Iranian Journal of Basic Medical Sciences
Vol. 10, No. 4, Winter 2008, 239 - 244
Received: November 10, 2007; Accepted: February 25, 2008

IJBMS

# Detection of *Pseudomonas* and *Flavobacterium* Species Harbouring Organophosphorus Degrading Elements from Environment

[1]* Aboalfazl Nazarian, [2] Bahram Amini

## Abstract

**Objective**
The extensive pollution of natural ecosystems has occurred by organophosphorus pesticides, being used for improvement of crop production in agriculture. Biodegradation is interested in application of multifunctional bacteria containing genetical elements which specially degrade organophosphorus compounds. In this paper the detection of two bacteria species, *Pseudomonas* and *Flavobacterium*, which harbouring organophosphorus degrading elements from environmental samples is reported.

**Materials and Methods**
Several resistant strains isolated from different cultures that previously were cultivated with related sources such as soils and waters. These bacteria had been isolated from organophosphorus enriched mineral solutions, and following inoculation, visible colonies were observed on mineral and MacConkey's agar medium. The resistant bacteria were identified by conventional procedures and monitored with certain properties like stability, tolerance, resistance to organophosphorus compounds and different antibiotics. The genetical elements for organophosphate degradation were confirmed by adding acridine orange to culture of resistant strains which could delete these capabilities.

**Results**
Fifty strains were optimally grown in presence of three classes of organophophoruses like guthion, dimethoate and methyl parathion with concentrations of 2.5, 4, and 8 $gl^{-1}$ respectively. The gram-negative bacteria were identified using conventional diagnostic procedures, as *Pseudomonas* and *Flavobacterium* species. These strains were harbouring organophosphorus degrading elements which were deleted by acridine orange as mutagen. Also, most of these bacteria were resistant to different antibiotics that used as biomarkers in discrimination of sensitive strains to organophosphoruses.

**Conclusion**
The genetical elements such as resistance to organophosphoruses and antibiotics were transferred to the sensitive bacteria by matting technique and cured as multifunctional organisms which had high capability for various organophosphoruses. The multi-degrading strains might be suggested as useful tools for detoxification of harmful organophosphoruses in agriculture.

**Keywords:** Biodegradation, Catalytic enzymes, Organophosphorus degrading elements, Resistant bacteria.

*1- Department of Clinical Biochemistry, Faculty of medicine, Zanjan University of Medical Sciences, Zanjan, Iran*
*Corresponding author: Tel: +98-241- 4240300-1; Fax: +98- 241-4249553; email: abolnaz@yahoo.com
*2- Department of Clinical Microbiology, Faculty of Medicine,* Zanjan *University of Medical Sciences, Zanjan, Iran*

## Introduction

Organophosphruses (OP) are a group of neuro-toxic compounds which are broadly used in different industries mainly for pest control as insecticides (1). OP is principally anti-choline esterases and represses the neuro-muscular activities of either human or insects. Such OP like dimethoate may cause high toxicity because it is easily absorbed through skin (2). OP residues possibly are distributed in environment after application. The documents of Iranian pest control centre in ministry of agriculture had indicated the wide use of OP insecticides as 25 folds more than other compounds (3). However, the various residues of OP were distributed through sources of waters that might highly contaminated the natural food chains. Bioremediation of OP is an effective method that was suggested by resistant bacteria because it could be improved as a tool for cleaning of natural environments (4). Certain bacteria would included hydrolytic enzymes which simply degraded such OP like methyl parathion so it could be break 2500 folds more than chemical routes (0.1 N NaOH), (4). The OP have different derivatives (ester, amide and thiol) of phosphates or phosphonates which usually alkyl or aryl groups directly attach to phosphorus (phosphonates) or bound via oxygen or sulfur (phosphates) (5). Three kinds of OP were used: guthion (azinphos methyl), dimethoate (dimethon) and methyl parathion. These three OP were applicated as routine and representative of three classes of pesticides. *Pseudomonas* family has potentially exhibited as active species, harbouring genetical elements (plasmids) for OP degradation. The manipulating of degrading potential has developed by transferring of organophosphorus degrading plasmids (OPD) to non resistant strains (3, 6, 7).

The gram-negative bacteria containing OPD plasmids were isolated from the environmental samples of soil and waters, where highly polluted with OP.

## Materials and Methods

Mineral solution which were used in this project included different salts; $CaCl_2$: 0 .02 g, $MgCl_2$: 0.2 g, $K_2HPO_4$: 1.0 g, $Na_2HPO$: 1.0 g, $NH_4NO_3$: 1.0 g, $FeCl_{l3}$: 0.0001 g, (Merck), Distilled $H_2O$ up to 1 L. These compounds were consecutively dissolved in 800 ml distilled water and adjusted to 1 liter at pH of 7.2- 7.4. The mineral solutions (50 ml) sterilized in 100 ml flasks (duplicated) and used for culture growth in biochemistry lab (IBB, Institute of Biochemistry and Biophysics)

### *Isolation of resistant gram-negative Strains*

1) The samples were obtained from area which contaminated with organophosphoruses. Seven types of environmental samples (soil and water) randomly collected from depth of 10 cm and transferred to laboratory. 10 g (ml) of each sample including different soils (A, B, C, D and E) and water (F) which previously contaminated by OP, aerobically incubated in duplicated series of 50 ml sterilized mineral solution containing: 0.0, 0.02, 0.04 and 0.08 ($gl^{-1}$) of three class of OP (guthion, dimethoate and methyl parathion) for 1 week. These bacterial cultures were continuously shook (50 rpm) at room temperature.

2) The growth quantity was monitored by measuring turbidity (500 nm) and viability every 48 hrs. The optimal growth cultures were re-incubated again at similar conditions for 1 week, but with 10 folds more OP concentrations than the previous steps.

3) The resistant cultures of bacteria against OP with optimal growth were incubated in mineral solution containing (0, 0.2, 0.4, 0.8) $gl^{-1}$ of the above mentioned three OPs at different temperatures (4, 24, 37, 50 $^{o}$ C) for 1 week and measured for optimal viability. The $10^{-6}$ cell dilution of culture, platted on MacConkey's agar at room temperature for 48 hr, the pure colonies were observed by gram staining using a light microscope.

### *Study of OP resistant bacteria*

**1) Stability:** The stability of resistant bacteria against OP was continually studied. The $10^{-6}$ cell dilution of resistant cultures inoculated in to a semi-solid mineral medium containing low concentrations of OP were continuously

incubated at room temperature for 48 hrs monthly.

**2) Tolerance:** The optimal viability for each resistant strain showed its specific tolerance against a class of OP. The pure colonies were inoculated in nutrient broth including 0.0, 2.0, 4.0 and 8.0 $gl^{-1}$ of the three mentioned OP at room temperature for 24 hrs and the $10^{-6}$ cell dilution of each resistant strain was showed suitable viability.

**3) Resistance to antibiotics:** The resistance against different antibiotics was also determined. The $10^{-6}$cell dilution of pure bacterial cultures were platted in Muller-Hinton agar and checked with antibiotic disks (Ampicilline (Amp), Amikacine (Amn), Chloramphenicol (Chl), Gentamicine (Gen), Kanamicine (Kan), Furadantoin (FN), Streptomycin (Stp), Nalidicsic acid (Nal)) (8).

**4) Identification:** The strains which were optimally degraded OP, idetified by two procedures:

**a)** The conventional biochemical procedures which were examined on routine methods (8).

**b)** The specific multi diagnostic kits for enterobacteriaceae that named Entrotube II or API - 20E kit were used (Rosch).

**5) Stock culture:** A slant semi-solid minimal medium containing low concentration of OP and 0.2 $gl^{-1}$ thymine was prepared in screw capped vials and inoculated with pure identified strains every 2 months.

**6) Confirmation of OPD plasmids:** The optimal growing strains in the presence of OP were incubated in nutrient broth including 0.0, 0.2, 0.3, 0.4 $mg.ml^{-1}$ of acridine orange as chemical mutagen at room temperature for 48 hrs in shaking condition. The $10^{-6}$ cell dilution of culture was platted on semi solid medium, then the pure colonies transferred to medium with and without OP by the replica technique (9, 10).

## Results

***Isolation of Resistance gram-negative strains:*** During three steps different bacteria from environmental samples, like soil and water exhibited their degrading capability against three classes of OP. A minimal salt solution containing 0.0, 0.02, 0.04, 0.08 $gl^{-1}$ concentrations of guthion, dimethoate and methyl parathion was aerobically used in 3 intervals of 1 week. The OP concentrations were increased to 10 folds in 2nd and 3rd step. Also different temperatures of 4, 24, 37 and 50 $^{o}C$ were used at 3rd step. The bacterial growth was quantified with turbidity measurement at 500 nm absorption against control culture (without OP). Different soils (A, B, C, D & E) & water (F) samples were incubated at three successive steps in the presence of 3 selected OP (Dimethoate, methyl parathion, guthion) with different concentrations (step1: 0.0, 0.02, 0.04, 0.08 $gl^{-1}$, step2 and 3: 0.0, 0.2, 0.4, 0.8 $gl^{-1}$) duplicated at room temperature showed optimal growth in expose to above mentioned OP, (Figure 1). Some samples in the final step showed excellent resistance against OP, (Figure 2). The viability was confirmed by platting of bacterial culture with $10^{-6}$ cells/ml in semi solid mineral medium (Figure 3). The most OP degrading bacteria were selected among studied strains.

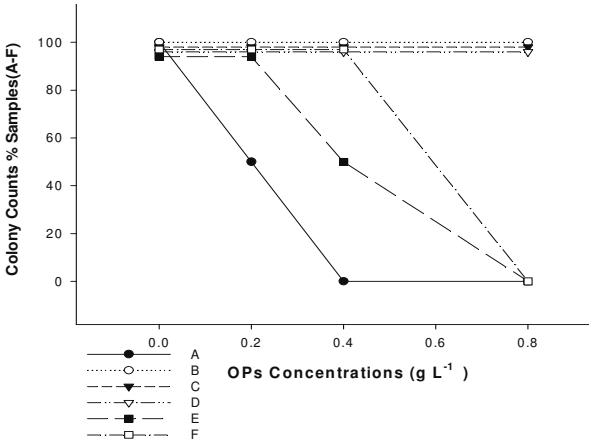


Figure 1. Resistance induction. Different soils (A, B, C, D & E) & water (F) samples were incubated at three successive steps in the presence of 3 selected Organophosphates (OP), (Dimethoate, Methyl Parathion, Guthion) with different concentrations (step1: 0.0, 0.02, 0.04, 0.08 $gl^{-1}$, step 2 and 3: 0.0, 0.2, 0.4, 0.8 $gl^{-1}$) duplicated at room temperature. Some samples in final step were showed excellent resistance against OP (Viable counts in dillution: $1\times 10^{-3}$/ every 48 hr). The OP resistance was decreased in some mineral solution.

The gram negative resistant strains typically formed colonies on MacConkey's Agar containing OP, after 48 hrs incubation (Figure 4).

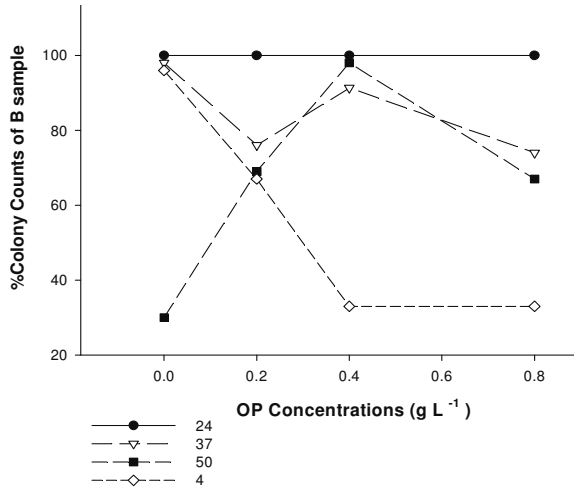


Figure 2. Optimal Growth Temperature, The soil sample (B) was incubated in the presence of different concentrations (0.0, 0.2, 0.4, 0.8 $gl^{-1}$) of Organophosphates (OP), (Guthion, Methyl Parathion, Dimethoate) at different temperatures (24, 37, 50 & 4° C) that were showed excellent resistance and growth (viable counts with dilution :1× $10^{-3}$ /ml) .This sample also was showed high resistance at various temperatures (4, 24, 37 & 50° C). The growth curves of the resistant bacteria in exposure to three different OP were typically the same.

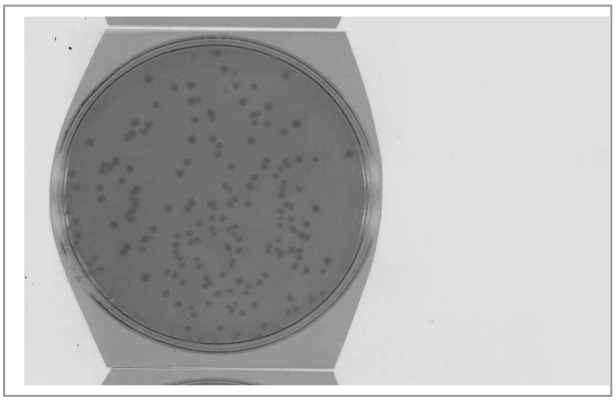

Figure 3. The resistant strains were typically formed Colonies on MacConkey's Agar containing OP after 48 hr incubation.

### *Study of resistant bacteria*

1) First of all about 100 different colonies showed degrading capability against various OP, but about 50% of them lost this ability by continuous incubation on semi solid medium, then only the complete stable strains were studied.

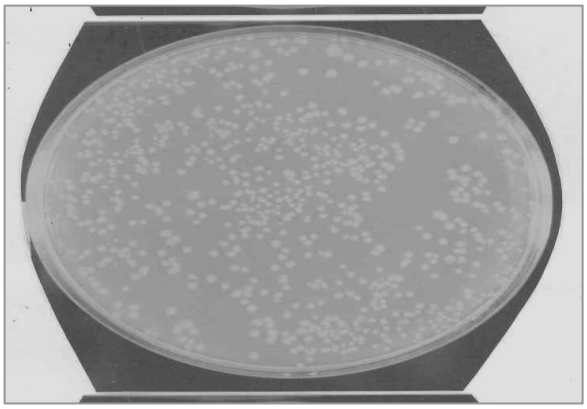

Figure 4. The resistant strains colonies on semi solid mineral medium containing Organophosphates.

2) The resistant strains optimally tolerated guthion, dimethoate and methyl parathion at high concentration (2.5, 4.0 and 8.0 $gl^{-1}$) respectively. The cultured strains showed high turbidity and formed high or uncountable colonies.

3) The studied strains were identified by two different methods that showed similar results and belonged to *Pseudomonas* and *Flavobacterium* families. They also, observed by light microscopic, gram staining and Scanning Electron Microscopy (SEM), (Figure 5).

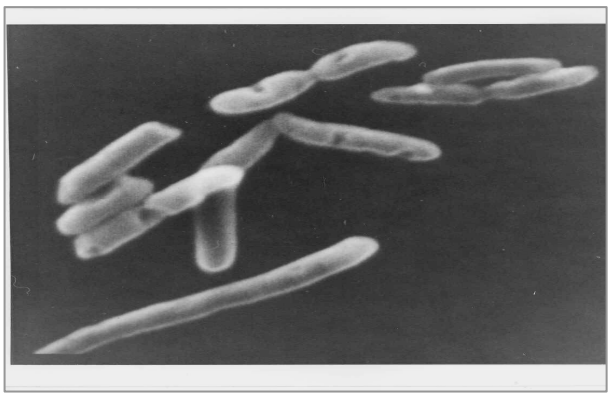

Figure 5. *Pseudomonas putida* by Scanning Electron Microscopy (SEM) magnified. X 100000.
Electron Microscopy Lab Institute of Biochemistry and Biophysics (IBB).

4) The OP resistant strains were stored in special semi solid medium containing OP and thymine in slant position. These cultures continually renewed every 2 months.

5) The role of OPd elements (plasmids) was confirmed with concentration of 0.3 $gl^{-1}$ acridine orange, which five resistante strains lost their capability to OP degradation. The

sensitive strains were differentiated by replica plating procedure on mineral medium with and without OP.

## Discussion

Bacterial utilization of OP as anti nervous system and high toxic chemical were studied in this research. The wild type strains isolated from different environmental samples like soils and water. The studied bacteria were highly resistant against three different classes of OP (Dimethoate, Methyl Parathion, and Guthion) in high concentrations. The stability, tolerance and optimal growth of these species were monitored with growth turbidity and viability. The gram-negative bacteria were isolated by parallel platting on semi- solid mineral medium and MacConkey's agar medium. These bacteria showed multi – capability for resistance against different OP and antibiotics. The studied resistant bacteria belonged to two important families (*Pesudomonas* and *Flavobacterium*).

The multi- potential strains were isolated by enriched culture with different OP at various levels. The OP induced the hydrolytic enzymes specified to OP degradation. It looks a long time for the complete degradation, of OP like guthlon, but other groups like dimethoate and methyl parathion were simply utilized as sole phosphorus and energy source. However, it was supposed that certain genetical elements (plasmids) could express the degradation potential. The resistant strains lost the OP degrading plasmids by acridine orange as mutagen (9, 10). The OPD plasmids were transformed to sensitive strains by mixing culture of sensitive and resistance bacteria. The OP sensitive strains had lost the OP degrading capabilities and differentiated from OP resistant strains by replica platting procedure in mineral medium with and without OP sequentially.

These data indicated that certain genes had been engaged in various catabolic pathways. Further researches have suggested the catabolic genes coding for specialized enzymes for xenophobic chemicals (11, 12).

Also specific models have been studied for biochemical pathways (13, 14). *Pseudomonas* and *Flavobacterium* which were identified as the high potent and resistant species also, have been studied by one of us (3) and others previously, (15). Antibiotics resistance also showed another linked degrading capability which was used in differentiation and diagnosis of resistant species. Several aerobic gram-negative exhibited the high ability for OP degradation. Similar works have found that hydrolytic enzymes are involved in the biodegradation of man-made compounds such as parathion derivatives and dimethoate in microorganisms from natural environment (16, 17) which are also resistant to some antibiotics (18, 19). These OPD plasmids were transferred by matting between resistant and sensitive strains and confirmed with replica platting technique on mineral medium containing OP. However, OP degrading plasmids which were specified to multi-potent strain are excellent candidate for enzymatic source, as effective tools in bioremediation and organophosphate detoxification.

## Acknowledgement

We would like to acknowledge the helps of Miss Ahmadian and Miss Jamshidi in SEM lab and Mr Fallah in photography room at International Biochemistry and Biophysics Institute (IBB) for their best effort. This paper was financially supplied by the grant of Iranian Ministry of defense and logistics and International Biochemistry and Biophysics Institute (IBB).

## References

1. Mathur S C. The pesticides industry in India .In chemical weekly Annual Ed Raghavan R:.NewDelhi: Pesticide Association of India publication; 1992.185-192.
2. Al-Jughbir MT, Salhab AF, Hamarsheh F A. Dermal & Inhalation exhibition to dimethoate. Archives Environment Contam Toxicol 1992; 22:358-361.

3. Nazarian A. Utilization of Dimethoate by Wild Type Pseudomonas Putida from polluted Sites in Iran. Pak J Bio Sci 2007; 10(4): 664-667.
4. Munnecke DM. Hydrolysis of organophosphate insecticides by an immobilized-enzyme system. Biotechnol Bioenginee 1979; 21(12): 2247-2261.
5. Anonymous, Environmental health criteria 63, Organophosphorus Insecticides. WHO, Geneva, 2004. ISBN 9241542632.
6. Deshpande N M, Kanekar PP. Plasmid – mediated degradation in Pseudomona*s* aeroginos*a* MCMB-427. Letters in Applied Microbiology 2001; 33:275-279.
7. Hayatsu M,Hirano M,Tokuda S.Involvement of two Plasmids in Fenitrothion degradation by Burkhoderia sp strain NF100.Appl Environ Microbiol 2000*;* 54:288-293.
8. Finegold S M,Martin WJ. Diagnostic Microbiology, 6th Ed. London: Mosby; 1982. 249-279.
9. Ying Yong Sheng Tai Xne Bao. Isolation and determination of silver resistant bacterial plasmids 2007; 17(2):305-8. Article in Chinese. PMID: 16706059 [Pud Med – indexed for MEDLINE].
10. Spengler G, Molnar A, Schelz Z, Amaral L, Sharples D Monlar J. The mechanism of plasmid wring in bacteria. Curr. Drug Targets 2006; 7 (7): 823 – 41.
11. Bertanie I, Kojic M, Venturi V. Regulation of the p-hydroxybenzoic acid hydroxylase gene (pobA) in plant-growth-promoting Pseudomonas Putida WCS358. Microbiology 2001; 147**:** 1611-1620.
12. Chen Y,.Zhang X, Liu H, Wang Y, Xia X. Study on Pseudomonas sp. WBC-3 capable of complete degradation of methylparathion. Wei Sheng Wu Xue Bao*,* 2002; 42(4):490-7.
13. Van der Meer JR, De Vos WM ,Harayama S , Zehnder A J. Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol Rev 1992; 56(4):677-694.
14. Morales GJF, Linares A, Beloso JP, Albar JL, Martinez, Rojo F. The Pseudomonas Putida Crc. Global Regulator Controls the Expression of Genes from Several Chromosomal Catabolic Pathways for Aromatic Compounds. J Bacteriol 2004; 186(5): 1337 – 1344.
15. Chauddhry GR, Ali AN,Wheeler WB. Isolation of a methyl parathion-degrading Pseudomonas sp. That possesses DNA homologous to the opd gene from a Flavobacterium sp. Appl Environ Microbiol 1988; 54(2): 288-93.
16. Aislabie J G. Lloyd-Jones .A review of bacterial degradation of pesticides. Australian Journal of Soil Research 1995; 33(6): 925-942.
17. Eduardo D, Ferrandez A, Prieto M A, Garcia J L. Biodegradation of Aromatic Compounds by Escherichia coli. Microbiol Molecular Biol Reviews 2001; 65(4): 523-569.
18. Lewinson O, Bibi E.Evidence for simultaneous binding of dissimilar substrates by the Escherichia coli multidrug transporter MdfA. Biochemist 2001; 40(42):12612-8.
19. Lewinson O. The Escherichia coli multidrug transporter MdfA catalyzes both electrogenic and electroneutral transport reactions. Pro*c* Natl Acad Sci *USA* 2003; 100(4):1667-72.