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Bacterial Degradation of Chlorpyrifos in Pure Cultures and in Soil

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Chlorpyrifos [O,Odiethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a widely used insecticide effective against a broadspectrum insect pests of economically important crops. In rice, chlorpyrifos is intensively used for effective control of gall midge (*Orseolia oryzae*), leaffolder (*Nappalocroas medinalis*) and leafhopper (*Nephotettix virescens*). Chlorpyrifos is characterized by P-O-C linkage, as in diazinon, parathion, methyl parathion and fenitrothion. But, chlotpyrifos is not affected by accelerated biodegradation in retreated soils unlike diazinon (Sethunathan 1971; Sethunathan and Pathak 1972), parathion (Sethunathan 1973), methyl parathion (Misra et al. 1992) and fenitrothion (Misra et al. 1993). Therefore, efforts to isolate chlorpyrifos-degrading microorganisms from chlorpyrifos-acclimatized soils have not been successful (Racke et al. *1990).* A *flavobacterium* sp., isolated from diazinon-retreated rice fields (Sethunathan and Yoshida 1973), is probably the most versatile bacterium reported in literature, capable of hydrolyzing, with great ease, a variety of organophosphorus compounds [diazinon, parathion, methyl parathion, fenitrothion, coumaphos, diisopropyl fluorophosphate (a structural analogue of nerve gases, soman and sarin)], widely differing in side chain and ring moiety, but all characterized by a common P-O-C linkage (Sethunathan and Yoshida 1973; Adhya et al. 1981; Karns et al. 1986; Attavay et al. 1987). Recently, an *Arthrobacter* sp., isolated from a flooded soil retreated with methyl parathion, could hydrolyze not only methyl parathion, but also parathion and fenitrothion (Misra et al. 1992). Racke and Coats (1988) found that chlorpyrifos was neither metabolised nor cometabolised by an *Arthrobacter* sp. In view of the problems encountered in the isolation of chlorpyrifos-degrading microorganisms from chlorpyrifos-enrichments, it was thought worthwhile to determine whether bacteria, isolated from enrichments with related organophosphorus compounds with a common P-O-C linkage, can be used for detoxification of chlorpyrifos. In the present study, we report the rapid degradation of chlorpyrifos, added to a mineral salts medium as a sole carbon source or applied to the soil, by the *Flavobacterium* sp. ATCC 27551 [isolated from diazinon-retreated rice fields (Sethunathan and Yoshida 1973)] and the *Arthrobacter* sp. [isolated from a flooded soil retreated with methyl parathion (Misra et al. 1992)].

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MATERIALS AND METHODS

The *Flavobacterium* sp. ATCC 27551, isolated from diazinon-retreated rice fields (Sethunathan and Yoshida 1973) and the *Althrobacter* sp., isolated from a flooded soil retreated with methyl parathion (Misra et al. 1992) were used in this study. Chlorpyrifos was added to presterilized 100-mL Erlenmeyer flasks in 0.1 mL of acetone. After evaporation of acetone in 24 h, 10 mL of a mineral salts medium ($MgSO_4$. 7 H_2O, 0.2 g; K_2HPO_4, 0.1 g; $CaSO_4$, 0.4 g; $FeSO_4$. 7 H_2O, 0.001 g; distilled water, 1L, pH 6.5) were placed in 100-mL Erlenmeyer flasks and the flasks were shaken for 24 h on an orbital shaker. The medium was inoculated with a suspension of the cells of *Flavobacterium* sp. ATCC 27551 or *Arthrobacter* sp., grown on Wakimoto agar medium [$Ca(NO_3)_2$. 7 H_2O, 0.5 g; Na_2HPO_4.12 H^2O, 2 g; peptone, 5 g; sucrose, 15 g; $FeSO_4$. 7 H_2O, 0.5 g; agar, 15 g; distilled water, 1L] and MGSP medium ($MgSO_4$. 7 H_2O, 0.2 g; K_2HPO_4, 0.1 g; $CaSO_4$, 0.4 g; $FeSO_4$. 7 H^2O, 0.001 g; glucose, 10 g; peptone, 5 g; agar, 15 g; distilled water, 1 L), respectively for 48 h and prepared in sterile distilled water. Medium, not inoculated with bacterial suspension, served as control. Roth inoculated and uninoculated samples were incubated under intermittent shaking to provide aerobic conditions. At periodic intervals, the samples were withdrawn aseptically from duplicate flasks and analysed for chlorpyrifos by gas-liquid chromatography (g.l.c.) after its extraction in hexane. To determine the proliferation of *Flavobacterium* sp. and *Arthrobacter* sp. during incubation with chlorpyrifos in the mineral salts medium, 0.1 mL of the inoculated medium was withdrawn from the flasks at 0 (immediately after inoculation with the respective bacteria) and 24 h after inoculation and, after appropriate dilution, plated on Wakimoto agar medium (for *Flavobcterium* sp.) or MSGP medium (for *Arthrobacter* sp.). The bacterial colonies developed on agar plates were counted after 5 days of plating to determine the population of the bacteria.

Air-dried sieved (< 2 mm) alluvial soil [organic matter, 1.63%; total N, 0.11%; clay, 25.6%; silt, 12.6%; sand, 61.8%; CEC (mEq.100 g^{-1} soil), 18.6; pH, 5.91 from the experimental farm of Central Rice Research Institute, Cuttack was placed in 20g portions in presterilized 100-mL Erlenmeyer flasks. Soil samples were moistened with sufficient water to provide 60°h water holding capacity for nonflooded conditions. In another set, 20-g portions of the alluvial soil were placed in presterilized test tubes (200 mm x 25 mm) and flooded with 25 mL of sterile distilled water. After 10 days of incubation of soil samples under nonflooded and flooded conditions, chlorpyrifos was added to the soil samples in 0.1 mL of acetone. Nonflooded and flooded soil samples were inoculated with suspensions (prepared as described earlier) of the cells of *Flavobacterium* sp. or *Arthobacter* sp. in sterile distilled water. After incubation at room temperature (28 ± 2 °C), chlorpyrifos residues in soil samples from duplicate flasks were extracted in acetone-hexane and then analysed by g.l.c.

Chlorpyrifos residues in the mineral salts medium (inoculated and uninoculated) were extracted by shaking portions (1-2 ml) of the medium in duplicate flasks with 1-5 mL of hexane and 50 mg of sodium sulphate for 5 min. For extraction of chlorpyrifos residues from soil samples, flooded soil samples in each of two

duplicate tubes were transferred to 250 mL volumetric flasks with 50 mL of acetone and shaken for 1 h on an orbital shaker. After 1 h of equilibration with acetone, 20 mL of hexane were added to the flasks and the contents shaken again for 1 h followed by addition of 2% sodium sulphate in distilled water to make the volume to 250 mL. For extraction of chlorpyrifos residues from nonflooded soil, 25 mL of water were added to nonflooded soil to bring the same soil-water ratio as in flooded soil before extraction of chlorpyrifos as described above.

Chloripyrifos, extracted in hexane from the mineral salts medium or from the soil samples were analysed in a Varian gas chromatograph model 3400, equipped with a Ni^{63} detector and a metal column (2 m length, 1/8" OD) packed with 35 OV-17 chrom WHP 80/100 mesh. The operating conditions were carrier gas (95% argon in 5% methane) flow, 20 mL. mL^{-1}, column temperature, 220 °C, injector temperature, 240 °C, and detector temperature, 240 °C. Under these conditions, the retention time for chlorpyrifos was 4.05 min and recovery was around 85% and 800/6 of the applied chlorpyrifos from the mineral salts medium and soil samples, respectively.

RESULTS AND DISCUSSION

Both *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. effected very rapid degradation of chlorpyrifos, added to the mineral salts medium as a sole carbon source (Table 1).

Table 1. Degradation of chlorpyrifos in a mineral salts medium inoculated with *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. under aerobic conditions

Treatment	Chlorpyrifos" recovered (µg mL^{-1})[b] from medium		
	Incubation (h)		
	6	24	48
Uninoculated	4.86	4.02	3.12
Inoculated with			
Flavobacterium sp.	3.45 ± 0.19	1.82 ± 0.07	0
Arthrobacter sp.	3.03 ± 0.26	0	0

[a] Chlorpyrifos was added to the medium in hexane to provide 10 µg mL^{-1} medium.

[b] Mean of duplicate estimations ± deviation.

Arthrobacter sp. appeared to be more effective than the *Flavobacterium* sp. in degrading chlorpyrifos. Because, it took 24 h for *Arthrobacter* sp. and 48 h for *Flavobacterium* sp. to effect complete loss of all the added insecticide form the medium. During the corresponding period, there was some loss of chlorpyrifos from the uninoculated medium as well, probably due to its chemical hydrolysis in the mineral salts medium. It is pertinent to mention here that the *Flavobacterium* sp. was isolated from diazinon-retreated rice field (Sethunathan and Yoshida 1973) while *Arthrobacter* sp. was isolated from a flooded alluvial soil retreated with methylparathion (Misra et al. 1992). Chlorpyrifos is related to diazinon and methylparathion in having a common P-O-C linkage and was, therefore, degraded, evidently by hydrolysis at P-O-C linkage as with diazinon (Adhya et al. 1981) and parathion (Sethunathan and Yoshida 1973). In the absence of its authentic hydrolysis products, 3,5,6-trichloro-2-pyridinol and diethyl thiophosphoric acid, it was not possible to characterize the products of bacterial degradation of chlorpyrifos. This is probably the first report on such rapid degradation of chlorpyrifos as a sole carbon source in pure cultures of bacteria. There are reports on the resistance of chlorpyrifos to degradation in pure (Hirakoso 1969) and mixed (Sethunathan and Pathak 1972) cultures of microorganisms. There is evidence that phosphotriesterases of the *flavobacterium* sp., used in this study, could hydrolyse several phosphorothioate insecticides including chlorpyrifos (Brown, 1980). Interestingly, a 3-4 fold increase in the density of bacterial cells occurred in 24 h during degradation of chlorpyrifos as a sole carbon source by the *Arthrobacter* sp. (Table 2). Proliferation of *Flavobacterium* sp. was, however, negligible during the same period. Evidently, chlorpyrifos was used as a substrate for growth by the *Arthrobacter* sp., and not by the *Flavobacterium* sp. This would explain the more rapid degradation of chlorpyrifos by the *Arthrobacter* sp. than that by the *Flavobacterium* sp.

Table 2. Proliferation of *Flavobactetrum* sp. ATCC 27551 and *Arthrobacter* sp. in mineral salts supplemented with chlorpyrifos

Incubation (h)	Treatment	Medium inoculated with	
		Colony forming unit x 10^7	
		Flavobacterium sp.	*Arthrobacter* sp.
0	Without chlorpyriios	69	59
	With chlorpyrifos	71	63
24	Without chlorpyrifos	75	67
	With chlorpyrifos	88	289

Data are mean of three replicates.

There is no evidence for proliferation of microorganisms in soils or in pure cultures of bacteria using chlorpyrifos as energy source (Racke et al. 1990).

Table 3. Degradation of soil-applied chlorpyrifos by *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. under flooded conditions.

Treatment	Chlorpyrifos[a] recovered ($\mu g\ g^{-1}$)[b] from soil						
	Incubation (days)						
	0	2	6	12	15	20	28
Uninoculated	9.32 ± 0.04	7.35 ± 1.0	7.95 ± 0.15	4.39±0.23	4.67 ± 0.23	3.10 ± 0.20	1.15 ± 0.35
Inoculated with							
Flavobacterium sp.	8.60 ± 0.07	7.05 ± 0.05	3.15 ± 0.06	0.70±0.30	0	0	0
Arthrobacter sp.	9.40 ± 0.15	8.60 ± 0.08	5.40 ± 0.0	1.25±0.05	1.10 ± 0.38	0.20 ± 0.10	0

[a] Chlorpyrifos was added to the soil in hexane to provide 10 $\mu g\ mL^{-1}$ medium. [b] Mean of duplicate estimations ± deviation.

Table 4. Degradation of soil-applied chlorpyrifos by *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. under nonflooded conditions.

Treatment	Chlorpyrifos[a] recovered ($\mu g\ g^{-1}$)[b] from soil						
	Incubation (days)						
	0	2	6	12	15	20	28
Uninoculated	9.66 ± 0.04	8.20 ± 0.60	6.60 ± 0.20	3.85 ± 0.35	4.20 ± 0.08	3.60 ± 0.0	1.65 ± 0.05
Inoculated with							
Flavobacterium sp.	7.60 ± 0.42	6.80 ± 0.0	4.64 ± 0.06	1.75 ± 0.14	1.26 ± 0.14	1.00 ± 0.10	0
Arthrobacter sp.	9.20 ± 0.40	7.05 ± 0.15	5.52 ± 0.33	3.55 ± 0.35	3.10 ± 0.24	2.61 ± 0.09	0.40 ± 0.06

[a] Chlorpyrifos was added to the soil in hexane to provide 10 $\mu g\ mL^{-1}$ medium. [b] Mean of duplicate estimations ± deviation.

According to an earlier study, *Arthrobacter* sp., used in this study, could readily proliferate in the presence of methyl parathion as a sole carbon source (Misra et al. 1992) attributed to its ability to utilize the pnitrophenol formed from methyl parathion for growth. Ring moieties of methyl parathion and chlorpyrifos are not structurally related. Probably, diethyl thiophosphoric acid, the hydrolysis product of chlorpyrifos, was utilized by the *Arthrobacter* sp. for its proliferation.

In another experiment, the ability of the *Flavobacterium* sp. and the *Atthrobacter* sp. to degrade soil-applied chlorpyrifos under nonflooded and flooded conditions. Both *Flavobacterium* sp. and *Arthrobacter* sp. effected rapid degradation of chlorpyrifos, applied to the alluvial soil, under nonflooded conditions (Table 3) as well as under flooded conditions (Table 4). Degradation of chlorpyrifos in inoculated soil samples proceeded more rapidly under flooded conditions than under nonflooded conditions. Thus, chlorpyrifos disappeared totally within 15 days under flooded conditions and within 28 days under nonflooded conditions in soil inoculated with the *Flavobacterium* sp. In soil inoculated with *Arthrobacter* sp., it took less than 28 days under flooded conditions and more than 28 days under nonflooded conditions for complete loss of chlorpyrifos. The concentration of chlorpyrifos decreased also in uninoculated soil samples, but certainly to a less extent than in inoculated soil samples. *Flavobacterium* sp. appeared to be more effective than the *Arthrobacter* sp. in degrading soil-applied chlorpyrifos. But, in mineral salts medium supplemented with chlorpyrifos as a sole carbon source, *Arthrobacter* sp. was more effective than the *Flavobacterium* sp. in degrading chlorpyrifos. The reason for this discrepancy in the chlorpyrifos-degrading ability of two bacteria in soil and mineral salts medium is not clear. Probably, *Flavobacterium* sp. was more efficient than the *Arthrobacter* sp. in competing with the indigenous microorganisms in the complex soil environment. The data presented in this study provide the convincing evidence for exceptionally rapid degradation of (i) chlorpyrifos in pure cultures of bacteria (*Flavobacterium* sp. and *Arthrobacter* sp.) and (ii) soil-applied chlorpyrifos by these bacteria.

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