Metabolism of γ -hexachlorocyclohexane by *Arthrobacter citreus* strain BI-100: Identification of metabolites

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Growth characteristics of the aerobic bacterial strain *Arthrobacter citreus* BI-100 in mineral salts medium with γ -hexachlorocyclohexane (γ -HCH) as the sole source of carbon and degradation of γ -HCH by the strain are reported. The highest yield of the bacteria is observed at a γ -HCH concentration of 100 mg/L. At this concentration, the bacteria entered the exponential phase of growth without any lag. At 8 h of growth, no residual HCH, but its metabolites, was detectable in the medium. The bacterium attained its stationary phase at 48 h and at 72 h; no metabolite of γ -HCH could be detected by gas chromatography. Six metabolic intermediates of γ -HCH produced by *A. citreus* BI-100 at different periods of growth were characterized by using gas chromatography-mass spectrometry and high-performance liquid chromatography, which furnished evidence for the presence of γ -1,3,4,5,6-pentachlorocyclohexene, tetrachlorocyclohexene, trichlorocyclohexa-diene, 2-chlorophenol, phenol, and catechol, among others.

Key Words—*Arthrobacter citreus*; γ -hexachlorocyclohexane; metabolism; pentachlorocyclohexene; tetrachlorocyclohexene; trichlorocyclohexa-diene

Introduction

γ -Hexachlorocyclohexane (γ -HCH), also known as lindane, has been used worldwide since the 1940s for agriculture and public health purposes (Lal and Saxena, 1972). Although it has been banned in many countries because of its toxicity and recalcitrant nature (Johnson and Talbot, Jr., 1983; Ray et al., 1992), numerous sites still remain contaminated throughout the world (Bachmann et al., 1988; Marks et al., 1989), and newer sites are being generated because of its continued use in other countries even today. Residual pesticides remaining in the field enter the food chain (Joshi,

1986; Singh et al., 1986) and get concentrated in the fatty tissues, thus causing major health hazards (Chandra et al., 1992; Dikshith et al., 1989; Krishna et al., 1989; Pandey et al., 1990; Ray and Gupta, 1987). Moreover, besides killing the target pests, the pesticide causes damage to a wide variety of organisms and thus affects the ecology and the environment.

Several reports are available on the degradation of γ -HCH (Castro and Yoshida, 1971; Sethunathan, 1973; Sethunathan et al., 1983) by anaerobic bacteria such as *Clostridium* species and *Citrobacter freundii* (Jagnow et al., 1977; MacRae et al., 1969; Ohisa and Yamaguchi, 1978). The pathways of degradation of γ -HCH by these bacteria have also been worked out (Heritage and MacRae, 1977a,b; Jagnow et al., 1977; Ohisa et al., 1980, 1982). In contrast, reports on the degradation of γ -HCH by aerobic bacteria are limited

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(Imai et al., 1989; Matsumura et al., 1976; Sahu et al., 1990; Tu, 1976).

In a previous study, four strains of bacteria were isolated from soil by an enrichment technique in our laboratory, using γ -HCH as the sole source of carbon (Datta et al., 1998). Of these, BI-100 was found to be the most potent one for γ -HCH degradation. The bacterium has been identified as *Arthrobacter citreus* with a laboratory strain designation BI-100 in the present study and has been catalogued as *A. citreus* MTCC 3148 in Microbial Type Culture Collection, Chandigarh, India. In the present study, the identification of metabolites of aerobic degradation of γ -HCH by the bacterial strain *A. citreus* BI-100 (=MTCC 3148) is reported. To our knowledge, this seems to be the first report of degradation of γ -HCH by a strain of *Arthrobacter*.

Materials and Methods

Chemicals. γ -HCH (analytical grade, 97% purity) and 2-chlorophenol were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade and used as such without further purification.

Culture conditions and growth of *A. citreus* BI-100. Isolation of the strain BI-100 with the ability of degrading γ -HCH was described in a previous communication (Datta et al., 1998). It has been classified as *A. citreus* by Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, and laboratory strain designation is BI-100 (=MTCC 3148). The strain was routinely grown at 28°C in mineral salts medium containing γ -HCH (100 mg/L) as the sole source of carbon, unless otherwise stated.

To monitor the degradation of γ -HCH and to characterize the metabolites produced by BI-100, 8×10^8 cells were inoculated to 100 ml of mineral salts medium (Datta et al., 1998), supplemented with γ -HCH to a final concentration of 100 mg/L in 500 ml conical flasks. The flasks were incubated at 28°C on a rotary shaker (150 rpm). Growth of the strain was monitored in the culture by a measurement of OD₆₀₀, and residual HCH was measured at intervals by gas chromatography (GC) equipped with an electron capture detector. For characterization of metabolites, culture filtrates at different periods of growth were extracted thrice each time with an equal volume of ethyl acetate. The extracts were combined, dried under a gentle stream of nitrogen, and analyzed by gas chromatography-mass

spectrometry (GC-MS).

To study the degradation of γ -HCH, 2-chlorophenol, and phenol by resting cells of BI-100, the bacteria were first grown in mineral salts medium supplemented with γ -HCH (100 mg/L) as the sole source of carbon at 28°C on a rotary shaker at 150 rpm, harvested during the late log phase by centrifugation (10,000 $\times g$; 10 min at 4°C), washed with 100 mM phosphate buffer (pH 6.0), and resuspended in mineral salts medium. The suspension (20 ml; OD₆₀₀ 5.0) in a conical flask (100 ml) was incubated with the desired substrate (final concentration 100 mg/L) at 28°C on a rotary shaker for different periods.

Assay for γ -HCH degradation activity with cell-free extract. Cells harvested during the late log phase of growth from 100 ml of culture were frozen (−20°C, 1 h) and ground with neutral alumina (about 0.5 g). To the cell paste, 2 ml of 100 mM phosphate buffer (pH 6.0) was added, mixed thoroughly, and centrifuged (15,000 $\times g$; 20 min); the supernatant was used as the crude cell-free extract. The cell-free extract (10 μ l containing about 200 μ g of protein) was added to 100 μ l of assay solution (100 mg/L γ -HCH in mineral salts medium). It was incubated at 37°C for different periods. The assay mixture was extracted by the addition of 100 μ l of ethylacetate and vortexing for 5 min. After centrifugation (15,000 $\times g$; 5 min), the ethylacetate layer was recovered, dried under a gentle stream of nitrogen, and analyzed by GC-MS.

GC-MS of metabolites of γ -HCH. A GC-MS of metabolites produced by the strain BI-100 from γ -HCH was performed by using a Shimadzu QP 2000 gas chromatograph. Metabolites were separated on an ULBON HR-1 equivalent to an OV-1 fused silica capillary column (0.25 mm $\times$ 50 m; 0.25 μ m film thickness) with helium as the carrier gas (at 2 ml/min). The column temperature was held at 100°C for 7 min, then increased to 250°C at a rate of 5°C/min. The mass spectrometer was operated at an electron ionization energy of 70 eV. A 0.5 μ l solution containing 10 μ g of sample was injected in each case.

High-performance liquid chromatography (HPLC) of metabolites of γ -HCH. Metabolites of 2-chlorophenol or phenol produced during incubation with γ -HCH grown cells of BI-100 were resolved on a Nova-Pak C18 reverse phase column (3.9 $\times$ 150 mm; particle size 5 μ m) of an HPLC system equipped with a UV detector (Waters 486) at 254 nm. A 10 μ l solution containing 10–20 μ g of sample was injected in each case. Water :

methanol (60:40) and water:acetic acid:methanol (59.9:0.1:40) were used as the mobile phase. The flow rate was maintained at 1 ml/min. Two micrograms of standard sample dissolved in 10 μ l solvent were injected into the system where the minimum detection limit is 1 μ g.

Results

Classification of the strain BI-100

Based on morphological, cultural, and biochemical characteristics, the strain has been classified as *A. citreus* BI-100 (=MTCC 3148) by Microbial Type Culture Collection, Chandigarh, India. The bacteria are Gram-positive rods arranged singly with a succession of changes in the cell form accompanying growth. In stationary phase cultures, the cells are spherical and uniform in size. The colonies are opaque, round with entire margin, smooth surface, and yellow coloration. Growth temperature of the strain ranges from 15 to 37°C with an optimum at 28°C, and growth pH ranges from 5.0 to 11.0 with an optimum at pH 6.0. The bacteria are tolerant to 5% NaCl and are methyl red- and Voges-Proskauer test-negative. They do not reduce nitrite or nitrate and show negative reaction in cytochrome oxidase, arginine dihydrolase, and phenylalanine deamination tests. The bacteria are positive for growth on MacConkey agar as nonlactofermenter, utilize citrate, hydrolyze casein, and are catalase-, lysine decarboxylase- and ornithine decarboxylase-positive. They produce acid from galactose and sucrose, but not from adonitol, arabinose, dextrose, lactose, maltose, melibiose, raffinose, salicin, sorbitol, trehalose, or xylose. The bacteria have the murein diamino acid lysine and the sugar galactose as the cell wall carbohydrate. Based on these properties, the bacteria have been classified as *A. citreus* with a strain designation of BI-100.

Growth of the strain *A. citreus* BI-100 on γ -HCH

In preliminary experiments, the yield of the strain as measured by OD₆₀₀ was observed to increase with increasing concentrations of γ -HCH, reaching a maximum at 100 mg/L, whereupon the yield gradually decreased (data not shown). As such, all further experiments were carried out by using γ -HCH at 100 mg/L, unless otherwise stated. At this concentration of γ -HCH, the bacteria entered the exponential phase of growth without any detectable lag, and the population

Fig. 1. Growth of *Arthrobacter citreus* BI-100 in mineral salts medium with γ -HCH as the sole source of carbon and energy.

Inset: Percent residual γ -HCH at different hours of growth of the strain BI-100 in mineral salts medium containing γ -HCH at a concentration of 100 mg/L.

of bacterium increased almost 40-fold (Fig. 1). Figure 1 (inset) shows the degradation kinetics of γ -HCH by *A. citreus* BI-100 during growth in mineral salts medium. At 8 h, γ -HCH in the culture appears to be degraded completely to its metabolites, and no γ -HCH in the culture filtrate could be detected. The strain attained its stationary phase at 48 h, and thereafter there was little change in optical density of the culture until 72 h.

Detection of metabolites by gas chromatography

Figure 2 shows the gas chromatographic profiles of extracts of culture filtrate of *A. citreus* BI-100 grown on γ -HCH for different periods. As expected, Fig. 2A shows only the peak of γ -HCH present in the culture at 0 h of growth. Extract of the culture filtrate of BI-100 grown for 1 h yielded a metabolite that appeared as a new peak, f (Fig. 2B), in the profile in addition to the peak of γ -HCH. A profile of the extract of culture grown for 2 h (Fig. 2C) shows that although there was a sizable decrease in peak height of γ -HCH, two new peaks, d and e, emerged while the peak height of f increased slightly. Extracts of culture grown for 4 h furnished yet another new peak, b, and peaks d, e, and f still increased (Fig. 2D). At 8 h, peaks a and c appeared concomitant to a complete disappearance of peak for γ -HCH with an increase in peaks b, d, e, and

Fig. 2. Profiles of degradation products of γ -HCH (100 mg/L) by *A. citreus* BI-100 at different periods during growth in mineral salts medium as detected by gas chromatography with an electron capture detector.

The strain was grown for (A) 0 h, (B) 1 h, (C) 2 h, (D) 4 h, (E) 8 h, (F) 32 h, and (G) 72 h.

f (Fig. 2E). At 32 h, peaks a, b, and c greatly increased, of which peak c was the most pronounced one with a disappearance of peaks d, e, and f (Fig. 2F). At 72 h, however, no peaks could be detected (Fig. 2G), and the profile appeared to be only basal as a straight line.

In a separate experiment, the peaks b, a, and c, which appeared in GC profiles as metabolites of γ -HCH during different periods of growth of *A. citreus* BI-100, were identified to be 2-chlorophenol, phenol, and catechol, respectively, by comparison of their retention time with those of authentic samples. Metabolites of degradation of γ -HCH by resting cells and by crude cell-free extract of BI-100 were also similarly characterized by GC (data not shown). The results corroborated the sequences of appearances and disappearances of peaks of metabolites as observed with growing cells.

Identification of metabolite M-1

The first product, metabolite M-1 (peak f in GC profile, Fig. 2B), furnished a mass spectrum exhibiting the base peak at m/z 183. The molecular ion peak (M^+) appeared at m/z 252 with low intensity (Table 1). The other major peaks corresponded to the resultant ions, which were possibly produced by dehydrochlorination and/or dechlorination of γ -PCCH (Table 1). The compound was identified as γ -1,3,4,5,6-pentachlorocyclohexene (γ -PCCH, Fig. 3) by comparison of its mass spectrum with that of an authentic sample reported elsewhere (Imai et al., 1989).

Identification of metabolite M-2

The metabolite M-2 (peak d in GC profile, Fig. 2C) furnished a mass spectrum with a molecular ion peak M^+ at m/z 218 (Table 1). The base peak appeared at m/z 181. Other major peaks at m/z 145, 111, and 75 possibly resulted from dechlorination and/or dehydrochlorination of the molecular ion of M-2. The metabolite M-2 produced was tentatively identified as tetrachlorocyclohexene (TCCH, Fig. 3). However, the fragmentation pattern of the compound obtained was found to be different in certain respects from that of γ -3,4,5,6-TCCH produced by the metabolism of γ -HCH by *Clostridium rectum* (Ohisa and Yamaguchi, 1978). It may be mentioned that four probable positional isomeric structures are possible for this compound M-2, but the isomer produced by *A. citreus* BI-100 (Fig. 3) could not be identified with certainty in this study.

Table 1. GC-MS data of products formed from γ -HCH by the strain *Arthrobacter citreus* BI-100.

Metabolite	GC R_t (min)	m/z of major ion peaks (abundance)	Suggested compound
M-1	32.26	254 (11.6), 252 (M^+ , 10.87), 219 (80.43), 217 (62.8), 183 (100), 181 (98.91), 145 (20.11), 111 (64.13), 109 (64.13), 75 (27.17)	γ -1,3,4,5,6-Pentachlorocyclohexene (γ -PCCH) ^a
M-2	24.33	218 (M^+ , 18.48), 183 (94.02), 181 (100), 147 (30.97), 145 (31.52), 111 (38.04), 75 (35.87)	Tetrachlorocyclohexene (TCCH) ^b
M-3	24.36	183 ($M^+ + 1$, 100), 181 (92.4), 145 (47.83), 111 (59.8), 109 (21.74)	Trichlorocyclohexa-diene (TCCD) ^b
M-4	9.10	128 (M^+ , 100), 92 (30.43), 64 (83.15)	2-Chlorophenol ^c
M-5	8.46	94 (M^+ , 63.04), 93 ($M^+ - 1$, 100), 66 (48.91)	Phenol
M-6	18.66	110 (M^+ , 100), 82 (19.02), 81 (42.4), 55 (42.4), 53 (53.26)	Catechol ^c

^a Identification is based on mass spectrum and comparison with that reported elsewhere.

^b Identification is based on mass spectrum.

^c Identification is based on mass spectrum, also by comparison of retention time with those of authentic samples on GC and HPLC and identification of products of metabolism of 2-chlorophenol, as well as of phenol.

Fig. 3. Structures of metabolic intermediates of HCH identified at various periods of growth of *A. citreus* BI-100.

Identification of metabolite M-3

The mass spectrum of the compound M-3 (peak e in GC profile, Fig. 2C) showed the base peak at m/z 183, which corresponds to $M^+ + 1$. There were three minor peaks at m/z 145, 111, and 75 (Table 1), which were possibly formed by dehydrochlorination and/or dechlorination of the molecular ion. The product M-3 was tentatively identified as trichlorocyclohexa-diene (TCCD, Fig. 3) which is consistent with the mass data.

Identification of metabolite M-4

Metabolite M-4 resolved by GC (peak b, Fig. 2D)

and tentatively identified as 2-chlorophenol (Fig. 3) by comparison with an authentic sample was further characterized by MS analysis. Its mass spectrum (Table 1) showed a molecular ion peak at m/z 128, which happened to be the base peak. The formation of ions at m/z 92 and 64 was thought to be due to subsequent dehydrochlorination followed by loss of CO from M-4. The mass spectral fragmentation pattern of M-4 was found to be identical with that of an authentic sample of 2-chlorophenol.

Fig. 4. High-performance liquid chromatography elution profiles of degradation products of 2-chlorophenol or phenol by γ -HCH grown *A. citreus* BI-100.

(A) Degradation products of 2-chlorophenol; (B) degradation products of phenol; Peaks: (a) catechol; (b) phenol; (c) 2-chlorophenol.

Identification of metabolite M-5

The metabolite M-5 resolved by GC (peak a, Fig. 2E), tentatively identified as phenol (Fig. 3) by comparison with an authentic sample, was further characterized by mass studies. It furnished molecular ion peak at m/z 94 in its mass spectrum. Other fragments at m/z 66 and 40 may be ascribed to the loss of CO and C_2H_2 , respectively (Table 1). This spectral pattern was found to be quite similar to that of an authentic sample of phenol.

Identification of metabolite M-6

The metabolite M-6 resolved by GC (peak c, Fig. 2E) and tentatively identified as catechol (Fig. 3) by comparison of its retention time with that of an authentic catechol was further confirmed by GC-MS data (Table 1). Its molecular ion peak appeared at m/z 110, which happens to be the base peak. Subsequent fragments appeared at m/z 82 ($M^+ - CO$) and 81 ($M^+ - CO - H^+$). The latter one possibly underwent loss of CO giving rise to a peak at m/z 53 (Table 1). The mass spectral pattern of the metabolite M-6 was identical to that of authentic catechol.

Identification of metabolites of 2-chlorophenol or phenol by HPLC

HPLC analysis revealed that γ -HCH-grown resting cells of *A. citreus* BI-100 incubated with 2-chlorophenol for 1 h yielded the products phenol and catechol along with some other unidentified metabolites (Fig. 4A). On the other hand, when the cells were incubated with phenol for the same period, the products obtained were catechol and the other unidentified metabolites (Fig. 4B). The peaks of the unidentified metabolites gradually disappeared with the increased time of incubation. However, the peaks corresponding to the ones present in the profile of control experiment where the cells were incubated without any γ -HCH or its subsequent metabolite as substrate remained unaltered (data not shown).

Discussion

Among the four strains of bacteria previously isolated in our laboratory by enrichment culture, the strain BI-100 was found to be the most potent one for biodegradation of γ -HCH (Datta et al., 1998). The strain has been identified as *A. citreus* BI-100 by its morphological, cultural, and biochemical properties. The highest yield of cells as recorded by OD_{600} was observed at 100 mg γ -HCH/L. The present studies show that BI-100 degraded γ -HCH to its metabolic products completely within 8 h. However, the organism continued to grow and divide until 48 h with a 40-fold increase in cell density, suggesting that γ -HCH is transformed to its metabolites faster than it can be converted to biomass.

In the present study six of the metabolites of γ -HCH of the strain BI-100 were identified, which include γ -PCCH, TCCH, TCCD, 2-chlorophenol, phenol, and catechol produced at different periods of growth. The first peak of metabolic product of γ -HCH that appeared was identified as γ -1,3,4,5,6-PCCH by GC-MS analysis. It was detected within 1 h of growth of the strain BI-100 on γ -HCH in mineral salts medium. γ -PCCH was reported to be the first product of γ -HCH metabolism in many microorganisms (Benezet and Matsu-mura, 1973; Engst et al., 1974; Imai et al., 1989; Matsumura et al., 1976; Sahu et al., 1990; Sweeney, 1969; Tu, 1976). Aerobic degradation of γ -HCH by *Pseudomonas paucimobilis*, renamed as *Sphingomonas paucimobilis* (Imai et al., 1989), and a strain of *Pseudomonas* sp. No. 62 (Tu, 1976) was shown to

result in γ -PCCH as the first product of metabolism, whereas in *Pseudomonas putida* (Matsumura et al., 1976), two patterns of γ -HCH degradation were observed, one being the dehydrochlorination pathway in which γ -PCCH is formed. On the contrary, an anaerobic degrader such as *Clostridium* sp. degrades γ -HCH via γ -TCCH (Ohisa and Yamaguchi, 1978). However, BI-100 appears to degrade γ -HCH via γ -PCCH to TCCH, which is then transformed to other metabolites. But the fragmentation pattern obtained for TCCH produced by BI-100 was found to be different in certain respects from that reported for γ -3,4,5,6-TCCH produced by *Clostridium rectum* (Ohisa and Yamaguchi, 1978), indicating structural differences. It may be mentioned that four positional isomeric structures of TCCH are possible to be produced. However, the isomer produced by BI-100 (Fig. 3) could not be ascertained in this study. TCCH was possibly formed by dechlorination of γ -PCCH in this organism. This is in contrast to the formation of tetrachlorocyclohexa-diene as a transient product by dehydrochlorination of γ -PCCH during the metabolism of γ -HCH by *Sp. paucimobilis* (Nagata et al., 1994). TCCH produced appears to be subsequently converted by *A. citreus* BI-100 to TCCD by dechlorination, although TCCH and TCCD both appeared within 2 h of growth of BI-100. It is interesting to note the formation of TCCD, in contrast to the production of 1,2,4-trichlorobenzene, as a dead-end product by *Sp. paucimobilis* during the metabolism of γ -HCH (Nagata et al., 1994). However, no dead-end product was found to accumulate during γ -HCH metabolism by BI-100 in this study, evidenced because the culture extract at 72 h exhibited no peak of metabolite in GC profile. Subsequent to TCCD, 2-chlorophenol appeared to be the next identifiable compound appearing in the GC profile during the degradation. Formation of 2-chlorophenol from TCCD might occur via the formation of transient intermediate(s), which could not be detected. Catechol is identified as one of the metabolites to be produced in the late phase of growth of the organism. The appearance of catechol suggests the possibility of its formation by reductive dechlorination followed by hydroxylation of 2-chlorophenol. This conjecture was supported by the observation that the incubation of 2-chlorophenol with γ -HCH-adapted cells of BI-100 resulted in the formation of phenol and catechol along with some other unidentified metabolites (Fig. 4A). However, only catechol and the other unidentified products were pro-

duced when phenol was used as the substrate (Fig. 2B). Thus it is concluded that 2-chlorophenol is converted to phenol and then to catechol presumably by reductive dechlorination (DeWeerd and Sufflita, 1990), followed by monooxygenase reaction. Furthermore, the test organism could degrade 2-chlorophenol under acidic conditions, producing phenol and catechol in contrast to the microorganisms that metabolize chlorophenols to produce chlorocatechols at elevated pH (Heritage and MacRae, 1977b; Johnson et al., 1985; Kishino and Kobayashi, 1995; MacRae et al., 1969). At 72 h of bacterial growth, no peak of metabolites was detectable by GC; a profile similar to that of the control culture receiving no γ -HCH indicates that catechol was further catabolized and mineralized.

This study indicates that some γ -HCH metabolites produced by *A. citreus* BI-100 identified are quite different from those produced from γ -HCH by any other single microorganism reported in the literature (Benezet and Matsumura, 1973; Francis et al., 1975; Matsumura et al., 1976; Nagata et al., 1993; Tu, 1976; Yule et al., 1967). The spent culture of γ -HCH at 72 h showed an absence of the pesticide or its metabolites in the GC profile, suggesting complete mineralization. The complete mineralization of γ -HCH by a single microbe appears to be a rare phenomenon, and as such the strain *A. citreus* BI-100 might be useful in cleaning up the environment from γ -HCH build-up.

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