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ORIGINAL PAPER

Degradation of β-hexachlorocyclohexane by haloalkane dehalogenase LinB from γ-hexachlorocyclohexane-utilizing bacterium *Sphingobium* sp. MI1205

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Abstract The technical formulation of hexachlorocyclohexane (HCH) mainly consists of the insecticidal γ-isomer and noninsecticidal α-, β-, and δ-isomers, among which β-HCH is the most recalcitrant and has caused serious environmental problems. A γ-HCH-utilizing bacterial strain, *Sphingobium* sp. MI1205, was isolated from soil which had been contaminated with HCH isomers. This strain degraded β-HCH more rapidly than the well-characterized γ-HCH-utilizing strain *Sphingobium japonicum* UT26. In MI1205, β-HCH was converted to 2,3,5,6-tetrachlorocyclohexane-1,4-diol (TCDL) via 2,3,4,5,6-pentachlorocyclohexanol (PCHL). A haloalkane dehalogenase LinB ($LinB_{MI}$) that is 98% identical (seven amino-acid differences among 296 amino acids) to LinB from UT26 ($LinB_{UT}$) was identified as an enzyme responsible for the two-step conversion of β-HCH to TCDL. This property of $LinB_{MI}$ contrasted with that of $LinB_{UT}$, which catalyzed only the first step conversion of β-HCH to PCHL. Site-directed mutagenesis and computer modeling suggested that two of the seven different amino acid residues (V134 and H247) forming a catalytic pocket of LinB are important for the binding of PCHL in an orientation suitable for the reaction in $LinB_{MI}$. However, mutagenesis also indicated the involvement of other residues for the activity unique to $LinB_{MI}$. Sequence analysis revealed that MI1205 possesses the IS*6100*-flanked cluster that contains two copies of the $linB_{MI}$ gene. This cluster is identical to the one located on the exogenously isolated plasmid pLB1, suggesting that MI1205 had recruited the *linB* genes by a horizontal transfer event.

Keywords Haloalkane dehalogenase · Hexachlorocyclohexane · Environmental pollutant · Sphingomonads

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Introduction

The technical formulation of hexachlorocyclohexane (t-HCH), a popular insecticide prior to the 1990s, mainly consists of the insecticidal γ-isomer (10–12%) and the non-insecticidal α- (60–70%), β- (5–12%), and δ- (6–10%) isomers (Li et al. 2003). The use of t-HCH has now been banned in many countries because of its toxicity and persistence in soil, but numerous contaminated sites are still present throughout the world. Among the isomers of t-HCH, β-HCH is the most recalcitrant; it is usually the predominant isomer remaining in contaminated soils and in animal tissues and fluids (Willett et al. 1998). Its six chlorines are all in equatorial positions, which seem to confer the greatest chemical stability to this isomer.

Bacteria that degrade HCH can be a good model for understanding the way bacteria adapt to their environment. Only 60 years have passed since the first release of HCH into the environment (Walker 1999; Li et al. 2003). Thus, it is probable that the genes and enzymes for HCH degradation have evolved within this short period (Phillips et al. 2005). Exploring new genes and enzymes involved in the

degradation of β-HCH will lead us to a better understanding of how catalytic enzymes have evolved toward the recalcitrant xenobiotics.

To date, a number of bacterial strains that are able to aerobically degrade one or more HCH isomers have been isolated and their degrading activities toward each isomer have been investigated (Sahu et al. 1990; Siddique et al. 2002; Boltner et al. 2005; Kumar et al. 2005; Manickam et al. 2006; Mohn et al. 2006). With respect to the degradation pathway of HCH isomers, only that of γ-HCH has been comprehensively investigated whereas first understanding of α-HCH degradation is beginning to appear (Suar et al. 2005). *Sphingobium japonicum* (formerly *Sphingomonas paucimobilis*) (Pal et al. 2005) UT26, which utilizes γ-HCH as a sole source of carbon and energy, is the only strain whose degradation pathway of γ-HCH has been elucidated in detail. In this bacterium, γ-HCH is converted to β-ketoadipate by sequential reactions catalyzed by LinA to LinF (Imai et al. 1991; Nagata et al. 1993b, 1994; Miyauchi et al. 1998, 1999; Endo et al. 2005). The *linA, linB*, and *linC* genes are constitutively expressed and dispersed on the UT26 genome, while the *linD* and *linE* genes form an inducible operon under control of the transcriptional regulator, LinR (Nagata et al. 1999b, 2006; Miyauchi et al. 2002). LinA requires a 1,2-*biaxial* HCl pair on a substrate molecule, so that LinA catalyzes the dehydrochlorination of α- and δ-HCH besides γ-HCH, but not that of β-HCH (Trantirek et al. 2001). Several other HCH-degrading strains have been found to possess nearly identical *lin* genes (Thomas et al. 1996; Kumari et al. 2002; Dogra et al. 2004; Ceremonie et al. 2006), suggesting that the degradation pathways of γ-HCH in those strains are similar to that in UT26.

The genes and enzymes for the degradation of β-HCH have been of great interest owing to the difficulty of its degradation and the importance of its practical application. We reported recently that LinB from UT26 ($LinB_{UT}$), which catalyzes the second step in the degradation of γ-HCH, converts β-HCH to 2,3,4,5,6-pentachlorocyclohexanol (PCHL) by hydrolytic dehalogenation (Nagata et al. 2005). In UT26, however, we could not detect PCHL degradation activity. In this study, we isolated a γ-HCH-utilizing strain, *Sphingobium* sp. MI1205, which converts not only β-HCH to PCHL but also PCHL to 2,3,5,6-tetrachlorocyclohexane-1,4-diol (TCDL). Although LinB from MI1205 ($LinB_{MI}$) was 98% identical (seven amino-acid differences among 296 amino acids) to $LinB_{UT}$, the former enzyme was found to catalyze the two-step conversion of β-HCH to TCDL. Two independent studies which were published very recently by other groups (Sharma et al. 2006; Wu et al. 2007) have reached the same conclusion that LinB2 from *Sphingomonas* sp. BHC-A or $LinB_{B90A}$ from *Sphigobium indicum* B90A, respectively, both of which are identical to $LinB_{MI}$, converted not only β-HCH to PCHL but also PCHL to TCDL. We further describe (i) stereochemical characterization of TCDL, (ii) quantitative evaluation of the difference between $LinB_{UT}$ and $LinB_{MI}$, (iii) mutational analysis, and (iv) prediction of molecular mechanism by molecular modeling.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria–Bertani broth (LB), and the sphingomonad strains were grown at 30°C on 1/3LB agar medium (3.3 g of Bacto Tryptone per liter, 1.7 g of yeast extract per liter, 5 g of sodium chloride, 15 g of agar per liter) or 1/10 W medium (KH_2PO_4, 170 mg; Na_2HPO_4, 980 mg; $(NH_4)_2SO_4$, 100 mg; $MgSO_4$, 48.7 mg; $FeSO_4$, 0.52 mg; MgO, 10.75 mg; $CaCO_3$, 2.0 mg; $ZnSO_4$, 0.81 mg; $CuSO_4$, 0.16 mg; $CoSO_4$, 0.15 mg; and H_3BO_3, 0.06 mg per liter) containing an appropriate carbon source. Ampicillin and tetracycline were used at a final concentration of 50 and 20 μg/ml, respectively.

Enrichment and isolation of γ-HCH-utilizing bacteria from soil sample

The soil used in this study was sampled from a field in Miyagi prefecture, Japan. It had been contaminated with t-HCH and still contained low levels of HCH isomers (0.03–0.1 mg/l, mainly α-HCH). The cell densities of the heterotrophic bacteria and γ-HCH-utilizing bacteria in the soil sample have been previously estimated (Miyazaki et al. 2006). The bacterial cell fraction was collected from 1 g (wet weight) of soil matrix in 2 ml of 1/10 W medium by vortexing and centrifugation (1,000×*g*, 30 min). One ml of the supernatant was inoculated into 100 ml of W medium (Imai et al. 1989) or 1/10 W medium containing 33 μM γ-HCH. In the further steps for enrichment, only 1/10 W medium was employed because no significant decrease in γ-HCH was observed in the W medium even after prolonged incubation. After static incubation at 25°C for 8 days, 1 ml of the primary enrichment culture was transferred into 100 ml of fresh 1/10 W medium, and the resultant secondary enrichment culture was incubated under the same condition for 2 days. The procedure of transfer and incubation was repeated once, and serial dilutions of the tertiary enrichment culture were spread on 1/10 W agar plates containing 1.8 mM γ-HCH as a sole source of carbon and energy. After incubation at 25°C for 10 days, a number of colonies with a

Table 1 Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Sphingobium sp. MI1205	HCH^{+a}	This study
Sphingobium japonicum UT26	HCH^{+}; Nal^{r}	Imai et al. (1989)
E.coli		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lacZYA-argF) Φ80lacZΔM15*	Sambrook et al. (1989)
XL1-Blue MR	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gylA96 relA1 lac*	Stratagene
BL21	F^{-}, *ompT*, *hsd*SB, *gal*	Novagen
Plasmids		
pKS13	RK2 replicon; *cos* Mob^{+}, Tc^{r}	Kimbara et al. (1989)
pUC18	pMB9 replicon; Ap^{r}	Vieira and Messing (1982)
pUC19	pMB9 replicon; Ap^{r}	Vieira and Messing (1982)
pAQN	pMB9 replicon; $lacI^{q}$ aqn^{b} Ap^{r}; *tac* promoter	Terada et al. (1990)
pAQNM	pMB9 replicon; $lacI^{q}$ aqn^{b} Ap^{r}; *tac* promoter	Endo et al. (2005)
pSH43	pKS13 with about 24 kb of MI1205 DNA containing *linB*	This study
pSH1801	pUC18 containing a 10-kb *Eco*RI-*Eco*RI fragment downstream of *linB*	This study
pSH1805	pUC18 containing a 2.2 kb *Eco*RI–*Eco*RI fragment containing *linB*. *linB* is orientated in the same direction as the *lac* promoter	This study
pSH1903	pUC19 containing a 7 kb *Bam*HI–*Eco*RI fragment upstream of *linB*	This study
pSH1904	pUC19 containing a 5 kb *Bam*HI–*Eco*RI fragment containing *linB*	This study
pMLBH6	pAQN containing His-tagged $linB_{UT}$	Nagata et al. (1999a)
pUTI134V	pAQN containing His-tagged $linB_{UT}$ mutant (I134V)	This study
pUTA247H	pAQN containing His-tagged $linB_{UT}$ mutant (A247H)	This study
pUTW	pAQN containing His-tagged $linB_{UT}$ mutant (I134V + A247H)	This study
pMIWT1	pAQNM containing His-tagged $linB_{MI}$	This study
pMIV134I	pAQNM containing His-tagged $linB_{MI}$ mutant (V134I)	This study
pMIH247A	pAQNM containing His-tagged $linB_{MI}$ mutant (H247A)	This study
PMIW	pAQNM containing His-tagged $linB_{MI}$ mutant (V134I + H247A)	This study

[a] Growth on γ-HCH

[b] Aqualysin I gene of *Thermus auaticus*

cleared zone, which is an indicator of γ-HCH degradation, were picked up. Single-colony isolation was repeated several times on γ-HCH minimal media. Several colonies with detectable γ-HCH degradation activity were stocked.

Monitoring the degradation of β-HCH and its degradation products in sphingomonads

The colonies grown on 1/3 LB plates were picked up and washed twice with 1/10 W medium. Then the bacterial cells were suspended at an amount of 5 mg (wet weight)/ml in 1/10 W medium containing 17 μM of β-HCH, and the concentrations of β-HCH and its metabolites were monitored as described below. One unit of β-HCH degradation activity was defined as the activity required for the transformation of 1 μmol of β-HCH per min. The activity was estimated from the linear range of the β-HCH disappearance rate.

GC analysis

GC conditions

The analysis was conducted using a Shimadzu GC-17A gas chromatograph with an ^{63}Ni electron capture detector (ECD) and Rtx-1 capillary column (30 m × 0.25 mm × 0.25 μm; Restek, http://www.restekcorp.com). The column temperature was increased from 160 to 200°C at a rate of 4°C/min for the separation of the peak of PCHL from that of TCDL, and then from 200 to 260°C at a rate of 20°C/min. Gas flow rate was 30 ml/min. We were successfully able to separate the two polar metabolites under this condition. The retention time of PCHL was 9.7 min while that of TCDL was 9.5 min. Ten μM of 2,4,5-trichlorophenol was used as the internal standard.

Estimation of PCHL and TCDL concentrations

The concentration of TCDL was estimated from the peak area of TCDL produced from 17 µM of *β*-HCH by a large amount of purified $LinB_{MI}$ (510 µg protein/ml). When 17 µM of *β*-HCH was converted by this high amount of $LinB_{MI}$ for 2 h, no remaining *β*-HCH or PCHL was observed. The concentration of TCDL thus obtained from 17 µM *β*-HCH was regarded as 17 µM. The concentration of PCHL was similarly estimated from the peak area of PCHL produced from 17 µM of *β*-HCH by a large amount of purified $LinB_{UT}$ (285 µg protein/ml) for 2 h. In this case, however, a small amount of *β*-HCH remained and a small amount of TCDL was produced because the activity of $LinB_{UT}$ to dehalogenate *β*-HCH is by far lower than that of $LinB_{MI}$. Thus the concentration of PCHL produced from 17 µM of *β*-HCH was subtracted by the remaining *β*-HCH and the small amount of TCDL formed and subsequently used as a standard.

GC-MS analysis

We performed GC-MS analysis for the identification of the metabolites of *β*-HCH. Approximately 10^8–10^9 cells of *Sphingobium* sp. MI1205 were recovered from colony material on a plate, suspended in 500 µl of 1/10 W medium containing 33 µM *β*-HCH, and incubated at 37°C for 1–3 h. One-hundred µl of the suspension was extracted with an equal volume of ethyl acetate after acidification by adding 10 µl of HCl, and the extract was analyzed by GC-mass spectrometry (MS) with a QP2010 spectrometer (Shimadzu, Kyoto, Japan) and a DB-17 capillary column (30 m × 0.25 mm × 0.25 µm; J&W Scientific). The column temperature was increased from 100 to 260°C at a rate of 10°C and then held at 260°C. The carrier gas flow was 14.0 ml/min.

NMR analysis

Preparation and purification of TCDL

Approximately 1 g (wet weight) of MI1205 cells were inoculated into 600 ml of 1/10 W medium containing 68 µM of *β*-HCH, and incubated for 12 h at 30°C. After removal of the cells by centrifugation, the supernatant was acidified by adding HCl and extracted twice with a half volume of ethyl acetate. After evaporation, the reaction mixture was suspended in a small volume of ethyl acetate and the suspension was spotted onto the TLC plates (Partisil K6; Whatman, Middlesex, UK). The solvent system for the separation of the metabolite from other contaminants consisted of hexane-acetone (7:3, vol/vol). After development, the TLC plates were dried and exposed to iodine vapor. A slight brownish-colored fraction of silica gel was collected and extracted with ethyl acetate, and then the extracts were evaporated again.

^{1}H-NMR spectrum

^{1}H-NMR was performed at 500.00 MHz on JEOL α-500 (Tokyo, Japan) at 23°C. The metabolite was dissolved in 0.5 ml of $(CD_3)_2SO$ (Darmstadt, Germany). ^{1}H chemical shifts were reported on the *δ*-scale (parts per million) by assigning the residual solvent peak to 2.49 ppm. The acquisition parameters were: spectral width 10,000 Hz, 65536 data points, 1.0 s relaxation delay, 6.5536 s acquisition time, and 32 scans. The analysis was followed by a homospin decoupling experiment.

DNA manipulations and DNA sequence analysis

Established methods were used for the preparation of plasmid DNA, its digestion with restriction endonucleases, ligation, agarose gel electrophoresis, and transformation of *E. coli* cells (Maniatis et al. 1982). Isolation of genomic DNA from *Sphingobium* sp. MI1205 was performed as described previously (Nagata et al. 1993a) with the exception of the culturing step; *Sphingobium* sp. MI1205 cells were grown on 1/3LB plates. A genomic library of *Sphingobium* sp. MI1205 was constructed using broad-host-range cosmid vector pKS13 in *E. coli* XL-1 blue MR as described previously (Imai et al. 1991). The library in *E. coli* was transferred into *S. japonicum* UT26 by electroporation. The UT26 cells grown on 1/3 LB plates for 2 days were harvested and washed four times with 1 mM MOPS buffer kept in wet ice (0°C) containing 10% glycerol. The cells were then collected by centrifugation for 2 min at 3,000×*g* and resuspended in the same buffer. A 50 µ l aliquot of the cell suspension and plasmid DNA was transferred to an electroporation cuvette (Bio-Rad; 2 mm-wide gap) and pulsed using a Gene Pulser (Bio-Rad) at 2.5 kV, 25 *µ*F, and 800 Ω. After electroporation, the cells were incubated for 6 h in 1 ml of 1/3LB and plated on 1/3LB agar plates containing 20 µg/ml of tetracycline. Nucleotide sequences were determined with an ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA) and an ABI Prism Big Dye Terminator kit (Applied Biosystems), and analyzed with the genetyx program, version 12 (SDC Inc., Tokyo, Japan). Homology searches were performed with BLAST programs available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Screening of the MI1205 genomic library

The MI1205 genomic library in *S. japonicum* was assayed for production of TCDL from *β*-HCH. Approximately 10^8

cells from each colony were picked and suspended in 100 µl of the assay solution (1/10 W medium containing 33 µM β-HCH). The solution was incubated for 12–18 h at 30°C, 100 µl of ethyl acetate was added, and vortexed for 1 min. After centrifugation, the ethyl acetate layer was recovered. One µl of the recovered extract was used for gas chromatography equipped with an electron capture detector (ECD). For assay of the PCHL degradation activity, 1/10 W medium containing PCHL was prepared by biosynthesis: ca. 0.5 g (wet weight) of UT26 was inoculated into 10 ml of 1/10 W medium containing 34 µM of β-HCH, and incubated for 12 h at 30°C. After removal of the cells by centrifugation, the supernatant was used as the assay solution for PCHL degradation activity.

Site-directed mutagenesis

Six LinB mutants, $LinB_{UT}$ I134V, $LinB_{UT}$ A247H, $LinB_{UT}$ I134V + A247H, $LinB_{MI}$ V134I, $LinB_{MI}$ H247A, and $LinB_{MI}$ V134I + H247A were constructed using inverse PCR as described previously (Chaloupkova et al. 2003). The oligonucleotides used here are listed in Table 2. For efficient expression of the protein products *in E. coli*, a canonical Shine-Dalgarno sequence (TAAGGAGG) (Makrides 1996) was added to the oligonucleotide primer 'LinB SD Eco'.

Expression and purification of the His-tagged proteins

The His-tagged $LinB_{UT}$ and $LinB_{MI}$ proteins and their mutant proteins were expressed in *E. coli* under the control of the *tac* promoter and $lacI^q$ by using vectors pAQN and pAQNM. pAQN has the same structure as pAQI (Terada et al. 1990) except for the differences in the aqualysin I-coding region. The *E. coli* BL21 cells containing these plasmids were cultured in LB at 37°C. When optical density at 600 nm of the culture reached 0.6, the protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. After the induction for 8 h at 25°C, the cells were harvested and disrupted by sonication using a Sonopuls GM70 (Bandelin, Berlin, Germany), and centrifuged at 100,000×*g* for 1 h. The supernatant was further purified by using BD TALON Metal Affinity Resins (BD Biosciences, Mountain View, CA). The His-tagged enzymes were bound to the resin in the equilibrating buffer (20 mM potassium phosphate buffer (pH 7.5) containing 0.5 M sodium chloride, 10% glycerol, and 10 mM imidazole). Unbound and weakly bound proteins were washed out with buffer containing 10 mM imidazole. The target enzyme was then eluted by the same buffer containing 500 mM imidazole. The active fractions were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol. The enzyme was stored in the same buffer.

Enzymatic assays

Enzymes were incubated with 17 µM of β-HCH in 50 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol at 30°C. 100 µl of the mixture was extracted with an equal volume of ethyl acetate and then analyzed by GC (ECD). The concentration of each enzyme in the reaction mixtures was 100 µg/ml. The concentrations of PCHL and TCDL were measured as described above.

Evaluation of kinetic parameters from experimental data

Kinetic data were fitted to the irreversible two-step reaction structure of HCH conversion to TCDL via PCHL

Table 2 Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′)	Use
63f	CAGGCCTAACACATGCAAGTC	Amplification of 16S rRNA gene
1387r	GGGCGGWGTGTACAAGGC	Amplification of 16S rRNA gene
LinB SD Eco	GCCGAATTC**TAAGGAGG**AATATCGATGAGCCTC	Amplification of $linB_{MI}$
LinB His Bam	GCCGGATCCTTA*GTGATGGTGATGGTGATG*TGCTGGCGCAA	Amplification of $linB_{MI}$
LinB I134V f	GAAGCGGTCGCCATGCCGATC	Site-directed mutagenesis of $linB_{UT}$
LinB I134V r	CATATAGGCAATCCCCTGTAC	Site-directed mutagenesis of $linB_{UT}$
LinB A247H f	GAGCCGGGACACCTGACCACG	Site-directed mutagenesis of $linB_{UT}$
LinB A247H r	GGCGTTGATGAAGAGTTTCGG	Site-directed mutagenesis of $linB_{UT}$
LinB V134I f	ATCACCATGCCGCTCGAATGGGC	Site-directed mutagenesis of $linB_{MI}$
LinB V134I r	CGCTTCCATATAGGCAATCCCCTG	Site-directed mutagenesis of $linB_{MI}$
LinB H247A f	GCCCTGACCACGGGCCGAATACGCG	Site-directed mutagenesis of $linB_{MI}$
LinB H247A r	TCCCGGCTCGGCGTTGATGAAGAG	Site-directed mutagenesis of $linB_{MI}$

Recognition sites for restriction enzymes used for cloning are underlined. A canonical Shine-Dalgarno sequence (Makrides 1996) is expressed in boldface type. A sequence for the additional six-histidyl tail is expressed in italics

(Structure 1) by using the GEPASI 3.2 software (Mendes 1997). The specificity constants for both reaction steps (k_1 and k_2) were obtained from the calculation. An evolutionary programming (Baeck et al. 1997) was used to optimize the kinetic constants during the fitting of the kinetic data to the Structure 1.

Structure 1

$$\mathrm{E + HCH} \xrightarrow{k_1} \mathrm{E + PCHL} \xrightarrow{k_2} \mathrm{E + TCDL}$$

Homology modeling and *in silico* mutagenesis

The $\mathrm{LinB_{MI}}$ and I134V + A247H mutant of $\mathrm{LinB_{UT}}$ ($\mathrm{LinB_{UT\text{-}MI}}$) were prepared by homology modeling using SWISS-PDB VIEWER 3.71 (SPDBV) and SWISS MODEL2 (Guex and Peitsch 1997), and by *in silico* mutagenesis using PYMOL 0.9 and SPDBV.

Molecular docking

PCHL was docked onto $\mathrm{LinB_{UT}}$, $\mathrm{LinB_{MI}}$ and $\mathrm{LinB_{UT\text{-}MI}}$ by using AUTODOCK 3.05 as described previously (Kmunicek et al. 2003). The orientations of PCHL in the active site of the enzyme were clustered according to the feasibility of dehalogenation from the *ortho*, *meta* and *para* positions, respectively.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rRNA gene of *Sphingobium* sp. MI1205 and the IS*6100-linB* gene cluster with its flanking regions determined in this study have been deposited in DDBJ/EMBL/GenBank databases under the accession numbers AB277549 and AB278602, respectively.

Results

Enrichment, isolation and phylogenetic analysis of a γ-HCH-utilizing bacterium

To enrich γ-HCH-utilizing soil bacteria, the indigenous bacterial cells were collected from the soil and inoculated into 1/10 W medium. The γ-HCH in the medium completely disappeared within 8 days. From the tertiary enrichment culture, in which γ-HCH had completely disappeared within 2 days, some γ-HCH-utilizing candidates were obtained. Among them, one isolate forming a brownish–yellow-pigmented colony on 1/3 LB plates exhibited strong activity for the γ-HCH degradation. Its 16S rRNA gene sequence showed 99% identity to that of *Sphingobium herbicidovorans* (Takeuchi et al. 2001). The isolate was designated *Sphingobium* sp. MI1205. This strain degraded β-HCH, but could not grow on β-HCH as a sole carbon source.

Degradation of β-HCH in MI1205

The activity of MI1205 to degrade β-HCH was assayed and compared to that of UT26 (Fig. 1). MI1205 degraded β-HCH at a rate of 8.3×10^{-5} U/mg of cells during the first 30 min while UT26 at a rate of 4.0×10^{-6} U/mg of cells during the first 90 min, indicating that the activity of MI1205 was approximately 20 times higher than that of UT26. A distinctive difference was observed between the two strains: two metabolites, M1 and M2, were detected in the degradation of β-HCH in MI1205, whereas only one metabolite, PCHL, was detected in

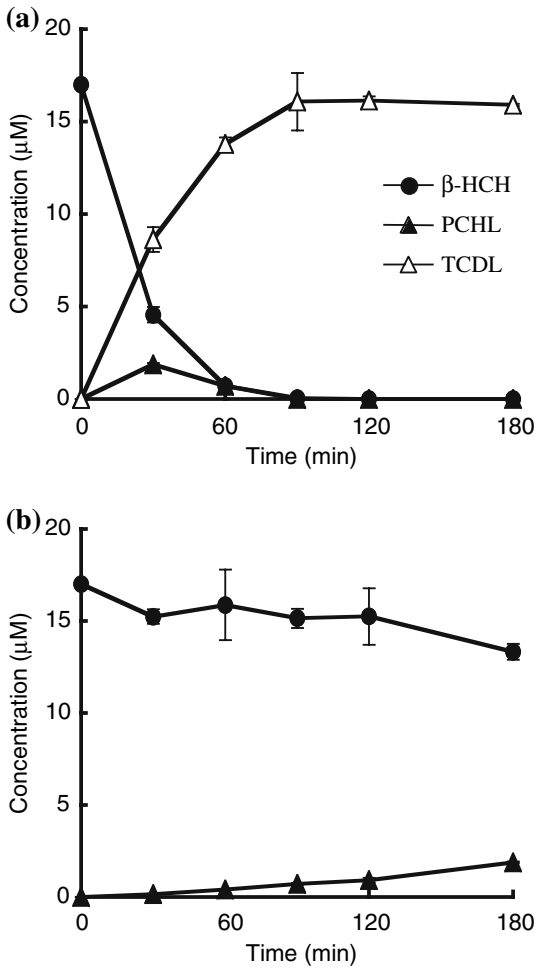

Fig. 1 Degradation of β-HCH and appearance of its degradation products in reaction mixtures with MI1205 (**a**) or UT26 (**b**). Values given are the mean of triplicate. *Error bar* means standard deviation. The concentration of β-HCH at time zero is 17 μM

UT26. M1 was identified as PCHL by comparing the retention time and MS spectrum with those of PCHL produced by UT26. In the culture with MI1205, PCHL rapidly disappeared with a concomitant accumulation of M2. When PCHL was incubated with MI1205, the rapid production of a metabolite whose retention time and MS spectrum were identical to those of M2 was observed with a concomitant sharp decrease in PCHL (data not shown), indicating that PCHL is further converted to M2 in MI1205.

Identification of the metabolite M2

The mass spectrum of M2 (Fig. 2a) was interpreted in detail with the help of the spectra interpretation software Mass Frontier 1.0 (HighChem, Slovak Republic). The ion at m/z 217, which was the heaviest ion in this spectrum, is an even-electron ion and cannot be a molecular ion. The isotopic cluster at m/z 217–223 indicates three chlorine atoms. Counting mass of the fragment m/z 217, six carbon, six hydrogen and three chlorine atoms account together for 183

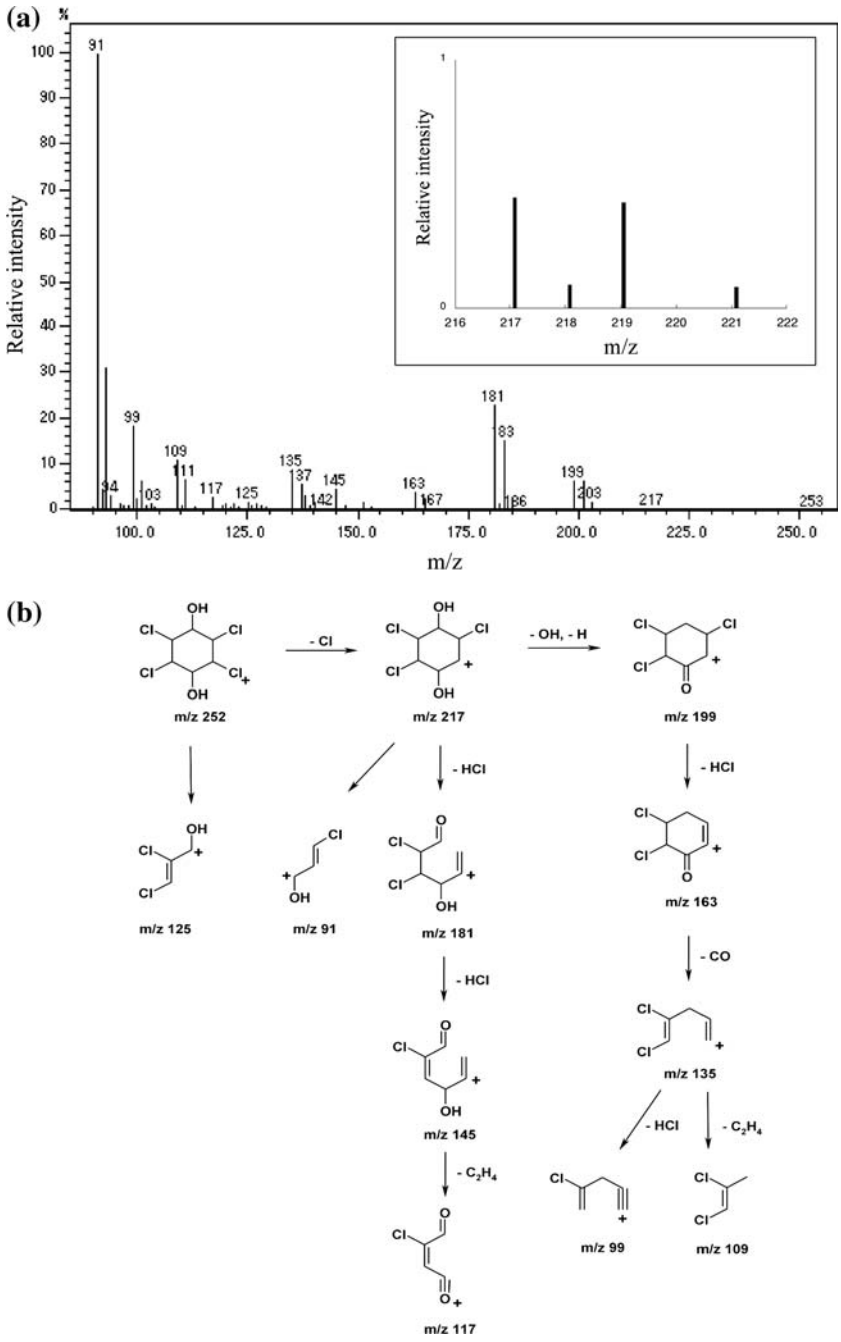

Fig. 2 Identification of the metabolite M2 (2,3,5,6-tetrachlorocyclohexane-1,2-diol) using GC-MS. **a** Mass spectrum of the metabolite M2. The inset shows the enlarged spectrum around m/z = 219. **b** Predicted fragmentation pattern of the metabolite M2 (electron impact, 70 eV)

atomic mass units and the remaining 34 atomic mass units suggest the presence of two hydroxyl groups (OH, m/z 17) in the fragment m/z 217. The m/z 217 seems to have originated from the molecular ion m/z 252 of tetrachlorocyclohexanediol ($C_6H_8Cl_4O_2$) by a loss of a chlorine atom m/z 35 (Fig. 2b). The isotopic cluster of the lower-mass ion m/z 199 also indicates three chlorine atoms in the molecule. The difference of 18 m/z between m/z 217 and 199 suggests the loss of one hydroxyl group and one hydrogen atom. The fragment m/z 199 has further lost HCl, forming m/z 163. The fragment m/z 135 has appeared by the clevage of CO from the m/z 163. The fragment m/z 135 has further released C_2H_4 or HCl to form the fragments m/z 109 and m/z 99, respectively. The isotopic cluster of m/z 181 indicates two chlorine atoms in the molecule. The difference of 36 m/z and the change in the proportion of isotopic peaks intensity indicates a loss of HCl from m/z 217 to m/z 181. The fragments m/z 145 and 117 have their origin in m/z 181; the former has been formed from m/z 181 by a loss of HCl and the latter from m/z 145 by a loss of C_2H_4. The fragment ions m/z 125 ($C_3H_2Cl_2OH$) and m/z 91 (C_3H_3ClOH) have been formed by splitting of parental ions m/z 252 and m/z 217, respectively. In addition, the Mass Frontier software suggests that the base peak m/z 91 can be formed only from *ortho*- or *para*- substituted diol; there was no pattern forming the m/z 91 fragment from the *meta* isomer. Consequently, the most probable structures to account for this spectrum are 3,4,5,6-tetrachlorocyclohexane-1,2-diol and 2,3,5,6-tetrachlorocyclohexane-1,4-diol with m/z 252.

M2 was further analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Fig. 3) to completely determine the structure of this compound. Each proton resonance was assigned on the basis of the peak areas, proton–proton coupling constants, multiplicities and homo spin decoupling experiments. The spectrum contained three resonances belonging to M2 with relative areas of 2, 1 and 1, indicating that a total of eight protons of this compound (six protons and two OH groups) are divided into three types with different chemical environments: four protons (singlet), two protons (doublet) and two OH groups (doublet). This clearly indicates that M2 is a *para*-substituted compound, since *ortho*- and *meta*-substituted compounds do not have any four-chemically equivalent protons and thus cannot yield the singlet indicating four protons. Homo-spin decoupling experiments proved that the broad doublet at 6.39 ppm was coupled with the sharp doublet at 4.11 ppm and was also very slightly coupled with the broad singlet (data not shown), allowing us to assign each doublet. The explanation for the very slight coupling of 1H and 4H with 2H/6H and 3H/5H, respectively, is that the dihedral angles comprised of the vicinal protons are close to rectangular in shape, probably due to the influence of the functional groups, in which case coupling constants are known to be close to zero (known as 'Karplus relationships') (Karplus 1959; Karplus 1963). This means that 1H and 4H are in the equatorial positions since the other protons are in the axial positions. Consequently, we concluded that M2 is 2,3,5,6-tetrachlorocyclohexane-1,4-diol (TCDL) with 1H and 4H in the equatorial positions and with 2H, 3H, 5H and 6H in the axial positions.

Isolation of a gene for the conversion of PCHL to TCDL

To obtain a gene responsible for the conversion of PCHL to TCDL, we constructed a genomic library of MI1205 in *E. coli* using a broad-host-range cosmid vector. The resultant library was transferred into *S. japonicum* UT26 and screened for activity to produce TCDL from *β*-HCH. Since $LinB_{UT}$ converts *β*-HCH to PCHL, the UT26 cells accumulates PCHL when incubated with *β*-HCH. Consequently, the UT26 derivative carrying the target MI1205 gene was

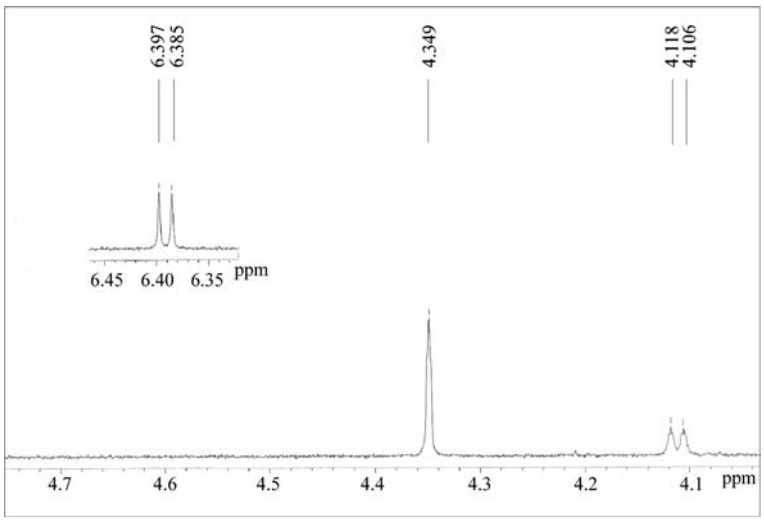

Fig. 3 ^{1}H-NMR spectrum of the metabolite M2 (500.00 MHz). The broad doublet (J = 6.0 Hz) at 4.11 ppm, the broad singlet at 4.35 ppm, and the doublet (J = 6.0 Hz) at 6.39 ppm have the relative area of 1, 2 and 1, respectively

expected to produce TCDL in the presence of β-HCH. This screening successfully resulted in the isolation of a cosmid (pSH43) having a gene for the activity. pSH43 had a ca. 24 kb insert, and its double digestion with *Eco*RI and *Bam*HI produced four fragments with sizes of 10, 7, 4.8, and 2.2 kb (Fig. 4). All of these fragments were subcloned into pUC18 or pUC19, and the recombinant plasmids were introduced into *E. coli*. The in vivo assay of the transformants revealed that the 2.2 and 4.8 kb inserts on pSH1805 and pSH1904, respectively, exhibited the activity to convert PCHL to TCDL (Fig. 4). It is noteworthy that the two inserts also showed the activity to convert β-HCH to PCHL. The 7.2 kb nucleotide sequence covering the whole insert of pSH1805 and its flanking regions revealed an IS*6100-linB*-IS*6100-linB*-IS*6100* cluster (Fig. 4), named the IS*6100-linB* cluster, identical to that located on the exogenously captured plasmid pLB1 (Miyazaki et al. 2006). The sequence of the region outside of the leftmost IS*6100* copy was also identical to pLB1, while that of the rightmost IS*6100* copy was different (Fig. 4). The two *linB* genes in the cluster were identical to each other and *linB* from *S. indicum* B90A, a well-characterized HCH-degrading bacterium isolated in India. Further deletion analysis revealed that the *linB* gene from MI1205 is responsible for the conversions of both β-HCH and PCHL (data not shown). The *linB* genes from MI1205 ($linB_{MI}$) and UT26 ($linB_{UT}$) shared 99 and 98% identities at the nucleotide and amino-acid sequence levels, respectively. Interestingly, there are no synonymous mutations between the two *linB* genes: the eight nucleotide differences result in seven amino-acid substitutions (the amino-acid substitution at position 247 was caused by two neighboring nucleotide substitutions). To confirm the activity of $LinB_{MI}$ to catalyze the two-step conversion of β-HCH, we purified the His-tagged $LinB_{MI}$ protein expressed in *E. coli* and assayed its activity (Fig. 5e). $LinB_{MI}$ actually converted β-HCH to TCDL via PCHL, whereas $LinB_{UT}$ converted β-HCH to PCHL but almost not to TCDL (Figs. 5a, e, 6).

Site-directed mutagenesis

The amino acid-sequences of $LinB_{MI}$ and $LinB_{UT}$ differ at positions 81, 112, 134, 135, 138, 247, and 253, and the residues at positions 134 and 247 of $LinB_{UT}$ are located in the catalytic pocket (Marek et al. 2000). On the basis of the assumption that these two residues might have a significant effect on the difference of the catalytic activities of the two enzymes, we constructed three mutants of $LinB_{UT}$ ($LinB_{UT}$ I134V, $LinB_{UT}$ A247H, and a double mutant $LinB_{UT}$ I134V + A247H) and three mutants of $LinB_{MI}$ ($LinB_{MI}$ V134I, $LinB_{MI}$ H247A and a double mutant $LinB_{MI}$ V134I + H247A). We monitored the dehalogenation of β-HCH by the six mutants accompanied by the appearance of PCHL and TCDL (Fig. 5). The values of k_{cat} and K_m of the enzymes could not be calculated because of the limited solubility of β-HCH in water; however, from these data we evaluated the specificity constants of $LinB_{UT}$, $LinB_{MI}$ and their mutants in both the first and the second dehalogenation reaction (Table 3). $LinB_{UT}$ I134V and $LinB_{UT}$ I134V + A247H exhibited higher activity for the conversion of PCHL than wild-type (wt) $LinB_{UT}$, although the single mutation A247H unexpectedly gave rise to lower catalytic activity toward β-HCH than wt $LinB_{UT}$. In addition, both of the substitutions, V134I or H247A, into wt $LinB_{MI}$ caused the reduction of the activities for the second step. These results strongly suggested that V134 and H247 play important roles in the second dehalogenation reaction. Furthermore, the double mutant $LinB_{MI}$ V134I + H247A significantly decreased the activities for both steps, indicating that V134 and H247 have an additive effect on the activities for the conversion of β-HCH to PCHL and PCHL to TCDL when the backbone structure of the enzyme is $LinB_{MI}$-type. However, it is noteworthy that the double mutant $LinB_{MI}$ V134I + H247A still maintained significant activity for the second step. These results clearly indicate that all or some of the other five amino-acid residues that

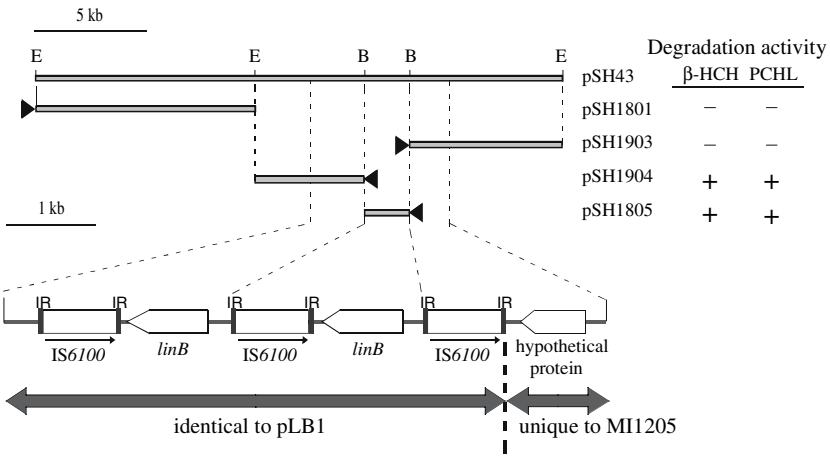

Fig. 4 Restriction map, deletion analysis and partial gene organization of the *linB* genes in the cloned fragment of strain MI1205 in pSH43. The directions of transcription by *lac* promoter are indicated by arrowheads. The directions of IS*6100 tnp* genes of are indicated by arrows under the map. *IR* inverted repeat, *E Eco*RI, *B Bam*HI

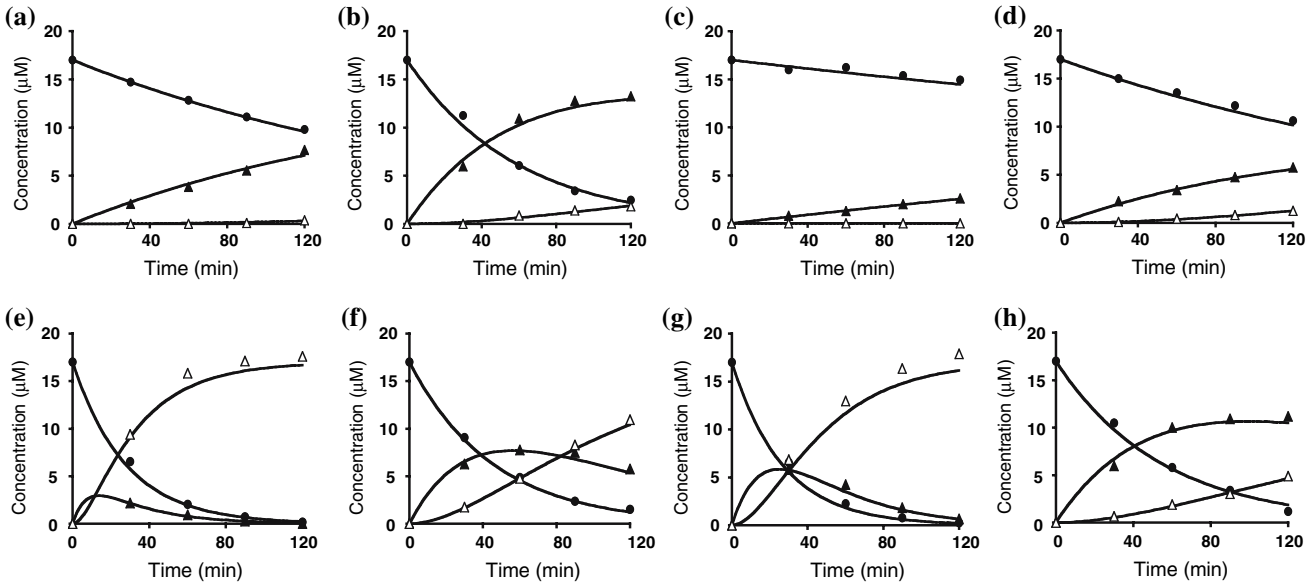

Fig. 5 Degradation of *β*-HCH and appearance of its metabolites in reaction mixtures with 100 μg/ml of $LinB_{UT}$ wild type (**a**), $LinB_{UT}$ I134V (**b**), $LinB_{UT}$ A247H (**c**), $LinB_{UT}$ I134V + A247H (**d**), $LinB_{MI}$ wild type (**e**), $LinB_{MI}$ V134I (**f**), $LinB_{MI}$ H247A (**g**), and $LinB_{MI}$ V134I + H247A (**h**). Values given are the mean of triplicates. The concentration of *β*-HCH at time zero is 17 μM. *Symbols* are as shown in Fig. 1

Fig. 6 *β*-HCH transformations catalyzed by $LinB_{MI}$ and $LinB_{UT}$

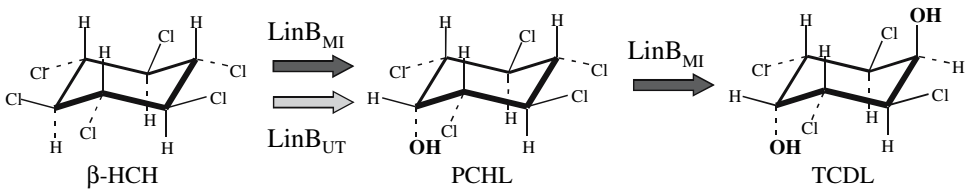

Table 3 Specificity constants (mM^{-1} s^{-1}) of $LinB_{UT}$, $LinB_{MI}$ and their mutants

Enzyme	Step 1 HCH → PCHL	Step 2 PCHL → TCDL
$LinB_{UT}$ wild type	0.0271 ± 0.0002	0.0036 ± 0.0006
$LinB_{UT}$ I134V	0.0969 ± 0.0017	0.0099 ± 0.0008
$LinB_{UT}$ A247H	0.0077 ± 0.0007	–[a]
$LinB_{UT}$ I134V + A247H	0.0243 ± 0.0002	0.0183 ± 0.0010
$LinB_{MI}$ wild type	0.205 ± 0.005	0.716 ± 0.052
$LinB_{MI}$ V134I	0.124 ± 0.005	0.080 ± 0.003
$LinB_{MI}$ H247A	0.210 ± 0.015	0.240 ± 0.021
$LinB_{MI}$ V134I + H247A	0.104 ± 0.003	0.027 ± 0.001

[a] Not determined

are not located in the catalytic pocket are also involved in the difference of activity.

Molecular modeling

The binding of PCHL in *ortho1*, *ortho2*, *meta1* and *para* orientations (Fig. 7a) in $LinB_{UT}$, $LinB_{MI}$ and $LinB_{UT}$ I134V + A247H, named $LinB_{UT\text{-}MI}$, was observed by molecular docking. The *Meta2* binding mode of PCHL did not meet the criteria of a productive binding mode. In $LinB_{UT}$, the population of the *meta1* orientation was major in the active site (Fig. 7b), possibly due to energetically favorable formation of a hydrogen bond between the *meta1*-PCHL-OH and D108-$O_{\delta 1}$ (Fig. 7a). D108-$O_{\delta 1}$ performs a nucleophilic attack in the first step of the dehalogenation reaction, and a negative partial charge is required on this atom for the attack. This charge will be significantly reduced by electron withdrawal of the hydroxyl group of *meta1*-PCHL, making the first step of the dehalogenation of *meta1*-PCHL hardly feasible. However, in $LinB_{MI}$ and $LinB_{UT\text{-}MI}$, the population of *meta1* orientation was reduced and the *para* orientation prevailed (Fig. 7b), indicating that the binding of PCHL to *para* orientation is energetically more favorable than to *meta1* orientation in these variants. The substitution A247H seems to change the shape of the active site cavity, making it more suitable for a *para* orientation than for a *ortho*- or *meta* one. Furthermore, an extra hydrogen bond between *para*-PCHL-OH and H247-$N_{\varepsilon}H_{\varepsilon}$ in the '*' conformation was observed (Fig. 7c), providing additional energetic advantage for this reactive binding mode.

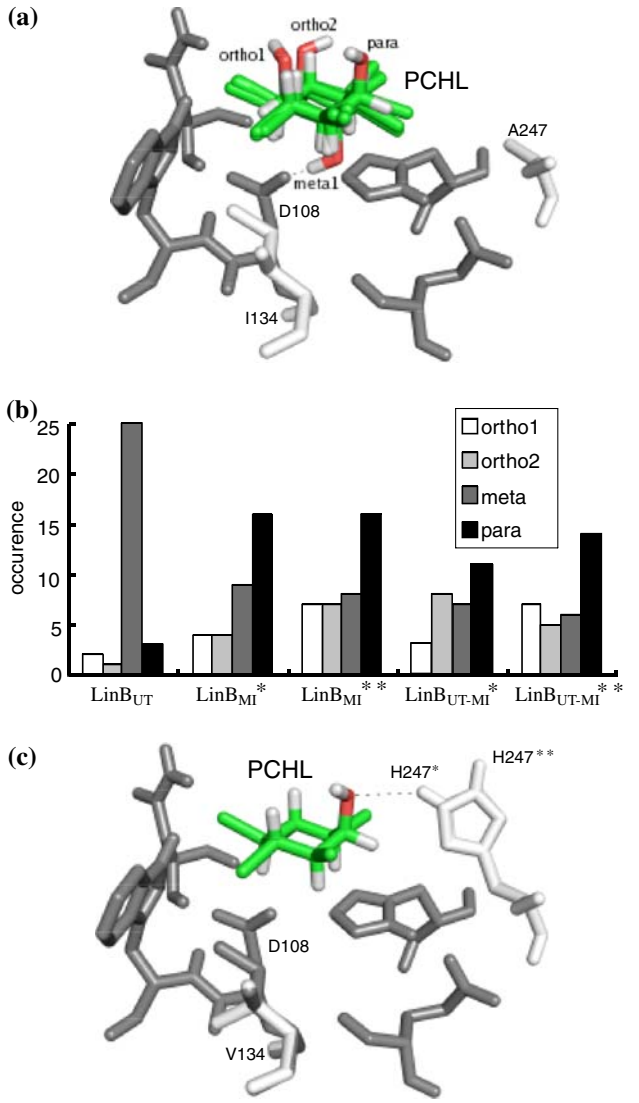

Fig. 7 Computational analysis of PCHL binding to the active site of LinB$_{UT}$, LinB$_{MI}$, and LinB$_{UT-MI}$. **a** Orientations of PCHL in the active site of LinB$_{UT}$ obtained from the molecular docking. PCHL molecules in different orientations spatially align except for the positions of hydroxyl substituents indicated by labels *ortho1*, *ortho 2*, *para*, and *meta 1*. Catalytic residues are shown in *dark gray*, and the residues I134 and A247 *in white*. Hydrogen bond between *meta1*-PCHL-OH and D108-$O_{\delta 1}$ is indicated by *dashed line*. **b** Population of productive PCHL binding modes in LinB variants among 50 docking runs. Since two different conformations of H247 (H247* and H247** in *panel c*) are possible, two structures were analyzed for each LinB$_{MI}$ and LinB$_{UT-MI}$. **c** Favored *para*-orientation binding of PCHL in LinB$_{MI}$ and LinB$_{UT-MI}$. Hydrogen bond between H247* and PCHL is marked by *dashed line*

Discussion

We demonstrated in this study that haloalkane dehalogenase LinB$_{MI}$ from *Sphingobium* sp. MI1205 catalyzes the two-step dehalogenation of *β*-HCH. Our definite identification of the product TCDL provides an explanation for the mechanism of the two-step conversion of *β*-HCH by LinB$_{MI}$. Haloalkane dehalogenases cleave the carbon-halogen bond and replace a halogen with a hydroxyl group with an inversion of configuration in an S_N2-type substitution mechanism (Verschueren et al. 1993; Damborsky and Koca 1999). The stereochemistry of TCDL indicates that 1Cl and 4Cl of *β*-HCH were replaced with hydroxyl groups with inversion of configuration (Fig. 6). Together with the fact that the proposed catalytic triad of LinB$_{UT}$ (Hynkova et al. 1999) was conserved in LinB$_{MI}$, it was strongly suggested that the two-step dehalogenation proceeds via the same mechanism as the one reported for LinB$_{UT}$ (Prokop et al. 2003).

The specificity constants clearly showed that LinB$_{MI}$ and LinB$_{UT}$ have different enzymatic properties (Table 3) despite having only seven amino-acid differences. Reports on the functional difference between two 'wild' enzymes for the degradation of xenobiotics with a small number of amino-acid differences (more than 90% identity) have been limited (Seffernick et al. 2001; Liu et al. 2005), for which reason LinB becomes an interesting addition. Considering the potential use of LinB for HCH bioremediation, the property of LinB$_{MI}$ is useful because *β*-HCH is the most problematic environmental pollutant among HCH isomers and the conversion to TCDL may more easily lead to productive and complete disappearance. To reveal the unique reaction specificities of LinB$_{MI}$, we focused on two amino acid residues, V134 and H247, because these two residues are located in the catalytic pocket (Marek et al. 2000). Mutational analyses in this study demonstrated that these two residues seem to determine the rate of the second dehalogenation reaction of PCHL to TCDL in LinB$_{MI}$ (Fig. 5). Molecular modeling suggested that the two residues aid in preferential binding of PCHL in the reactive *para* orientation, simultaneously reducing the possibility to form the non-reactive *meta* orientation (Fig. 7). Hydrogen bonding is critical for the determining reactive orientations of PCHL in the active site of LinB variants. However, mutagenesis results suggested that at least some of the other five residues, T81, V112, T135, L138, and I253, which are located outside of the catalytic pocket, are important for the two-step dehalogenation of *β*-HCH. Wu et al. (2007) in a similar recent study also suggested important role of these amino acid residues in dehalogenation but could not conclusively demonstrate this. Further understanding on the molecular differences between LinB enzymes should come from some structural analysis.

It is quite noteworthy that all eight substitutions between *linB*$_{MI}$ and *linB*$_{UT}$ are nonsynonymous. This may reflect the history that LinB$_{MI}$ and LinB$_{UT}$ diverged from the common ancestral LinB relatively recently, undergoing strong positive selection (Vacquier and Lee 1993; Endo et al. 1996), although more studies are needed to elucidate the physiological significance of the activity unique to LinB$_{MI}$.

Our recent study showed that the identical IS*6100-linB* cluster in MI1205 is located on pLB1, a plasmid exogenously isolated from the same soil sample used in this study (Fig. 4) (Miyazaki et al. 2006). pLB1 is self-transmissible at least among the α-proteobacterial strains, strongly suggesting that MI1205 recruited the IS*6100-linB* cluster by a horizontal transfer event. However, the nucleotide sequence of MI1205 just located at one neighboring region of the IS*6100-linB* cluster was different from pLB1. The difference observed outside of an inverted repeat of IS*6100* suggests that the IS*6100*-mediated transposition and/or rearrangement occurred in the soil environment.

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