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Biodegradation of Chlorpyrifos by *Klebsiella* sp. Isolated from an Activated Sludge Sample of Waste Water Treatment Plant in Damascus

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ABSTRACT. A chlorpyrifos (CPY)-degrading bacterial strain was isolated from an activated sludge sample collected from the *Damascus Wastewater Treatment Plant*, Syria. The isolation of *Klebsiella* sp. was facilitated by the addition of CPY at a rate of 3.84 g/L of sludge weekly (selection pressure). Identification of *Klebsiella* sp. was done using major staining and biochemical differentiation tests (Gram stain, cytochrome oxidase and some relevant saccharide fermentation tests using biochemical assays). *Klebsiella* sp. was maintained by culturing in a poor medium consisting of mineral salts and CPY as the sole carbon source. When 3 activated sludge samples were incubated in the presence of CPY (13.9 g/L sludge), 46 % of added CPY were degraded within 4 d. By comparison, within 4 d the isolated *Klebsiella* sp. was found to break down 92 % of CPY when co-incubated in a poor mineral medium in which CPY was the sole carbon source (13.9 g/L poor medium). Isolated *Klebsiella* sp. was able to tolerate up to 17.3 g of CPY in the poor medium.

Abbreviations

CPY	chlorpyrifos [(*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphothioate]		
TCP	*O*-(3,5,6-trichloro-2-pyridyl)phosphothioate		
DWTP	*Damascus Wastewater Treatment Plant*	GC	gas chromatography
EC	emulsifiable concentrate	MSM	mineral salt medium

CPY is a broad spectrum insecticide used worldwide in agriculture (Cho *et al*. 2002). It was introduced in 1965 (Hayes and Laws 1990), and originally used to control mosquitoes in the immature larval stage; however, it is no longer registered for this purpose. CPY is effective in controlling a variety of insects, including cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice (*US EPA* 1986). It was used as an insecticide on grain, cotton, fruit, nut and vegetable crops, as well as on lawns and ornamental plants (Hayes and Laws 1990); it is available in emulsifiable concentrate, dust, flowable, pellet, spray, granular and wettable powder formulations (Meister 1992).

Microbial degradation of organophosphate pesticides is of particular interest because of the high mammalian toxicity of such compounds and their widespread and extensive use (Singh *et al*. 2004). Some organophosphate pesticides, such as parathion, were shown to be degraded by bacteria (Singh *et al*. 2003). Moreover, a strain of *Pseudomonas putida* capable of hydrolyzing methylparathion and of using 4-nitrophenol as the sole carbon source was isolated from soil (Rani and Kumari 1994). Although CPY has been in use as an agricultural household insecticide for a long time, attempts to isolate a CPY-degrading bacterial strain have until recently failed. The resistance of CPY to enhanced degradation in soil was attributed to this failure (Singh *et al*. 2004). It was suggested that the accumulation of TCP (with antimicrobial properties) prevents the proliferation of CPY-degrading microorganisms in soil (Racke *et al*. 1990). CPY was reported to be cometabolically degraded in liquid media by *Flavobacterium* sp. and *Escherichia coli* clone (Richnis *et al*. 1997; Wang *et al*. 2005). However, these microbes do not utilize CPY as energy source. The degradation of CPY was demonstrated in mineral-salt medium by *Arthrobacter* sp. which was initially isolated from methylparathion-enriched soil (Mallick *et al*. 1999).

The objective of the present study was to isolate and identify CPY-resistant bacteria from activated sludge samples collected from DWTP. Also, the study sought to assess the ability of the isolated strain to degrade CPY and to determine the tolerance level of the isolated strain to CPY.

MATERIALS AND METHODS

Chemicals. CPY (EC) for commercial use, 480 g/L, was obtained from the local market. Technical grade CPY (99.3 %) was obtained from the *Institute of Industrial Organic Chemistry*, Poland. Solvents were of residue grade and were obtained from *Merck*.

CPY extraction. CPY was extracted using the liquid–liquid extraction method. Fifty mL of CH_2Cl_2 were added to 5 mL of CPY-containing culture medium in a separation funnel. The mixture was vigorously shaken for 5 min and left to stand until phase separation took place. The CH_2Cl_2 layer (bottom layer) was passed through anhydrous Na_2SO_4 on a *Whatman* filter paper no. 1 into a 200-mL round-bottom flask. The sample was extracted twice, the extracts were combined and concentrated on a rotary evaporator at 40 °C under vacuum. The extract was evaporated to dryness using N_2 stream, the volume being adjusted to 1 mL with Me_2CO. CPY extraction efficiency was determined by preparing a 250-mL sludge sample spiked with 3.84 g/L CPY in 3 replicates and extracting them (see *above*). The extraction efficiency of CPY from sludge was 79, 81, and 80 % for the 3 replicates.

CPY analysis. The extracts containing CPY were analyzed by GC using the Agilent 6890 apparatus; the initial column temperature was 60 °C with the initial hold time of 1 min; temperature raised by 25 K/min up to 200 °C with a final hold time of 7 min; injector temperature 200 °C, N_2 flow rate 3 mL/min, injection volume 1 μL; injections were delivered into a DB-35MS GC column: 30 m × 0.32 mm i.d., 0.25 mm film thickness. Detection of CPY was achieved by using a flame photometric detector: H_2 flow rate 75 mL/min, air flow rate 100 mL/min, detector temperature 250 °C.

Isolation and characterization of a CPY-degrading bacterium. A sample of activated sludge was collected from the DWTP. The sludge sample was exposed to CPY-selection pressure by weekly addition of 3.84 mg/mL of CPY to activated sludge sample (250 mL). The choice to use a high concentration of CPY for selection pressure (3.84 mg/mL per week) was to enhance the selection pressure, thereby reducing the number of surviving species and only to obtain the organisms that were able to withstand such a high concentration. At weekly intervals and before the weekly addition of CPY, an activated sludge sample was streaked on Mackonkey (*HiMedia* M001) and nutrient agar media (*HiMedia* M081) for anaerobic and aerobic bacteria cultivation, respectively. The isolated species were identified and differentiated by observing the colors and morphological characteristics indicative of their colonies according to the *HiMedia* product information manual descriptions.

For the isolation, detection, and distinguishing of some relevant important coliform species (such as *Enterobacter* sp., *Klebsiella* sp. and *E. coli*), Mackonkey agar was used. Incubations were carried out at 37 °C for 1 d according to *APHA* (1995). Confirmation of the isolated bacteria and identification by some major staining and biochemical differentiation tests, including Gram staining, cytochrome oxidase and some fermentation tests of relevant saccharides were done according to *APHA* (1995). The isolated strain was initially examined by means of Gram smear and colonial morphology. Cytochrome oxidase test was performed to determine if the organism produces this enzyme if present in a large amount; cytochrome oxidase will react visibly with *N*,*N*,*N*′,*N*′-tetramethyl-1,4-benzenediamine which gets oxidized into indophenol producing a deep purple color. Therefore, the test reagent is soaked into a *Whatman* filter paper no. 1, a loopful of the organism is rubbed on the filter paper; the presence of cytochrome oxidase is indicated by the formation of a purple color. Saccharide fermentation test was performed by the inoculation of the isolated strain into a medium consisting of a saccharide, basal nutrients, and phenol red. The inoculated media were incubated for 1 d at 37 °C. The persistence of red color in the inoculation medium indicates that the isolate is a non-fermenter, whereas the development of yellow color with no bubble formation indicates the occurrence of a fermentation process with no acid production. The development of yellow color and the formation of bubbles indicates a fermentation process with acid production.

Confirmation of the identity of the bacterial isolate was done using the Thermolab Reader and a Bacterial Sensitivity Program (*Merlin*, Finland).

Degradation of CPY by activated sludge vs. *isolated* Klebsiella *sp*. The MSM used to investigate the capability of an isolated strain of *Klebsiella* sp. to degrade CPY consisted of (mg/L): $MgSO_4$ 0.15, KCl 0.5, Na_2HPO_4 0.01 dissolved in 1 L of distilled water and sterilized in the autoclave. For degradation study, 3 replicates of 250 mL of MSM were inoculated with 10^5 cells per mL of isolated strain and supplemented with 2 mL of CPY (EC), its final concentration being 3.84 g/L. Controls were 3 replicates of 250 mL of MSM supplemented with 2 mL of CPY (EC) but not inoculated with *Klebsiella* sp. For activated sludge degradation capability, 3 replicates of activated sludge (250 mL each) were supplemented with 2 mL of CPY (EC). Control consisted of 3 replicates of sterilized activated sludge supplemented with 2 mL of CPY (EC). Sterilization of activated sludge was achieved by placing it in an autoclave (120 °C, 103 kPa, 20 min). The two experiments, *i.e.* comparison of activated sludge CPY degradation *vs*. the control, and *Klebsiella* sp. de-

gradation of CPY *vs*. the control were performed in a single run. Samples were placed in a water bath with shaking at 37 °C. Five mL of each replicate were taken for CPY analysis every day. Percentages of CPY degradation were calculated based on the amount present in each sampling period for each replicate compared with the initial amount of CPY.

Tolerance of isolated CPY-degrading bacterium to CPY. For determining the tolerance of isolated CPY-degrading bacterium to chlorchlorpyrifos, 10^5 cells per mL of isolated, acclimated strain were inoculated into 100 mL of MSM medium. CPY (9.6 g/L) was added to the inoculated medium daily. The viability of the acclimated strain was detected daily by culturing on Mackonkey agar.

Statistical analysis. Biodegradation of CPY in the 2 sets of control samples and those of *Klebsiella* sp. and activated sludge were subjected to repeated measures ANOVA analysis using StatView Statistical Program (*Abacus Concepts* 1996). Results of sludge degradation of CPY were compacted with their corresponding controls and compared with the results of *Klebsiella* sp. degradation of CPY with their corresponding controls. ANOVA test was performed with Fisher's LSD test at a 1 % level.

RESULTS

A bacterium was isolated from an activated sludge sample that was able to withstand a selection pressure of weekly addition of CPY (3.84 g/L). During 4 weeks, the selection of CPY pressure (*i.e.* the addition of 3.84 g of CPY in total) yielded only one isolate that continued to grow on Mackonkey agar medium. The isolated strain was described as G^- mucoid rods. It is a sugar-fermenting bacterium and a non-acid gas producer. Biochemical tests showed that it belongs either to *Aerobacter* sp. or to *Klebsiella* sp. However, the gelatin test and bacterial sensing-plate-reader tests confirmed that it belongs to *Klebsiella* sp. Isolated bacteria continued to grow under selection pressure conditions by culturing in a poor medium containing CPY. Four d following inoculation of mineral culture medium (poor medium) supplemented with CPY (3.84 g/L) with 10^5 cells per inoculum, the concentration of cells increased to reach the order of 10^8, an indication that the strain was able to utilize CPY as a source of carbon for growth and multiplication. This was accompanied by degradation of CPY.

Table I *top* shows the fate of CPY when incubated with activated sludge samples. Mean percentages of CPY degradation in the controls, where CPY was incubated with sterilized activated sludge, was very low and it ranged from 0.8 % for the 1st d to 3.5 % for the 4th and final day. However, when incubated with activated sludge (treatment), the mean percentages of CPY degradation were 12, 19, 33 and 49 % for days 1, 2, 3, and 4.

Table I. Chlorpyrifos (concentration *c*, g/L; mean ± SD) degradation by an activated sludge sample[a–c]

Time, d	Control		Treatment*	
	c	degradation, %	*c*	degradation, %
Activated sludge sample				
0	3.84	0	3.84 e	0 e
1	3.81 ± 0.004	0.80 ± 0.10	3.37 ± 0.06 d	12.3 ± 1.53 d
2	3.78 ± 0.01	1.50 ± 0.34	3.11 ± 0.04 c	19.0 ± 1.00 c
3	3.74 ± 0.005	2.67 ± 0.12	2.56 ± 0.06 b	33.3 ± 1.53 b
4	3.70 ± 0.01	3.53 ± 0.15	1.97 ± 0.06 a	48.7 ± 1.53 a
***Klebsiella* sp.[d, e]**				
0	3.84	0	3.84 e	0 e
1	3.80 ± 0.01	0.97 ± 0.15	2.69 ± 0.04 d	30.0 ± 1.00 d
2	3.79 ± 0.01	1.40 ± 0.20	1.80 ± 0.04 c	53.0 ± 1.00 c
3	3.75 ± 0.01	2.33 ± 0.21	1.04 ± 0.04 b	73.0 ± 1.00 b
4	3.71 ± 0.005	3.33 ± 0.12	0.29 ± 0.06 a	92.3 ± 1.53 a

[a]From *Damascus Wastewater Treatment Plant*. [b]Chlorpyrifos was added to activated sludge samples.
[c]Three replicates per treatment. [d]Isolated from an activated sludge sample.
[e]Cultured in poor medium (mineral salts) with chlorpyrifos as the sole carbon source.

*Values followed by different letters within each column are significantly different at 1 % level according to the LSD test.

Incubation of CPY with *Klebsiella* sp. isolated from activated sludge following selection pressure exerted by the constant addition of CPY resulted in a higher degradation of CPY and at a faster rate. Table I *bottom* shows the mean percentages of CPY degradation by isolated *Klebsiella* sp. grown on mineral (poor) culture medium supplemented with CPY as the sole carbon source. By the 1st d an average of 30 % of added CPY has already been degraded compared to 12 % degradation observed in the 1st d when CPY was incubated with activated sludge. Mean percentages of CPY degradation by the isolated *Klebsiella* sp. increased to reach 53, 73 and 92 % for the 2nd, 3rd and 4th d, respectively. Differences in CPY degradation between the days were significantly different at a 1 % confidence level according to the LSD significance test. For the controls, CPY degradation ranged between 0.97 and 3.3 % only.

Isolated *Klebsiella* sp. grown on a poor medium supplemented with CPY as carbon source was able to withstand relatively high concentrations of CPY. It could grow in the presence of 173 g of CPY in 1 L of poor culture medium (*i.e.* 173 g/L) to which CPY was added at a rate of 9.6 g/L per day.

DISCUSSION

Activated sludge is a suitable medium for growth of various microbial species. We used a selection pressure of CPY in order to find CPY-degrading bacteria. The use of selection pressure is a common approach to isolate pesticide-degrading organisms. It was used for different applications ranging from anaerobic biodegradation of chlorophenols in acclimated sludge (Boyd and Shelton 1984), to isolation from soil of bacteria and fungi capable of degrading various types of pesticides (Richards *et al*. 1997; Singh *et al*. 2003; Hashimi *et al*. 2004; Wang *et al*. 2005). Some papers reported the isolation of CPY-degrading organisms from soil (Singh *et al*. 2004; Yang *et al*. 2005; Ajaz *et al*. 2005; Yu *et al*. 2006).

Our study is the first report on the isolation of CPY-degrading bacteria from activated sludge. Although CPY is said to be resistant to enhanced degradation (Racke *et al*. 1990; Singh *et al*. 2003), the extended use of CPY for many years may permit some opportunistic microorganisms to develop the capability to use this toxic compound as an energy source for their survival as reported with organochlorine pesticides (Singh *et al*. 2000) and with a bacterial strain capable of degrading CPY that was isolated from Australian soil where CPY had been in use for 15 years (Singh *et al*. 2004). In Syria, CPY was introduced 20 years ago (*Directorate of Plant Protection*, personal communication), and it is used extensively in agricultural and in public health application. Our bacterial strain was isolated from activated sludge taken from DWTP, in which drainage water from Damascus and greater Damascus area is treated.

The selection pressure applied by us has facilitated the isolation of one of these organisms, and improved its capability to use this compound as energy source compared to its capability before selection pressure. Mean percentage of CPY degradation by isolated *Klebsiella* sp. reached 92 % within 4 d (Table I *bottom*), compared to 48 % degradation within the same period of CPY exposure to activated sludge (Table I *top*). Singh *et al*. (2004) reported that a soil-isolated *Enterobacter* strain was capable of complete degradation of CPY at a concentration of >250 mg/L in less than 2 d. In this paper, 53 % of CPY at 3.84 g/L were degraded within 2 d, this being equivalent to >2 g/L. The difference in the degradation capability of *Klebsiella* sp. observed here compared to that of *Enterobacter* sp. (Singh *et al*. 2004) may be attributed to the difference in the selection pressure to which each of the two bacteria was subjected. Singh *et al*. (2004) isolated their *Enterobacter* strain from a soil that was subjected to 25 mg/kg CPY, thrice at an interval of 9 d, totaling 75 mg/kg on the 27th d after which they isolated their *Enterobacter* sp. that was capable of degrading 250 mg/L in less than 2 d.

In the present study, activated sludge was subjected to 3.84 g/L CPY, at weekly intervals for 4 weeks, totaling 12.4 g in 4 weeks after which a *Klebsiella* sp. strain was obtained and was capable of degrading 2 g/L in 2 d. *Klebsiella* sp. isolated by us showed a high tolerance to CPY in comparison with tolerance reported by Ajaz *et al*. (2005). They reported the isolation of 3 bacterial strains capable of CPY degradation, and that the isolated strains, *viz. Pseudomonas*, *Aeromonas* and *Klebsiella* tolerated 2, 4, and 8 g/L of CPY, respectively. *Klebsiella* sp. isolated here tolerated 173 g/L of CPY in MSM. This difference might be attributed to the high amount of CPY (3.84 g/L per week) used in the selection pressure which made the isolated *Klebsiella* sp. more tolerant and adapted to high concentrations of CPY.

A follow-up of this work should concentrate on whether the strain is capable of degrading TCP, to examine the degrading capability of the strain using ^{14}C-labelled CPY to determine the amount of CPY which is degraded to $^{14}CO_2$, the performance of the strain in the presence of other carbon sources, the best temperature and pH for optimum performance of the isolate and the enzymic activity of the *Klebsiella* strain in terms of CPY degradation.

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