Molecular identification and cloning of organophosphate degradation gene in some bacterial isolates

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ABSTRACT

Seventeen local bacterial isolates which can hydrolyze a wide range of organophosphate (OP) insecticides were purified. They were reduced to ten different isolates based on their RAPD pattern and protein profile and termed as ASM-1 through ASM-10. Chemical assay and bioassay revealed that the isolate ASM-5 was the best isolate in chlorpyrifos degradation. The morphological and molecular identification characterized ASM-5 as Agrobacterium tumefaciens. A gene encoding a protein involved in OP hydrolysis was cloned and sequenced from A. tumefaciens ASM-5. This gene (named opdA) had sequence similarity about 98.7% with the A. tumefaciens opd gene. The coding sequence of the gene was sub cloned down stream an inducible expression promoter to evaluate the gene-enzyme system responsible for its OP-hydrolyzing activity. The biological activity of OPDA protein became more efficient compared to OPDA native protein.

Keywords: Organophosphorus, degrading-bacteria, opdA gene.

INTRODUCTION

Organophosphorus compounds (OP) are widely used as pesticides to control agricultural and household pests. Overall, OP compounds account for ≈38% of total pesticides used globally (Post, 1998; Singh and Walker, 2006). The excessive use of natural resources and large scale synthesis of OP compounds have generated a number of environmental problems such as contamination of air, water and terrestrial ecosystems, harmful effects on different biota, and disruption of biogeochemical cycling (Cisar and Snyder, 2000; Tse *et al.*, 2004). OP compounds poisoning is a world-wide health problem with around 3 million poisonings and 200 000 deaths annually (Karalliedde and Senanayake, 1999 and Sogorb *et al.*, 2004). The compounds have been implicated in several nerve and muscular diseases in human beings (Ragnarsdottir, 2000). In the third-world countries, they remain the major insecticides in agricultural pest management. Therefore, there is a need for economical and dependable methods of OPs detoxification from the environment. Current methods to detoxify OP pesticides mainly rely on chemical treatment, incineration and landfills (Richins *et al.*, 1997). Chemical methods, although feasible, are problematic due to production of a large volume of acids and alkali that subsequently must be disposed. Landfills function adequately, but leaching of pesticides into surrounding soil and ground water supplies is a big issue of concern.

Incineration, which is the most reliable method for destruction of these compounds has met serious public opposition because of the potentially toxic emissions and is economically restrictive (Richins *et al.,* 1997).

Soil microflora is one of the basic agents for detoxification of pesticides. Some investigators found that soil contaminated with pesticides could be possible decontaminated by inoculation with specifically adapted microorganism (Cho *et al.,*2000). Enzymatic detoxification of OP insecticides by some bacterial species has received a considerable attention (diSloudi *et al.,* 1999; Chen-Goodspeed *et al.,* 2001; Gilbert *et al.,* 2003). A variety of OP pesticide-degrading bacteria have been isolated from environments that have come in contact with these chemicals (Cheng *et al.,* 1993; Laurence *et al.,* 2000; Masahito *et al.,* 2000).

These bacterial strains have the ability to degrade OPs using different types of enzymes. The most widely characterized enzymes are phosphotriesterase (PTEs) or organophosphate hydrolase (OPH) and Organophosphate degrading (OPDA). The OPDA or OPH enzyme is capable of hydrolyzing a wide range of OPs (Shimazu *et al.,* 2001).The OPDA enzyme is encoded by the *opd* gene. The bacterial *opd* genes have a similar sequence in different bacterial strains (Horne *et al.,* 2002).

The goals of this investigation were to isolate the bacterial strains having the ability to degrade the chlorpyrifos (OPs insecticide) from agricultural soil and wastewater near area where OPs are heavily used. In addition, the identification and characterization of the gene-enzyme system responsible for OP-hydrolyzing activity was the second goal. Finally, the nucleotide sequence of the *opdA* gene from one of these bacterial isolates will be cloned, expressed in *E.coli* expression sytem and evaluated for its ability for OPs bioremediation.

MATERIALS AND METHODS

Bacterial strains and plasmids
The local bacterial isolates (17 isolates) that used in this study were collected from chlorpyrifos polluted soil and wastewater polluted samples from different governorates (Dakahliyah, Gharbiyah, Minufiyah, Qalyubiyah and Kafr El-Shaykh). Samples were transferred into sterile bottles and stored at 4 $^{\circ}$C untill used.

Escherichia coli DH5α and M15 strains were used as hosts for the cloning and expression studies, respectively. they were kindly provided by Prof. Dr. Naglaa Abdallah, Fac. of Agric., Cairo Univ. The plasmid pGEM-T Easy (Promega,USA) was used to clone the PCR products. The plasmid pQE-30 (QIAGEN, USA) was used as an expression vector. Strains harboring plasmids were grown in Luria-Bertani (LB) medium, at 37 °C and vigorously shaked (300 rpm). Bacteria harboring expression vector were grown to an OD_{600}=0.4 (for 4-5 hr) before induction with 1 mM IPTG as recommended by the manfacturer.

Isolation of Chlorpyrifos - degrading bacteria
Water and soil samples collected as previously described, were diluted serially and sample of each dilution was plated (in duplicate) on LB agar medium that was previously overlaid with 100 ppm Chlorpyrifos. Plates were incubated for 3 to 5 days at two different temperatures (30 °C and 37 °C) till bacterial colonies appeared.

Chemical assay
The bacterial isolates were inoculated (5.6x10^8 cfu/ml) onto M9 minimal medium

supplemented with 100 ppm chlorpyrifos as the sole carbon source. Chlorpyrifos was dissolved in acetone (250 mg / 300 µl), then added to 100 ml M9 media. The bacterial isolate was grown in triplicates at each treatment. After 14 incubation days, residual chlorpyrifos was determined in the culture extract of the bacterial isolates. The amount of chlorpyrifos was measured by Gas Liquid Chromatography and High Performance Liquid Chromatography (HPLC) according to Chukwudebe *et al.* (1989).

Insect bioassay

In order to determine the ability of bacterial isolates to OPs degradation, bioassay of bacterial isolates was performed on first instar larvae of the cotton leaf worm, *Spodoptera littoralis*. After incubating bacterial strains with chlorpyrifos (100 ppm) in M9 minimal media, the diluted supernatant (1:10) was applied uniformly over the food surface in each cup and allowed to dry. Ten larvae were used per dilution and three cups were used for each dilution. The cups were then incubated at 28°C, and mortality was scored.

Identification of isolated bacteria

Single colonies were streaked on LB agar plates for characterization. Selected seventeen different colonies, isolate-1 through isolate-17 were restreaked on LB agar plates for further purification. The purified colonies were stained with Gram's and endospore stain and then examined microscopically to determine the shape and spore forming ability of the selected isolates. Identification was done by using the Biolog MicroLog3 4.20 System (Auto Identification System) at the Plant Pathology Research Institute, Giza, Egypt. Furthermore, biochemical and physiological identification were carried out as described by John *et al.* (1994). Molecular tools such as 16S ribosomal RNA gene was also applied to characterize the selected isolates (Horne *et al.,* 2002).

Random Amplified Polymorphic DNA (RAPD)

RAPD-PCR was carried out according to the procedure published by Williams *et al.* (1990). RAPD-PCR experiments were done using ten OPERON oligonucleotide primers (Table 1). PCR reaction was carried out in a total volume of 25 µl. Each reaction mixture contained 25 ng genomic DNA, 0.25 µM decamer oligonucleotide primer, 1 unit of Taq DNA polymerase, 2.5 µl of l0X buffer and deionized dd H_2O. PCR amplification was performed for 40 cycles after an initial denaturation step for 3 min at 94 °C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 36 °C for 1 min, extension at 72 °C for 2 min. An extension step was performed for 5 min at 72 °C in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel. DNA patterns were analysed, compared and DNA patterns were combined using Diversity Database (Version 2) software (Bio-Rad). The levels of similarity between DNA profiles were calculated using Dice coefficient.

Table (1): The sequences of the primers used in the RAPD-PCR

Primer	Sequence
A-01	5'-CAGGCCCTTC-3'
A-03	5'-AGTCAGCCAC-3'
A-04	5'-AATCGGGCTG-3'
A-07	5'-GAAACGGGTG-3'
A-11	5'-CAATCGCCGT-3'
A-19	5'-CAAACGTCGG-3'
C-05	5'-GATGACCGCC-3'
C-11	5'-AAAGCTGCGG-3'
C-18	5'-TGAGTGGGTG-3'
O-02	5'-ACGTAGCGTC-3'

SDS–PAGE analysis of whole-cell proteins

All bacterial isolates were characterised by 10% SDS–polyacrylamide gel electrophoresis of vegetative stage whole-cell proteins according to the method described by Costas (1992). The protein profiles similarity was calculated using Dice coefficient.

PCR analysis

A loopful of purified bacterial colony was suspended in 100 µl of sterile distilled water and DNA was released from the bacterial cells by boiling in a water bath for 5 minutes to lyse the cells (Elnagdy, 2004). The tubes were spun briefly to collect the condensate. The PCR was performed in a 50 µl reaction volume containing 1X buffer, 200 µM each of dGTP, dATP, dCTP and dTTP, 2.5 units of *Taq* DNA polymerase, 100 pmol of each primer and 5 µl of the DNA template. The target DNA sequences were amplified in a programmed Thermal Cycler by using the step-cycle program as follows: denature the DNA at 94°C for 45 sec, anneal the primers and the DNA at different temperature (50 and 58°C) for 45 sec, and extend the newly synthesized DNA at 72°C for 7 min for a total of 35 cycles.

PCR was carried out to amplify fragments of *16S rRNA* and *opdA* genes. Two specific primers, F-16SA (5`-GCGGCAGGCTTA-ACACATGCAA-3`) and R-16SA (5`-GGTTCCCCTA-CGGCTACCTTGT-3`), were designed to amplify the *16s rRNA* gene. Also, the upstream and downstream oligonucleotide primers, F-opdA (5`-GGATCCATGCAAACGAGAAGAGATGCACTT-3`) and R-opdA (5`-AAGCTTTCATCGTTCGGTATCT-TGACGGGG-3`), were used for amplification of the *opdA* gene. The F-opdA and R-opdA primers were designed to contain, respectively, a *Bam*HI restriction site at the *opdA* start codon and a *Hin*dIII restriction site at the stop codon (underlined bases).

Construction of plasmids for OPDA overexpression

The PCR fragment was cloned into the pGEM-T Easy to generate the recombinant plasmid pGEM-opdA. The complete nucleotide sequencing of the recombinant plasmid pGEM-opdA was determined using automated DNA sequencing to confirm that the *opdA* gene was cloned. The *opdA* gene was *Bam*HI and *Hin*dIII digested from pGEM-opdA and inserted into corresponding sites at the pQE-30 to generate the construction plasmid pQE-opdA. The pQE-30 expression

vector provides a high level expression in *E. coli* of proteins containing a histidine affinity tag at the NH_2 terming. The fused protein is under the control of tightly regulated T5 promoter and Lac operator to allow induction with IPTG according to recommended method.

RESULTS AND DISCUSSION

Phenotypic characterisation

In this study, the seventeen local chlorpyrifos hydrolyzing bacterial isolates were purified from agricultural waste water and soil samples. These isolates were capable of growing on minimal salt media containing 100 ppm chlorpyrifos as a sole carbon source. The isolates were divided into seven different groups based on colony and cell shape. Within each group, the bacterial isolates were similar in morphological, physiological and biochemical characteristics and no differences were observed between them. However, more than one method is usually needed to differentiate the similar isolates.

### Differentiation of OPs-degrading bacterial isolates by RAPD-PCR

RAPD-PCR analysis was performed with 10 different primers to differentiate the isolates which have the same morphological shape. The results indicated that isolate 1 exhibited different RAPD profile with isolate 3 in contrast to the same banding pattern observed with isolate 2 with all tested primers (Fig. 1). Matrix of RAPD profile showed that the similarity between isolates 1 and 2 was 100 % but it was 36.1 % between isolates 1 or 2 and 3. The same results were observed with isolates 4, 5 and 6 which indicated that isolate 4 has the same banding pattern as isolate 5, but different banding pattern with isolate 6. Moreover, the similarity matrix of RAPD profile between isolates 4 and 5 was 100 %, but it was 16.7 % between isolate 4 or 5 and 6.

Isolates 7 and 8 produced the same pattern but their patterns were clearly different from isolate 9 profile. In addition, the similarity matrix of RAPD pattern between isolates 7 and 8 was 100 %, but it was 32.8% between either of them and isolate 9.

RAPD profile was identical between isolates 10 and 11, isolates 12 and 13, isolates 14 and 15, and isolates 16 and 17. Furthermore, similarity matrix was 100 % between isolates 10 and 11, isolates 12 and 13, isolates 14 and 15, and isolates 16 and 17.

SDS-PAGE electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is used for further differentiating between the seventeen isolate genotypes, because this method is relatively easy and many samples can be analyzed at the same time. It is also cheaper than other fingerprinting methods. The SDS-PAGE was carried out for all bacterial isolate genotypes as illustrated in Fig. (2). Bands with different molecular weight were detected and ranged from 12 kDa to 121.9 kDa, while the total number of bands ranged from 13 in isolate 9 to 25 in isolates 14 and 15. Moreover, the results of protein profile were similar to the results of RAPD-PCR. To obtain more balanced values for genetic similarity among OPs-degrading bacterial isolates, the data of RAPD and protein profile were combined (Table 2). Based on the morphological characterizations, the seventeen isolates were divided into seven groups, however the molecular analyses were more sensitive and divided them into ten different isolates.
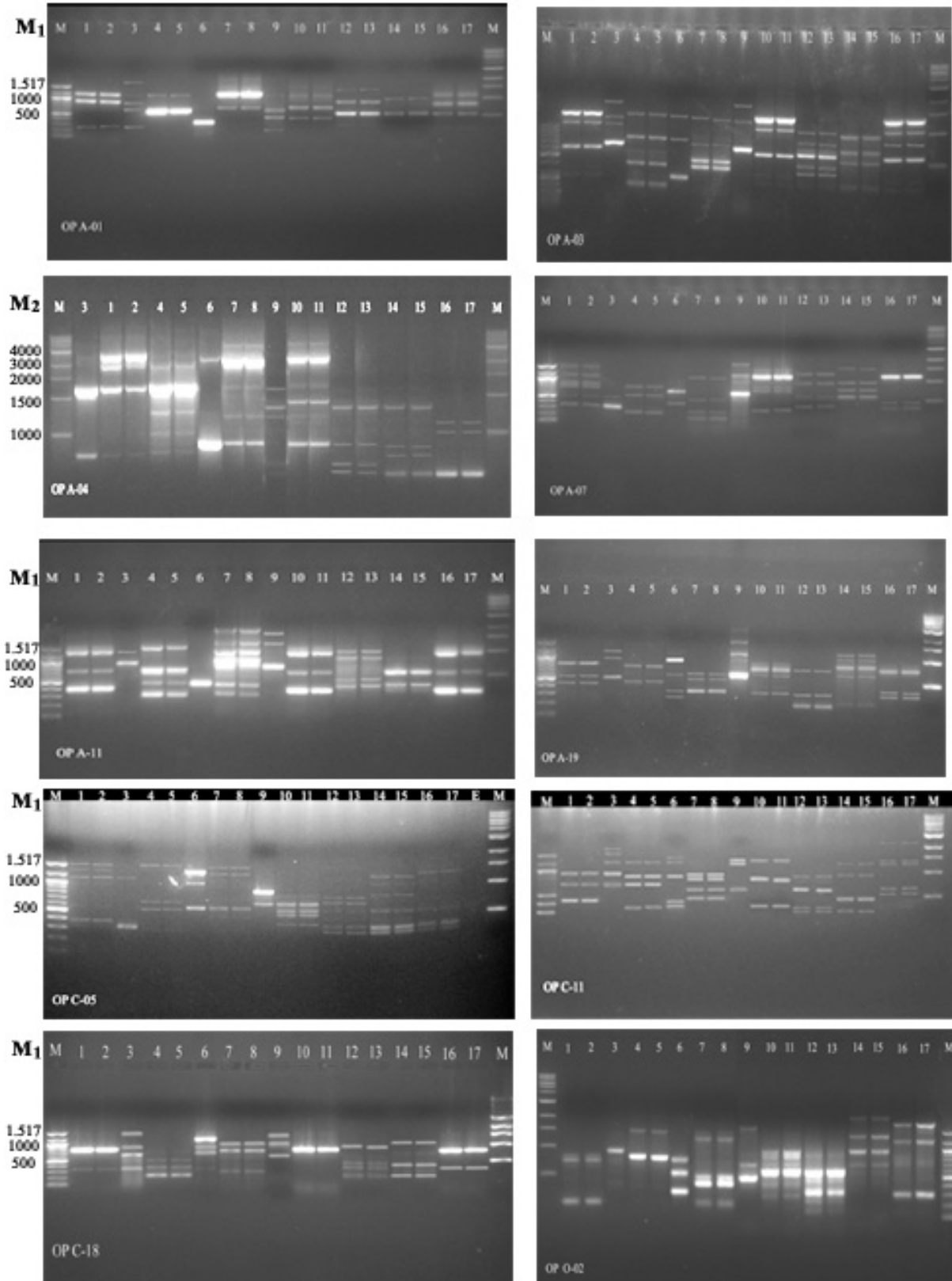
Fig. (1): DNA patterns of OPs-degrading local bacterial isolates (seventeen isolates) produced by RAPD using ten operon primers. M_1: 100 bp DNA ladder and M_2: 1 kb DNA ladder (BioLabs)
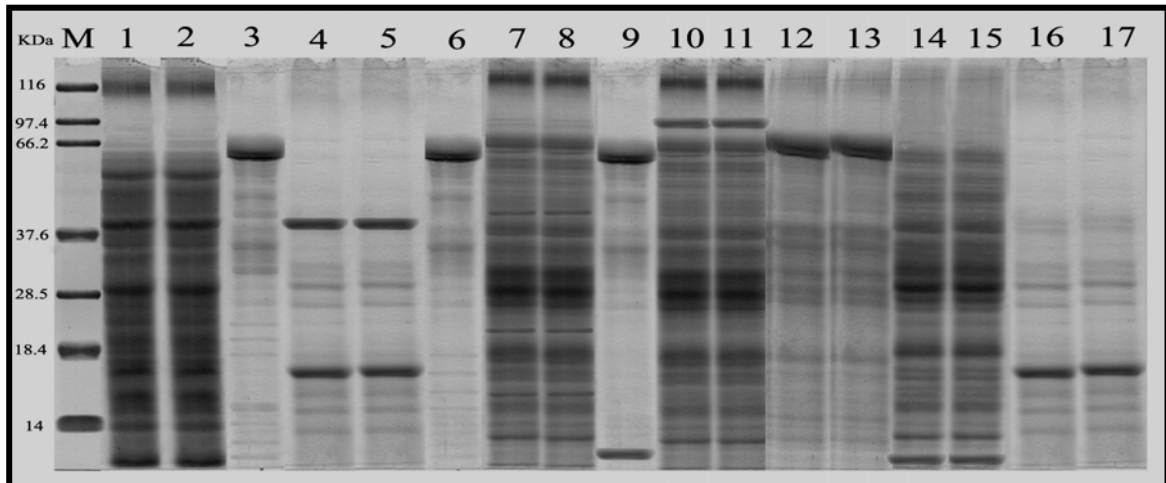
Fig. (2): Whole-cell protein profiles of OPs-degrading bacterial isolates (from 1 to 17). M: protein molecular weight marker (MoBiTec)

Table (2): Summary of similarity matrix % based on RAPD, protein profile and combined data analysis.

Comparison	RAPD similarity (%)	Protein profile similarity (%)	Combining data	Conclusion
Isolates 1 and 2	100.0	100.0	100.0	identical genotype
Isolates 1 or 2 and 3	36.10	44.40	39.60	closely different
Isolates 4 and 5	100.0	100.0	100.0	identical genotype
Isolates 4 or 5 and 6	16.70	50.00	30.00	closely different
Isolates 7 and 8	100.0	100.0	100.0	identical genotype
Isolates 7 or 8 and 9	32.80	35.90	34.00	closely different
Isolates 10 and 11	100.0	100.0	100.0	identical genotype
Isolates 12 and 13	100.0	100.0	100.0	identical genotype
Isolates 14 and 15	100.0	100.0	100.0	identical genotype
Isolates 16 and 17	100.0	100.0	100.0	identical genotype

classical identification methods (Abdel-Salam, 1994 and 1999). Molecular tools such as protein banding pattern (SDS-PAGE) and DNA fingerprinting (RAPD-PCR) were applied for differentiation and merging similar bacterial isolates. RAPD-PCR eliminates the need for pure DNA; only a small amount of template is required for amplification reaction. It was advisable to analyze multiple isolates to differentiate genetic profiles as evidence by the results of this study.

the isolates (1 and 2; 4 and 5; 7 and 8; 10 and 11; 12 and 13; 14 and 15; and 16 and 17) have identical genotypes. These results provided sufficient information to differentiate the bacteria isolates into seven major groups containing ten genotypes. Therefore, the number of bacterial isolates could be minimized from seventeen isolates to ten isolates and designated as ASM-1 through ASM-10.

Residual determination of chlorpyrifos in bacterial isolates inoculated media

Percentage of residual chlorpyrifos was determined in a liquid culture of bacterial isolates. The data showed a considerable removal of chlorpyrifos with elapsed time in inoculated media. During the first seven days, more than 50 ppm of the chlorpyrifos were degraded to other compounds by isolate ASM-

2 or ASM-5 or ASM-8 compared to 95.9 ppm in non-inoculated media (Fig. 3). Fourteen days later, however, chlorpyrifos was reduced to 20.2 ppm, 16.4 ppm and 23.05 ppm in ASM-2, ASM-5 and ASM-8 inoculated culture, respectively, compared to 90.3 ppm in non-inoculated media. These results indicated that ASM-5 isolate was the best bacterial isolates capable of chlorpyrifos degradation.
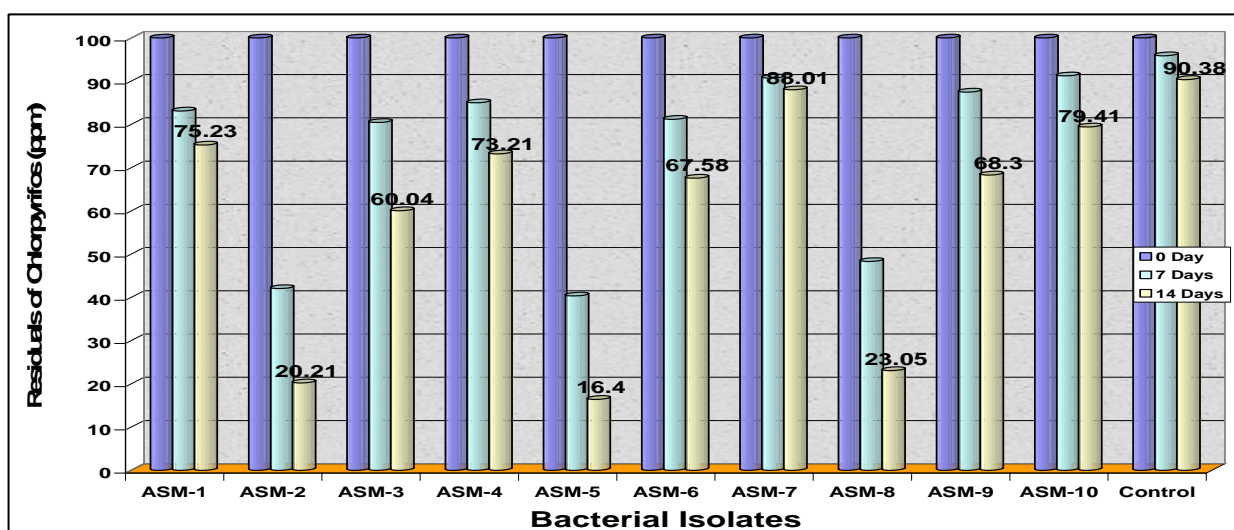

Fig. (3): Percentage of recovery of residual chlorpyrifos in free M9 minimal media in comparison with M9 media inoculated with bacterial strains.

Bioassay of chlorpyrifos

To assess the toxicity of chlorpyrifos against *Spodoptera littoralis,* technical grade chlorpyrifos and culture filtrates of bacterial isolates-inoculated and chlorpyrifos fortified cultures, respectively, were incorporated into artificial diets. The neonates' larvae were placed in each cup and three cups were used for each dilution. Scoring of larval mortality was recorded after incubation time (Fig. 4). The mortality percentages of bacterial isolates were 30%, 20% and 36% with ASM-2, ASM-5 and ASM-8, respectively, compared to 100% in the control. These results showed that ASM-

5 has the best effect in chlorpyrifos degradation, among the ten tested isolates.

Identification of isolated bacteria

The use of Biolog System apparatus had identified ASM-5 as *A. tumefaciens.* In addition, biochemical and physiological identification had characterized ASM-5 as *A. tumefaciens* which was a gram-negative, catalase-positive, oxidase-positive and rod-shaped bacterium. Also, cells are single or pair rod shapes. They are non-spore-forming. Optimum temperature at 25-28°C. Colonies is usually convex, circular, smooth, and non pigmented.

In order to identify and confirm that the isolate ASM-5 is belong to *A. tumefaciens*, eleven 16S rRNA genes *from A. tumefaciens* strain LMG383 (GenBank accession no. AJ130719), strain O363 (GenBank accession no. AJ389908), strain 0362 *(*GenBank accession no. AJ389907), strain CIP43-76 (GenBank accession no. AJ389900), strain CFBP2884 (GenBank accession no. AJ389894), strain CIP127-76 (GenBank accession no. AJ389898), strain CIP28-75 (GenBank accession no. AJ389899), strain ICPB T37 (GenBank accession no. AJ389906), strain C58 (GenBank accession no. AJ012209), strain Mushin6 (GenBank accession no. AJ389905) and strain Zutra F/1 (GenBank accession no. AJ389909) were aligned for identifying the conserved DNA sequences of *16S rRNA gene*. Two conserved sequences were selected for primer designs (F-16sA and R-16SA primers).

To identify the ASM-5 at the molecular level, the *16s rRNA* gene has been amplified using *16s rRNA* primers (Fig. 5) and the amplified fragment was cloned into pGEM-T Easy cloning vector. Screening of the hybrid plasmid carrying the *16s rRNA* fragment was carried out by digestion using *Eco*RI endonucleases (Fig. 6) and other methods. Recombinant plasmid was subjected to nucleotide sequencing and characterization of the cloned gene. The sequence of approximately 1,432 bp of the *16S rRNA* gene of ASM-5 showed 96.4 % similarity with that of the 16S rRNA gene of *A. tumefaciens* strain ICPBT37, strain Mushin6, strain C58, strain AHL7 and strain ZutraF/1 and 95.8 % similar to that of the 16S rRNA gene of *A. tumefaciens* strain 0363. Computer-aided analysis was done using the DNA Star software provided by DNA star Inc. (Madison, WI, USA) and DNA Blast web site.
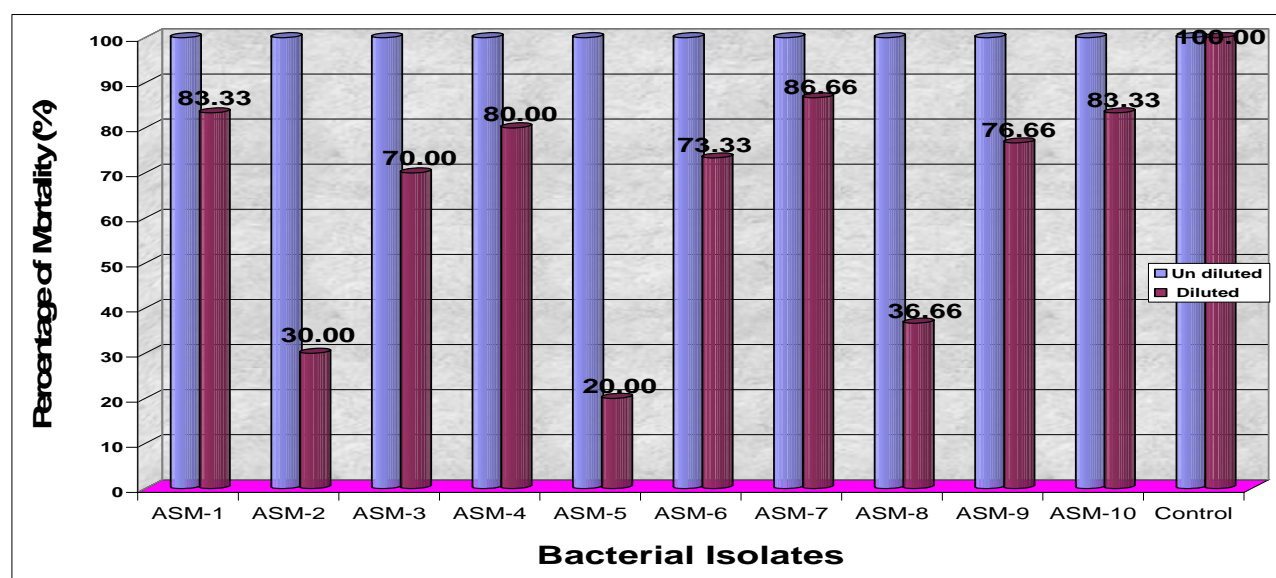
Fig. (4): Determination of mortality percentage against Spodoptera littoralis using undiluted and diluted (1:10) supernatant after incubating bacterial strains with chlorpyrifos (100 ppm) in M9 minimal media.

Cloning of the *opdA* gene from ASM-5 strain

Based on the DNA sequence of *A. tumefaciens opdA* gene (GenBank accession no. AY043245), the specific primers for *opdA* gene (F-opdA and R-opdA) were designed. PCR amplification of the *opdA* gene was carried out using F-opdA and R-opdA primers to amplify the complete coding sequence of
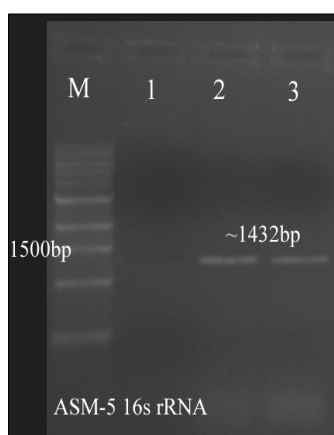

Fig. (5):1.5 % agarose gel resolving the PCR amplification of the 16s rRNA fragment of ASM-5 isolate (lane 2 and 3). M: 1 kb DNA ladder (BioLabs).

The complete nucleotide sequencing of the pGEM-opdA plasmid was carried out using automated DNA sequencer to confirm the correct orientation of the gene. The sequences of the 5´ and 3´ ends were obtained using T7 as a forward primer and SP6 as a reverse primer, respectively. *opdA* gene encoded a protein composed of 384 amino acids with an estimated molecular weight of 41426 Daltons. Moreover, the nucleotide sequence alignment of the *A. tumefaciens*

the gene about ~1155 bp fragment size (Fig. 7).The *opdA* amplified fragment was cloned into pGEM-T Easy cloning vector. Double digestion using *Bam*HI *and Hind*III endonucleases (Fig. 8) was done to screen the positive colonies. Recombinant plasmid carrying the *opdA* gene was named as pGEM-opdA.
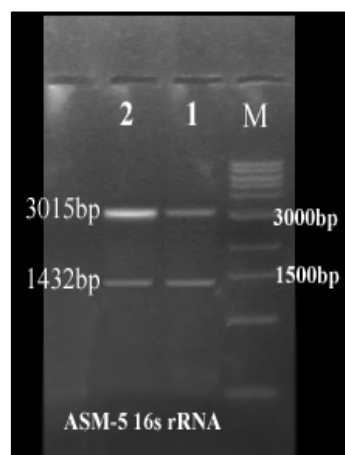
Fig. (6): Screening for the positive colonies of the hybrid plasmids by digestion using EcoRI endonucleases (lane 1 and 2). M: 1 kb DNA ladder (BioLabs).

ASM-5 *opdA* gene revealed that *opdA* gene has an identity of 98.7 % with the GenBank *A. tumefaciens opdA* gene published sequence.

Cloning of *opdA* gene in *E. coli* expression system

The *opdA* gene was released as a ~ 1155 bp *Bam*HI */Hind*III fragment from pGEM-opdA and sub-cloned at the corresponding sites of the pQE-30 expression vector (Fig. 9). Recombinant plasmid was screened using PCR experiment (Fig. 10).
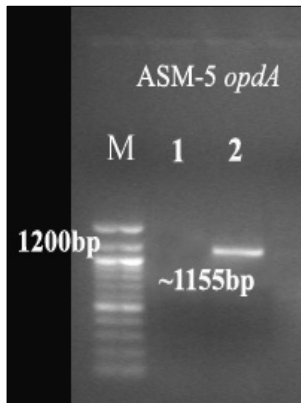
Fig. (7): 1.5 % agarose gel resolving the PCR amplification of the opdA fragment of ASM-5 isolate (lane 2). M: 1 kb DNA ladder (BioLabs).
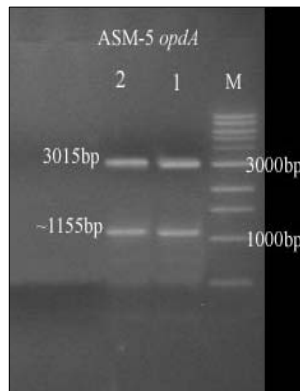
Fig. (8): Screening for the positive colonies of pGEM-opdA plasmid by digestion using BamHI and HindIII endonucleases (lane 1 and 2). M: 1 kb DNA ladder (BioLabs).
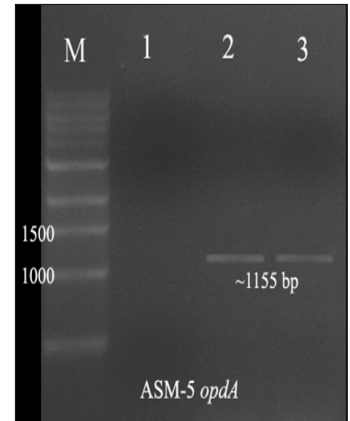
Fig. (10): Screening for the positive colonies of pQE-opdA plasmid by PCR (lane 2 and 3). M: 100 bp DNA ladder (BioLabs).
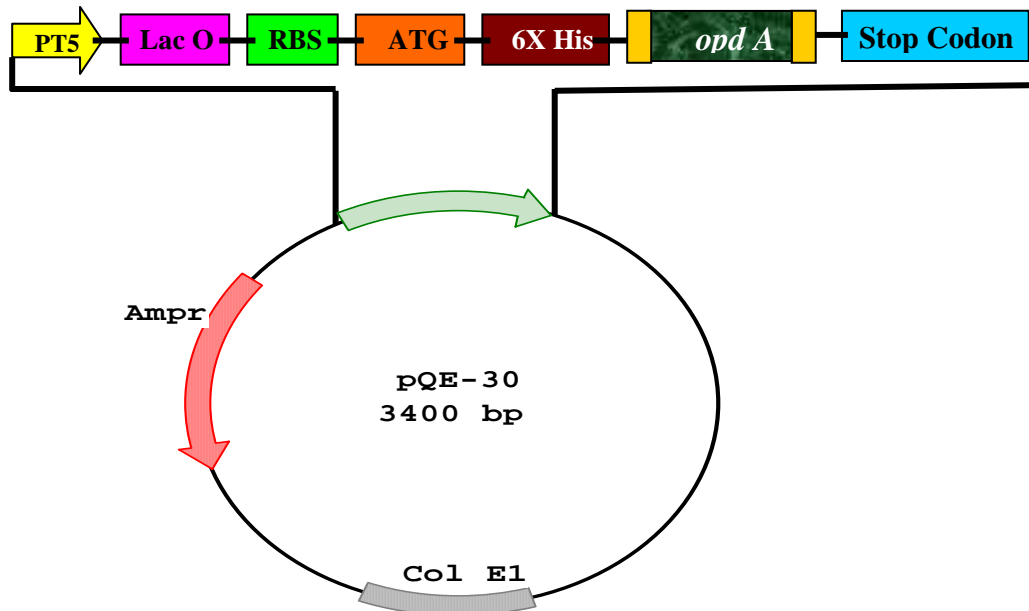
Fig. (9): Physical map of the pQE-opdA expression plasmid.

Evaluating of expression system of the *opdA* gene

Evaluation of the expression for the *opdA* gene was carried out either by using chemical assay or bioassay as rapid methods for measuring the activity of the expression of the gene. The percentage of residual chlorpyrifos was scored 5.25 ppm compared to 93.5 ppm in a non-inoculated medium. After expression of *opdA* gene within pQE-opdA host cells, the mortality percentage was 13.3%, compared to 20% with native gene. These results indicated that the expression of *opdA* gene became more efficient under T5 promoter compared to the *A. tumefaciens* ASM-5 *opdA* native gene. Consequently, the rate of

chlorpyrifos degradation for the *opdA* gene with the expression vector showed high a rate compared to the degradation rate of *A. tumefaciens* ASM-5.

The bacterial biodegradation and utilization of OP compounds as the sole carbon source were reported by some investigators, (Singh *et al.*, 2003; Singh *et al.*, 2004; Singh *et al.*, 2005). In a recent study, chlorpyrifos degradation by ASM-5 in the absence of other growth substrates was investigated. The results indicated that chlorpyrifos supported the growth of ASM-5 as a sole source of carbon. Consistent with these results, different bacteria have shown to grow on parathion and methyl parathion as the sole carbon source (Shimazu *et al.*, 2001; Zhongli *et al.*, 2001). The growth of ASM-5 in this poor media was due to the biodegradation of this insecticide by the enzyme machinery that already exists inside the cell. This enzymatic machinery is largely hydrolytic to such compounds and plays a major role in utilization of these materials as nutrient sources. This finding was also confirmed by those obtained by Bhaskaran *et al.* (1973) who found that some soil microorganisms especially *Streptomyces* sp., can utilize the OP insecticides under the *in-vitro* conditions. Similar results were obtained by Nelson (1982).

The results of chemical and bioassay revealed that the ASM-5 isolate was the best bacterial isolates in chlorpyrifos degradation. Therefore, morphological and molecular identification of ASM-5 isolate was done. The morphological (automated and manual) and the molecular identification (amplification, cloning and sequencing of *16s rRNA* gene) had characterized and identified the ASM-5 isolate as *A. tumefaciens*. Horne *et al.* (2002) have obtained similar results. They isolated a bacterial strain, *Agrobacterium radiobacter*

P230 that has the ability to hydrolyze a wide range of OP insecticides. Also, they identified this isolate on morphological and molecular (*16S rRNA* gene) levels. Further, a methyl parathion-degrading bacterial strain M6 was isolated and identified as *Plesiomonas* sp. (Zhongli *et al.*, 2001). In accordance with the above results, Singh *et al.* (2004) had isolated a bacterial strain with the ability to hydrolyze a chlorpyrifos. They identified this strain as a *Enterobacter* strain B-14.

OPs contain three phosphoester linkages and are hence termed phosphotriesters. In general, hydrolysis of one of the phosphoester bonds reduces the toxicity of OPs (Horne *et al.*, 2002). Enzymatic detoxification of OPs has become the focus of many studies, because other means of removing OP residues are impractical, costly or environmentally hazardous. The most widely studied bacterial enzyme is the organophosphate degrading enzyme from *A.radiobacter* (OPDA) is very similar in sequence to an organophosphate hydrolase (OPH) from *Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551. OPDA enzyme catalyzes the hydrolysis of a wide range of organophosphate pesticides (Shimazu *et al.*, 2001). Recent advances in molecular biology have spent up new avenues to move towards the goal of engineering microbes or enzymes to function as designer biocatalyst (Chen and Mulchandani, 1998). Several authors have identified the *opd* and *opdA* genes in organisms isolated from different geographic locations (Sethunathan and Yoshida, 1973; Chaudhry *et al.*, 1988; Harcourt *et al.*, 2002). Moreover, many authors reported the cloning of organophosphate degradation genes from different bacteria using methods different from the one mentioned in this study. Horne *et al.* (2002) reported the isolation of an *A. radiobacter* strain P230, which has the ability

to hydrolyze wide range OP insecticides. In addition, they were able to clone the gene involved in OPs hydrolysis which was 1155 bp and encoded a protein composed of 384 amino acids. Also, Zhongli *et al.* (2001) reported the cloning of a novel organophosphate hydrolase gene designated *mpd* (GenBank accession no. AF338729) from a strain of *Plesiomonas* by shotgun cloning.

In this study, the *opdA* gene had been identified in an organism isolated from Egyptian environment. The size of *opdA* gene was found to be approximately 1155 bp and encoded a protein composed of 384 amino acids. After cloning and sequencing, the nucleotide sequence alignment of the ASM-5 *opdA* gene revealed that *opdA* gene has an identity of 98.7 % with the GenBank *A. tumefaciens opd* gene. This is due to that the *opd* genes having the same DNA sequence and encoding the same protein sequence were acquired by *B. diminuta* MG, *Flavobacterium* sp. strain ATCC 27551 (Harper *et al.,* 1988) and *A. radiobacter* P230 (Horne *et al.,* 2002).

Horne *et al.* (2002) overexpressed the OPDA in *E. coli* DH10β by using the pMAL protein fusion and purification system, which resulted in expression of a maltose-binding protein (MBP). By the same way, *opdA* gene was sub-cloned into pQE-30 expression vector to generate the recombinant plasmid pQE-opdA which resulted in expression of a 6xHis-OPDA fusion protein. Moreover, the expression of *opdA* gene became more efficient under T5 promoter control compared to *A. tumefaciens* ASM-5 *opd* native gene.

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الملخص العربي

التعريف الجزيئي و كلونة جين هدم المبيدات الفوسفورية العضوية في بعض العزلات البكتيرية

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تم عزل سبع عشرة عزلة بكتيرية لها القدرة على هدم وتحليل مدى واسع من المبيدات الفوسفورية العضوية ، و نظراً لتشابه هذه العزلات مع بعضها البعض في الصفات المورفولوجيةو الميكروسكوبية . تم إستخدام تكنيك RAPD and SDS-PAGE لتوضيح الأختلافات بين هذه العزلات و إختزال عددها إلى عشرة عزلات مختلفة سميت ASM-1 حتى ASM-10 . وأوضحت نتائج التحليلات الكيميائية و البيوحيوية Chemical assay and Bioassay أن أفضل هذه العزلات في تكسير مبيد الكلوربيروفوس (كنموذج للمبيدات الفوسفورية) هي العزلة ASM-5 . وقد تم تعريف هذه العزلة على المستوى المورفولوجى و الجزيئى على أنها Agrobacterium tumefaciens و إستخدمت هذه السلالة لعزل و كلونة و قراءة تتابع الجين المسئول عن هدم وتكسير المبيد. وهذا الجين يسمى opdA وأثبتت النتائج أن التتابعات الوراثية للجين تتشابه بدرجة عالية مع تتابعات جين A. tumefaciens opd . و تم تقيم التعبير الجينى المسئول عن هدم المبيدات الفوسفورية العضوية عن طريق إجراء كلونة مرة أخرى لهذا الجين في ناقل تعبيرى تحت تحكم محفز مستحث.