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Degradation of Parathion by Bacteria Isolated from Flooded Soil

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Two bacteria, *Bacillus* sp. and *Pseudomonas* sp., were isolated from parathion-amended flooded alluvial soil which exhibited parathion-hydrolyzing ability. *Bacillus* sp. readily liberated nitrite from the hydrolysis product, *p*-nitrophenol, but not from intact parathion. *Pseudomonas* sp. hydrolyzed parathion and then released nitrite from *p*-nitrophenol. These studies establish bacterial degradation of parathion past the *p*-nitrophenol stage to the end product, nitrite.

Folidol, a commercial formulation of parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate), is extensively used in India for control of common insect pests of rice. Although parathion is known to be relatively less persistent than chlorinated hydrocarbon insecticides, recently this compound was reported to persist for more than 16 years in a sandy loam soil (13). Parathion undergoes rapid degradation in flooded rice soils either via reduction of the nitro group (12) or via hydrolysis at the P-O-C linkage after repeated applications (10). Degradation of parathion by *Flavobacterium* sp. isolated from diazinon-amended flooded rice soil ceased at the *p*-nitrophenol stage (11). This paper reports more extensive degradation of parathion past the *p*-nitrophenol stage by a single bacterium isolated from flooded alluvial soil and identified as *Pseudomonas* sp. Formation of nitrite as an end product in the degradation of *p*-nitrophenol by a species of *Bacillus* was also investigated.

MATERIALS AND METHODS

Preparation of parathion-hydrolyzing enrichment culture. One milliliter of aqueous 1,000-ppm solution of parathion was added to 20 g of an alluvial soil from the Institute farm at 2-week intervals. The soils contained in test tubes (25 by 220 mm) were flooded with 24 ml of distilled water. Within 24 to 48 h after the third addition, the standing water over the soils in certain tubes turned yellow, indicating the hydrolysis of parathion to *p*-nitrophenol (10). This standing water, together with soil suspension exhibiting parathion-hydrolyzing ability, was pooled from several tubes and employed as an enrichment culture.

Isolation of bacteria. A dilution (10^{-4}) of the enrichment culture was mixed with molten modified Wakimoto nutrient agar medium (8) and incubated. Isolates from the agar medium were transferred to a sterile mineral solution [$(NH4)_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; K_2HPO_4, 0.1 g; $Ca(NO_3)_2$, 0.01 g; distilled water, 1,000 ml] containing parathion or *p*-nitrophenol as the sole carbon source. None of the isolates decomposed parathion, but a species of *Bacillus* capable of decomposing *p*-nitrophenol was isolated (10).

In another experiment, the enrichment culture was serially diluted, and 1 ml of each dilution was incubated with parathion in a sterile mineral solution following the methods described for diazinon-hydrolyzing *Flavobacterium* sp. (8). The lowest dilution (10^{-6}) which exhibited parathion-hydrolyzing ability was chosen for further studies. The active parathion-hydrolyzing agents in the 10^{-6} dilution were multiplied by incubating 1 ml of this dilution with 4 ml of sterile mineral solution containing parathion as the sole carbon source. After 48 h, this solution was streaked on a modified Wakimoto agar medium (8). Individual bacterial isolates developing on the agar medium were transferred to a sterile mineral solution containing parathion. The medium which was incubated with isolate P-6 turned yellow within 24 h, and the yellow color faded within the next 24 h. This isolate was further purified and identified as *Pseudomonas* sp.

Degradation studies. The ability of *Pseudomonas* sp. to decompose parathion was tested as follows. The mineral solution containing aqueous parathion was passed through a membrane filter (Millipore Corp.; 0.45 μm pore size), and 25-ml samples of this sterile solution (pH 7.1) were distributed in 250-ml Erlenmeyer flasks. The medium was inoculated with 0.1 ml of bacterial suspension in sterile distilled water prepared from 3- to 7-day-old cultures. The incubation mixture was incubated at 27 C in a biological oxygen demand (BOD) incubator. Uninoculated media served as control.

Methods for extraction (11) and analysis (10) of parathion residues in the incubation mixture have been described earlier. Residues in the medium were extracted three times with 20 ml of chloroform-diethyl ether (1:1), and the solvent fraction was pooled. The residues were evaporated to dryness at room temperature and then dissolved in 2 ml of methanol. The

residues spotted on 300-μm-thick Silica Gel G plates were developed for a distance of 15 cm by employing hexane-chloroform-methanol (7:2:1) as a developing agent. After drying, the authentic compounds of parathion and *p*-nitrophenol were located by spraying the chromatoplate with 0.5% palladium chloride in 2% HCl followed by 2.5 N NaOH. The silica gel areas of the samples corresponding to parathion were scraped carefully and transferred to a test tube. One milliliter of 2.5 N NaOH was added to each tube, and parathion was converted to *p*-nitrophenol by alkaline hydrolysis in a water bath for 1 h. After cooling, the volume was made up to 25 ml, silica gel was removed by centrifugation, and the supernatant was read in a Klett-Summerson colorimeter employing a 420-nm blue filter. The amount of parathion in the samples was obtained by multiplying the values for *p*-nitrophenol by 2.094.

p-Nitrophenol in the silica gel areas of the samples opposite to the authentic compound was directly eluted in 0.1 N NaOH. After centrifugation of the silica gel suspension, *p*-nitrophenol in the supernatant was determined colorimetrically against an appropriate blank as described earlier.

In a test to determine whether nitrite was formed during the bacterial decomposition of *p*-nitrophenol, 20-ml samples of sterile mineral solution without $(NH4)_2HPO_4$ and $Ca(NO_3)_2$ supplemented with *p*-nitrophenol were inoculated with 0.1 ml of a suspension of *Pseudomonas* sp. in sterile water. The samples were drawn periodically, and nitrite in the samples was analyzed colorimetrically by using sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride (2).

Bacillus sp. which decomposed *p*-nitrophenol as the sole carbon source (10) was tested for its ability to release nitrite from *p*-nitrophenol as described for *Pseudomonas* sp. The same bacterium was also tested for its ability to liberate nitrite from intact parathion.

RESULTS

The bacterial isolate P-6 was gram negative, rod, and aerobic, and was identified as a nonfluorescent species of *Pseudomonas*. The isolate showed many characteristics of *P. multivorans* Stanier et al. and appeared to belong or to be closely related to this species. Large inclusions, which were presumably polyhydroxy butyrate, were very clear with the Gram stains. All tests for spores were negative.

When *Pseudomonas* sp. was incubated with parathion, the insecticide was rapidly degraded. The color of the incubation medium turned yellow within 4 h of incubation, indicating the formation of *p*-nitrophenol. At 20 h, however, the yellow color disappeared, evidently because of the further metabolism of *p*-nitrophenol. Quantitative analysis of parathion and *p*-nitrophenol confirmed these findings. At 4 h, about 50 μg of added parathion was hydrolyzed and 19 μg of *p*-nitrophenol was recovered as the major metabolite (Table 1). No other metabolite could be detected in the thin-layer chromatogram of the solvent extract. At 20 h, however, parathion was completely destroyed and no *p*-nitrophenol could be detected. No appreciable degradation of parathion occurred in the uninoculated control during 20 h of incubation. When the bacterium was grown in a nitrogen-free medium with *p*-nitrophenol as the sole carbon source at 27 C ± 2 C in a BOD incubator, nitrite nitrogen was released from the organic nitro molecule (Table 2). The amount of nitrite formed was proportional to the amount of *p*-nitrophenol degraded. Within 16 h of incubation, 157 μg of *p*-nitrophenol was decomposed, liberating 51 μg of nitrite. In the uninoculated control, nitrite was not detected. In a preliminary resting-cell experiment, nitrite was formed when living resting cells of *Pseudomonas* sp. were exposed to parathion in phosphate buffer (pH 7.1). These studies indicated that nitrite was formed from *p*-nitrophenol.

Bacillus sp., isolated from flooded alluvial soil, was reported earlier to utilize *p*-nitrophenol as a sole carbon source (10). In a test to determine the end product formed during the breakdown of *p*-nitrophenol by *Bacillus* sp., the bacterium was incubated with *p*-nitrophenol in a mineral solution as described for *Pseudomonas* sp. Within 24 h of incubation, 166 μg of *p*-nitrophenol was metabolized, releasing 43 μg of nitrite (Table 3).

To test whether *Bacillus* sp. could release

TABLE 1. *Degradation of parathion by Pseudomonas sp. isolated from flooded soil*

Incubation (h)	Recovery (μg/25 ml of medium)	
	Parathion	*p*-Nitrophenol
0	87	0
0.5	75	5
4	36	19
20	0	0

TABLE 2. *Liberation of nitrite from p-nitrophenol as the sole carbon source by Pseudomonas sp. isolated from flooded soil*

Incubation (h)	Recovery (μg/20 ml of medium)	
	p-Nitrophenol	Nitrite[a]
0	157	0
8	84	11
16	0	52
24	0	56

[a] Theoretical value of nitrite formed from 157 μg of *p*-nitrophenol, 52 μg.

TABLE 3. *Liberation of nitrite from p-nitrophenol as the sole carbon source by Bacillus sp. isolated from flooded soil*

Incubation (h)	Recovery (μg/20 ml of medium)	
	p-Nitrophenol	Nitrite[a]
0	166	0
6	143	1
12	58	14
24	0	43

[a] Theoretical value of nitrite formed from 166 μg of *p*-nitrophenol, 55 μg.

nitrite from intact parathion, the bacterium was incubated with the insecticide for 120 h. *p*-Nitrophenol and nitrite were not formed.

Pseudomonas sp. was subcultured repeatedly on parathion- or *p*-nitrophenol-free modified Wakimoto agar. After the fifth subculture, the bacterium was tested for its ability to degrade parathion or *p*-nitrophenol as the sole carbon source in a mineral solution as described earlier. The bacterium retained its ability to hydrolyze parathion within 3 h of incubation and then release nitrite from *p*-nitrophenol within 24 h despite five transfers on parathion- and *p*-nitrophenol-free media.

DISCUSSION

Parathion metabolism in plant and insect systems via oxidation, reduction of nitro group, or hydrolysis is well established (5), but the stepwise degradation of this insecticide in microorganisms has not been investigated (1). Until recently, the major pathway of parathion metabolism in soils and microorganisms appeared to be the reduction of the nitro group. Now, we have clear evidence that parathion can be hydrolyzed biologically at the nitrophenyl C–O–P bond, both in flooded soils after its repeated additions (10) and in pure culture by *Flavobacterium* sp. (11, 12). Degradation of parathion by *Flavobacterium* sp., however, ceased at the *p*-nitrophenol stage (11). The results presented in this study clearly established the degradation of parathion past the nitrophenol stage by *Pseudomonas* sp., leading to the formation of nitrite as an end product. Nitrite formed appeared to persist in pure culture studies with *Bacillus* sp. and *Pseudomonas* sp. during the 24-h incubation period. However, in flooded soil nitrite does not accumulate (6), because of its rapid denitrification to molecular nitrogen in an anaerobic environment. Various nitrophenols are known to be metabolized, liberating nitrite in the process. *Corynebacterium simplex* formed nitrite from phenolic substrates containing the nitro group in the *para* position (3). A species of *Arthrobacter* released nitrite from a herbicide 3,5-dinitro-*o*-cresol (4).

Similarly, both *Bacillus* sp. and *Pseudomonas* sp. used in this study readily released nitrite from *p*-nitrophenol, utilizing the latter as a sole carbon source (Table 2, 3). This finding is in agreement with that of Raymond and Alexander (7). *Pseudomonas* sp., in addition, possessed an enzyme system(s) capable of hydrolyzing parathion. This result is apparently the first report on the degradation of parathion to *p*-nitrophenol and then to nitrite by the same bacterium.

Metabolism of parathion in flooded soil or in pure cultures of microorganisms isolated from flooded soil involves (i) nitro group reduction, leading to the formation of aminoparathion as a major metabolite (9); (ii) hydrolysis to *p*-nitrophenol as a major metabolite which resists further degradation (11); and/or (iii) hydrolysis to *p*-nitrophenol followed by the formation of nitrite (this report). The findings reported in this paper show that microorganisms in flooded soils contribute to the rapid breakdown of parathion via the hydrolytic pathway to the end product, nitrite.

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