NOTES

Isolation and Characterization of a Novel γ-Hexachlorocyclohexane-Degrading Bacterium

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The natural biotic capacity of soils to degrade γ-hexachlorocyclohexane (γ-HCH, lindane) was estimated using an enrichment technique based on the ability of soil bacteria to develop on synthetic media and degrade the xenobiotic compound, used as the sole source of carbon and energy. Bacterial inocula from relatively highly contaminated soils (from wood treatment factories) were found to promote efficiently the degradation of γ-HCH, which subsequently permitted isolation of a competent γ-HCH-degrading microorganism. The decrease of γ-HCH concurrently with the release of chloride ions and the production of CO2 demonstrated the complete mineralization of γ-HCH mediated by the isolate. This was confirmed by gas chromatography-mass spectrometry analyses showing that degradation subproducts of γ-HCH included an unidentified tetrachlorinated compound and subsequently 1,2,4-trichlorobenzene and 2,5-dichlorophenol. The two linA- and linB-like genes, respectively, for a γ-HCH dehydrochlorinase and a dehalogenase were characterized by using a PCR strategy based on sequence homologies with previously published sequences from Sphingomonas paucimobilis UT26. Nucleotide sequence analysis of the linA-like region revealed the presence of a 472-bp open reading frame exhibiting high homology with the linA gene from S. paucimobilis, while a preliminary study also indicated strong homology among the two linB genes. All enzymes involved in the γ-HCH degradative pathway appear to be extracellular and encoded by genes located on the chromosome, although numerous cryptic plasmids have been detected.

Halogenated aliphatic compounds are often considered to be relatively recalcitrant in many subsurface environments, such as soils, sediments, and groundwaters, due in part to their chemical stability and in part to the lack of appropriate microbial activity for their degradation (32). Moreover, the lack of selective pressure, which is related to the compound concentration and availability, might also play a role in their relative recalcitrance. Therefore, the possibility exists of finding indigenous soil microflora exposed to chlorinated pesticides at different concentrations, low in agricultural applications, medium at wood treatment sites, for example, and high at spill sites, and their adaptation potential could be different due to the type of exposure. Several authors have reported the degradation of a chlorinated cycloaliphatic compound, γ-hexachlorocyclohexane (γ-HCH, lindane) under anaerobic conditions by mixed bacterial cultures in flooded soils, lake sediments, and sludges (9, 18) and by pure bacterial strains such as Clostridium spp. (13, 17, 23). γ-HCH was also recently shown to be degraded in aerobic soils (5, 22), which provided the source for microorganisms capable of at least partial lindane degradation and in some cases its use as the sole source of carbon and energy (24, 28, 31). Earlier, two microorganisms classified as Pseudomonas sp. (31) and Escherichia coli (7) have been isolated and described as possessing only the ability to transform γ-HCH to 2,3,4,5,6-pentachloro-1-cyclohexene (γ-PCCH). In addition, α-HCH has been, like lindane, observed to degrade under aerobic conditions in soil (1, 2), which subsequently was used as inoculum for isolating a α-HCH-degrading aerobe identified as Pseudomonas vesicularis (10). Recently, Nagasawa et al. (20) reported the isolation from an upland experimental contaminated field (28) of a bacterial strain, classified as Sphingomonas paucimobilis SS86, which used γ-HCH as the sole source of carbon and energy. The proposed pathway for γ-HCH degradation by this strain includes dehydrochlorination as a preliminary step, resulting in the production of γ-PCCH.

Bacterium enrichment and strain isolation. In this study, we investigated under laboratory conditions, soil samples from sites where γ-HCH has been applied every 2 years for about 20 years for agricultural purposes. Fifty grams of soil was placed in a 200-ml continuously stirred Erlemeyer flask at 30°C containing 1 g of γ-HCH (Merck-Schuchardt, München, Germany) per liter in the presence of dimethyl sulfoxide (DMSO) (15% wt/vol) (Sigma, Saint Quentin Fallavier, France) as the sole source of carbon in mineral medium (pH 7.4) containing per liter: 85 mg of KH2PO4, 217.5 mg of KHPO4, 334 mg of NaHPO4 · 2H2O, 5 mg of NH4Cl, 22.5 mg of MgSO4 · (H2O), 36.4 mg of CaCl2 · (H2O)2, and 0.25 mg of FeCl3 · (H2O). These experiments conducted over a 2-year period never permitted detection of a degradative microbial activity even with the addition of potential cometabolites such as glucose or succinate. Subsequent attempts to isolate adapted bacteria

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from these samples failed (results not shown). The role of the selective pressure applied to microbial communities exposed to these compounds has not been addressed extensively. We cannot conclude from our experiments whether soil bacteria capable of degrading γ-HCH are totally lacking or present at a very low frequency or whether the concentration of the chlorinated pesticide compound in agricultural applications remains too low to permit an effective selective pressure. For instance, previous reports demonstrate that a biodegradation activity was detected in some of the soils which had experienced higher concentrations of γ-HCH (10, 28). In these cases, the competent bacteria were successfully isolated, identified, and genetically investigated (11). Nevertheless, bacteria isolated from some soils did not demonstrate γ-HCH dehydrochlorinase activity, which would be restricted to some S. paucimobilis (22, 28), P. vesicularis (10), or E. coli (7) isolates.

Flasks were also inoculated with bacteria extracted from samples in which the γ-HCH concentration had been higher than in agricultural use (mixed soils from wood treatment sites). For this purpose, 5 g of soil from each of the wood treatment facilities were mixed with 10 g of each of Biolyte CX80 and CX85 and 100 ml of Ringer (1/4 solution) and then placed on a shaker table for 1 h followed by 10 min of ultrasonication. One milliliter of the supernatant was then added to each of four Erlenmayer flasks containing 3 liters of the following medium: one had only mineral medium and γ-HCH (0.5 mM); one had mineral medium, γ-HCH (0.4 mM), and a surfactant (Symeronic); one had mineral medium, γ-HCH (0.5 mM), and cyclohexanol (1.7 mM); and the last one had mineral medium, γ-HCH (0.5 mM), and glucose (1.7 mM). All four were outfitted with continuous-flowing carbon dioxide-free air and carbon dioxide traps (0.025 N BaOH) on the air outlet. The continual addition of γ-HCH and its subsequent dechlorination led to an acidification of the medium, requiring pH adjustments with time using a 1 N NaOH solution. After the enrichments were well established, suspension samples were taken and plated onto SMA medium agar plates (pH 7.0) containing, per liter, 5 g of Bio try case (Biomerieux, Marcy l’Etoile, France), 2.5 g of yeast extract, and 1 g of glucose in order to isolate pure cultures. In this set of experiments γ-HCH degradation was detected, as demonstrated by the production in 45 days of about 70% of the expected theoretical maximum amount of carbon dioxide (Fig. 1). The enrichment in the flask containing only γ-HCH as substrate was maintained over 18 months and was continually supplied with γ-HCH when needed. Moreover, aliquots were periodically extracted from some soils did not demonstrate γ-HCH dehydrochlorinase activity, which would be restricted to some S. paucimobilis (22, 28), P. vesicularis (10), or E. coli (7) isolates.

FIG. 1. Evolution of chloride and carbon dioxide production in the enrichment flask inoculated with a soil sample mixture from two wood treatment factories. (○, chloride; □, carbon dioxide), measured during a 45-day period during which γ-HCH was periodically added (1.7 mmol per flask at day 0 and immediately after each measurement for a total of five additions). Production of chloride and carbon dioxide were equivalent to about 60% of the added γ-HCH (8.6 mmol, producing about 51 mmol each of chloride and carbon dioxide). Evolution of the chloride and carbon dioxide production in the flask containing the yellow translucent bacteria added in presence of γ-HCH as the sole source of carbon and energy (●, chloride; ■, carbon dioxide). γ-HCH was added three times, at day 0 and after each of the two intermediate measurements. Each time 0.13 mmol was added, and over the 50-day period an equivalent of about 20 mmol of chloride and carbon was added. The initial chloride concentration at day 0 was due to previous γ-HCH additions before the start of this datum collection.

3 h) but decreased in liquid mineral medium supplemented with γ-HCH, resulting in a doubling time of about 3 days. γ-HCH degradation kinetics. The γ-HCH degradation efficiency of the isolated bacterium was observed by its incubation in liquid mineral medium containing γ-HCH. Experiments under conditions similar to the initial enrichment culture were carried out in a flask with 1 g of γ-HCH per liter as substrate and the degrading isolate as inoculum (10^6 bacteria per ml) and were maintained for 18 months. Subsamples were taken periodically for analyses. Chloride concentration in the mineral medium was monitored by filtering 2 ml of the bacterial suspension with a 0.22-μm-pore-size membrane (Sartorius, Göttingen, Germany). The filtrate was acidified with few drops of HNO_3 (50% [wt/vol]) and mixed with 45 ml of acetone. Chloride concentration was determined with Ag-AgCl-specific electrodes in the presence of AgNO_3 (0.1 M) and monitored with a potentiometric titer computer. These experiments demonstrated that the degradation of γ-HCH was more or less complete in less than 3 days. The concentration of the released chlorides was consistent with the carbon dioxide production, and no significant lag was observed between the release of chlorides and the production of carbon dioxide (Fig. 1). The bacterial concentration remained constant at approximately 3 × 10^5 CFU/ml during the experiment. Transformation of γ-HCH to 1,2,4-trichlorobenzene (1,2,4-TCB) in the SMA medium was monitored by extracting 5 ml of the bacterial suspension with 2 ml of hexane. The UV absorption spectrum between 250 and 320 nm of the hexane extract (1 ml) was determined on a Kontron 930 spectrophotometer. The spectrum obtained was then compared with that of the standard 1,2,4-TCB (Aldrich, Saint Quentin Fallavier, France) after addition and reextraction from the SMA medium. Compounds were extracted from 2 ml of culture in SMA medium by vigorous shaking in the presence of 1 ml of hexane (analytic grade; Aldrich, Saint Quentin Fallavier, France). After centrifugation (3,835 × g for 5 min), the hexane layer was recovered and 1 μl of this extract was analyzed by gas chromatography.
Phenotypical identification of the \( \gamma \)-HCH-degrading isolate. The \( \gamma \)-HCH-decomposing bacterium was found to form dark yellow flat colonies with clean edges on SMA medium agar plates (results not shown). After about 4 days of growth on SMA medium, colonies were recovered from the petri plates to be resuspended with a sufficient quantity of 0.9% (wt/vol) NaCl buffer to reach 10\(^9\) bacteria per ml. This suspension was then used to inoculate the API 20 NE strip (Biomerieux, Marcy l’Etoile, France) and Biolog microplate (Biolog, Hayward, Calif.) bacterial identification systems according to the manufacturer’s recommendations. According to the API identification system the isolate would belong to the species \( P. \) vesiculatis, while Biolog identified it as a member of the genus \( Xanthomonas \). Interestingly, an \( \alpha \)-HCH-degrading bacterium isolated from a polluted soil (10) was found to develop similar colonies on SMA medium and to produce a yellow pigment and was also classified as \( P. \) vesiculatis according to an API test. Comparison at the molecular level of the two isolates but also of representatives of the genus \( Xanthomonas \) via DNA-DNA hybridization and 16S rRNA gene sequence analyses would be of interest to assess the phylogenetic relationships among these bacteria.

The pigment of the \( \gamma \)-HCH-degrading isolate was extracted from colonies by mixing them vigorously with 2 ml of acetone. The acetone layer containing pigments was separated by centrifugation, and the visible absorption spectrum of the acetone extract was determined on a Kontron 930 spectrophotometer at wave lengths between 400 and 550 nm. The yellow pigment, once extracted with acetone, exhibited the characteristic absorbance spectrum of the carotenoid “Nostoxanthine,” with absorption maxima at 428, 452, and 489 nm. A similar pigment was previously described in \( S. \) paucimobilis (14) and \( Flavobacterium \) sp. (4).

Cloning of a \( linA \)-like gene encoding a \( \gamma \)-HCH dehydrochlorinase activity. Total DNA from the \( \gamma \)-HCH-degrading isolate was digested with several restriction endonucleases alone or in combination and hybridized on Southern blots with a \( linA \) PCR probe. This probe was obtained by PCR, using two primers (FGPlinA, 433–69, 5’-CCCTAGACAAAGCGCCAGG-3’; FGPlinA, 434–411, 5’-GGTGAATATGTGTCGATC-3’) complementary to the \( linA \) gene from \( S. \) paucimobilis (11). PCR amplifications (29) were performed in a total volume of 50 \( \mu \)l, each reaction mixture containing 0.07 mM dioxigenine-11-dUTP (Boehringer Mannheim, Meylan, France), 0.15 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 0.5 \( \mu \)M each primer, and 1.5 U of \( Taq \) DNA polymerase (Gibco BRL, Cergy-Pontoise, France) in a buffer containing 10 mM Tris-HCl (pH 8.3), 125 mM KCl, 1.5 mM MgCl\(_2\), and 0.01% (wt/vol) gelatin. The DNA used as the template was from the recombinant plasmid pUC118-linA (12), containing the \( linA \) gene from \( S. \) paucimobilis (11). PCR products with the expected sizes were obtained by electrophoresing 1/10 of the PCR products on a 0.8% (wt/vol) agarose gel, suggesting that a gene possessing a strong homology with the \( linA \) gene from \( S. \) paucimobilis exists in the \( \gamma \)-HCH-degrading isolate.

Total DNA from the \( \gamma \)-HCH-degrading isolate was digested either with \( BamHI \) or \( HindIII \) and with both enzymes and then hybridized at 37\(^\circ\)C for 18 h in the presence of 50% formamide with the \( linA \) and \( linB \) PCR probes. Standard protocols (25) were used for DNA electrophoresis and blotting on nylon filters (GeneScreen Plus; Dupont, Boston, Mass.). Nonradioactive hybridizations and detection were carried out by using the chemiluminescent substrate CSPD from a nonradioactive detection kit (Boehringer Mannheim) according to the manufacturer’s instructions.

Hybridizing fragments were extracted from the gel by using a GeneClean II kit (Bio 101, Vista, Calif.) and ligated into \( BamHI-HindIII \)-digested Bluescript SK\(^-\) (Stratagene, La Jolla, Calif.). The ligated DNA was used to transform maximum...
efficiency E. coli DH5α competent cells (Gibco BRL, Cergy-Pontoise, France). E. coli cells were plated onto LB selective plates in the presence of 1 g of γ-HCH per liter in DMSO in order to detect recombinant clones that exhibited a γ-HCH degradation halo. Plasmid extraction, subcloning, and sequencing procedures were as described earlier, using FGPlinA1, 433–69, FGPlinA2, 434–411, T3, and T7 as sequencing primers. A positive hybridization fragment of 4.3 kb (results not shown) was detected when total DNA was digested with HindIII and BamHI enzymes used in combination. DNA fragments of the corresponding sizes were extracted from the gel and cloned, and the recombinant E. coli clones harboring 4.3-kb inserts hybridized with the linA probe. Screening was based on the detection of a clear zone around colonies plated onto LB selective agar plates in the presence of 1 g of γ-HCH per liter in DMSO. Among the 514 recombinant clones tested, 6 exhibited a dehydrochlorination activity, converting γ-HCH to 1,2,4-TCB, as estimated by a UV spectrometrical analysis, and possessed the same pLINA1 plasmid harboring the 4.3-kb recombinant DNA fragment. The partial restriction map of this fragment was determined (Fig. 3). This permitted subcloning of part of the 4.3-kb fragment, yielding plasmids pLINA2 carrying a 1.4-kb Clal-BamHI fragment and pLINA3 carrying a 1.2-kb HindII fragment (Fig. 3). The bacterial clones with the pLINA3 plasmid exhibited the same level of γ-HCH dehydrochlorinase activity as plasmid pLINA1-containing clones, while the pLINA2-containing bacteria exhibited a weaker activity. This might be due to the promoter region of the linA-like gene being located on the DNA region between the Clal and HindII sites. The nucleotide sequence of the 1.2-kb HindII region of the pLINA3 plasmid (Fig. 4) was determined on both strands by using Qiagen midiprep columns (Qiagen, Düsseldorf, Germany) to prepare plasmids, the dideoxy-chain termination method of Sanger et al. (26), the T7 sequencing kit (Pharmacia, LKB, Uppsala, Sweden), and primers T3 and T7. A unique open reading frame (ORF) of 472 bp was found within the 1.2-kb DNA region, the ORF being preceded by a putative Shine-Dalgarno sequence. Putative -10 and -35 regions were found in the region upstream of the Shine-Dalgarno sequence (Fig. 4). This linA-like gene encodes for a 17.3-kDa polypeptide that consists of 157 amino acid residues, and the gene has a G+C content of 53%. Moreover, a strong homology was found with the linA gene from the S. paucimobilis UT26 strain (11), with the only exception being a 3-bases insertion resulting in the production of an additional amino acid. This means that two bacterial strains isolated from distant soils (Japan and France) would possess similar γ-HCH dehydrochlorinase activities encoded by a nearly identical gene, indicating that both the protein and the corresponding gene or their antecedents have been highly conserved throughout bacterial evolution.
Detection of a linB-like gene encoding a haloalkane dehalogenase activity. In order to determine whether other genes involved in the degradation pathway were also conserved among the two γ-HCH-degrading isolates, the same strategy as the one developed above was used for detecting a linB-like gene in the γ-HCH-degrading isolate DNA. Primers FG-PlinB1435–18 (5′-GCCATTTGGCGAGAAGAA-3′) and FG-PlinB2436–729 (5′-GGCGTTGATGAAGAGTTT-3′) complementary to part of the linB gene from S. paucimobilis UT26 according to the published sequence (21) were used in PCR reactions with the γ-HCH-degrading isolate DNA as the template. This resulted in the production of an expected 709-bp DNA fragment. When digested with the enzymes NlaIII, PvuI, and NciI the PCR products exhibited patterns similar to those obtained with the linB gene from S. paucimobilis UT26 (results not shown). Moreover, the use of this PCR fragment as a probe produced positive hybridization results with a 3.5-kb HindIII, a 3.4-kb PstI, and a 1.7-kb BamHI fragment, confirming the presence of a linB-like gene in this isolate and suggesting a strong homology with the analogous gene in the S. paucimobilis UT26 strain.

Characterization of the linA-like gene products. In order to overproduce γ-HCH dehydrochlorinase, the linA gene encoding this activity was inserted into pKT230 (3), a broad-host-range, high-copy-number RSF1010-derived vector. Gene linA from the isolated γ-HCH-degrading bacterium, which encodes the transformation of γ-HCH to 1,2,4-TCB, was inserted into the HincII cloning site. The resultant plasmid was cloned into E. coli and Pseudomonas fluorescens R2f (30). However, the sizes of the halos surrounding the developing recombinant colonies were larger in P. fluorescens after 2 days than in E. coli even after 6 days (results not shown), suggesting an increased expression of linA in P. fluorescens.

The R2f cells containing pKT230:linA were cultured in 500 ml of LB medium supplemented with γ-HCH. At regular time intervals, 50 ml of culture was centrifuged for 15 min at 5,500 × g, and the supernatant was filtered (pore size, 0.22 μm). Proteins from the sterile supernatant were precipitated with solid ammonium sulfate (80% [wt/vol] of saturation) at 4°C for 2 h and centrifuged for 15 min at 5,500 × g. The pellet was resuspended into 1 ml of 50 mM potassium phosphate buffer, pH 7.5. The product of linA was measured by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, using the discontinuous buffer system of Laemmli (16). Eighty micrograms of SDS-solubilized protein samples were heated in boiling water for 2 min before electrophoresis on a 0.75-mm-thick, 12% (wt/vol) polyacrylamide gel overlaid with an SDS-4.5% (wt/vol) polyacrylamide stacking gel. The weight of the proteins was determined using a standard protein marker.
degradation enzyme was determined by using the following M₄ standards (Sigma): \( \alpha \)-lactalbumin (M₄ 14,200), trypsin inhibitor (M₄ 20,100), trypsinogen (M₄ 24,000), carbonic anhydrase (M₄ 29,000), glyceraldehyde-3-phosphate dehydrogenase (M₄ 36,000), egg albumin (M₄ 45,000), and bovine albumin (M₄ 66,000). Gels were stained with 0.1% (wt/vol) Coomassie blue R250 in 50% (vol/vol) methanol–7.5% (vol/vol) acetic acid and destained with 30% (vol/vol) ethanol–10% (vol/vol) acetic acid. A major protein band was detected on the gels, but this band disappeared when \( \Phi \) pseudomonas R2F cells were cultured without \( \gamma \)-HCH (data not shown). This suggested induction of the expression of the \( \text{linA} \)-like gene by the substrate. This was confirmed by the increasing amount of the specific protein detected all along the growth kinetics when identical amounts of concentrated proteins (80 \( \mu \)g) were electrophoresed (Fig. 5). Its molecular mass was estimated to be 15.4 kDa, in good agreement with the mass deduced from the nucleotide sequence of the \( \text{linA} \) gene (17.3 kDa corresponding to the mature enzyme plus its signal sequence).

**Extracellular enzyme activity.** The appearance of halos on agar plates containing precipitated \( \gamma \)-HCH around colonies of the original \( \gamma \)-HCH-degrading isolate or of the recombinant \( E. \ coli \) and \( \Phi \) pseudomonas clones harboring only the \( \text{linA} \) gene lead to the consideration of the extracellular nature of the enzymes responsible for \( \gamma \)-HCH dechlorination. Supernatants from actively dechlorinating cultures were collected by centrifugation and filtration through 0.2-\( \mu \)m-pore-size filters. The original cultures, the supernatant (tested for the absence of bacterial cells), and the mineral medium, all with \( \gamma \)-HCH added to them, were monitored for chloride production (Fig. 6). The supernatant and the cell cultures produced about the same quantity of chloride over the course of the experiment, roughly 90% of that added in the form of \( \gamma \)-HCH, confirming the extracellular nature of whole enzymes involved in the \( \gamma \)-HCH dechlorination activity.

**Detection of plasmids and localization of catabolic properties.** The degradative pathway for numerous chemical compounds has been found to be controlled and expressed by genes located on extrachromosomal replicons. These so-called “degradative” plasmids were found to be conjugative types ranging in size from 40 to more than 300 MDa (5). This was the case for the extrachromosomal location of the HCH degradation determinant in two \( \text{Pseudomonas} \) strains (15), confirming the value of detecting and studying degradative properties of plasmids in the \( \gamma \)-HCH-degrading strain isolated in this study. The analytical in situ hybridization procedure initially described by Eckhardt (6) as well as the preparative method of Schwingaman (27) permitted detection of seven different plasmids ranging in size from 20 to 180 MDa in the isolate (Fig. 7). Knowledge that catabolic functions are often carried by plasmids has stimulated our research directed toward defining their role in \( \gamma \)-HCH degradation. This was done by using the \( \text{linA} \) PCR probe to hybridize plasmid profiles resulting from both extraction methods. Total DNA from the isolate digested with the \( \text{BamHI} \) enzyme was also hybridized as a positive control. None of the plasmids exhibited a positive signal with the \( \text{linA} \) probe, while the 4.3-kb \( \text{BamHI} \)-hybridizing fragment from the chromosome gave a positive response. This indicates that such plasmids remain cryptic, the \( \gamma \)-HCH degradation function of the strain being presumably located on the chromosome, although the presence of larger and undetected plasmids cannot be totally excluded. In addition, several experiments demonstrated the stability of the degradation function. For example, this \( \gamma \)-HCH-degrading isolate was cultured over 16 generations on SMA medium (without \( \gamma \)-HCH) and did not lose its \( \gamma \)-HCH degradation activity or its plasmid pattern (results not shown).

Moreover, conjugation experiments between this strain and \( E. \ coli \) or \( \Phi \) pseudomonas never succeeded in transferring the capacity to degrade \( \gamma \)-HCH to the recipient strains (results not shown).

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**FIG. 7.** Detection of plasmid DNAs in the isolate according to the in situ gel method. Lanes: 1 and 2, \( E. \ coli \) VS17 (19) as size standards, with plasmids of 34, 5, 3, 6, 3, 2, 2, 4, 1.86, 1.49, and 1.3 kbp; 3, preparation from 1.2 ml of a 2-day liquid culture in rich medium.

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