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Analysis of the role of LinA and LinB in biodegradation of δ-hexachlorocyclohexane

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Summary

Commercial formulations of hexachlorocyclohexane (HCH) consist of a mixture of four isomers, α, β, γ and δ. All these four isomers are toxic and recalcitrant pollutants. *Sphingobium* (formerly *Sphingomonas*) sp. strain BHC-A is able to degrade all four HCH isomers. Eight *lin* genes responsible for the degradation of γ-HCH in BHC-A were cloned and analysed for their role in the degradation of δ-HCH, and the initial conversion steps in δ-HCH catabolism by LinA and LinB in BHC-A were found. LinA dehydrochlorinated δ-HCH to produce 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via δ-pentachlorocyclohexene (δ-PCCH). Subsequently, both 1,4-TCDN and δ-PCCH are catalysed by LinB via two successive rounds of hydrolytic dechlorinations to form 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) and 2,3,5-trichloro-5-cyclohexene-1,4-diol (2,3,5-TCDL) respectively. LinB could also catalyse the hydrolytic dechlorination of δ-HCH to 2,3,5,6-tetrachloro-1,4-cyclohexanediol (TDOL) via 2,3,4,5,6-pentachlorocyclohexanol (PCHL).

Introduction

Hexachlorocyclohexane (HCH) is an organochlorine insecticide, which has been banned in technologically advanced countries because of its toxicity and persistence in the environment. However, in some areas in the world, this insecticide is still in use for economic reasons and thus, new areas continue to be contaminated. Theoretically, HCH has eight possible stereoisomers, of which four (α-, β-, γ- and δ-HCH) predominate in the commercial products (Johri *et al*., 1996). γ-HCH is the best-known and effective insecticide component of HCH, and only 9–18% of commercial HCH consists of this γ-isomer. The remaining part consists of α-, β- and δ-HCH, which do not show insecticide activity (Langenhoff *et al*., 2002), but still persist in the environment with toxicity to insects, birds, mammals and other non-target organisms (Johri *et al*., 1996).

Several bacterial strains *Sphingobium japonicum* UT26, *Sphingobium indicum* B90A, *Sphingobium francense* Sp+, *Sphingobium* (formerly *Sphingomonas*) sp. BHC-A and *Sphingomonas* sp. α4-2 and γ1-7 which are able to degrade α-, β-, γ- and δ-HCH have been isolated (Nagasawa *et al*., 1993a; Kumari *et al*., 2002; Dogra *et al*., 2004; Ma *et al*., 2005; Mohn *et al*., 2006a), and the genes responsible for the degradation of HCH isomers have also been cloned and characterized (Nagata *et al*., 1993a; 1999a; Trantirek *et al*., 2001; Kumari *et al*., 2002; Suar *et al*., 2004; Endo *et al*., 2005; Suar *et al*., 2005; Sharma *et al*., 2006). In the case of γ-HCH, the complete degradation pathway under aerobic conditions in strain UT26 has been reported, which consists of *linA*, *linB*, *linC*, *linD*, *linE*, *linF*, *linR* and *linX* genes (Imai *et al*., 1991; Nagasawa *et al*., 1993a,b,c; Nagata *et al*., 1993a,b; 1994; 1999a,b; Miyauchi *et al*., 1998, 1999; Endo *et al*., 2005). The first step in the degradation of γ-HCH is initiated by two LinA catalysed dehydrochlorinations to produce 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via γ-pentachlorocyclohexene (γ-PCCH) (Imai *et al*., 1991; Nagata *et al*., 1993a; 1999b; Mohn *et al*., 2006b). Then LinB acts only on γ-PCCH and makes two hydrolytic dechlorinations to form 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) via 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL) (Nagata *et al*., 1993b). For α-HCH, the initial degradation steps are slightly different due to its two enantiomeric forms (Suar *et al*., 2005). Two variants of a dehydrochlorinase from strain B90A, LinA1 and LinA2, can enantioselectively transform chiral α-HCH to β-PCCH enantiomers due to their differences in substrate specificity (Suar *et al*., 2005). LinA1 preferentially converts the (+)-α-HCH enantiomer to (3S, 4S, 5R, 6R)-β-1,3,4,5,6-PCCH whereas LinA2 preferentially converts the (–)-α-HCH enantiomer to (3R, 4R, 5S, 6S)-β-1,3,4,5,6-PCCH (Suar *et al*., 2005). The β-PCCH enantiomers are then degraded through the same pathway as γ-PCCH (Lal *et al*., 2006). In the case of β-HCH, the first degradation step is initiated by two

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LinB catalysed hydrolytic dechlorinations, rather than two LinA catalysed dehydrochlorinations, to produce β-2,3,5,6-tetrachloro-1,4-cyclohexanediol (β-TDOL) via β-2,3,4,5,6-pentachlorocyclohexanol (β-PCHL) (Nagata *et al*., 2005; Sharma *et al*., 2006; Wu *et al*., 2007). While in the case of δ-HCH, the initial degradation steps seem more complex and both LinA and LinB seem to be capable of acting on δ-HCH (Sharma *et al*., 2006). Two types of dechlorination of δ-HCH have also been reported. One is the dehydrochlorination of δ-HCH to δ-PCCH by LinA from UT26 and the other is the hydrolytic dechlorination of δ-HCH to tetrachlorocyclohexanediol via PCHL by LinB from B90A (Nagata *et al*., 1993a; Trantirek *et al*., 2001; Sharma *et al*., 2006), which indicates metabolic diversity in the degradation of δ-HCH.

In this study, we investigated the metabolic diversity of δ-HCH in strain BHC-A. On the basis of the identification of metabolites, we found hidden conversion steps in δ-HCH catabolism by LinA and LinB in BHC-A.

Results and discussion

Cloning of the linA, linB, linC, linD, linE, linF, linR *and* linX *genes from* Sphingobium *sp. BHC-A*

Eight *lin* genes responsible for the degradation of γ-HCH were cloned from strain BHC-A using a polymerase chain reaction (PCR) strategy based on sequence homologies with previously published sequences from UT26 and B90A. The dehydrochlorinase (*linA*) gene from BHC-A shared the same nucleotide sequence with the *linA* gene of UT26, Sp+, DS2 and the *linA2* gene of B90A. The halidohydrolase (*linB*), dehydrogenases (*linC* and *linX*), reductive dechlorinase (*linD*), dioxygenase (*linE*), reductase (*linF*) and LysR-type transcriptional regulator (*linR*) genes from BHC-A had about 99% nucleotide similarity with the corresponding genes of UT26, Sp+, DS2 and B90A. These results suggest the existence of similar degradation pathway for γ-HCH in BHC-A as reported in UT26 and B90A.

Assay for the conversion activity of δ-HCH by LinB and identification of the metabolites

In a previous study, we reported LinB (formerly LinB2) from BHC-A, which showed only seven heterogeneous amino acid residues in similar sites of LinB from UT26, could hydrolytically dechlorinate β-HCH to produce TDOL via PCHL (Wu *et al*., 2007). In this study, we assayed the dechlorination activity of LinB with α-, γ- and δ-HCH, respectively, by gas chromatography-mass spectrometry (GC-MS). During the period of incubations, no disappearance of α and γ-HCH were observed. In contrast, obvious degradation of δ-HCH was detected at the approximate rate of 1.6 μmol min^{-1} mg^{-1} of protein (Fig. 1A). The concentration of δ-HCH decreased with a simultaneous increase in the concentration of one product designated M1 (retention time, 21.615 min). However, with prolonged incubation, an obvious disappearance of M1 was found with a simultaneous increase in the concentration of another novel metabolite designated M2 (retention time, 24.357 min). After 80 min, M1 completely disappeared with the concentration of M2 reaching maximal levels. M2 was the final product of this reaction and no disappearance of M2 was detected in prolonged incubations.

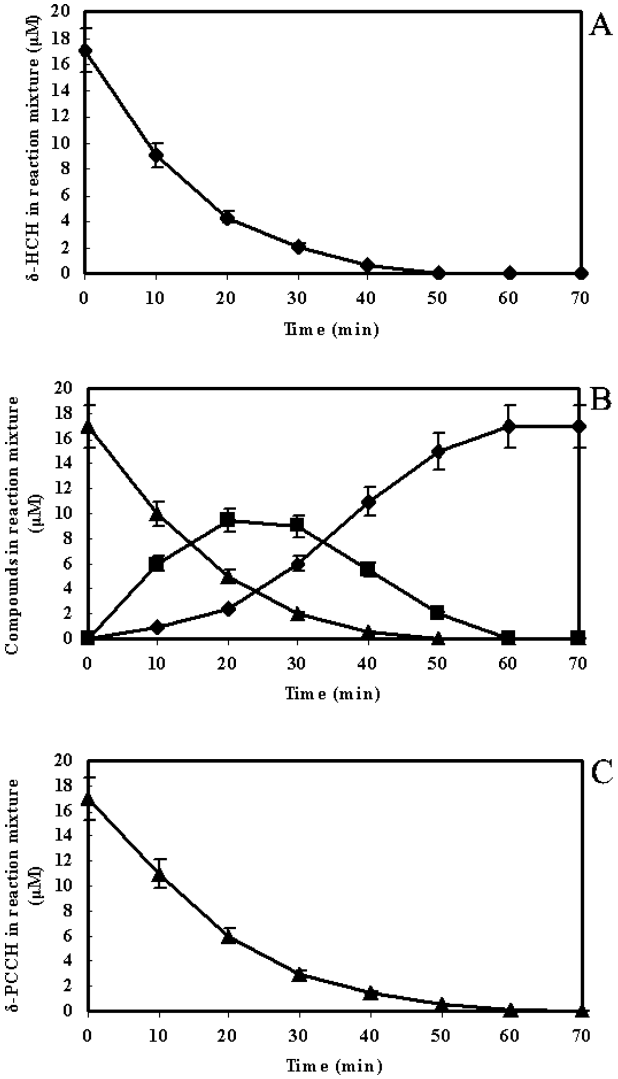


Fig. 1. Conversion of δ-HCH by LinB (A); ◆, δ-HCH. Conversion of δ-HCH by LinA (B); ▲, δ-HCH; ■, δ-PCCH; ◆, 1,2,4-TCB. Conversion of δ-PCCH by LinB (C); ▲, δ-PCCH. The error bars indicate standard deviations.

M1 was identified as PCHL because its mass spectrum showed the same fragmentation pattern described in the

literature (Wu *et al*., 2007). The fragmentation of M2 was consistent with that of TDOL and the one-dimensional ^{1}H nuclear magnetic resonance (NMR) data showed that only two signals of M2 were observed at 4.39 ppm (chlorometine, 4H) and 4.27 ppm (hydroxymetine, 2H), respectively, which indicated that the two OH functional groups on the cyclic carbon of M2 were situated in para-position. Thus, M2 was identified as TDOL. The proposed conversion steps of δ-HCH by LinB were dechlorination of δ-HCH to produce TDOL via PCHL. However, we noticed the retention times of PCHL and TDOL in this study were not identical to those of β-PCHL and β-TDOL converted from β-HCH (data not shown). This was possibly caused by differences in their configurations. Due to the lack of availability of authentic samples of δ-PCHL and δ-TDOL, we could not compare the configuration of metabolites of δ-HCH by LinB with authentic samples and determine their configuration.

Assay for the conversion activity of δ-HCH by LinA and identification of the metabolites

In previous studies, the conversion of δ-HCH to δ-PCCH by LinA from strain UT26 was reported and δ-PCCH was deemed as the only product in the dehydrochlorination of δ-HCH (Nagata *et al*., 1993a; Trantirek *et al*., 2001). In this study, we performed the conversion reaction by using His-tagged LinA. During the period of incubations, an obvious decrease in the concentration of δ-HCH at the approximate rate of 1.4 μmol min^{-1} mg^{-1} of protein and a simultaneous increase in the concentration of one product designated M3 (retention time, 13.716 min) was detected by GC-MS (Fig. 1B). M3 was identified as δ-PCCH because of its identical retention time and mass spectrum as those of an authentic sample of δ-PCCH (Imai *et al*., 1989). However, we found δ-PCCH was not the only product of dehydrochlorination of δ-HCH as reported. In prolonged incubations, δ-PCCH could be converted to another product designated M4 (retention time, 9.396 min). After 60 min, δ-HCH and δ-PCCH completely disappeared and only M4 could be detected. The retention time and fragmentation of M4 was consistent with those of 1,2,4-TCB (Imai *et al*., 1991). Thus, M4 was identified as 1,2,4-TCB, which meant consecutive elimination of two HCl-groups per δ-PCCH molecule. It was similar to the conversion of γ-PCCH to 1,2,4-TCB, in which γ-PCCH is dehydrochlorinated by LinA to form the unstable intermediate tetrachlorocyclohexadiene (TCCH), which can dehydrochlorinate spontaneously to produce 1,2,4-TCB (Nagasawa *et al*., 1993a,b,c). In light of these observations, we deduced the TCCH was the intermediate of the conversion of δ-PCCH to 1,2,4-TCB, although it was not detected by GC-MS, and that the dehydrochlorination of TCCH to 1,2,4-TCB was also a spontaneous reaction. The results suggested LinA could catalyse two subsequent dehydrodehalogenations of δ-HCH to produce TCCH.

Indirect identification of TCCH and the dechlorination reaction of TCCH by LinB

In the degradation pathway of γ-HCH, LinB can convert TCCH produced from γ-PCCH by LinA (Nagasawa *et al*., 1993a,b,c). In this study, we assayed the transformation of TCCH, produced from δ-PCCH by LinA. In the conversion of δ-PCCH by LinA and LinB simultaneously, the concentration of δ-PCCH decreased with simultaneous increases in the concentration of not only 1,2,4-TCB, but also in three other products designated as M5 (retention time, 17.073 min), M6 (retention time, 10.262 min) and M7 (retention time, 14.599 min) respectively. M5 was identified as 2,5-DDOL because its mass spectrum showed the same fragmentation pattern as previously reported (Nagasawa *et al*., 1993a). In the degradation pathway of γ-HCH, it had been demonstrated that 2,5-DDOL is only produced from the dechlorination of the unstable intermediate 1,4-TCDN (one of TCCH) by LinB (Nagata *et al*., 1993b). Thus, 1,4-TCDN could be indirectly determined by the detection of 2,5-DDOL. Based on this, we deduced there was 1,4-TCDN in this reaction. LinA dehydrochlorinated δ-PCCH to produce 1,4-TCDN, while the dehydrochlorination of 1,4-TCDN to 1,2,4-TCB was a spontaneous reaction (Nagasawa *et al*., 1993a,b,c). The retention time and fragmentation of M6 was consistent with those of 2,5-DCP (Nagasawa *et al*., 1993a). Thus, M6 was identified as 2,5-DCP, with one spontaneous dehydrochlorination product of another unstable intermediate 2,4,5-DNOL formed in the conversion of 1,4-TCDN to 2,5-DDOL (Nagasawa *et al*., 1993a,b,c). Therefore, we could also indirectly determine there was 2,4,5-DNOL in this reaction. These results suggested LinB could also dechlorinate 1,4-TCDN to 2,5-DDOL via 2,4,5-DNOL.

However, the concentration of the dechlorination products 2,5-DDOL, 1,2,4-TCB and 2,5-DCP were lower than expected. Simultaneously, The mass spectrum of M7 showed a molecular ion peak of *m/z* 216 ($M7^{+}$) and major fragment peaks such as *m/z* 199 ($M7^{+}$-OH), *m/z* 180 ($M7^{+}$-HCl), *m/z* 163 ($M7^{+}$-OH-HCl), *m/z* 145 ($M7^{+}$-HCl-Cl), *m/z* 135 ($M7^{+}$-OH-HCl-C_2H_4) and *m/z* 120 ($M7^{+}$-Cl-Cl-C_2H_2), which were not identical to those of any known products. The isotopic cluster of the lower-mass ion *m/z* 216–222, found in the spectrum, indicated three chlorine atoms in M7. These observations suggest that M7 was one dechlorination product and there was one novel dechlorination conversion of δ-PCCH in this reaction, which was possibly catalysed by LinB directly.

Assay for the conversion activity of δ-PCCH by LinB and identification of the metabolite

In the conversion of δ-PCCH by LinB, the decrease in the concentration of δ-PCCH at the approximate rate of 1.2 μmol min^{-1} mg^{-1} of protein (Fig. 1C) and a simultaneous increase in the concentration of one product (retention time, 14.599 min) were detected by GC-MS. After 70 min, δ-PCCH completely disappeared with the concentration of this product reaching a maximum. Gas chromatography-mass spectrometry showed that this product shared the same retention time and mass spectrum as M7. Hence, it was identified as M7. Because δ-PCCH contained five chlorine atoms while M7 contained three (see above), M7 must be the dechlorination product of δ-PCCH. The difference between these two compounds' molecular weights was only 36 (δ-PCCH molecular weight was 252; M7 molecular weight was 216); thus, two OH (OH molecular weight was 17) should replace two chlorine atoms (chlorine molecular weight was 35) in δ-PCCH to form M7. Nuclear magnetic resonance analysis was performed to determine the positions of the two OH functional groups in the carbon circle of M7. In one-dimensional ^{1}H NMR spectrometry, seven signals of M7 were observed at 4.2, 4.3, 4.4, 4.5, 5.7, 5.9 and 6.2 ppm, respectively (Fig. 2A), which were produced by the chemical shifts of seven protons in the M7 molecule. By deuterium exchange, we found signals at 5.7 and 6.2 disappeared, which suggested that the two protons belong to two hydroxyl groups respectively. The signal at 5.9 was produced by the chemical shift of a proton of olefinic. Other signals were produced by the chemical shifts of four protons in the carbon circle. HH-COSY two-dimensional ^{2}H NMR spectrum was shown (Fig. 2B) and the correlation of protons was analysed (Table 1). Based on these data, M7 was identified as 2,3,5-trichloro-5-cyclohexene-1,4-diol (2,3,5-TCDL).

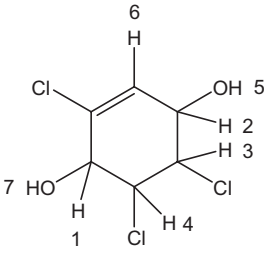
Interpretation of the conversion of δ-HCH by LinA and LinB

From the data presented above, several novel conversions of δ-HCH by LinA and LinB could be interpreted (Fig. 3). LinA dehydrochlorinated δ-HCH to produce 1,4-TCDN via δ-PCCH. Subsequently, both 1,4-TCDN and δ-PCCH were catalysed by LinB via two successive rounds of hydrolytic dechlorinations. 1,4-TCDN was dechlorinated to form 2,5-DDOL via 2,4,5-DNOL and δ-PCCH was dechlorinated to form 2,3,5-TCDL. LinB could also catalyse the hydrolytic dechlorination of δ-HCH to TDOL via PCHL. Through this series of reactions, two dead-end products, 1,2,4-TCB and 2,5-DCP, were produced from the unstable intermediates of 1,4-TCDN and 2,4,5-DNOL respectively. In theory, the conversion of δ-PCCH to 2,3,5-TCDL should contain two steps. δ-PCCH was first dechlorinated to produce one intermediate and then the intermediate was dechlorinated again to produce 2,3,5-TCDL. However, in our study, this intermediate was not isolated from the reactions. There were two possibilities: (i) the intermediate was unstable and could not be detected by GC-MS and (ii) the conversion process was too rapid. Once the intermediate was produced, LinB converted it to 2,3,5-TCDL immediately without accumulation. In spite of this, we were still able to indirectly deduce the intermediate from 2,3,5-TCDL. Due to the asymmetry of 2,3,5-TCDL, there were two possible intermediates, 2,3,4,5-tetrachloro-5-cyclohexene-1-ol (2,3,4,5-TCOL) and 2,4,5,6-tetrachloro-2-cyclohexene-1-ol (2,4,5,6-TCOL). One of these should be the intermediate. The role of LinA was redefined in this study; δ-PCCH was not the terminal product, but rather could be converted to 1,4-TCDN. This was very important because it not only extended the conversion ability of LinA but also provided the distinct substracts for LinB to produce metabolic diversity.

Checking these novel conversions of δ-HCH in Sphingobium *sp. BHC-A*

By homologous recombination, three kinds of mutants, exemplified by tbA (Δ*linA*), tbB (Δ*linB*) and tbAB (Δ*linA* and Δ*linB*), were obtained. Two kinds of complementation strains, exemplified by hbtbA and hbtbB, were also obtained by triparental mating. The degradation of

Table 1. HH-COSY two-dimensional ^{2}H NMR data analysis of M7.

Proton	Chemical shift (ppm)	Description	Correlation proton	Structural formula
1	4.2	1H (hydroxymetine)	4, 7	
2	4.3	1H (hydroxymetine)	3, 5, 6	
3	4.4	1H (chlorometine)	2, 4	
4	4.5	1H (chlorometine)	1, 3	
5	5.7	1H (hydroxyl)	2	
6	5.9	1H (olefinic)	2	
7	6.2	1H (hydroxyl)	1	

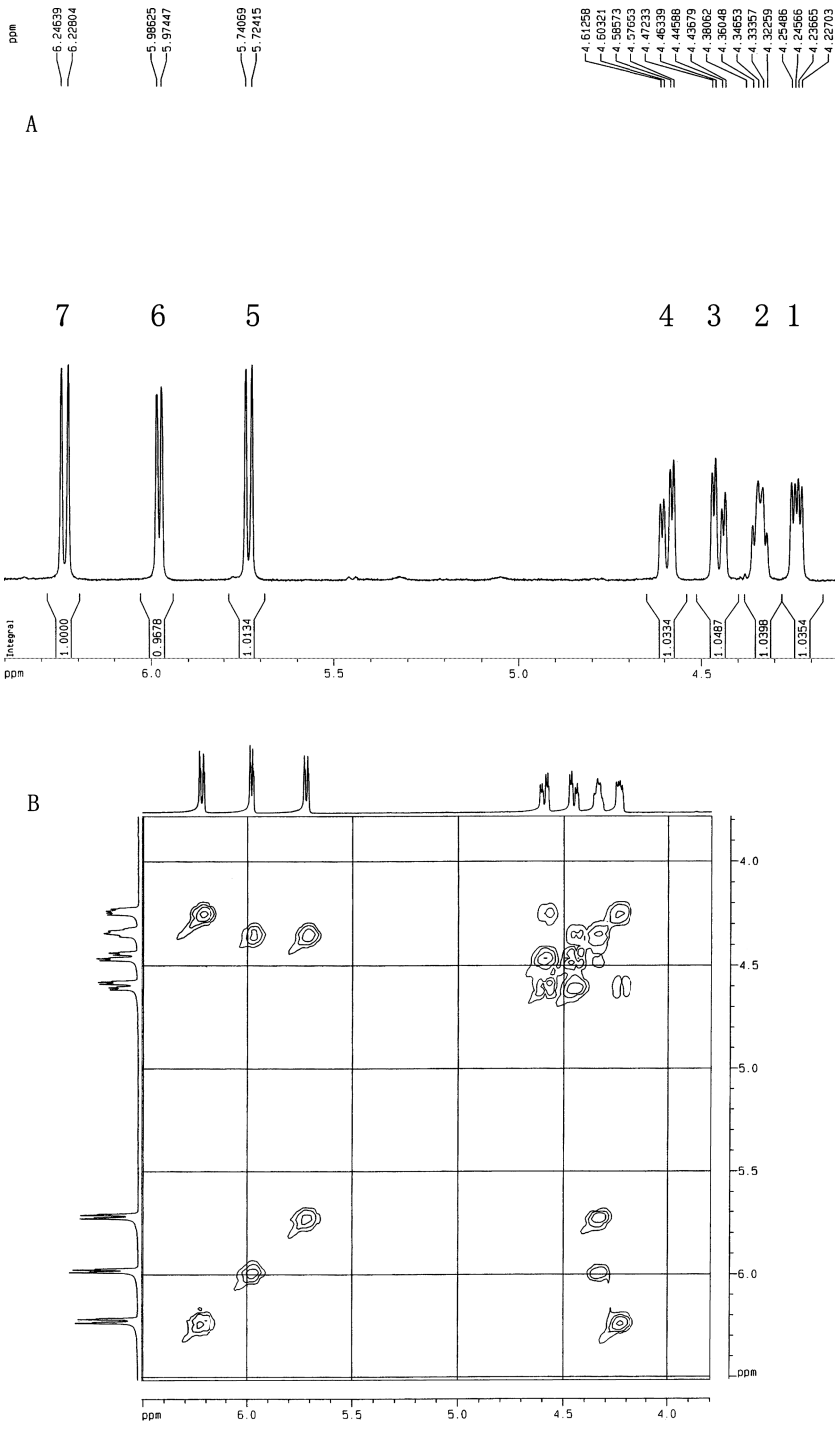


Fig. 2. One-dimensional ^{1}H NMR spectrum of M7 (A) and HH-COSY two-dimensional ^{2}H NMR spectrum of M7 (B).

δ-HCH in BHC-A and its derivative strains tbA, tbB, tbAB, hbtbA and hbtbB was detected, respectively, by GC-MS. The results showed all compounds belonging to these novel conversions could be detected from BHC-A cultures. TDOL and 2,3,5-TCDL could be transformed with prolonged incubation while 1,2,4-TCB, 2,5-DCP and 2,5-DDOL were not degraded anymore. Strain tbA lost dehydrochlorination activity of δ-HCH and only hydrolytically dechlorinated δ-HCH to TDOL via PCHL. No other compounds of these novel conversions were observed. By adding δ-PCCH in this reaction, the hydrolytic dechlorination of δ-PCCH to 2,3,5-TCDL could be detected. TDOL and 2,3,5-TCDL were also degraded continuously with prolonged incubation. Strain tbB lost hydrolytic dechlorination activity of δ-HCH and only dehydrochlorinated 17 μM δ-HCH to 17 μM 1,2,4-TCB via δ-PCCH; no

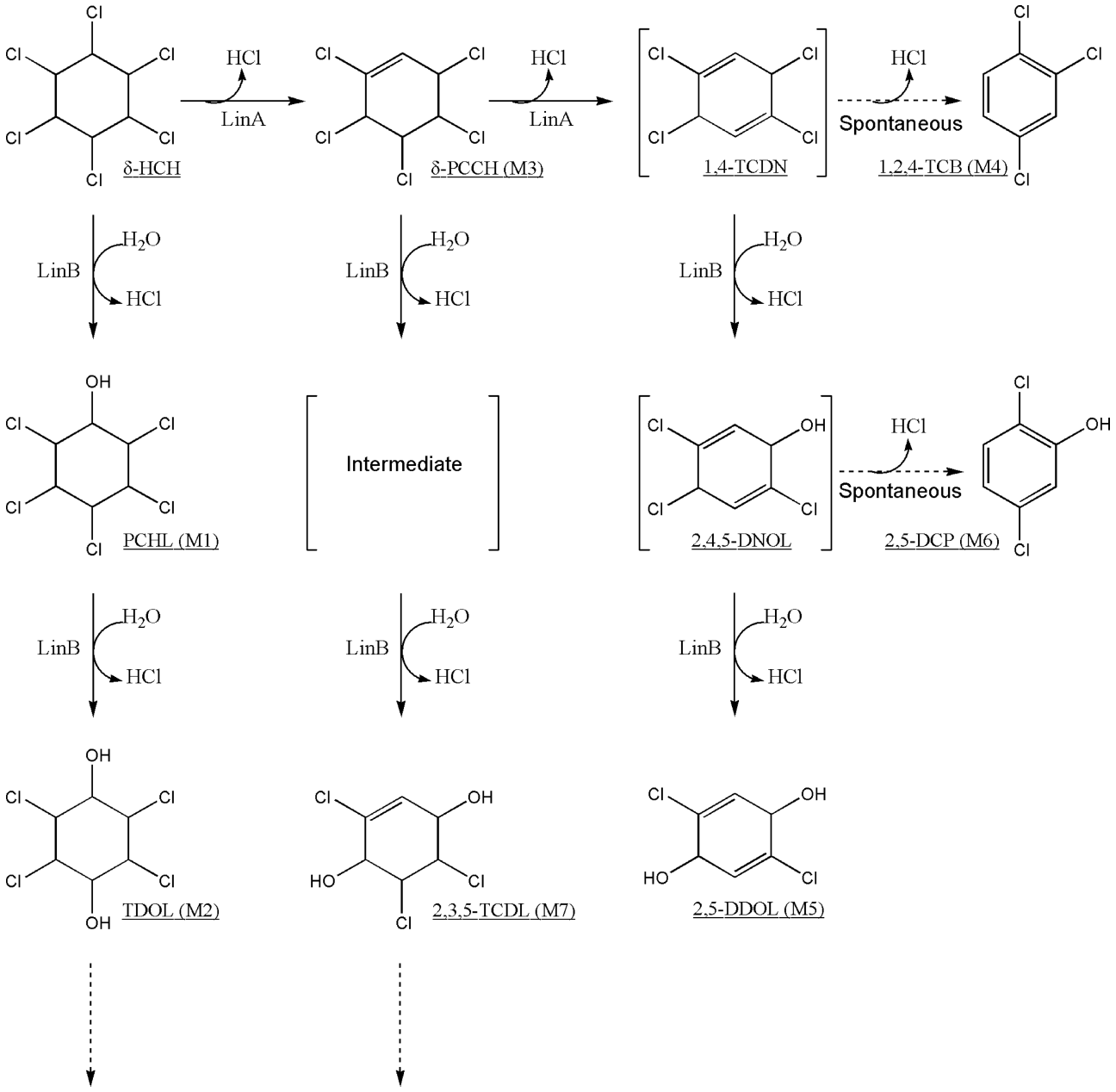


Fig. 3. Proposed conversions of δ-HCH by LinA and LinB.

other hydrolytic dechlorination products were detected. Strain tbAB lost the degradation activity of δ-HCH completely, while the complementation strains hbtbA and hbtbB could metabolize δ-HCH like strain BHC-A. According to the results of our study, the novel conversions obtained by *in vitro* experiments could be confirmed in *Sphingobium* sp. BHC-A. These results still indicated that LinC or LinD in BHC-A could not convert 2,5-DDOL derived from δ-HCH. Perhaps it was caused by the configuration of 2,5-DDOL, which was not identical to the configuration of 2,5-DDOL derived from γ-HCH.

No detection of the conversion activity of TDOL and 2,3,5-TCDL by LinC, LinD, LinE, LinF and LinX

In the above experiment, the conversion of TDOL and 2,3,5-TCDL, derived from δ-HCH, was observed in strain BHC-A. In this experiment, we assayed the conversion of TDOL and 2,3,5-TCDL by LinC, LinD, LinE, LinF and LinX, which are involved in the γ-HCH degradation pathway. No decrease of TDOL and 2,3,5-TCDL was detected in this study (the detection limit, 10 μg l^{-1}) (data not shown). This result suggests that other enzyme(s) are responsible for the conversion of TDOL and 2,3,5-TCDL in BHC-A.

Experimental procedures

Bacterial strains, plasmids and culture conditions

HCH-degrading strain *Sphingobium* sp. BHC-A (Amp^r, Str^r) (Ma *et al*., 2005;Wu *et al*., 2007) was grown at 30°C in Luria–Bertani (LB) medium (Maniatis *et al*., 1982). *Escherichia coli* strains were grown on LB medium at 37°C. *Escherichia coli* DH5α (F$^-$/*hsdR17*, *recA1*, *gyrA*) cells were used for cloning experiments. Aliquots (200 μl) were heat-shock transformed (Maniatis *et al*., 1982). *Escherichia coli* BL21 (B F$^-$*ompT hsdS* (r_B^- m_B^-) *gal dcm*) cells were used for expression experiments. *Escherichia coli* SM10 [*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^r)] cells were used as the host of homologous recombination plasmids (Espinosa-Urgel *et al*., 2000). *Escherichia coli* HB101 with plasmid RK600 (*Cm*^{r}) was used as a triparental conjugation helper (Espinosa-Urgel *et al*., 2000).

T-vectors pMD18-T (Amp^r) and plasmids pET29a (Km^r) were used for cloning and expression, respectively (Maniatis *et al*., 1982). The homologous recombination plasmids pEX18Gm and pEX18Tc were used for mutagenesis (Hoang *et al*., 1998). Plasmid pBBR1MCS-2 was used as the broad-host-range cloning vector for complementation of the mutant strains (Kovach *et al*., 1995).

Antibiotics were used at final concentrations of 50 μg ml^{-1} for ampicillin (Amp), streptomycin (Str) and kanamycin (Km) and 20 μg ml^{-1} for gentamicin (Gm), tetracycline (Tc) and chloramphenicol (Cm).

Chemicals and enzymes

All organic solvents were glass-distilled grade or high-performance liquid chromatography grade (Tedia, USA). Analytical grade α-, γ- and δ-HCH was purchased from Sigma-Aldrich USA (Genetimes Technology, China). Isopropyl-β-D-thiogalactopyranoside (IPTG) and Xgal were obtained from Shanghai Sangon Biological Engineering Technology and Service. Enzymes and kits necessary for DNA manipulations were purchased from Takara Biotechnology (Dalian), and New England Biolabs, Beijing, China.

Isolation of DNA

Genomic DNA from *Sphingobium* sp. BHC-A was isolated by high-salt concentration precipitation (Miller *et al*., 1988). Plasmid DNA of *E. coli* was isolated by the alkaline lysis method (Maniatis *et al*., 1982).

Polymerase chain reaction amplification of the lin genes responsible for the degradation of γ-HCH from Sphingobium *sp. BHC-A*

Based on sequence homologies with previously conserved *lin* genes sequences responsible for the degradation of γ-HCH from UT26 (Imai *et al*., 1991; Nagasawa *et al*., 1993a,b,c; Nagata *et al*., 1993a,b; 1994; 1999a,b; Miyauchi *et al*., 1998, 1999; Kumari *et al*., 2002; Endo *et al*., 2005), the primers were designed on request. Total genomic DNA from BHC-A was used as template for specific PCR amplifications. The PCR products were cloned in T-vectors and sequenced at Takara Biotechnology (Dalian).

Expression and purification of His-tagged LinA, LinB, LinC, LinD, LinE, LinF and LinX, respectively

Individually amplified *linA*, *linB*, *linC*, *linD*, *linE*, *linF* and *linX* genes were digested with EcoRI and XhoI and then introduced into multiple cloning site of pET29a to construct expression plasmids p29A, p29B, p29C, p29D, p29E, p29F and p29X, respectively, which could express the corresponding protein in high amounts containing 6-histidyl tails into the C-terminus in *E. coli* BL21. *Escherichia coli* BL21 containing these plasmids was incubated in 500 ml of LB medium at 30°C respectively. When the culture reached an optical density of 0.5 at 550 nm, IPTG was added to a final concentration of 1 mM. Cells were harvested after 4 h incubation, washed with 50 mM potassium phosphate buffer (pH 7.5), and resuspended in the buffer. The cells were disrupted by sonication (VCX600; Sonics, Newtown, CT, USA) and centrifuged at 20 000 *g* for 30 min. The supernatant was further purified by a 2-ml volume of NTA-Ni^{2+} agarose (QIAGEN) at 4°C respectively. The amount of His-tagged protein was determined by a protein assay kit purchased from Shanghai Sangon Biological Engineering Technology and Service, with bovine serum albumin as a standard. The purified enzymes were stored in the 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 10% glycerol at concentration about 1 mg ml^{-1} at 0–4°C, respectively (Maniatis *et al*., 1982; Wu *et al*., 2007).

Biochemical synthesis of δ-PCCH

Ten milligrams of δ-HCH of more than 99% purity and 100 ml of 50 mM potassium phosphate buffer (pH 7.5) were equilibrated at 30°C in a water-bath shaker. The enzymatic reaction was initiated by adding 200 μl of the purified His-tagged LinA (final concentration, 2 mg l^{-1}) and incubated at 30°C for 20 min. The reaction was then stopped by extraction with hexane. The product δ-PCCH was purified by preparative liquid chromatography with a preparative column (10 mm × 250 mm, BonChrom) packed by silica gel (5 μm). Dichloromethane (20%) in hexane was used as the mobile phase.

Enzyme reactions

In the present study, we evaluated substrate transformations by the purified His-tagged proteins, which were diluted with 50 mM potassium phosphate buffer (pH 7.5) to yield a final concentration of 0.5 mg l^{-1}. LinB was incubated with 17 μM α-, γ- and δ-HCH, respectively, at 30°C; LinA was incubated with 17 μM δ-HCH at 30°C; LinA and LinB were incubated with 17 μM δ-PCCH at 30°C simultaneously; LinB was incubated with 17 μM δ-PCCH at 30°C; LinC, LinD, LinE, LinF and LinX were incubated with 17 μM TDOL at 30°C, respectively, which was converted from δ-HCH by LinB; LinC, LinD, LinE, LinF and LinX were incubated with 17 μM 2,3,5-TCDL at 30°C, respectively, which was converted from δ-PCCH by LinB. Aliquots were taken out from these reactions periodically and extracted with an equal volume of ethyl acetate respectively. The mixture was vortexed for 2 min. After centrifugation (12 000 *g*, 5 min), the ethyl acetate layer was recovered; 1 μl of this extract was analysed by GC-MS.

Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry analysis was performed with a Varian-Saturn GC-MS (Varian-Saturn 2200), equipped with the CP-3800 GC, injection port split-splitless and the 2000 Series Ion Trap MS. The column used was a DB-1701 Low Bleed/MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent). The oven temperature was programmed as follows: hold time at 100°C, 5 min; ramp rate at 10°C min^{-1} to 260°C; hold time at 260°C, 30 min. The temperatures corresponding to the transfer line and the ion trap were 280°C and 220°C, respectively, and the ionization energy was 70 eV. The injection volume was 1 μl via a splitless injection at 280°C. Helium was used as a carrier at a flow rate of 1.0 ml min^{-1}.

Identification of the metabolites by NMR spectroscopy

Fifteen milligrams of δ-HCH and δ-PCCH, respectively, and 50 ml of phosphate buffer (pH 7.5) were equilibrated at 30°C in a water-bath shaker. Two enzymatic reactions were initiated by adding 1 ml of LinB (final concentration, 20 mg l^{-1}) to each reaction and incubated at 30°C overnight. The reactions were then stopped by extraction with ethyl acetate. Afterward, ethyl acetate was blown dry by nitrogen gas, and the sample converted from δ-HCH was dissolved in 99.99% CD_3OD and the sample converted from δ-PCCH was dissolved in 99.99% deuterated dimethylsulfoxide (DMSO). Nuclear magnetic resonance analysis was performed with a Bruker DRX 400 MHz NMR spectrometer.

Homologous recombination mutagenesis

About 300 bp of the *linA* gene fragment was obtained by PCR amplification of *Sphingobium* sp. BHC-A genomic DNA by using primers P1 added EcoRI site (5′-CCG GAA TTC CTA TTT GGT GGG ATG ATG CA-3′) and primers P2 added HindIII site (5′-CCC AAG CTT TCG TGC ATG CGT TGC GCT TA-3′). After digestion with EcoRI and HindIII, the fragment was introduced into the multiple cloning site of pEX18Gm (Hoang *et al*., 1998) to construct homologous recombination plasmid pEX18Gm-A.

About 500 bp of the *linB* gene fragment was obtained by PCR amplification of strain BHC-A genomic DNA by using primers P3 added EcoRI site (5′-CCG GAA TTC CGA TTC GGA CAA GCT CGA TC-3′) and primers P4 added HindIII site (5′-CCC AAG CTT TTT CGC TGA GCC AGC CGG CA-3′). After digestion with EcoRI and HindIII, the fragment was introduced into the multiple cloning site of pEX18Tc (Hoang *et al*., 1998) to construct another homologous recombination plasmid pEX18Tc-B. pEX18Gm-A and pEX18Tc-B were, respectively, transformed into *E. coli* SM10.

pEX18Gm-A was then transferred into strain BHC-A by triparental mating. Mutants (Δ*linA*) were selected from LB medium containing 50 μg ml^{-1} of Str and 20 μg ml^{-1} of Gm.

pEX18Tc-B was also conjugally transferred into strain BHC-A and the selected mutant (Δ*linA*), respectively, followed by the same mating procedures. Mutants (Δ*linB*) were selected from LB medium containing 50 μg ml^{-1} of Str and 20 μg ml^{-1} of Tc; Mutants (Δ*linA* and Δ*linB*) were selected from LB medium containing 50 μg ml^{-1} of Str and 20 μg ml^{-1} of Gm and Tc respectively.

Complementation of mutant strains

Individual *linA* and *linB* gene were excised from the expression plasmids p29A and p29B by digestion with KpnI and HindIII and cloned into the broad-host-range cloning vector pBBR1MCS-2, respectively, yielding pBBR1MCS-2A and pBBR1MCS-2B.

pBBR1MCS-2A and pBBR1MCS-2B were then transformed into *E. coli* DH5α and conjugally transferred into the mutants (Δ*linA*) and the mutants (Δ*linB*), respectively, followed by the same triparental mating procedures. Conjugants harbouring plasmid pBBR1MCS-2A and plasmid pBBR1MCS-2B were selected from LB medium containing 50 μg ml^{-1} of Str and Km.

Checking the novel conversions of δ-HCH by using Sphingobium *sp. BHC-A and its derivative strains*

The degradation of δ-HCH by strain BHC-A and its derivative strains was carried out in mineral salt medium (SM) containing (per litre) 1.0 g of NH_4NO_3, 1.0 g of NaCl, 1.5 g of K_2HPO_4, 0.5 g of KH_2PO_4 and 0.1 g of $MgSO_4$. Precultures of strains grown overnight in LB were transferred at 2% (v/v) into fresh medium containing 17 μM of δ-HCH. Each flask was incubated at 30°C on a rotary shaker. Appropriate controls containing medium plus the 17 μM of δ-HCH were prepared simultaneously. Aliquots were taken out periodically and extracted with an equal volume of ethyl acetate. One microlitre of this extract was analysed by GC-MS.

Nucleotide sequence accession numbers

The *lin* sequences of *Sphingobium* sp. BHC-A have been deposited in GenBank under the following accession numbers: *linA*, DQ372106; *linB*, DQ246619; *linC*, DQ462464; *linD*, DQ480725; *linE*, DQ399709; *linF*, DQ399710; *linR*, DQ399711; *linX*, DQ486136.

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