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Characterization of the novel HCH-degrading strain, *Microbacterium* sp. ITRC1

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Abstract A gram-positive *Microbacterium* sp. strain, ITRC1, that was able to degrade the persistent and toxic hexachlorocyclohexane (HCH) isomers was isolated and characterized. The ITRC1 strain has the capacity to degrade all four major isomers of HCH present in both liquid cultures and aged contaminated soil. DNA fragments corresponding to the two initial genes involved in γ-HCH degradative pathway, encoding enzymes for γ-pentachlorocyclohexene hydrolytic dehalogenase (*linB*) and a 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase (*linC*), were amplified by PCR and sequenced. Their presence in the ITRC1 genomic DNA was also confirmed by Southern hybridization. Sequencing of the amplified DNA fragment revealed that the two genes present in the ITRC1 strain were homologous to those present in *Sphingomonas paucimobilis* UT26. Both 16S rRNA sequencing and phylogenetic analysis resulted in the identification of the bacteria as a *Microbacterium* sp. We assume that these HCH-degrading bacteria evolved independently but possessed genes similar to *S. paucimobilis* UT26. The reported results indicate that catabolic genes for γ-HCH degradation are highly conserved in diverse genera of bacteria, including the gram-positive groups, occurring in various environmental conditions.

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Introduction

Gamma-hexachlorocyclohexane (γ-HCH, Gammexane, lindane), a chlorinated pesticide, has been widely used for crop protection and prevention of vector-borne diseases for many decades. During its production, four major isomers (Fig. 1) are formed: α-HCH (70%), β-HCH (8%), γ-HCH (13%), and δ-HCH (9%), but only the gamma isomer has insecticidal property. These isomers are highly hydrophobic, persistent, and widespread in the environment. They accumulate in the food chain (Deo et al. 1994) on account of their lipophilic properties, which leads to toxicity (ATSDR 1999). During their presence in the environment, they also volatilize and are transported to the atmosphere (Simonich and Hites 1995; Blais et al. 1998; Donald et al. 1999). For example, the presence and fluxes of α- and γ-HCH were determined in four rivers that flow to the North Sea in UK. It was reported that these four rivers export 30.0 kg/year of γ-HCH and 14.8 kg/year of α-HCH to the sea (Meharg et al. 1999). In addition, historical trends of production of HCH isomers and their application worldwide (Breivik et al. 1999; Li 1999) have been reported in detail. In India, the residues of HCH have been detected in surface and subsurface soils (Agnihotri et al. 1996), in food products (Kannan et al. 1992), dairy milk (John et al. 2001), and recently, in packaged drinking water (Mathur et al. 2003), having concentrations several folds higher than permissible limits.

Current practices to detoxify organochlorine pesticides rely on chemical treatment, incineration, and landfills, which are economically restrictive. Bioremediation, the removal of environmental pollutants by living organisms, has become a viable and promising means of restoring contaminated sites. Therefore, bacteria capable of degrading HCH isomers have received considerable attention as they provide the possibility to be utilized for in situ detoxification. Four sulfate-reducing bacteria (SRB) were reported for their transformation potential of γ-HCH from anaerobic marine sediments (Boyle et al. 1999). It was reported that SRB dehalogenate γ-HCH to tetracholoro-cyclohexane and monochlorobenzenes. It was observed

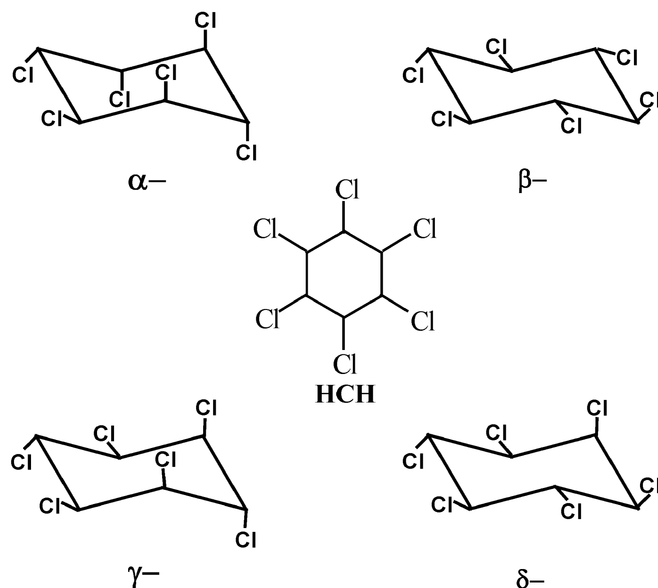

Fig. 1 Structures of four hexachlorocyclohexane (HCH) isomers

that α-HCH present in a contaminated soil was susceptible to biodegradation under different redox conditions, but β-HCH was recalcitrant (Bachmann et al. 1988).

A strain of *Pseudomonas paucimobilis* isolated from paddy field rhizosphere soil was demonstrated to degrade α-, γ-, and δ-HCH but not the β-isomer (Sahu et al. 1990). Another strain of *P. paucimobilis* SS86, which is now *Sphingomonas paucimobilis* UT26, that was able to use γ-HCH as the sole source of carbon and energy was isolated from an experimental field to which γ-HCH had been applied (Senoo and Wada 1989). Subsequently, a bacterium from the Rhine River in France identified as *Rhodanobacter lindaniclasticus* (Nalin et al. 1999) was isolated for its capability to degrade γ-HCH (Thomas et al. 1996). The three bacteria mentioned above were subjected to genetic analysis to identify the genes responsible for γ-HCH degradation. The genes from these bacteria were either partially (Thomas et al. 1996; Kumari et al. 2002) or completely (Nagata et al. 1999) cloned or sequenced. Degradation rates of the HCH isomers were investigated under a range of pH and temperature conditions using a *Pandorea* species (Siddique et al. 2002). Thus, mainly the degradative capabilities of only gram-negative bacterial genera for HCH isomers have been studied so far. Only one report involving the gram-positive *Bacillus* sp. (Gupta et al. 2000) is available. Here, we describe the identification of a gram-positive *Microbacterium* sp. (ITRC1 strain) that is capable of degrading all four major isomers of HCH. We also report the identification of genes involved in the γ-HCH degradation pathway.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

The *Microbacterium* sp. ITRC1 strain was isolated for its capability to degrade the HCH isomers as its sole carbon source. The detailed cultivation conditions on the mineral medium containing the HCH isomers are given in "Enrichment and isolation of a bacterial strain for HCH degradation," "Biodegradation of HCH isomers in liquid-culture conditions," and "Biodegradation of HCH isomers from contaminated soil." *Escherichia coli* DH5α (Bethesda Research Laboratories 1986), obtained from Stratagene, was cultivated aerobically with constant shaking in Luria–Bertani (LB) medium (Sambrook and Fritsch 1989) supplemented, if appropriate, with 100 µg/ml ampicillin. The properties of the plasmids and strains used in this study are summarized in Table 1.

Chemicals, enzymes, and DNA primers

Analytical-grade α-, β-, γ-, and δ-HCH were obtained from Riedel-deHaën, Germany. Technical HCH was procured from India Pesticides Ltd., Chinnhat Industrial Area, Lucknow, India. Mercuric thiocyanate, 2-phenoxy ethanol, and ferric ammonium sulfate were purchased from Sigma, USA. Enzymes for DNA manipulations were from Gibco BRL, and the nonradioactive labeling and detection kit was purchased from Roche Diagnostics GmbH, Mannheim,

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
E. coli DH5α	*supE44 Δlac U169 (80 lacZ ΔM15) hsdR recA1 endA1 gyrA96 thi relA1*	Gibco BRL/Bethesda Research Laboratories 1986
Microbacterium sp.	Aerobic bacteria, gram positive, motile, rod-shaped, yellow color; degrades four major isomers of α-, β-, γ-, and δ-HCH	This study
pBluescript II SK (±)	Phagemid derived from pUC19: P*lac lacZ′* Apr; fl(+) origin; *col*E1 origin	Stratagene/Altschul et al. 1990
pIMA2	Recombinant plasmid pUC118 carrying 1.2 kb *Eco*RI–*Bam*HI fragment for *linA* gene	Imai et al. 1991
pYNA4	Recombinant plasmid pUC119 carrying 1.3 kb *Bgl*I–*Hpa*I fragment for *linB* gene	Nagata et al. 1993
pBFR41	Recombinant plasmid pUC119 carrying 1.2 kb *Sal*I fragment for *linC* gene	Nagata et al. 1994
pLIG1	pBluescript II SK (±) carrying PCR-amplified 0.8 kb DNA fragment for *linB*	This study
PLIG2	pBluescript II SK (±) carrying PCR-amplified 0.5 kb DNA fragment for *linC*	This study

Germany. Oligonucleotide primers were custom synthesized from MWG-Biotech AG, Ebersberg, Germany. The test substrates (Table 3) were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were of analytical grade and were available commercially.

Enrichment and isolation of a bacterial strain for HCH degradation

Sediment samples, rhizosphere soil, and wastewater from around an industry (India Pesticides Ltd.) engaged in the manufacture of γ-HCH for more than 15 years. The samples were collected by using sterile 1-l Schott bottles (Schott, Mainz, Germany) that were sealed with screw caps. After transportation to the laboratory, the samples were processed immediately under aseptic conditions. Fifty-gram portions from the three different samples were pooled and stirred thoroughly. From this soil slurry mixture, a 10-g portion was added to 20 ml of the mineral medium (Ahuja et al. 2001) supplemented with 0.34 mM of γ-HCH as the sole carbon source. The flasks were incubated in a shaker at 28°C, and after 7 days, 1 ml of suspension from this enrichment culture was transferred to new flasks with similar conditions as mentioned above. Such enrichment process was repeated five times. During this process, aliquots were plated on LB agar plates periodically. After the fifth round of enrichment, the cells were plated on LB agar plates, and it was discovered that only one type of bacterial colonies, having a bright yellow color, could grow on the plates. These cells were used as an inoculum for further degradation studies.

Biodegradation of HCH isomers in liquid-culture conditions

To initiate the degradation, we each precoated 250-ml Erlenmeyer flasks with α-, β-, γ-, and δ-HCH, respectively, to the final concentration of 0.34 mM. The flasks were inoculated with the ITRC1 strain and incubated for 4 days. The cells were harvested by centrifugation (13,200×*g* for 10 min), washed, and resuspended in fresh mineral medium to give a final $OD_{600\ nm}$ of 0.1 in 20 ml. This medium was added to the flask containing 0.34 mM of each HCH isomer to follow the degradation. Flasks containing both the medium and the HCH isomers but without any inoculation served as controls. All the treatment flasks and controls were incubated at 28°C with shaking at 200 rpm for 4 days.

Biodegradation of HCH isomers from contaminated soil

For the degradation of HCH isomers in soil, 1 kg of contaminated soil from the same industrial site was collected, mixed thoroughly, and sieved with 2 mm mesh. Initial concentration of HCH was quantified by gas chromatography (GC) analysis from a 10-g soil sample done in triplicate by extraction with the mixture of hexane and acetone (1:1 v/v). The contaminated soil was diluted by the garden soil in a 1:1 ratio, and 20 g of soil was added to each beaker. To this, 10 ml mineral medium containing 2.0×10^7 cfu was added, and the slurry was mixed thoroughly. The control flasks were not augmented with the ITRC1 strain. The treatment and control beakers were incubated at 28±2°C. Both uninoculated and inoculated samples were extracted in triplicate after the second, third, and fourth weeks and analyzed by thin layer chromatography (TLC) and GC. Prior to the initiation of the degradation of HCH isomers from contaminated soil, we had spiked a soil from our institute's garden premises with 0.34 mM technical HCH. This soil had not been exposed to any pesticide a priori. Augmentation of this soil with the ITRC1 strain resulted in the significant disappearance of all isomers of HCH (data not shown).

Analytical techniques

Chloride estimation, chromatography

Chloride liberation into the liquid medium during the degradation of HCH isomers was estimated by the colorimetric method to be as follows (Bergmann and Sainik 1957). The residual HCH, after incubation, was extracted twice with an equal volume of hexane and acetone (1:1 v/v), followed once with hexane alone. A suitable aliquot was used for TLC and GC analyses. For TLC, a 2- to 10-µl sample was spotted on the plates (silica gel_{60} 20×20 cm, 0.20 mm thickness, Merck, Darmstadt, Germany), and the chromatograms were developed in cyclohexane and visualized by spraying the chromogenic reagent (Kovacs 1965), followed by UV exposure. The chlorinated compounds were detected as dark brown spots on TLC plates. GC was performed with a Shimadzu model equipped with Ni^{63} electron capture detector. The carrier gas was nitrogen with a flow rate of 60 ml/min. Temperature for column, injector, and detector was maintained at 190, 250, and 250°C, respectively. Retention time for α-, β-, γ-, and δ-HCH was 1.56, 2.03, 2.55, and 3.25 min, respectively.

Isolation of DNA

Plasmid DNA from *E. coli* was isolated using Pharmacia Flexiprep Kits (Amersham Pharmacia Biotech, Freiburg, Germany) as per the manufacturer's instructions. ITRC1 strain cells, grown in 50 ml mineral medium containing 100 µg/ml γ-HCH and 0.5% yeast extract, were incubated at 28°C for 4 days at 150 rpm. Genomic DNA was isolated from 4-day-old cultures as described by Eulberg et al. 1997.

Polymerase chain reaction to amplify dehalogenase gene

The oligonucleotide primers were designed based on three known reductive dehalogenase genes (*linA*, *linB*, and *linC*) from *S. paucimobilis* UT26 (Nagata et al. 1999) and used to amplify the homologous DNA fragments from the ITRC1 strain. The reaction mixture (50 µl) contained 50 pmol of each primer, 0.5 µg of chromosomal DNA as template, 100 µM deoxynucleoside triphosphates, 1× PCR buffer, and 0.7 U of DNA polymerase from *Thermus brockianus* F500 (Biometra GmbH, Göttingen, Germany). PCR was performed with 30 cycles of denaturing (95°C, 30 s), annealing (55°C 1 min), and polymerization (72°C 1 min), with an additional 3 min of denaturation during the first cycle and an additional 10 min of polymerization during the last cycle.

Southern blot hybridization

Genomic DNA (3 to 4 µg) was digested with *Pst*I (for *linB*) and *Eco*RI for 6 h at 37°C, loaded onto a 0.8% agarose gel, and electrophoresed for 7 h at 40 V. The separated DNA was transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) by using protocols described by the manufacturer. The *linA*, *linB*, and *linC* gene probes were labeled using random primed DNA labeling with digoxygenin-dUTP alkali-labile DIG DNA Labeling Kit (Boehringer-Mannheim, Mannheim, Germany). Prehybridization (2 h) and hybridization (10 h) were performed using a hybridization oven (Amersham Pharmacia Biotech). The membrane was washed twice for 15 min at room temperature (~20°C) with 2× standard saline citrate (SSC) buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and twice at 65°C with 0.1× SSC buffer containing 0.1% (wt/vol) SDS. The bound probe was detected by using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as colorimetric reagents according to the manufacturer's instructions (Boehringer-Mannheim).

General in vitro manipulations of DNA and cloning

The transformation of *E. coli* DH5α was achieved by the method of Inoue et al. 1990. The recombinant plasmids used in this study are described in Table 1. Insert DNA was eluted from gels for digoxygenin labeling with the use of a Bio 101 Gene Clean II Kit. The PCR products were ligated into a T-tailed vector (Marchuk et al. 1991) prepared from pBluescript II SK (±), yielding the plasmids pLIG1 and pLIG2 (Table 2).

16S rRNA amplification and sequencing

In an effort to identify the HCH-degrading isolate, ITRC1 strain, 16S rRNA-specific DNA was amplified by PCR (Biometra GmbH) and sequenced. Approximately 50 ng purified chromosomal DNA from the ITRC1 strain, 10 pmol of each primer, 250 pmol of each deoxyribonucleoside triphosphate, 5 µl of 10× PCR buffer, 10 µl of $MgCl_2$ (25 mM), and 1 U of Taq polymerase (Gibco BRL) were added. Sterile, distilled H_2O was mixed to give a final volume of 50 µl. The thermal cycling protocol used included initial denaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 7 min was also used. The universal eubacterial 16S rRNA gene-specific primer 27f plus 1522r (Lane 1991) was used. The PCR products were sequenced twice on both strands by the dideoxy method (Sanger et al. 1977), using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with an automated sequencing system.

Table 2 Oligonucleotide primers used in this study

Primer designation	Sequences	Length (bp)	Accession number/ Reference
fwlinA	5′-GGC CGC GAT TCA GGA CCT CTA CT-3′	23	D90355
revlinA	5′-CGG CCA GCG GGG TGA AAT AGT-3′	21	Imai et al. 1991
LIGAFw	5′-CAA GBC GGG CCG CNA TTC A-3′	19	D90355
LIGRev	5′-CCG GAC GGG GCR AAR TCD AT-3′	20	Imai et al. 1991
linAF	5′-CGT AGA CAA GCG CCA AGA GG-3′	20	D90355
linAR	5′-GGT GAA ATA GTT CGT GCA TC-3′	20	Imai et al. 1991
linBF	5′-ATG AGC CTC GGC GCA-3′	15	D14594
linBR	5′-TCG CCG GAC AAA CGC-3′	15	Nagata et al. 1993
fwlinC	5′-TGA GCG GCA AGA CGA TAA T-3′	19	D14595
revlinC	5′-CAG CGG CGG ATG CGG TGT TGA- 3′	21	Nagata et al. 1994

Sequence alignments of 16S rRNA and phylogenetic inference

The nucleotide sequence for 16S rRNA obtained from both the strands was aligned (CLUSTAL W). The sequence similarity searches for 16S rRNA were performed in the DDBJ/EMBL/GenBank data library using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1990). Alignments and calculations were done using the ARB package. The dendrogram was calculated with the neighbor-joining algorithm (Saitou and Nei 1987). All positions of the alignment were included in the calculation

of relationships among *Microbacteria*. An out-group was added using the parsimony tool (Ludwig et al. 2004) of the ARB package, taking only those positions in the alignment that are conserved in at least 50% of the gram positives with high G+C DNA.

The bacterial ITRC1 strain studied in this report has been deposited in the Microbial Type Culture Collection (MTCC), which is an International Depository Authority at the Institute of Microbial Technology, Chandigarh, India, with accession number MTCC B0017.

Nucleotide sequence accession number

The 16S rRNA, *linB*, and *linC* gene sequences from the *Microbacterium* sp. ITRC1 strain obtained in this study were deposited in the DDBJ/EMBL/GenBank database with the accession numbers DQ 143988, DQ 143989, and DQ 143990, respectively.

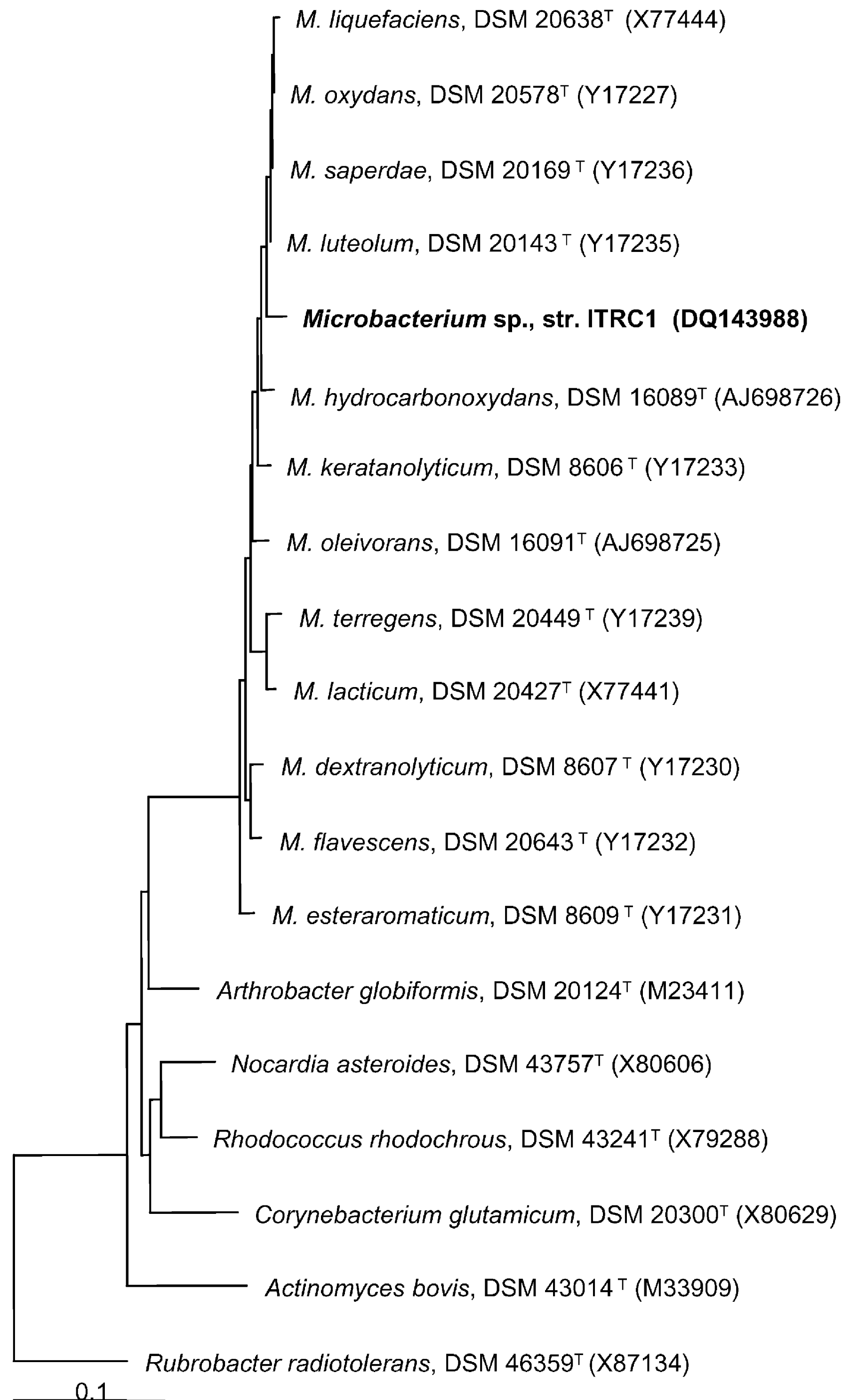

Fig. 2 Neighbor-joining tree based on 16S rRNA sequences showing the phylogenetic position of the ITRC1 strain along with other *Microbacterium* species. A *Rubrobacter radiotolerans* strain was used as the out-group. Accession numbers of the sequences retrieved from the databases are enclosed in parentheses. The *scale bar* indicates 0.1 estimated substitute per nucleotide

Results

Identification of the bacterium

The use of the primers corresponding to the 16S rRNA gene resulted in the amplification of a 1.5-kb product. The PCR products were sequenced and compared against complete 16S rRNA sequences using BLAST. The resulting analysis revealed >93 to 99% homologies to several *Microbacterium* species found in the GenBank database. Phylogenetic analysis (Fig. 2) showed that the HCH-degrading ITRC1 strain is a member of the *Microbacterium* sp., while the BLAST searches identified a similarity of more than 99% to *Microbacterium hydrocarbonoxydans*.

Biodegradation of α-, β-, γ-, and δ-isomers of HCH

The *Microbacterium* sp. ITRC1 strain utilizes HCH (α, β, γ, and δ) isomers (Fig. 1) as its sole carbon and energy source under aerobic conditions in liquid medium. The 0.34-mM concentration of individual HCH isomers supplemented in the medium was completely degraded in 4 days by this strain (Fig. 3). The ability of this bacterium to degrade these isomers resulted in (1) the rapid growth of the bacterium, (2) the release of covalently linked chloride in stoichiometric amount, and (3) the complete depletion of HCH isomers. During γ-HCH degradation, the formation of a product that reacts with Gibb's reagent (specific for phenols) was observed. Subsequently, it was identified as 2,5-dichlorophenol (2,5-DCP) on account of its comigration with the authentic 2,5-DCP, both in TLC and GC.

Degradation of HCH isomers from a contaminated soil

Soil from the vicinity of an industry manufacturing HCH for more than two decades was selected and deemed to be appropriate as it represented an aged HCH-contaminated soil sample. Total concentration of HCH isomers was 0.68 μmol/g soil (200 mg/kg); individually, the α-, β-, γ-, and δ-isomers constitute 39.4, 6.1, 48.2, and 6.3%, respectively. When the ITRC1 strain was incubated with the soil for 4 weeks, it resulted to a 94, 88, 96, and 82% degradation for α-, β-, γ-, and δ-HCH, respectively (Fig. 4a,b). On the other hand, more than 75% of each isomer remained in the uninoculated samples after 4 weeks. This means that around 90% of the HCH isomers were degraded under the above conditions in the ITRC1-inoculated samples.

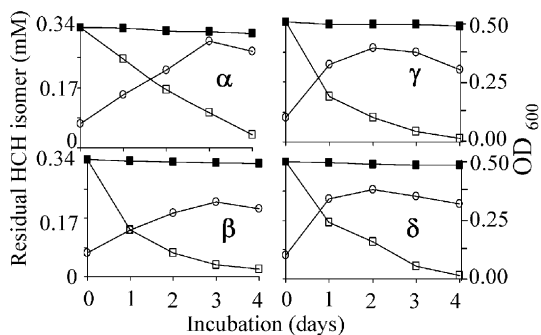

Fig. 3 Degradation of HCH isomers by the *Microbacterium* sp. ITRC1 strain. *Filled square*, uninoculated; *open square*, inoculated; *open circle*, growth of the ITRC1 strain. The initial concentrations of HCH isomers in liquid mineral medium were 0.34 mM, and the OD_{600} of the inoculum was 0.1

Growth on chlorinated hydrocarbons

The formation of a wide variety of chlorinated intermediates has been reported earlier during the degradation of γ-HCH (Nagata et al. 1999). We therefore tested the ability of the *Microbacterium* sp. ITRC1 strain to grow on these chlorinated hydrocarbons and to utilize these intermediates as its sole source of carbon and energy. We observed that the ITRC1 strain was able to grow on chlorophenols and hydroquinone but was unable to do so in the presence of chlorobenzenes (Table 3). More significantly, no growth was observed in the presence of 2,5-DCP, which was reported, in an earlier study, to be accumulating (Nagata et al. 1999).

PCR amplification of genes homologous to *linA*, *linB*, and *linC*: characterization of the inserts of pLIG1 and pLIG2

Two strategies, PCR amplification and Southern hybridization, were followed to identify and characterize the initial genes involved in γ-HCH degradation from *Microbacterium* sp. ITRC1. DNA of the ITRC1 strain was used

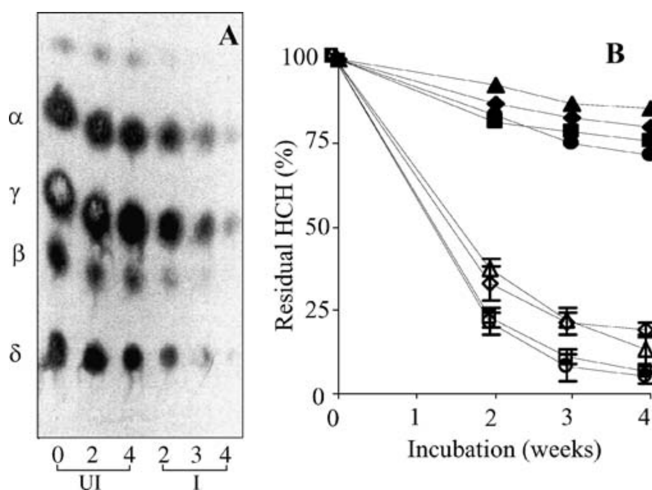

Fig. 4 **a**,**b** Degradation of HCH isomers from a contaminated soil by the *Microbacterium* sp. ITRC1 strain. Thin layer chromatography (TLC) of the biodegradation pattern after having been sprayed with the $AgNO_3$ chromogenic reagent. **b** Quantification of residual HCH isomers by gas chromatography (GC) analysis. *UI* uninoculated (*closed symbols*), *I* inoculated (*open symbols*). *Triangles*, β-HCH; *diamonds*, δ-HCH; *squares*, α-HCH; and *circles*, γ-HCH. The HCH isomers recovered at 0 time is taken as 100%

Table 3 Growth of the *Microbacterium* sp. ITRC1 strain on chlorinated compounds

Substrate[a]	Growth[b]
2,3,5-Trichlorophenol	+
2,4,5-Trichlorophenol	+
Hydroquinone	+
1,2,3,4-Tetrachlorobenzene	−
1,2,4-Trichlorobenzene	−
2,5-Dichlorophenol	−

[a]Substrates were supplied at 0.25 mM concentration in 20 ml medium
[b]Growth was measured at OD_{600} after 4 days of incubation at 28°C

as a template and yielded DNA fragments of 0.8 and 0.5 kb for *linB* and *linC* primer pairs (Table 2), respectively. However, under identical conditions, the *linA* primer (fwlinA and revlinA), as well as LIGAFw plus LIGARev and linAF plus linAR (Table 2), did not amplify any PCR product (Fig. 5a). DNA sequencing of the cloned PCR products showed that they were similar to the known hydrolytic dehalogenase (*linB*/pLIG1) and to a dehydrogenase (*linC*/pLIG2) homologous to the corresponding *linB* and *linC* genes present in *S. paucimobilis* UT26 (Nagata et al. 1999). The *linB* 800-bp PCR product was identical to the reported hydrolytic dehalogenase of *S. paucimobilis* UT26. In the case of *linC*, 5 out of 423 nucleotides were different when compared with UT26, which resulted to changes in 2 out of a total of 141 amino acid residues. In the *linC* gene of *Microbacterium* sp., a phenylalanine was substituted by a serine, while a glutamate was replaced by a glycine (Manickam et al. 2000).

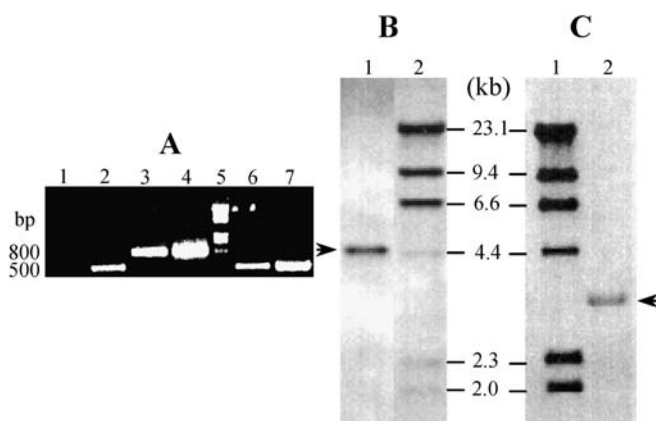

Fig. 5 **a** PCR amplification of genes homologous to *linA*, *linB*, and *linC*. *Lanes 1*, *3*, and *6* are the genomic DNA of the ITRC1 strain used as template; *lanes 2*, *4*, and *7* are *lin*A, *lin*B, and *lin*C recombinant plasmids, respectively, used as positive controls for amplification. **b** Southern blot using *linB* probe against the ITRC1 strain genomic DNA. *Lanes*: *1* ITRC1 genomic DNA digested with *Pst*I, *2* lambda DNA *Hind*III marker. **c** Southern blot using *linC* probe. *Lanes*: *1* Lambda DNA *Hind*III marker, *2* ITRC1 strain DNA digested with *Eco*RI

Southern hybridization

Southern blot analysis of the ITRC1 genomic DNA revealed the presence of a 4.3-kb *Pst*I fragment for the *linB* gene and a 3.0-kb *Eco*RI fragment for the *linC* gene (Fig. 5b,c). Similar results were also obtained using the original recombinant plasmids for the *linA*, *linB*, and *linC* genes (Table 1), and PCR probes.

Discussion

We present in this report the ability of a gram-positive *Microbacterium* species, ITRC1 strain, isolated from a contaminated industrial site, to degrade all the major isomers of HCH both in liquid culture and in a contaminated soil. The bacterium has the capability to utilize these isomers as its sole carbon source under the experimental conditions. Previously, an isolate, *S. paucimobilis* B90 (Sahu et al. 1990), has been reported to possess the ability to degrade the α-, γ-, and δ-isomers but not the β-isomers. This strain could also degrade 0.017-mM individual HCH isomers in 36 h (Sahu et al. 1993), and another bacterium, also an *S. paucimobilis* UT26 strain, was able to degrade 0.17 mM of γ-HCH alone (Nagasawa et al. 1993). In this study, we observe that the ITRC1 strain degrades HCH isomers two- and tenfold more than the above two bacteria, respectively. Moreover, all four isomers were degraded by our strain within 4 weeks in HCH-contaminated soil. Significantly, the highly persistent β-HCH isomer was also readily degraded by the bacterium both in contaminated soil and liquid cultures. The relatively longer time to degrade the isomers in contaminated soil can be attributed to low substrate solubility and bioavailability in the dissolved liquid state.

Our studies also demonstrate that the ITRC1 strain can grow on a broad spectrum of chlorinated compounds. In our studies, we did not observe any growth on 2,5-DCP. It is possible that this strain follows a degradation pathway similar to that in *S. paucimobilis* UT26 where the compound was found to be a dead-end product (Nagata et al. 1999).

The partial *linB* sequence reported here was found to be identical to that of a hydrolytic dehalogenase. On the other hand, the partial *linC* sequence obtained from the ITRC1 strain showed minor differences when compared with the sequence of a dehydrogenase enzyme belonging to the short-chain alcohol dehydrogenase family. It is not currently clear as to why we failed to get a PCR product using three different sets of *linA* primers. We assume that the *linA*-like gene may have more extensive sequence differences. Southern hybridization experiments carried out using a *linA* gene probe could also not detect a hybridizing DNA fragment.

Interestingly, the HCH-degrading bacterium isolated from HCH-polluted industrial soil belongs to a member of the genus *Microbacterium* as evidenced by its 16S rRNA sequencing. The members of the genus *Microbacterium* are known to have G+C values of 69 mol% (Takeuchi and

Yokota 1994). Recently, two novel members of *Microbacterium* sp. were also reported as having the capability to degrade crude oil (Schippers et al. 2005). This would imply, in the evolutionary viewpoint, that more gram-positive bacterial strains have adapted to degrade the highly chlorinated pesticides like HCH isomers and other hydrocarbons present in contaminated environments. Previously, the resistance to microbial degradation of HCH isomers has been ascribed to its highly chlorinated state. However, increasingly diverse bacterial populations with the ability to quantitatively dechlorinate recalcitrant xenobiotics like HCH are being isolated and demonstrated, such as those in the present study. To our knowledge, this is the first report of a gram-positive bacterium in pure culture possessing the ability to degrade all four major isomers of HCH. Significantly, this bacterium is likewise able to dechlorinate the most persistent β- and δ-HCH. The reported results suggest that the ITRC1 strain might be useful in bioremediation technology.

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