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Degradation of β-Hexachlorocyclohexane by Haloalkane Dehalogenase LinB from *Sphingomonas paucimobilis* UT26

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β-Hexachlorocyclohexane (β-HCH) is the most recalcitrant among the α-, β-, γ-, and δ-isomers of HCH and causes serious environmental pollution problems. We demonstrate here that the haloalkane dehalogenase LinB, reported earlier to mediate the second step in the degradation of γ-HCH in *Sphingomonas paucimobilis* UT26, metabolizes β-HCH to produce 2,3,4,5,6-pentachlorocyclohexanol.

The chlorinated insecticidal formation of a technical mixture of hexachlorocyclohexane (t-HCH) consisting of α-, β-, γ-, and δ-isomers has been used worldwide. Many countries, however, have now prohibited its use because of its toxicity and persistence in upland soil, but several contaminated sites remain throughout the world. Among these isomers, β-HCH is the most recalcitrant in the environment due to its chemical stability (1). Bacterial strains that degrade β-HCH have been reported (6, 20), but the enzyme involved in the degradation process remains unknown.

Sphingomonas paucimobilis UT26 utilizes γ-HCH as a sole source of carbon and energy under aerobic conditions (3). The degradation pathway of γ-HCH in this bacterium includes two steps of dehydrochlorination, two steps of hydrolytic dehalogenation, and one dehydrogenation step, catalyzed by LinA, LinB, and LinC, respectively, leading to the formation of 2,5-dichlorohydroquinone, which undergoes further degradation (16). The enzyme γ-HCH dehydrochlorinase LinA mediates the metabolism of α-, γ-, and δ-HCH but not that of the β-isomer (11). We demonstrate here that UT26 is able to transform β-HCH and that the activity is derived from the haloalkane dehalogenase LinB.

The activity of *S. paucimobilis* UT26 and its spontaneous *linA* deletion mutant YO5 (15) was assayed for the degradation of t-HCH (India Pesticides, Lucknow, India), which consists of α (67.4%), β (6.8%), γ (17.3%), and δ (7.4%)-isomers of HCH. Briefly, a small amount of a bacterial colony, grown on 1/3 Luria broth-agar medium (7) at 30°C, was picked and suspended (about 20 mg of cells/ml, final concentration) in the assay solution, 17 μM t-HCH in W medium (3). After incubation at 30°C for 12 h, the assay solution was extracted with an equal volume of ethyl acetate and analyzed by a Shimadzu GC-17A gas chromatograph (GC) equipped with an electron capture ^{63}Ni detector (Shimadzu, Kyoto, Japan) and an Rtx-1 capillary column (30 m by 0.25 mm by 0.25 μm; Restek). The column temperature was increased from 100 to 260°C at a rate of 20°C/min, and the gas flow rate was 30 ml/min. During the period of incubation, UT26 completely degraded all four HCH isomers, while YO5 degraded only β-HCH (data not shown). 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 calculated using linear range in the first several hours of reaction. When W medium containing 17 μM β-HCH was used as the assay medium, UT26 and YO5 cells degraded β-HCH linearly during the first 6 h at a rate of 1.5×10^{-6} and 1.6×10^{-6} U/mg of cells, respectively. No apparent difference in activity levels between these two strains suggested that an enzyme other than LinA is responsible for the metabolism of β-HCH. This observation is consistent with LinA's recognition of the *trans* and diaxial pairs of chlorine and hydrogen (9, 23), which are not present in β-HCH. In an earlier report, no degradation of β-HCH by UT26 was observed (6). We, however, could detect the very low activity in this study because a high concentration of cells was used for the reaction.

In our search for a gene responsible for the degradation of β-HCH, *Pseudomonas putida* PpY101 strains harboring cosmids pKSR1 (4), pKSR401 (10), pKSR501 (12), and pKSM1920 (8), containing the *linA*, *linB*, *linC*, and *linRED* operons, respectively, were assayed for their potential to degrade β-HCH. To our surprise, the strain *P. putida* PpY101(pKSR401), containing the *linB* gene, showed β-HCH degradation activity (3.1×10^{-7} U/mg of cells in the first 3 h). The activity for β-HCH degradation was further assayed in two more strains. The strain *Escherichia coli* DH5α(pULBH6) expressing the His-tagged LinB protein (14) showed β-HCH degradation activity (1.1×10^{-6} U/mg of cells in the first 3 h). On the other hand, *S. paucimobilis* UT26DB, whose *linB* gene had been disrupted by insertion of the kanamycin cassette that originated from pUC4K (22), did not show the activity. These results clearly indicate that LinB has β-HCH degradation activity.

LinB is a haloalkane dehalogenase of the α/β-hydrolase family of enzymes with relatively broad substrate specificity (5, 13). It has been the subject of crystallographic (17, 21), kinetic (19), mutagenesis (2), and computational (18) studies. In the present study, we evaluated the β-HCH transformation by the

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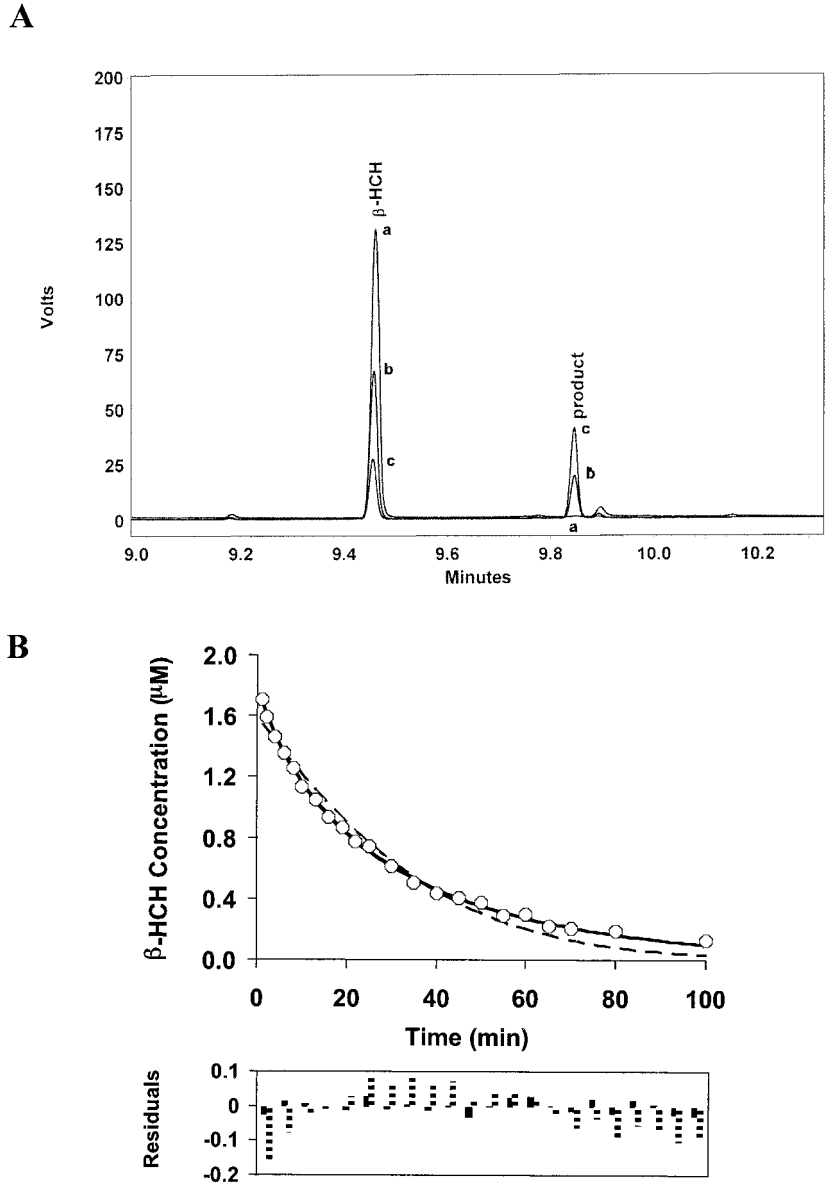

FIG. 1. Degradation of β-HCH by LinB. (A) GC analysis of the reaction mixture at 0 (a), 30 (b), and 80 (c) min. β-HCH (retention time = 9.45 min) was decreased with a simultaneous increase in reaction product M1 (retention time = 9.85 min). (B) Progress curve of β-HCH conversion by LinB. The dashed and solid lines (dashed and solid columns for residuals) represent the fit of Michaelis-Menten (R^2 = 0.9938) and product inhibition (R^2 = 0.9996) equations, respectively, to the kinetic data.

purified His-tagged LinB protein (14). The reaction was initiated by the addition of enzyme (20 mg/liter, final concentration) to the reaction mixture containing 2 μM β-HCH in 50 mM phosphate buffer (pH 7.5) at 37°C and was stopped by the addition of H_2SO_4 after 1 to 100 min. The hexane-extracted samples were analyzed on a GC equipped with a ZB-5 capillary column (30 m by 0.25 mm by 0.25 μm; Phenomenex) and an electron capture detector. The temperature program was isothermal at 60°C for 1 min, followed by an increase to 280°C at 20°C/min, and the carrier gas (He) flow rate was 1.8 ml/min. With the addition of LinB, β-HCH was decreased with a simultaneous increase in a metabolite (Fig. 1A), tentatively designated M1. The Michaelis-Menten and product inhibition equations were fitted by numerical integration to a progress curve obtained from the kinetic experiment by using MicroMath Scientist (MicroMath Research). The best fit was obtained using the product inhibition equation (Fig. 1B). This suggests that LinB can bind the product, which competes with the substrate for an enzyme active site. The unique values of k_{cat} and K_m could not be calculated from the data because of the limited solubility of β-HCH in water. Since the β-HCH concentration was lower than K_m, the substrate hydrolysis followed a first-order process. A first-order equation was fitted to the kinetic data (R^2 = 0.997) by using Origin 6.1 (OriginLab Corp.). The first-order rate constant for β-HCH decay, 0.041 ± 0.001 min^{-1}, and the k_{cat}/K_m ratio, 0.001 $s^{-1} \cdot \mu M^{-1}$, have been estimated. The k_{cat}/K_m ratio for β-HCH conversion by LinB is 2 orders of magnitude lower than that of the reaction of LinB with the best substrate 1-chlorohexane (k_{cat}/K_m = 0.16 $s^{-1} \cdot \mu M^{-1}$).

The mass spectrum of the product M1 was analyzed using a Trace MS 2000 mass spectrometer (Finnigan). The *m*/*z* 235, which was the heaviest ion found in the mass spectrum, is an even-electron ion and thus cannot be a molecular ion. The isotopic cluster at *m*/*z* 235 to 243 indicates four chlorine atoms, and the *m*/*z* 235-236 ratio suggests the presence of six carbon atoms in the molecule. The remaining 23 AMU suggest the presence of one oxygen atom and seven hydrogen atoms in the structure of the fragment. The isotopic cluster of lower-mass ion *m*/*z* 199, found in the spectrum, indicates three chlorine atoms in the molecule. The difference of 36 *m*/*z* between *m*/*z* 235 and *m*/*z* 199 is typical for chloroalkanes, indicating a loss of HCl. The same difference indicates the loss of HCl from *m*/*z* 199, forming *m*/*z* 163. In the second fragmentation route, the *m*/*z* 199 loses the oxygen and one carbon atom, forming the fragment *m*/*z* 170, which keeps the isotopic cluster of three chlorine atoms in the molecule. The ion *m*/*z* 170 further loses one chlorine (difference, *m*/*z* 35), and *m*/*z* 135 is found. The isotopic cluster of *m*/*z* 135 to 139 suggests the presence of two chlorine atoms in the fragment. The major peak at *m*/*z* 109 was formed by loss of two carbon atoms from the fragment ion *m*/*z* 135. The ion *m*/*z* 199 as well as *m*/*z* 156 contains three chlorine atoms. The difference of *m*/*z* 43 between these two ions also confirms the presence of one oxygen atom in the molecule. Because the carbon chain is closed in a circle and there is no indication of ions containing more than one oxygen atom, the most probable structure to account for this spectrum is 2,3,4,5,6-pentachlorocyclohexanol (PCHL) with *m*/*z* 270. The heaviest ion found in the spectrum *m*/*z* 235 was formed by breakaway of the first chlorine atom from the PCHL molecular ion.

In UT26, PCHL was not degraded even after its incubation for up to 2 days, indicating that the metabolism of β-HCH is restricted to the formation of this metabolite. PCHL has lower hydrophobicity and lower chemical stability than β-HCH, and the bacteria that degrade and utilize it may exist in the polluted environment, allowing the complete degradation of β-HCH by a combination of biological pathways.

In summary, it was concluded that, in the bacterial strain *S. paucimobilis* UT26, haloalkane dehalogenase LinB converts β-HCH to PCHL. This finding is extremely important, as it provides the first information regarding an enzyme to be used for metabolism of β-HCH, which is one of the most recalcitrant environmental pollutants. LinB is possibly also involved in the degradation of β-HCH in other reported bacteria (6, 20). Further experiments are necessary, however, to confirm the role of *linB* in *S. paucimobilis* strain B90, which seems to have stronger β-HCH degradation activity than UT26 (6).

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