*., 2003c, Mallick *et al.*, 1999). However, a limited literature is available pertaining to the genetic control of the bacterial degradation of chlorpyrifos. Guha *et al.,* (1997) reported the plasmid borne genes of a soil bacterium *Micrococcus* to be responsible for chlorpyrifos degradation. The first ever documented organophosphorus biodegrading gene was identified in *Pseudomonas diminuta* (Serdar *et al*., 1982). Singh *et al*., (2003c) reported a bacterial strain *Enterobacter* that could catabolically degrade chlorpyrifos. Bhagobaty & Malik (2008) have reported the *Pseudomonas* strains capable of degrading chlorpyrifos. These strains also harboured a single plasmid with a role in chlorpyrifos biodegradation. Studies based on the genetics of microbial degradation of chlorpyrifos have so far not been undertaken in Pakistan. We have been able to isolate and identify a chlorpyrifos degrading bacterium *Ps. putida* MAS-1 from a cotton grown soil. This research paper presents the data on the genetic control of chlorpyrifos degradation and attempts to electroporation mediated transfer of plasmid borne gene(s) from *Ps. putida* MAS-1 to *E. coli* DH5α.

Materials and Methods

Bacterial strains: An indigenous cotton soil chlorpyrifos degrading bacterial isolate *Ps. putida* MAS-1 was used in this study (for acridine mediated curing and as the plasmid 'donor'strain in transformation studies). *E. coli* DH5α, a plasmid free strain, was used as the recipient. Chlorpyrifos (Pakistan Agro Chemicals Pvt.), novobiocin (MP

Biomedicals, USA), acridine orange, ethidium bromide and agarose (Sigma) were used for the plasmid curing studies. Nutrient broth, brain heart infusion (BHI) broth, Luria basal broth (LBB) and agar (Oxoid) were used for growing the bacterial strains. Electroporation machine (Eppendorf 2510) was used for transformation experiments (transfer plasmid from the chlorpyrifos degrading bacterium *Ps. putida* MAS-1 into E. coli DH5α). Horizontal agarose gel electrophoresis apparatus (Bio Rad, USA) was used for plasmid elucidation.

Location of genetic determinants by curing technique: In order to determine the location of the pesticide resistance marker(s), the curing experiments were performed using fluorescent dyes (acridine orange, ethidium bromide) and antibiotic novobiocin. For this, the pesticide resistant culture *Ps. putida* MAS-1 was grown overnight in 5mL nutrient broth containing 2mg/mL of chlorpyrifos. Next day, 200µL of culture was added in 5mL nutrient broth (containing 2mg/mL chlorpyrifos) and incubated at 37°C in shakobator (Stuart scientific, UK) for 2 hours. The log phase 200µL culture was then added to 2mL nutrient broth containing different concentrations (20µg-10mg/mL) of acridine orange and ethidium bromide as described by Guha *et al.,* (1997) and novobiocin in 25-300µg/mL concentration as per Hooper *et al.,* (1984). Positive and negative controls were also run. Positive control contained only cells (without Ethidium bromide, acridine orange or novobiocin); while negative controls contained only Ethidium bromide, acridine orange or novobiocin. All the tubes were incubated (in dark) at 37°C for overnight. Next day, the tubes containing the lowest concentration of Ethidium bromide/acridine orange (in which growth was still visible) were selected and 100 µL volumes from these tubes was spread over plain nutrient agar plates and incubated at 37°C for overnight. A total of, 300 colonies were selected and streaked on the pesticide containing (10mg/mL concentration) and plain nutrient agar plates followed by incubation. Next day checked the presence and absence of colonies on both the plates. Replica plate technique (Salisbery *et al.,* 1972) was used in these experiments. Plasmid extraction was carried out by alkaline lysis method as described by Maniatis *et al.,* (1989).

Agarose gel electrophoresis: For this 30 mL of 1% agarose was melted in boiling water bath and 1µL of Ethidium bromide was added when temperature dropped to 45^{o}C. The melted agarose was poured after assembling the gel-casting tray with comb at one end and allowed to set. 1 x TAE was poured in the tank (Bio Rad, USA) to sub-merge the gel. 10 µL of the sample was loaded in the well(s) with and electrophoresis was carried out at 80 volts for 30-45 min. Thereafter, gel was removed and examined over UV transilluminator (UV Products, UK) for observing the plasmid DNA bands. The method described by Maniatis *et al.,* (1989) and modified by Ghazala (2000) was followed for agarose gel electrophoresis.

Transformation by Electroporation: Preparation of electrocompetent cells and electroporation of the cells were carried out by the methods described by Smith (1995) and Fiedler & Wirth (1988).

Results and Discussion

An indigenous chlorpyrifos degrading bacterial isolate *Ps. putida* was subjected to the genetic studies with particular reference to the location of genetic factors /

determinants for the control of chlorpyrifos degradation. Further, electroporation-mediated transformation was carried out to transform the plasmid genes (conferring chlorpyrifos degrading ability) from *Ps. putida* MAS-1 to *E. coli* DH5α competent recipient cells.

Ps. putida MAS-1 (a chlorpyrifos degrading soil isolate) was cured of the plasmid by acridine orange, whereas Ethidium bromide and novobiocin could not cure the plasmid genes responsible for chlorpyrifos degradation (Table 1). Naidu & Yadev (1997) successfully cured the siderophore production in *Aeromonas* sp., using acridine orange. Subhan (2005) also reported the acridine orange mediated curing of the hydrocarbon degrading plasmids in *Ps. aeruginosa* strains. Figure1 depicts the gel electrophoresis profile of chlorpyrifos resistance plasmid of *Ps. putida* MAS-1. It is clear that plasmid could not be extracted from the acridine orange treated bacterial strain (lane a) while 11kb plasmid (lane b) is visible in the uncured cells. Guha *et al.,* (1997) had also reported the absence of a plasmid in the acridine orange and SDS treated cured strains of *Micrococcus* sp.

Electroporation is a method of transformation that allows the introduction of foreign DNA into host cells (prokaryotic or eukaryotic) *via* the application of high-voltage electric pulses. The electric field induces pore formation in the cell wall and increases the permeability of the host cells to macromolecules, which allows the uptake of DNA (Lurquin,1997). Recombinant cell(s) could not be grown on chlorpyrifos containing LB agar plates after the triplicate trials of electroporation mediated transformation of the plasmid (isolated) from *Ps. putida* MAS-1 into *E. coli* DH5α (plasmid free) cells. The observations seemingly indicate the failure of plasmid transfer. However, to verify the mentioned possibility electroporated culture cells were plated on simple LB medium (not containing the insecticide) and incubated at the respective temperature for desired duration. Fifteen viable colonies (data not shown) were randomly picked for plasmid extraction. The gel profile of the extraction showed the presence of *Ps. putida* MAS-1 plasmid (~11Kbp) in two strains / colones of *E.coli* DH5α (Fig. 2.). However, lack of phenotypic expression (by the transformants) can be explained. The gene(s) responsible for this chlorpyrifos degradation are *mpd* and/or *opd*. Both *mpd* and *opd* genes have been isolated from a variety of organisms and located variably on chromosome and plasmid (Yang *et al*., 2006; Li *et al*., 2007). The failure of recombinant *E. coli* DH5α to give phenotypic expression suggests the presence the genes on chromosomes but in light of results obtained from plasmid curing experiments (where the cured organism lost its chlorpyrifos resistance property) imply the pleiotropic nature of the trait. It is possible that some of the regulatory or transporter genes are situated on chromosome while structural genes are present on plasmid. This will likely make cured and cloned organism unresponsive against the chlorpyrifos. Singh *et al.,* (2004) while working on the biodegradation of chlorpyrifos by *Enterobacter* strain B-14 have shown the possibility of this ability to be polygenic and chromosome based. Further, chlorpyrifos has been reported to be degraded cometabolically in liquid media by *Flavobacterium* sp., and by an *E.coli* clone with an *opd* gene (Wang *et al*., 2002). It is worth mentioning that *opd* gene(s) were found to be plasmid based in *Ps. diminuta* (Serdar *et al*., 1982), while Horne *et al.,* (2002) could isolate an *opd* gene from *Agrobacterium radiobacter*, which was located on the chromosome. It appears the role of transposon(s) is important in flip flop of the genes (including chlorpyrifos degrading genes). It would suggest that biodegradation of chlorpyrifos is mediated by split location of the genes (located on the plasmid and the chromosome) in the *Ps. putida* MAS-1 (a soil isolate).

Table 1. Location of genetic determinant for the pesticide resistance.

Curing agent	No. of colonies screened	No. of colonies cured	No. of colonies un-cured	Curing percentage
Ethidium bromide	300	0	300	0
Acridine orange	300	40	260	13
Novobiocin	300	0	300	0

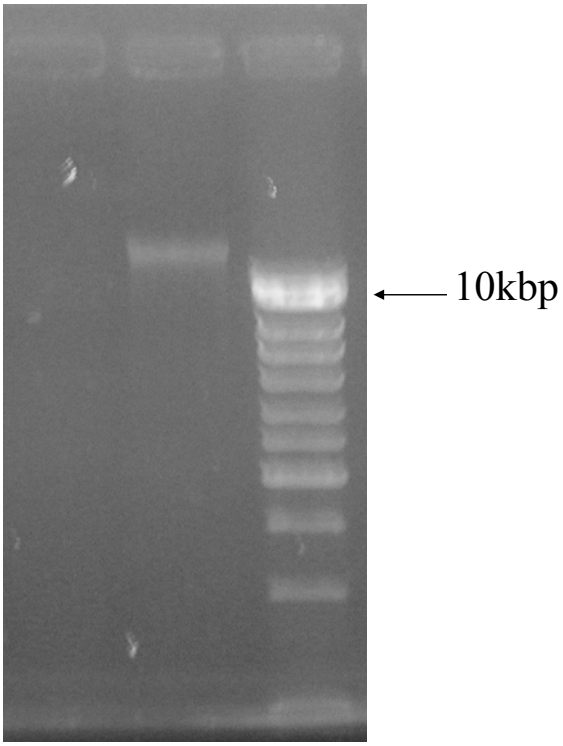

Fig 1. Agarose gel electrophoresis profile of pesticide resistance plasmid of *Pseudomonas putida* MAS-1 a: Cured, b: Uncured(>/~11Kbp) c: Marker.

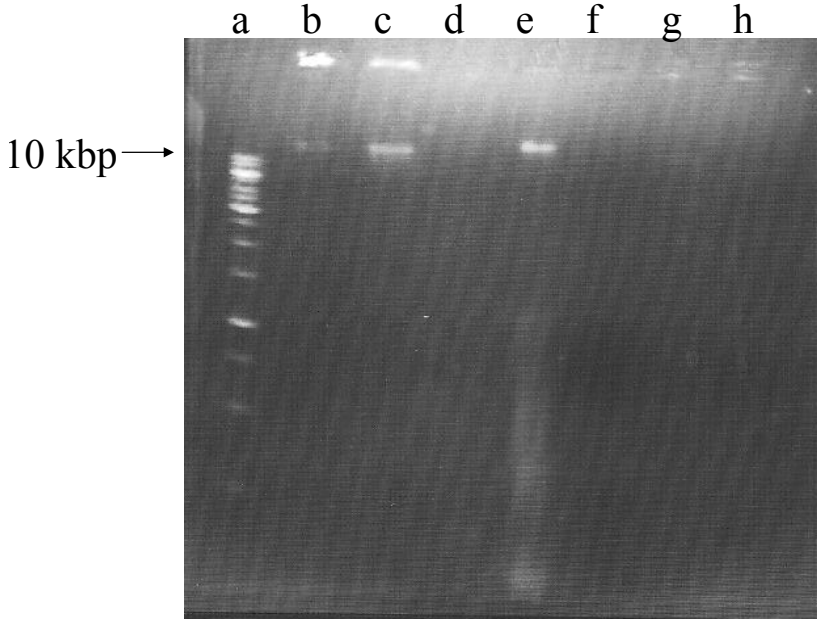

Fig. 2. Agarose gel electrophoresis profile of *Ps. putida* MAS-1 and cloned *E. coli* DH5α for plasmid: a - Marker; b, c, d, f, h are the plasmid extraction preparation of *E. coli* DH5α while "e" have plasmid extraction preparation of *Ps. putida*.

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