APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2002, p. 6021–6028 Vol. 68, No. 12
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.12.6021–6028.2002

Cloning and Characterization of *lin* Genes Responsible for the Degradation of Hexachlorocyclohexane Isomers by *Sphingomonas paucimobilis* Strain B90

Rekha Kumari,[1] Sanjukta Subudhi,[1] Mrutyunjay Suar,[1] Gauri Dhingra,[1] Vishakha Raina,[1] Charu Dogra,[1] Sukanya Lal,[1] Jan Roelof van der Meer,[2] Christof Holliger,[3] and Rup Lal[1]*

Department of Zoology, University of Delhi, Delhi-110007, India,[1] and Process of Environmental Microbiology and Molecular Ecotoxicology, Swiss Federal Institute for Environmental Science and Technology (EAWAG), CH 8600 Duebendorf,[2] and EPFL, ENAC-ISTE, Laboratory of Environmental Biotechnology, CH 1015 Lausanne,[3] Switzerland

Received 3 June 2002/Accepted 18 September 2002

Hexachlorocyclohexane (HCH) has been used extensively against agricultural pests and in public health programs for the control of mosquitoes. Commercial formulations of HCH consist of a mixture of four isomers, α, β, γ, and δ. While all these isomers pose serious environmental problems, β-HCH is more problematic due to its longer persistence in the environment. We have studied the degradation of HCH isomers by *Sphingomonas paucimobilis* strain B90 and characterized the *lin* genes encoding enzymes from strain B90 responsible for the degradation of HCH isomers. Two nonidentical copies of the *linA* gene encoding HCH dehydrochlorinase, which were designated *linA1* and *linA2*, were found in *S. paucimobilis* B90. The *linA1* and *linA2* genes could be expressed in *Escherichia coli*, leading to dehydrochlorination of α-, γ-, and δ-HCH but not of β-HCH, suggesting that *S. paucimobilis* B90 contains another pathway for the initial steps of β-HCH degradation. The cloning and characterization of the halidohydrolase (*linB*), dehydrogenase (*linC* and *linX*), and reductive dechlorinase (*linD*) genes from *S. paucimobilis* B90 revealed that they share ~96 to 99% identical nucleotides with the corresponding genes of *S. paucimobilis* UT26. No evidence was found for the presence of a *linE*-like gene, coding for a ring cleavage dioxygenase, in strain B90. The gene structures around the *linA1* and *linA2* genes of strain B90, compared to those in strain UT26, are suggestive of a recombination between *linA1* and *linA2*, which formed *linA* of strain UT26.

Hexachlorocyclohexane (HCH) is a chlorinated cyclic saturated hydrocarbon, also known as benzene hexachloride, or BHC. In the recent past, γ-HCH (lindane) and technical BHC (a mixture of HCH isomers) have been used extensively, particularly for the control of agricultural pests and mosquitoes. Commercially, technical BHC is prepared by chlorination of benzene in the presence of UV light. During its synthesis, primarily four isomers, α, β, γ, and δ, are formed in the ratio 65, 12, 12, and 7%, respectively (36). Among these, only γ-HCH has insecticidal properties, and its isolation requires several concentration and purification steps, thereby increasing its cost. Thus, primarily for economic reasons, technical BHC has been used widely and indiscriminately in several developing countries, including India. Although the use of lindane and BHC has been banned or severely restricted in a number of countries, including India, the ban has not stopped HCH residues from entering the environment. As a result, HCH residues are being detected in various environmental niches, not only in India but all over the world (4, 26, 27, 32). While γ-HCH is persistent in the environment but still degradable, the α and β isomers are the more problematic of the different HCH isomers in view of their very low solubility in water and thus their high potential for bioaccumulation. There is also evidence that the α and γ isomers of HCH are converted into the β isomer in living organisms (7). A survey for HCH residues in various samples conducted over many years showed that most are contaminated with α- and β-HCH (7, 26, 12).

The persistence of HCH isomers in aerobic environments is primarily due to the absence of microbes that can degrade them (8, 10, 11, 19). HCH isomers are known to degrade slowly under anaerobic conditions (10, 14, 28), but there are very few reports of HCH degradation under aerobic conditions. Matsumura et al. (15) first reported aerobic degradation of HCH by a *Pseudomonas* strain. Degradation of HCH by a *Pseudomonas paucimobilis* strain (later reclassified as *Sphingomonas paucimobilis* strain SS86) was reported in upland experimental fields in Japan where γ-HCH had been applied once a year for 12 years (31, 35). γ-HCH-degrading *S. paucimobilis* was also isolated from French soils (33). Despite the fact that these strains degraded α-, δ-, and γ-HCH, they were unable to degrade β-HCH.

The failure to discover microbes that could degrade β-HCH aerobically (2, 5, 6, 33) suggested that bacteria were unable to degrade β-HCH due to its unique chemical structure and stereochemistry. However, two reports described *S. paucimobilis* strain B90, isolated in Cuttack, India, from rice rhizosphere soil that had been treated repeatedly with technical BHC (3, 29), which was able to mineralize not only α-, δ-, and γ-HCH but also β-HCH (9, 29).

The genes and enzymes which trigger the degradation of

* Corresponding author. Mailing address: Department of Zoology, University of Delhi, Delhi-110007, India. Phone: 91-11-7666254. Fax: 91-11-7666541. E-mail: duzdel@del2.vsnl.net.in.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source[b]
E. coli DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ*U169 deoR*	Laboratory stock
E. coli BL21	B F$^-$ *ompT hsdS* (r_B^- m_B^-) *gal dcm*	Amersham Pharmacia
E. coli JM101	*supE thi* Δ(*lac-proAB*) F′[*tra D36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*]	Laboratory stock
S. paucimobilis B90	Aerobic, motile, rod shaped; degrades all four isomers of HCH (α, β, γ, and δ)	CRRI, Cuttack, India
S. paucimobilis UT26	Aerobic, motile, rod shaped; degrades α, γ, and δ-HCH but not β-HCH	Y. Nagata
pUC 13/19	2.7-kb; Amp^r; MCS internal to *lacZ* gene	Laboratory stock
pGEX-5X-3	GST fusion vector with *tac* promoter, *lacI*^{q}, factor Xa protease recognition site	Amersham Pharmacia
pIMA2	Recombinant construct carrying truncated *linA* gene	Y. Nagata
pYNA4R	Recombinant construct carrying truncated *linB* gene	Y. Nagata
pBFRU2	Recombinant construct carrying truncated *linC* gene	Y. Nagata
pKM2	Recombinant construct carrying truncated *linD* gene	Y. Nagata
pLIN14	pUC13 carrying 1.4-kb *Bcl*I fragment from genomic DNA of *S. paucimobilis* B90	This study
pLIN25	pUC13 carrying 2.5-kb *Bcl*I fragment from genomic DNA of *S. paucimobilis* B90	This study
pLINA1	pUC19 containing *linA1* gene	This study
pLINA2	pUC19 containing *linA2* gene	This study
pLINB	pUC19 containing *linB* gene	This study
pLINC	pUC19 containing *linC* gene	This study
pLIND	pUC19 containing *linD* gene	This study
pLINX	pUC19 containing *linX* gene	This study
pLINEA1	pGEX-5X-3 containing *linA1* gene	This study
pLINEA2	pGEX-5X-3 containing *linA2* gene	This study

[a] MCS, multiple cloning site; GST, glutathione *S*-transferase.
[b] CRRI, Central Rice Research Institute.

γ-HCH have been characterized mostly from *S. paucimobilis* UT26 (6, 19, 24), which is a nalidixic acid-resistant mutant of *S. paucimobilis* strain SS86. The primary enzyme for γ-HCH degradation in strain UT26 is HCH dehydrochlorinase, encoded by the gene *linA* (6, 22, 25). The remaining genes of the γ-HCH degradative pathway, i.e., *linB* (21, 23, 25), *linC* (20, 25), *linD* (16), and *linE* (17, 18), encode a halidohydrolase, a dehydrogenase, a reductive dechlorinase, and a dioxygenase, respectively, and have also been cloned and characterized from *S. paucimobilis* UT26. Parts of the genes for γ-HCH dehydrochlorinase and for the remainder of the HCH degradative pathway were amplified by PCR from DNA of the *S. paucimobilis* strain isolated from French soil (33), suggesting that HCH degradation is probably mediated by enzymes similar to those of *S. paucimobilis* UT26.

Since *S. paucimobilis* strain B90 degrades α-, β-, γ-, and δ-HCH, this organism is promising for developing bioremediation strategies for the decontamination of soils containing HCH residues. As a first step in this direction, we report here the degradation pattern of HCH isomers and the cloning and characterization of several *lin* genes for the HCH degradative pathway from *S. paucimobilis* B90.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *S. paucimobilis* B90 (29) was obtained from N. Sethunathan, Central Rice Research Institute, Cuttack, India, and *S. paucimobilis* UT26 (31, 35) was obtained from Y. Nagata (University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, Japan). Plasmids pMIA2, pYNA4R, pBFRU2, and pKM2 contain truncated fragments of the *linA*, *linB*, *linC*, and *linD* genes of *S. paucimobilis* UT26, respectively (Table 1), and were also obtained from Y. Nagata. *S. paucimobilis* B90 and *S. paucimobilis* UT26 were usually grown at 28°C in mineral salt medium (SM) containing (per liter) 0.5 g of $(NH_4)_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of K_2HPO_4, 0.01 g of $Ca(NO_3)_2$, and 0.01 g of $FeSO_4 \cdot 7H_2O$, supplemented with 1% glucose. *Escherichia coli* DH5α and JM101 were used as general host strains for plasmid cloning. The *E. coli* strains were grown routinely at 37°C in Luria broth (LB) or on LB agar (30) supplemented with the appropriate antibiotics when necessary.

Chemicals and enzymes. Analytical grade α-, β-, γ-, and δ-HCH were obtained from Ehrenstorfer GmbH (D-86199, Augsburg, Germany). Pentachlorocyclohexene (PCCH) was chemically synthesized from α-, γ-, and δ-HCH according to the method described by Trantirek et al. (34). Enzymes for DNA manipulations were purchased from Bangalore Genei (Bangalore, India), New England Biolabs Inc. (Beverly, Mass.), and Boehringer Mannheim (Mannheim, Germany). The Nonradioactive DNA Labeling and Detection kit was purchased from Boehringer Mannheim, and [α-^{32}P]dATP was obtained from the Bhabha Atomic Research Centre (Mumbai, India).

Analytical techniques. The degradation of α-, β-, γ-, and δ-HCH in *S. paucimobilis* B90, *S. paucimobilis* UT26, and recombinant *E. coli* containing plasmids with the *lin* genes was carried out either in SM supplemented with 1% glucose or in LB. Precultures of *S. paucimobilis* or *E. coli* grown overnight in SM plus glucose or LB were transferred at 1% (vol/vol) to fresh medium containing 5 μg of each HCH isomer/ml. Each flask containing α-, β-, γ-, or δ-HCH was incubated at 28°C (37°C for *E. coli*) on a rotary shaker. Appropriate controls containing medium plus the HCH isomer or medium plus the HCH isomer and *E. coli* containing pUC13-pUC19 or pGEX-5X-3 were kept simultaneously. Aliquots (200 μl) were taken out periodically and extracted twice with 500 μl of hexane. The concentration of HCH isomers was measured by subjecting samples to GC on a chromatograph equipped with an electron capture ^{63}Ni detector (GC 17A; Shimadzu, Kyoto, Japan) on a column containing 3% OV 17 (Chromatopak Analytical Instrumentation Pvt. Ltd, Mumbai, India). The column, injector, and detector temperatures were maintained at 200, 220, and 250°C, respectively, with a flow rate of carrier gas (nitrogen) of 27 ml/min.

DNA manipulation. Plasmid DNA from *E. coli* was isolated by the alkaline lysis method (30). The genomic DNA of *S. paucimobilis* was isolated by standard methods from cells pelleted from 100-ml cultures grown to stationary phase in SM containing 1% glucose (30). Southern hybridizations with fragments of the *linA*, *linB*, *linC*, and *linD* genes of *S. paucimobilis* UT26 (Table 1) were carried out by established procedures (30). DNA sequences were determined using standard methodologies for double-stranded plasmid DNA on an automated DNA sequencer (ABI PRISM model 377, version 3; Applied Biosystems) at the Department of Biochemistry, South Campus, University of Delhi, Delhi, India. The sequences were analyzed using the DNASIS package (Pharmacia). The BLAST program (1) was used for homology searches within GenBank, and ClustalW was used for multiple alignments of sequences.

Cloning of *lin* genes. The *lin* genes were cloned from *S. paucimobilis* B90 as follows. Two *Bcl*I fragments of 1.4 and 2.5 kb hybridizing to the *linA* probe from strain UT26 were eluted from *Bcl*I-digested strain B90 genomic DNA and ligated with *Bam*HI-digested pUC13. After ligation and transformation, colonies hybridizing positively to the *linA* probe were recovered. Genomic DNA of *S. paucimobilis* B90 was partially digested with *Sau*3AI and size fractionated on a

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′-3′)[a]	Added restriction site	Designation	Source or accession no.
1	GCGGATCCGCATGAGTGATCTAGACAGACTT	*Bam*HI	*linA*-sense	D90355
2	GCCTCGAGTTATGCGCCGGACGGTGCGAAATG	*Xho*I	*linA1*-antisense	This study
3	GCCTCGAGTCACGATTTTTGCAACAGAGC	*Xho*I	*linA2*-antisense	This study
4	GCGGATCCGCATGAGCCTCGGCGCAAAGCCA	*Bam*HI	*linB*-sense	D14594
5	GCCTCGAGTTATGCTGGGCGCAATCGCCGGAC	*Xho*I	*linB*-antisense	D14594
6	GCGGATCCGCATGTCTGATTTGAGCGGC	*Eco*RI	*linC*-sense	D14595
7	GCCTCGAGTCAGATCGCGGTAAAGCCGCCGTC	*Xho*I	*linC*-antisense	D14595
8	GCGAATTCAATGAGCGCTGATACAGAA	*Eco*RI	*linD*-sense	D89733
9	GCCTCGAGTTAGGCGTTGCTCAGGAGATGGAT	*Xho*I	*linD*-antisense	D89733
10	GCGGATCCGCATGGCTAACAGACTCGCAGGCA	*Bam*HI	*linX*-sense	D23722
11	GCCTCGAGTCAAACACCCACGGACCAGCCTCC	*Xho*I	*linX*-antisense	D23722
12	AGGAATTCCATGATGCAACTGCCCGAA	*Eco*RI	*linE*-sense	AB021867
13	AGCTCGAGCTCAAATGACGATCGGATC	*Xho*I	*linE*-antisense	AB021867
14	TGGGATCCCCGTGAATATAGATGACCTGG	*Bam*HI	*linR*-sense	AB021860
15	GGGTCGACTCACATCCGCGCGGACAG	*Sal*I	*linR*-antisense	AB021860
16	GCGGATCCGCTCTGTTGCAAAAATCGTGAAGC	*Bam*HI	Tn*610*-sense	X536535
17	GCGGATCCGATGACCATGATTACGCC	*Bam*HI	Tn*610*-antisense	X536535

[a] Restriction sites are underlined.

sucrose density gradient. DNA fragments of 35 to 45 kb were ligated to the *Bam*HI-digested cosmid pWE15 (Stratagene, La Jolla, Calif.). The cosmid library of *S. paucimobilis* B90 was screened with the *linA*, *linB*, *linC*, and *linD* gene probes described above. Positive clones were regrown, and the cosmids were isolated. Total genomic DNA from *S. paucimobils* B90 or cosmid clones containing appropriate inserts were also used as templates for PCR amplification with conserved *lin* gene primers (Table 2). PCR amplification was performed with a Nugene Thermocycler (Techne, Progene, Cambridge, United Kingdom) according to the specifications of the supplier. The PCR products were cloned in pUC13 and in the *E. coli* expression vector pGEX-5X-3 (Stratagene) in order to study the possible expression and activities of the *lin* gene products.

HCH dehalogenase activity in *linA1* and *linA2* clones. *E. coli* containing pLINEA1 or pLINEA2 was grown in LB plus 50 μg of ampicillin/ml and 5 μg of each HCH isomer/ml. Samples (200 μl) were withdrawn periodically with a micropipette, extracted twice with 500 μl of hexane, and subjected to GC as described above. Cell extracts were prepared from the same *E. coli* clones grown in 50 ml of LB medium containing ampicillin at an optical density at 600 nm of 1. The cells were harvested by centrifugation at 4,500 × *g* for 10 min at 4°C. The pellet was resuspended in sonication buffer (0.25 M glucose, 5 mM dithiothreitol, 2 mM Na_2EDTA, 150 mM NaCl, 50 mM Tris HCl, 100 mM phenylmethylsulfonyl fluoride, and 100 μg of lysozyme/ml, pH 7), and the cells were lysed by sonication in an Ultrasonicator (Misonix; Microson, New York, N.Y.). The lysed cells were centrifuged for 20 min at 5,000 × *g* and 4°C. Dehydrochlorinase activity in the supernatant was assayed by incubating 1 ml of cell extract (induced and uninduced *E. coli* cultures) with 5 μg of HCH isomer at 28°C with shaking. After regular time intervals, samples were withdrawn, extracted with hexane, and analyzed by GC as described above. The protein content of the cell extract was estimated by the method of Lowry et al. (13). Appropriate controls containing *E. coli* BL21 with pGEX-5X-3 were handled simultaneously.

Nucleotide sequence accession numbers. The *lin* sequences of strain B90 have been deposited in GenBank under the following accession numbers: *linX* and *linA1*, AY150579; *linA2*, AY150580; *linB*, AY150581; *linC*, AY150582; and *linD*, AY150583.

RESULTS AND DISCUSSION

Degradation of HCH by *S. paucimobilis* B90. *S. paucimobilis* B90 was tested for its ability to degrade α-, β-, γ-, and δ-HCH separately at 5 μg/ml in SM medium containing 1% glucose. Strain B90 was found to degrade α-, γ-, and δ-HCH rapidly (Fig. 1a). β-HCH was also degraded, but it did not disappear completely from the medium (Fig. 1a). No α-, γ-, or δ-HCH was detectable in the medium after 24 h. No complete degradation took place at levels beyond 50 μg/ml (not shown). The rates of degradation of HCH isomers by *S. paucimobilis* strain

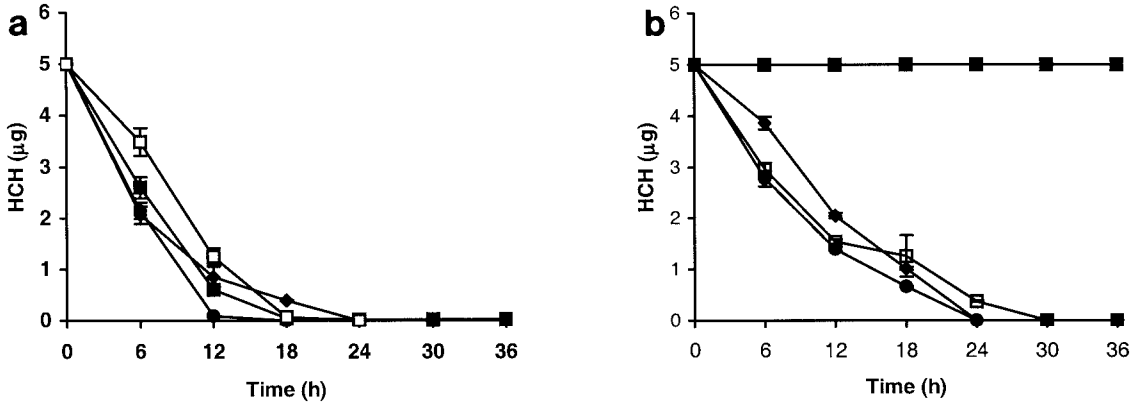

FIG. 1. Degradation of HCH by *S. paucimobilis* B90 (a) and *S. paucimobilis* UT26 (b) in SM plus 1% glucose. An initial inoculum of 0.5 ml (10^8 cells/ml) was added to 50 ml of medium, and simultaneously, each HCH isomer was added separately (5 μg/ml). Samples were withdrawn periodically, extracted with hexane, and analyzed on a gas chromatograph equipped with an electron capture detector. ♦, α-HCH; ■, β-HCH; ●, γ-HCH; □, δ-HCH. The error bars indicate standard deviations.

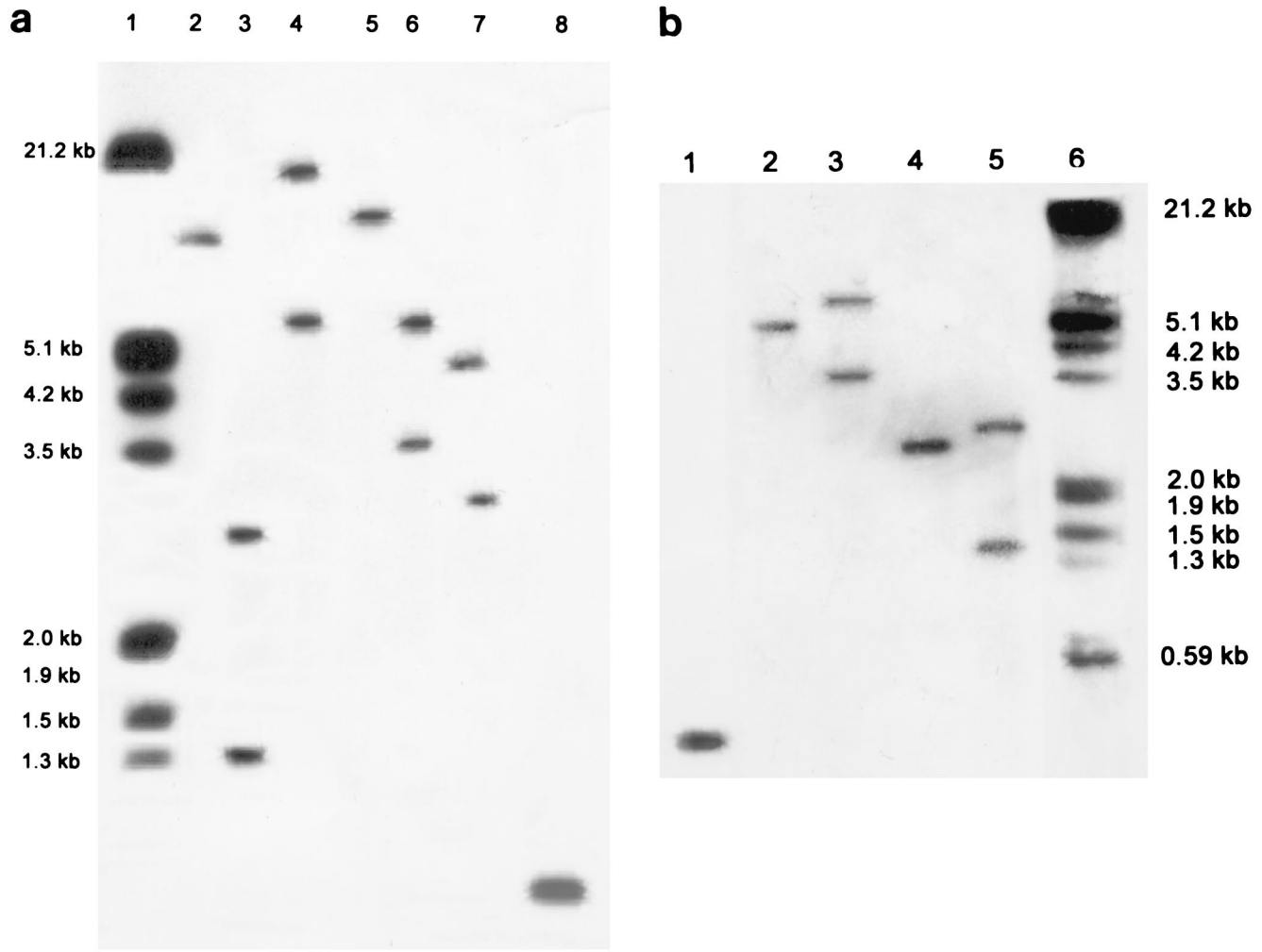

FIG. 2. Southern blot hybridization of genomic DNA of *S. paucimobilis* B90 (a and b) and strain UT26 (b) with an [α-^{32}P]dATP-labeled truncated fragment of *linA*. (a) Lane 1, lambda DNA digested with *Eco*RI and *Hin*dIII; lanes 2 to 7, B90 DNA digested with *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, and *Sal*I, respectively; lane 8, amplified *linA1* (462 bp) as a positive control. (b) Lane 1, amplified *linA1* (462 bp) as a positive control, lanes 2 to 5, genomic DNA digested with *Hin*dIII (UT26), *Hin*dIII (B90), *Bcl*I (UT26), and *Bcl*I (B90), respectively; lane 6, lambda DNA digested with *Eco*RI and *Hin*dIII.

B90 were slightly higher than those for *S. paucimobilis* UT26 (Fig. 1b). However, as expected, *S. paucimobilis* UT26 did not degrade β-HCH. Chromatograms obtained after GC analysis of the samples from incubations with α-, γ-, and δ-HCH revealed similar intermediates for *S. paucimobilis* B90 and *S. paucimobilis* UT26. The main types of intermediates corresponded to α-, γ-, and δ-PCCH, which was confirmed by PCCH standards produced after chemical conversion of each HCH isomer (α, γ, and δ; not shown). During β-HCH degradation, a very predominant peak appeared in GC chromatograms (at 6.5 min compared to β-HCH at 5 min) from samples taken after 36 and 72 h (not shown), which persisted in prolonged incubations but eventually disappeared. This intermediate had a different retention time than chemically synthesized β-PCCH. Further identification of this intermediate was outside the scope of the present work.

Cloning of HCH dehydrochlorinase genes in *E. coli*. Genomic DNA of *S. paucimobilis* B90 produced strong positive hybridization signals when hybridized with a *linA* gene fragment of *S. paucimobilis* UT26 (Fig. 2). For a number of digests, two hybridizing bands were observed (Fig. 2b, lanes 3 and 5). The sequences of the two cloned *S. paucimobilis* B90 *Bcl*I fragments with sizes around 1.4 and 2.5 kb predicted two nonidentical *linA*-homologous genes (Table 3), named *linA1* and *linA2*. The open reading frame (ORF) of the *linA1* gene encoded a 154-amino-acid polypeptide with amino acids 88% identical to those of *linA* from strain UT26. The peptide predicted from the *linA2* ORF encompassed 158 amino acids, with only the first two (extra) residues different from the *linA* gene product. The DNA sequence upstream and downstream of the *linA1* gene differed from the *linA2* region. Interestingly, the region upstream of *linA1* showed strong similarity to the upstream region of *linA* in strain UT26 containing the *linX* gene and ORFUP (Fig. 3). Downstream of *linA1*, no homology to UT26 was found. Instead, the nucleotide sequence showed nucleotides 99% identical to the DNA sequence of Tn*610* of *Mycobacterium fortuitum* (accession no. X536535). The 3′ end of the *linA1* gene and the Tn*610* sequence (only 608 nucleo-

TABLE 3. Comparison of various HCH degradative genes in *S. paucimobilis* B90 and *S. paucimobilis* UT26

Gene	Length (bp)		Peptide length (aa)[a]		Homology (%)	Function	Stability[b]	
	B90	UT26	B90	UT26			B90	UT26
linA1	462	468[c]	154	156	96	Dehydrochlorinase	+	−
linA2	474	468[c]	158		100[d]	Dehydrochlorinase	+	−
linB	888	888	296	296	99	Halidohydrolase	+	−
linC	750	750	250	250	99	Dehydrogenase	+	−
linD	1,038	1,038	346	346	99	Reductive dechlorinase	+	−
linE		963		321		Ring cleavage dioxygenase	ND	+
linR		909		303		Transcriptional regulator	ND	+

[a] aa, amino acids.
[b] +, stable; −, unstable; ND, not detected.
[c] *linA* gene of *S. paucimobilis* UT26
[d] Percent identical nucleotides between *linA* and *linA2*

tides of the total nucleotide sequence of Tn*610*) appeared to overlap (Fig. 3). Although we did not isolate the Tn*610* element completely from strain B90, hybridization of its genomic DNA with the DNA fragment from pLINA14 containing the Tn*610*-homologous DNA gave three hybridizing signals (data not shown), which indicated that there could be more than one copy of this element in the genome of *S. paucimobilis* B90. PCR amplification with primers based on the Tn*610* sequence located downstream of the *linA1* gene (primers 16 and 17 [Table 2]) resulted in a product of the expected size (655 bp) from *S. paucimobilis* B90 DNA but no amplification product with *S. paucimobilis* UT26 DNA (not shown). Also, genomic DNA of strain UT26 did not hybridize to the Tn*610*-like probe from plasmid pLIN14 in Southern hybridizations. This suggested that no Tn*610*-like element is present in strain UT26.

Conversely, the upstream sequence of the region of the *linA2* gene did not show any homology to that of *linA1* or *linA* of *S. paucimobilis* UT26, whereas its 3′ end completely overlapped with the DNA sequence downstream of *linA* of strain UT26 (Fig. 3). The present structure of the *linA1* and *linA2* regions in strain B90 suggests that the genetic organization of *linX* and *linA* in strain UT26 might have been the result of a recombination between the 5′ ends of (predecessor) *linA1* and *linA2* sequences. The few amino acid differences at the C-terminal end of LinA1 compared to those of LinA and LinA2 did not result in any obvious difference in dehydrochlorinase activity. Apparently, changes outside the catalytic dyad formed by His-73 and Asp-25 can be accommodated (34). Similar to *linA* of strain UT26 (6), the G+C contents of the *linA1* and *linA2* genes of *S. paucimobilis* B90 were lower than those of *linX*, *linB*, *linC*, and *linD*. Thus, as suggested earlier, the *linA* genes in strain B90 might have had a different origin than the other *lin* genes and perhaps were acquired by horizontal gene transfer (24).

Stability of the lindane degradation pathway. A few observations made in our laboratory suggest that the lindane degradation phenotype is more stable in strain B90 than in strain UT26. While *linA1*, *linA2*, *linB*, *linC*, and *linD* could always be amplified from colonies of *S. paucimobilis* B90 by the primers based on the corresponding *lin* genes from strain UT26 (Table 2), amplification could not be consistently achieved from colonies of *S. paucimobilis* UT26 (Fig. 4). When nearly 500 colonies of *S. paucimobilis* B90 were screened by GC analysis for degradation of HCH isomers, no lindane-negative mutants were detectable, whereas the same experiment with *S. paucimobilis* UT26 colonies resulted in 10 such mutants. The lack of the *linA* gene was confirmed in these UT26 mutants by PCR

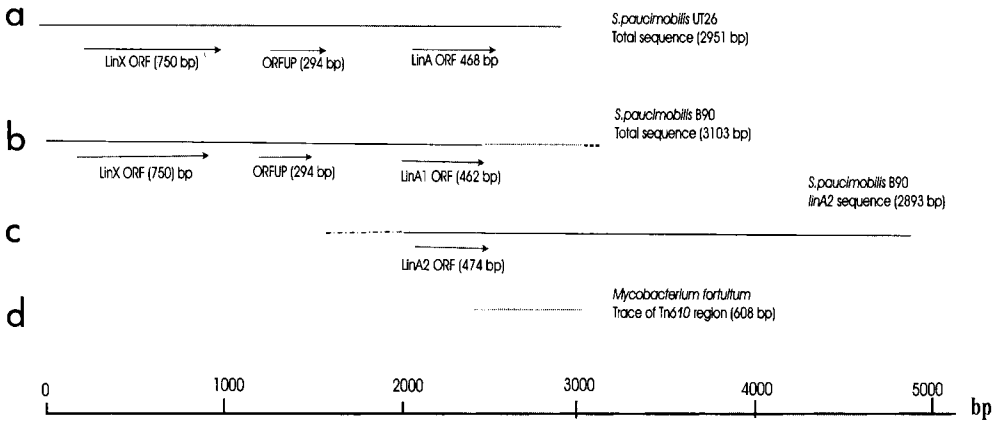

FIG. 3. Schematic drawings of the regions from *S. paucimobilis* UT26 and B90 comprising the *linA* genes. (a) Reconstructed drawing from the published DNA sequences of *linX* (accession no. D23722) (20) and *linA* (accession no. D90355) (6) of *S. paucimobilis* UT26. (b) reconstructed drawings based on the sequences of *linA1* and *linX* of *S. paucimobilis* B90. (c) The *linA2* region. (d) Trace of Tn*610* of *M. fortuitum* (accession no. X536535) (14a). Similar drawings (bold and dotted lines) indicate regions of DNA sequence homology between different genes or regions.

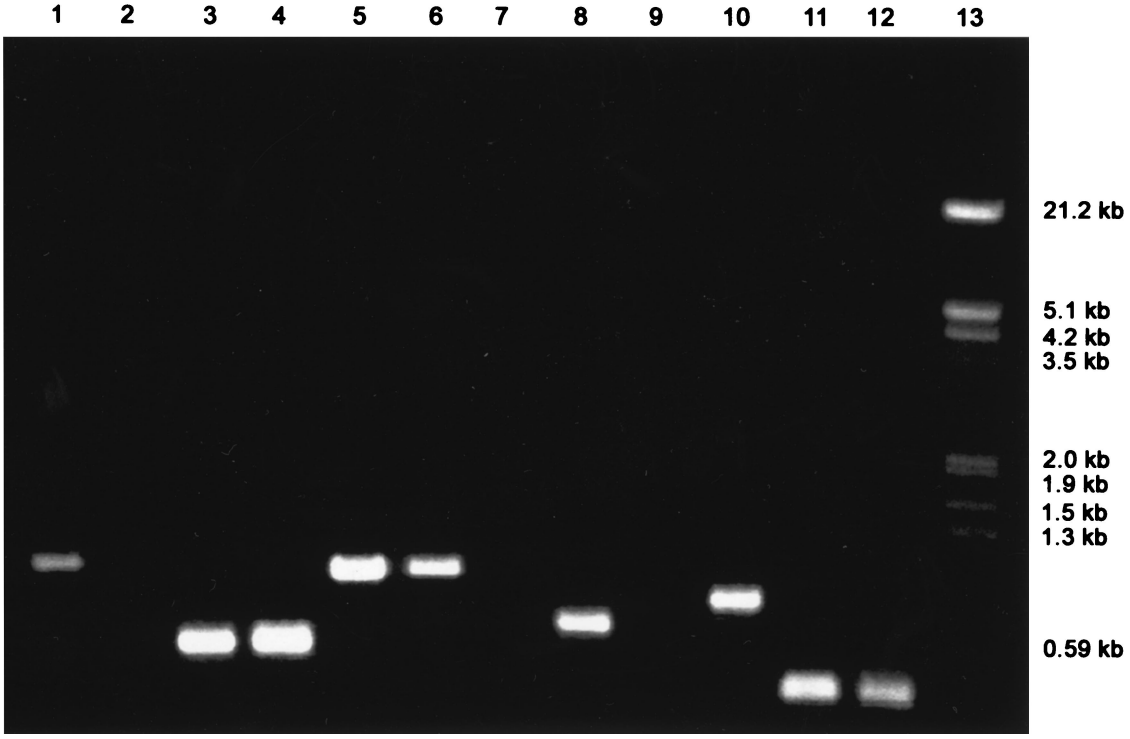

FIG. 4. Detected instability of *lin* genes in strain UT26. Shown are products of PCR amplification with primers for *linA*, *-B*, *-C*, *-D*, and *-X* and the Tn*610*-like sequence on total DNA of *S. paucimobilis* B90 and UT26 isolated from individual clones. Lanes: 1 and 2, Tn*610* detection in strain B90 (lane 1) and UT26 (lane 2); 3 and 4, *linX* in UT26 (lane 3) and B90 (lane 4); 5 and 6, *linD* in UT26 (lane 5) and B90 (lane 6); 7 and 8, *linC* in UT26 (lane7) and B90 (lane 8); 9 and 10, *linB* in UT26 (lane 9) and B90 (lane 10); 11 and 12, *linA* in UT26 (lane 11) and B90 (lane 12); 13, lambda DNA digested with *Eco*RI and *Hin*dIII.

amplification with primers 1 and 2 and DNA-DNA hybridization with the α-^{32}P-labeled *linA* as a probe (data not shown).

Demonstration of functionality of both *linA* copies in strain B90. By determining the disappearance of HCH from growing cultures of *E. coli* with plasmid pLINEA1 or pLINEA2, it was found that *E. coli* containing *linA1* or *linA2* degraded γ-HCH to γ-PCCH (Fig. 5a). No disappearance of γ-HCH was detected in control cultures of *E. coli* BL21 with pGEX-5X-3 alone. This demonstrated that the conversion was specific for the *linA1* and *linA2* inserts. Similar conversions of α- and δ-HCH to α- and δ-PCCH, respectively, were observed with *E. coli* BL21(pLINEA1) or BL21(pLINEA2) as well (not shown).

Interestingly, the rate of degradation of γ-HCH was much lower in the beginning (up to 6 h) in *E. coli* BL21 containing pLINEA1 than that of pLINEA2 cultures (Fig. 5a). Whether this difference can be attributed to the differences between the amino acids in LinA1 and LinA2 has yet to be determined. β-HCH was not measurably converted by the *E. coli linA1* or *linA2* clone. The PCCH intermediates disappeared from growing cultures of *E. coli* BL21(pLINEA1) or BL21(pLINEA2), as

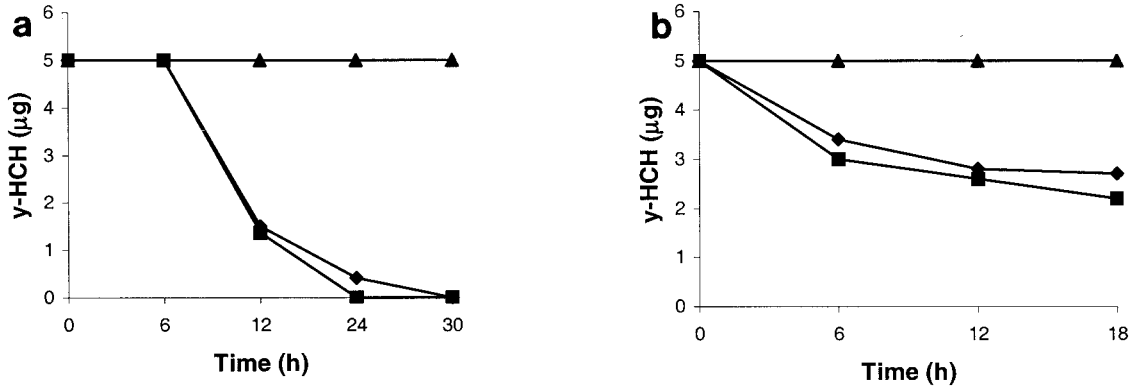

FIG. 5. Degradation of γ-HCH by *E. coli* BL21(pLINEA1) or BL21(pGEX-5X-3). (a) Conversion of 5 μg of γ-HCH/ml by pLINEA1 and pLINEA2 in *E. coli* BL21 growing in LB medium. Samples were drawn periodically and analyzed by gas chromatography. ▲, control; ◆, pLINEA1; ■, pLINEA2. (b) Degradation of γ-HCH by cell extracts of *E. coli* BL21(pLINEA1) expressing the *linA1* gene. The cell extracts contained 5 mg of protein/ml, to which 5 μg of γ-HCH/ml was added. ▲, control; ◆, pLINEA1; ■, pLINEA2.

TABLE 4. Hybridization mapping of cosmid clones with *linA*, *linB*, *linC*, *linD*, *linX*, and Tn*610* as probes

DNA probe	Hybridization with cosmid clone[a]:					
	3	5	6	10	23	27
linA1	−	−	+	+	+	+
linA2	−	−	+	+	+	+
linB	−	+	−	−	−	−
linC	−	−	+	−	+	+
linD	+	−	−	−	−	−
linX	−	−	+	−	+	+
Tn*610*	−	−	+	−	+	+

[a] +, hybridization; −, no hybridization.

well as in *E. coli* cell extracts upon prolonged incubation, which suggests their further dehydrochlorination.

Conversion of γ-HCH also took place in cell extracts of *E. coli* containing pLINEA1 or pLINEA2 (with PCCH as an intermediate), but not with *E. coli* BL21(pGEX-5X-3) (Fig. 5b). Again, only α-, γ-, and δ- but not β-HCH could be converted by the *E. coli* BL21(pLINEA1) or BL21(pLINEA2) cell extracts. The cell extracts prepared from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced culture did not degrade α-, γ-, and δ-HCH, which reflected the fact that induction results in improper folding of proteins, making them inactive.

All of the results with respect to dehydrochlorinase activity and transient PCCH accumulation by LinA1 and LinA2 are consistent with those for LinA published by others (6, 23, 34). It might be that both *linA* copies in strain B90 result in slightly higher rates of HCH degradation than in strain UT26 (Fig. 1). Contrary to our expectations, the presence of both *linA* copies in strain B90 seemed not to be important for β-HCH conversion. Since degradation and activity toward α-, γ-, and δ-HCH did occur in *E. coli* BL21, this seems to rule out the possibility that the LinA protein is not correctly localized in *E. coli*. From these results, we conclude at this point that at least initial degradation of β-HCH proceeds through a different enzyme than LinA1 or LinA2.

Cloning of the *linX*, *linB*, *linC*, and *linD* genes of *S. paucimobilis* B90. On the basis of presumed homology to the *lin* genes of strain UT26, the *linX*, *linB*, *linC*, and *linD* genes could also be identified and cloned from strain B90 by using primer sets listed in Table 2. These genes were highly similar to their counterparts in strain UT26 (Table 3). In contrast, no amplification of *linE*- and *linR*-like sequences in *S. paucimobilis* B90 could be obtained (data not shown). Hybridizations, restriction mapping, and DNA sequencing of cosmid clones containing *linA*, *linB*, *linC*, *linX*, and *linD* DNA fragments revealed that no single cosmid contained a DNA insert encompassing all of the isolated *lin* genes from strain B90 (Table 4). The *linA1* copy associated with the Tn*610*-like fragment and *linX* and *linC* (Fig. 3), whereas the *linA2* gene and the other *lin* genes (*linB* and *linD*) were not associated and were present at different positions on the B90 genome. The strong homology of the other *lin* ORFs of strain B90 with those of strain UT26 (Table 3) strongly suggests that they carry out the same functions in strain B90. Therefore, it is very likely that the lindane degradation pathway in strain B90 is similar to that of strain UT26, at least up to the step of hydroquinone (Fig. 6). Since we did not obtain amplification or hybridization of *linE* or *linR*, the ring cleavage oxygenase and regulatory element for the lower pathway might be different from that of strain UT26 (24).

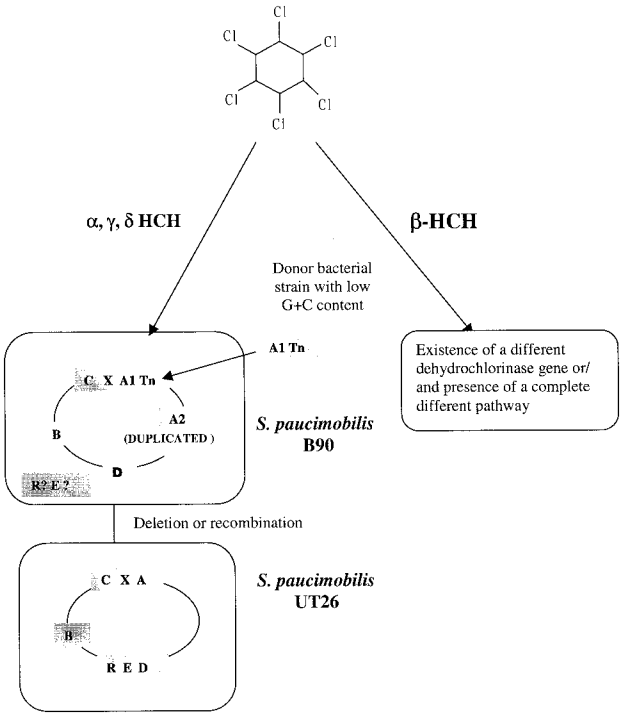

FIG. 6. Possible evolutionary origin of HCH degradative genes and pathways in *S. paucimobilis* B90 and *S. paucimobilis* UT26.

It is definitely intriguing and hopeful that at least three different aerobic lindane-degrading bacteria isolated at different positions on the globe (*S. paucimobilis* B90 from India, *S. paucimobilis* UT26 from Japan [6], and *S. paucimobilis* from France [33]) carry very similar if not identical genetic information for lindane degradation. Such microorganisms may lead to the spontaneous disappearance of HCH residues in the environment or might be used as a basis for achieving targeted bioremediation of HCH isomers.

ACKNOWLEDGMENTS

We thank Y. Nagata, Department of Biotechnology, University of Tokyo, for providing the plasmids and *S. paucimobilis* UT26. Thanks are also due to R. Eichenlaub and K. H. Gartemann for valuable suggestions and help. We also thank Kirsten Lawlor for her help in providing the *linE* probe and her valuable suggestions for the detection of *linE* and *linR*.

Part of this work was supported by grants under the Indo-Swiss Collaboration in Biotechnology.

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