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Degradation of Alpha-, Beta-, and Gamma-Hexachlorocyclohexane by a Soil Bacterium under Aerobic Conditions

SIDDHARTHA K. SAHU,[1] KAMALA KUMARI PATNAIK,[1] MANIKONDA SHARMILA,[2] AND N. SETHUNATHAN[2]*

Department of Botany, Ravenshaw College, Cuttack 753 003,[1] and Laboratory of Soil Microbiology, Central Rice Research Institute, Cuttack 753 006,[2] India

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A *Pseudomonas* sp., isolated from sugarcane rhizosphere soil, readily metabolized not only alpha and gamma isomers of hexachlorocyclohexane, but also the thermodynamically more stable beta isomer, under aerobic conditions. Bacterial degradation of the three isomers led to the accumulation of a transitory metabolite and eventual release of covalently linked chlorine as chloride in stoichiometric amounts.

Commercial formulations of hexachlorocyclohexane (HCH), a widely used insecticide in India and many tropical countries of the Third World, contain a mixture of isomers which may include alpha, beta, gamma, and other isomers. Until very recently, HCH isomers were considered to be highly persistent in aerobic environments, but they readily undergo rapid degradation in predominantly anaerobic ecosystems such as flooded soils and lake sediments (11, 13). A strict anaerobe, *Clostridium* sp., isolated from a flooded soil degraded alpha- and gamma-HCH, but not beta- or delta-HCH, under anaerobic conditions (6, 10).

Recently, aerobic biomineralization of alpha-HCH was demonstrated in a soil slurry from an HCH-contaminated site in the Netherlands (1, 2). In a long-term field experiment, gamma-HCH degraded slowly in the first 2 years after being applied to an upland soil, but in the third year more than 80% of the insecticide disappeared within 1 month (K. Senoo, H. Wada, and Y. Takai, Abstr. 5th Int. Symp. Microb. Ecol., abstr. no. S-20-3, p. 41, 1989). An aerobic bacterium, *Pseudomonas paucimobilis*, isolated from retreated upland soil readily degraded gamma-HCH. However, there are no reports on the degradation of recalcitrant beta-HCH in pure cultures of microorganisms under either anaerobic (9) or aerobic conditions. We studied the degradation of alpha-, beta-, and gamma-HCH by a bacterium isolated from sugarcane rhizosphere soil.

Analytical-grade (99.1% purity) alpha-, beta-, and gamma-HCH were obtained from Lachat Chemicals, Mequon, Wis. The authentic gamma isomer of pentachlorocyclohexene (PCH), used for identification of the metabolite formed from gamma-HCH, was a gift of N. Kurihara, Kyoto University, Kyoto, Japan.

For isolation of the HCH-degrading bacterium, sugarcane sets, treated with a commercial formulation of HCH to prevent termites and other insect pests, were planted in soil. After about 8 months, the sugarcane plants were uprooted and the roots were gently tapped to remove large soil clods. The roots (10 g [fresh weight] along with rhizosphere soil) were then shaken with 100 ml of sterile distilled water for 5 min, and the resulting suspension served as the rhizosphere soil suspension. A mineral salts medium [$(NH_4)_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; K_2HPO_4, 0.1 g; $Ca(NO_3)_2$, 0.01 g; distilled water, 1 liter (pH 7.0)] supplemented with 17 to 24 μM gamma-HCH was inoculated with 0.1 ml of sugarcane rhizosphere soil suspension and then incubated under aerobic conditions at room temperature (28 ± 2°C). Gamma-HCH disappeared from the inoculated medium in 5 days. Then 5 ml of this medium was again mixed with 5 ml of mineral salts medium containing 17 to 24 μM gamma-HCH and incubated for 5 days for selective enrichment of gamma-HCH-degrading microorganisms. This was repeated five times. Serial dilutions of this enrichment culture after the fifth transfer were plated on the same mineral salts medium containing 2% agar, 1% glucose, and 17 μM gamma-HCH. One of the bacterial isolates appearing on the medium readily degraded gamma-HCH that was added to the mineral salts medium as the sole source of carbon. This bacterium was further purified by several transfers on mineral salts-glucose agar slants and was used in this study.

To determine the ability of the bacterium to degrade gamma-HCH, 10-ml portions of the sterilized mineral salts solution containing 28 μM gamma-HCH as the sole source of carbon were placed in sterile 100-ml Erlenmeyer flasks and then inoculated with a suspension of the bacterium (13×10^4 cells) in 0.1 ml of sterile distilled water. Uninoculated medium served as the control. Inoculated and uninoculated media were incubated at 30 ± 1°C in a biological oxygen demand incubator with intermittent shaking to provide aerobic conditions. One-milliliter portions of the medium were withdrawn from triplicate flasks at 12-h intervals and analyzed for gamma-HCH by gas-liquid chromatography. To determine the proliferation of the bacterium in the presence of gamma-HCH as the sole source of carbon, serial dilutions (10^{-2} to 10^{-4}) of the inoculated medium with and without gamma-HCH were simultaneously plated on the same mineral salts medium supplemented with 1% glucose, 2% agar, and 17 μM gamma-HCH. The agar plates were incubated at 30 ± 1°C, and the number of CFU appearing on the duplicate plates was counted after 3 days.

To determine the ability of the bacterium to degrade alpha-, beta- and gamma-HCH and the amount of chloride released from these isomers, the isomers were separately added, in 0.1 ml of acetone, to 100-ml Erlenmeyer flasks containing 10 ml of deionized water (in lieu of mineral salts medium because of the interference of salts in chloride analysis). Each flask was inoculated with 0.1 ml of a suspension of the bacterium (9×10^5 cells) in sterilized deionized water. At 0 and 24 h after incubation at 30 ± 1°C, inoculated and uninoculated samples containing each isomer were

* Corresponding author.

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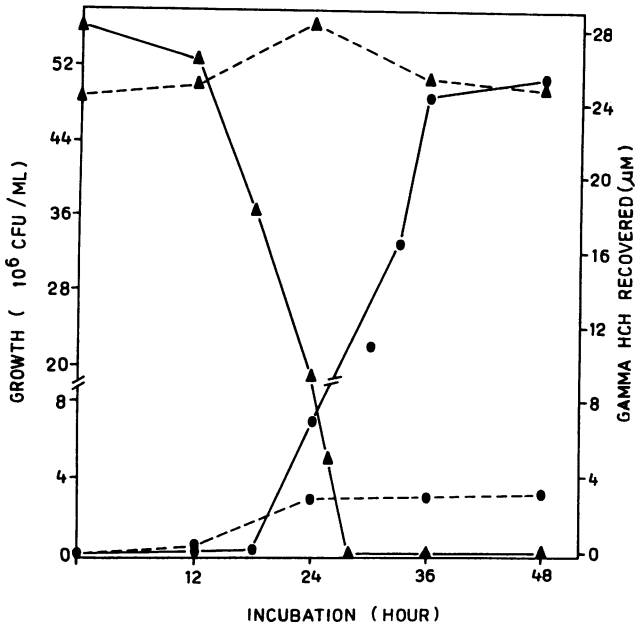

FIG. 1. Degradation of gamma-HCH, added to a mineral salts medium as the sole source of carbon, by a *Pseudomonas* sp. under aerobic conditions. Symbols: ▲----▲, gamma-HCH recovered from uninoculated medium; ▲——▲, gamma-HCH recovered from inoculated medium; ●----●, growth in medium without gamma-HCH; ●——●, growth in medium supplemented with gamma-HCH.

analyzed in triplicate for the respective isomer and the chloride content. For the beta isomer, samples were also analyzed at 72 h.

At 24-h intervals, 1- to 2-ml portions of the medium were withdrawn from triplicate samples and then shaken with 5 ml of hexane for 10 min. Residues extracted in hexane were analyzed in a Perkin-Elmer gas chromatograph equipped with a ^{63}Ni detector. Gas-chromatographic conditions used were as follows: column, 5% QF 1 on Chromosorb W, mesh 60/80; argon (95%)-methane (5%) flow at 13 ml/min; column temperature, 180°C; injector temperature, 210°C; and detector temperature, 230°C. Under these conditions, the retention times were 2.0 min for alpha-HCH, 2.8 min for beta-HCH, and 2.42 min for gamma-HCH. The recovery of the three isomers by this method was more than 95%.

For chloride analysis by colorimetry (3), 2-ml portions of the inoculated and uninoculated samples were treated with 0.2 ml of 0.25 M ferric ammonium sulfate in 9 M nitric acid and 0.2 ml of a saturated solution of mercuric thiocyanate in ethyl alcohol. The color that developed in 10 min was read at 460 nm against a blank that was formed from reagents alone.

The HCH-degrading bacterium used in this study was isolated from the rhizosphere soil of HCH-treated sugarcane. The bacterium was a strict aerobe and was present as motile, gram-negative, nonsporeforming, straight or curved rods that were catalase and oxidase positive. On the basis of these and other biochemical characteristics, the bacterium was identified as a *Pseudomonas* sp. (4).

The *Pseudomonas* sp. was tested for its ability to degrade gamma-HCH that had been added to the mineral salts medium as the sole source of carbon. Within 28 h after inoculation of the bacterium into the medium under aerobic conditions, gamma-HCH (initial concentration, 28 μM) disappeared completely and there was a concomitant increase in the population of the bacterium (Fig. 1). The population increase continued even after 28 h, suggesting that the bacterium was utilizing the degradation products of gamma-HCH for its growth. During the 48-h incubation period, the decrease in the concentration of gamma-HCH in the uninoculated control was negligible. During bacterial degradation of gamma-HCH, another peak of electron affinity appeared on the gas chromatogram with a retention time of 0.67 min. This intermediate was, however, unstable and disappeared in 28 h. Gamma-PCH is a key intermediate of gamma-HCH metabolism in enzyme preparations from houseflies (7), in soil (15), and in microbial cultures (5, 14). The retention time of the metabolite formed during bacterial degradation of gamma-HCH was compared with that of authentic gamma-PCH under gas-chromatographic conditions described above and found to be identical (0.67 min).

Earlier reports (5, 8, 12) have shown that degradation of gamma-HCH by strict and facultative anaerobic bacteria occurred only in nutrient-rich media supplemented with glucose, yeast extract, or peptone, suggesting that cometabolism was occurring. In the present study, gamma-HCH was readily utilized by the gamma-HCH-degrading *Pseudomonas* sp. as the sole source of carbon and energy.

The bacterium degraded not only gamma-HCH but also alpha- and beta-HCH added to deionized water under aerobic conditions (Table 1). The three isomers almost completely disappeared within 24 h of inoculation, and there was a concomitant release of chloride almost in stoichiometric amounts. In uninoculated controls, no chloride was detected. Release of chloride from inoculated samples suggests that the three isomers, including the beta isomer, were being mineralized by the bacterium. Transitory metabolites with retention times of 1.5 and 4.0 min were detected during bacterial degradation of alpha- and beta-HCH, respectively.

There were reports of degradation of either or both alpha-

TABLE 1. Chloride released from HCH isomers by the *Pseudomonas* sp.

Incubation time (h)	Amt of compound recovered (μM)[a]								
	Alpha-HCH			Beta-HCH			Gamma-HCH		
	Uninoculated[b]	Inoculated		Uninoculated[b]	Inoculated		Uninoculated[b]	Inoculated	
		Parent isomer[b]	Chloride		Parent isomer[b]	Chloride		Parent isomer[b]	Chloride
0	3.61 ± 0.03	3.65 ± 0.28	0	6.71 ± 0.17	6.74 ± 0.14	0	4.03 ± 0.24	4.13 ± 0.07	0
24	3.58 ± 0.07	0	2.95 ± 0.56	5.95 ± 0.07	0.45 ± 0	2.44 ± 1.78	4.03 ± 0.17	0	2.81 ± 0.05
72	ND[c]	ND	ND	4.82 ± 0.17	0	3.43 ± 0.33	ND	ND	ND

[a] Mean of duplicate estimations ± standard deviation.
[b] Parent isomers recovered.
[c] ND, Not determined.

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and gamma-HCH in pure cultures of strict anaerobes and facultative microorganisms (13). However, exceptionally rapid aerobic degradation of alpha-, beta-, and gamma-HCH by the same bacterium, as demonstrated in this study, has not been reported previously. What is particularly noteworthy is the extreme susceptibility of beta-HCH, a recalcitrant pollutant of great concern, to aerobically mediated bacterial degradation. This is the first report of the degradation of thermodynamically stable beta-HCH in a microbial culture.

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