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STUDY OF BACTERIAL RESISTANCE TO ORGANOPHOSPHOROUS PESTICIDES IN IRAN

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ABSTRACT

The broadness application of organophosphorus compounds has abounded the number of its polluted areas. Bioremediation has widely focused on insitu bacterial degradation of organophosphorus residues in the world. Therefore, in this research six numbers of samples from two different sources, soil and water randomly were isolated using different organophosphorus pesticides containing mineral solution without supplementation. More than 100 isolated strains were selected according to their simultaneous optimal growth on mineral medium with organophosphorus and Mac Conkey's agar. More than 50 percent of them were lost above resistance. The resistant strains were identified by two methods, the biochemical convention and API 20E procedure with positive agreement. The identified strains belonged to *Pseudomonas* and *Flavobacterium* species. The maximum tolerant concentrations of different organophosphorus pesticides by these resistant strains were 2.5, 4 and 8 g/L of guthion, methyl parathion and Dimethoate, respectively. The resistance to these pesticides due to organ phosphorous degrading plasmids had the ability to express hydrolytic enzymes. Resistant bacteria lost these plasmids by acridin orange and could translocate to sensitive strains. Thus, certain environmental bacteria could be used as protection tools against antinerve agents.

Key words: Bacterial resistance, bioremediation, organophosphorous pestisides, antinerve agents

INTRODUCTION

Organophosphorus (OP) compounds are a group of highly toxic chemicals which are extensively used throughout the world in various industries especially for agricultural control of wide range of insect species (Jaffery *et al.*,1989; Mathur, 1992), because these compounds inhibit acetyl choline esterase, an essential enzyme for neuromuscular activities of either human or insects (Anonymous ,2004) and have LC50 value (in rats) of as low as 14-24 mg/kg of body weight (methyl parathion), (Chaudhry *et al.*, 1988). Dimethoate is highly toxic and easily absorbed through the skin (Al-Jaghbir *et al*., 1992). this pesticide can be dangerous as a result of runoff from site of application. Studies of micro organisms in bioremediation are important in improvement of technologies for detoxification of OPs. (Hayatsu *et al.*, 2000). The principal knowledge of degradation is a basis for cleaning up the environmental OP contamination. The rate of enzymatic hydrolysis of parathion, an OP ester commonly used in pesticides, was found to be nearly 2,500 times the rate of hydrolysis in 0.1 normal sodium hydroxide, this hydrolytic enzyme is drived from soil bacteria (Munnecke, 1979). Among environmental resistant strains pseudomonas species have exhibited high potential for dimethoate. Finding of certain mechanisms by which biodegradation occur, induction and translocation to sensitive strains have developed (Deshpande, *et al.,* 2001). Application of OPs has been increased to improve crop production in Iran. According to the reports of the Pest

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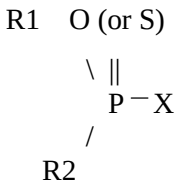
Control Center of Iranian Ministry of Agriculture, OP pesticides comparably have been used 25 folds more than other pesticides. The present study, determined and characterized the OP resistant strains which belonged to pseudomonas and flavobacterium species and harbored special OP degrading plasmids. P putida , one of identified wild type resistant strain lost OP degrading plasmids by acridin orange.

MATERIALS AND METHODS

The OP compounds are normally esters, amides, or thiol derivatives of phosphoric or phosphonic acid: where R1 and R2 are usually simple alkyl or aryl groups, both of which may be bonded directly to phosphorus (in phosphinates), or linked via O, or S (in phosphates), or R1 may be bonded directly ,and R2 may bonded via one of the above groups(phosphonates). The three selected OPs were: guthion (Azinphos Methyl), Dimethoate (*Dimeton*) and Methyl Parathion, which belonged to 3 different families of OPs as representative pesticides, (*Merk*) (Anonumous, 2004). Minimal salt media containing the following salts: $CaCl_2$: 0.02 g $MgCl$:0.2 g, K_2HPO_4:1.0g, KH_2PO_4:1.0g, NH_4NO_3:1.0g and $FeCl_3$: trace, *(Sigma)* in DH_2O, with pH = 7.2-7.4 up to 1 liter.



(1)

Selection of OPs Resistant Bacteria

The resistant bacteria to 3 different Ops were isolated at 3 steps: The soils (A, B, C, D, E) and water (F) samples from OPs contaminated fields of Majnoon island were randomly collected up to depth of 10 cm and transferred to lab.10 g (mL) of each sample aerobically were incubated in 50 ml sterile salt media into duplicate 100 ml cotton plugged flasks containing 0. 0,0.02,0.04,0.08 g/L of OPs with continuous shaking at room temperature for 1 week and observed for turbidity and viability every 48 hr. The samples with optimum turbidity or growth were reincubated in 50 ml sterile salt media containing 0.0,0.2,0.4,0.8 g/L of OP s at room temperature for 1 week. Only soil sample (B) with excellent growth and viable count 10^5 cell/ml were reincubated in sterile salt media containing 0.0,0.2,0.4,0.8 g/L OP s at 4 different temperatures (4,24,37 and 50 °C)for two weeks and observed for turbidity and viable counting every 48 hr.

Isolation of Gram- negatives bacilli

The most active samples were isolated as pure individual colonies on salt medium agar and Mac Conkeys agar as gram negative bacilli.

Identification of OPs resistant bacteria

Conventional bacterial mediums (Merk) and API20E Kit (rosch) were used in this study. The isolated bacteria were identified by conventional method and API20E kit system (Finegold and Martin, 1982) and were observed with gram staining by light microscopy and also electron scanning microscope.

Sensitivity to antibiotics

Bacterial interaction to several antibiotics with different concentrations were determined in Mueller–Hinton agar at 37 °C in 24 hr. The following antibiotics were used in this study : Ampiciline(Amp), Amikacine (Amn),Chloraphenicol (Chl), Gentamycine(Gen), Kanamycine(Kan), Foradantoine (Fn), Nalidixic acid (Nal), Streptomycin(*Stp.*), (Tolidaro drug co) and control (no antibiotics) with determined concentrations (Finegold and Martin 1982).

Maximum Tolerating Concentration of OPs

The maximum tolerating potential of resistant bacteria was assayed for OPs in nutrient broth containing different concentrations of OPs, like guthion (1, 2, 2.5) g/L, dimethoate(1 , 2, 4) g/L, methyl Parathion(2, 4, 8) g/L.

OPs Plasmid deletion

Resistant bacteria having specific plasmids that contained gene(s) for OPs degradation which were incubated in 10 mls BH medium plus 300 ìg glucose Containing 50,150,200,300 and 450 ìg acridine orange (Merk) for 48 hr, and 10^{-3}, 10^{-5}

and 10^{-7} dillutions of bacterial growth inoculated on semi solid mineral medium, and the sensitive colony detected by replica plating technique on medium containing OPs.

Stock culture

The 10 mls sterile semi solid mineral medium containing OPs and 10 ìg thymine (Merk) were prepared in slant shape in screw capped vials for deep inoculation of active and resistant bacteria. Bacterial stock cultures could be maintained for 2 months.

RESULT

Selection of the most resistant bacteria

Direct recording of growth turbidity with parallel plating from different soil and water samples resulted in colony counts which were plotted in Table 1. Different soil samples especially B had been possibly exhibited high growth. Accordingly the viable counts were high and gradually increased with rising of OP concentration at second week. Among different microorganisms only the high resistant bacteria were selected in mineral medium which had enriched by OPs (guthion, dimethoate and methyl parathion), in 3 steps of each 1 week incubation. However such resistant strains had been showed low viability in absence of OPs. Temperature were affected the growth of high resistant strain in B sample that can be seen in Table 2. The optimal growth temperature was arranged 24-37 °C.

Table 1: Mean colony count

Concentrations (g/L)	Samples A	B	C	D	E	F
0.000	100	100	98	96	94	90
0.020-0.20	50	100	98	96	94	40
0.040-0.40	0	100	98	96	50	10
0.080-0.80	0	100	98	96	0	10

*Different soils(A, B, C, D and E) and water (F) samples were incubated at two successive steps in the presence of 3 selected Ops (dimethoate ,methyl parathion ,guthion with different concentrations : (1: 0.0, 0.02,.04,.08g L^{-1} ,2: 0.2,0.4,0.8 g L-1) duplicate at room temperature. B sample in final step was showed excellent resistance to OPs (Viable counts in Dillution:1×10^{-3}/ every 48 hr).

Table 2: Optimal growth temperature

Concentrations (g/L)	Temprature (°C) 24	37	50	4
0.000	100	98	30	96
0.020-0.20	100	79	69	67
0.040-0.40	100	91.3	98	33
0.080-0.80	100	74	67	33

*The soil sample (B) was incubated in the presence of different concentrations (0.0,0.2,0.4,0.8 g/L) of OPs (guthion, methyl parathion, dimethoate) at different temperatures (24,37,50 and 4 ºC) that showed excellent resistance and growth (Viable counts with Dillution :1×10^{-3}/ml. This sample also was showed high resistance at variant temperatures(4,24, 37 and 50 ºC).

Identification of resistant bacteria

Identification of resistant bacteria was done by two procedures conventional method and API20E kit system which were specified to Entrobacteriaceae. These two methods showed high agreement (Table 1). The Identified species were studied by light and scanning electron microscopy (Table 2).

Sensitivity/Resistance to antibiotics and plasmid manipulation

Most of strains are resistant to antibiotics (Amp.Chl.Fn. and Nal.), but sensitive to antibiotics (Amn. Gen., Kan. And *Stp.*). The specific OP degrading plasmids were deleted by acridine orange.

Maximum tolerating concentration

The highest OPs concentrations were tolerated by optimal growth of selected bacteria determined in different concentrations of 3 group of OPs, The low concentrations of OPs (0.2,0.4,0.8 g/L) were enriched for optimum growth at first step , but at second step, Maximum Tolerating Concentration increased to 2.5,4 and 8 g/L for guthion ,dimethoate and methyl parathion, respectively. More than 100 aerobic gram negative bacilli exhibited high resistance in different concentration of OPs. Such resistance to these toxic chemicals seems to be unstable and about 50% of which has lost resistance abilities.

DISCUSSION

The organisms in the environment contaminated with OPs may adapt to use of novel chemicals as nutrients and energy requirements. The adaptation for more toxic OP such as guthion is required longer time than a lower toxic like dimethoate. An induction possibly was observed by OPs in resistant strains. Sequence information further suggests divergence of catabolic genes coding for specialized enzymes in degradation of xenobiotic chemicals. These specialized enzymes evolved from more common isozymes only after the introduction of xenobiotic chemicals into the environment (Bertani *et al.*, 2001, Chen *et al.*, 2002). The identified strains showed high potentials for extenive degradation of three selected OPs which were the representative of three different and related chemical structures. Similar findings have been reported for a kind of homology in OPs degrading gene in Pseudomonas sp.and Flavobacterium sp. (Chaudhry *et al.*, 1988). The interaction of antibiotics could be used as genetics markers and differential tools of resistant bacteria. The specific degrading plasmids have been determined for different OPs. These plasmids can be deleted by acridine orange which confirmed the genetic potential for resistant strains. The genetic characterization of an increasing number of aerobic pathways for degradation of (substituted) aromatic compounds in different bacteria has made it possible to compare the similarities in genetic organization and in sequence which exist between genes and proteins of these specialized catabolic routes and more common pathways (Van der Meer *et al.*,1992, Morales *et al.*, 2004). The inductions of metabolic pathway enzymes were promoted the resistant strains for incubation in high concentrations. Specialized enzyme systems and metabolic pathways for the degradation of man made compounds such as Parathion derivatives and dimethoate have been found in microorganisms isolated from geographically separated areas of the world (Aislabie and Lloyd Jones 1995, Eduardo et *al.*, 2001). The optimum temperature for resistant strains was between 25 to 37 °C (Table 2); high temperatures might affect the xenobiotics degradation but the termophillus strains naturally need high temperatures. The most resistant strains were also resistant to some antibiotics (Amp,Fn, Nal. and Chl) as multi potentials for known bacteria that have recently suggested by authors (Lewinson and Bibi 2001, Lewinson *et al.*, 2003). However , bacterial resistance to OPs is suggested; the high ability of its degradation/ reduction of anticholine esterase activity in identified strains needs more study and development such as relevant enzymatic application. However, there is virtually no information concerning the rates at which these mechanisms are operating in bacteria living in nature and the response of such rates to the presence of potential (xenobiotic) substrates. Quantitative data on the genetic processes in the natural environment and the effect of environmental parameters on the rate of evolution are needed.

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