

# Enantioselective Transformation of $\alpha$ -Hexachlorocyclohexane by the Dehydrochlorinases LinA1 and LinA2 from the Soil Bacterium *Sphingomonas paucimobilis* B90A

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*Sphingomonas paucimobilis* B90A contains two variants, LinA1 and LinA2, of a dehydrochlorinase that catalyzes the first and second steps in the metabolism of hexachlorocyclohexanes (R. Kumari, S. Subudhi, M. Suar, G. Dhingra, V. Raina, C. Dogra, S. Lal, J. R. van der Meer, C. Holliger, and R. Lal, Appl. Environ. Microbiol. 68:6021–6028, 2002). On the amino acid level, LinA1 and LinA2 were 88% identical to each other, and LinA2 was 100% identical to LinA of *S. paucimobilis* UT26. Incubation of chiral  $\alpha$ -hexachlorocyclohexane ( $\alpha$ -HCH) with *Escherichia coli* BL21 expressing functional LinA1 and LinA2 S-glutathione transferase fusion proteins showed that LinA1 preferentially converted the (+) enantiomer, whereas LinA2 preferred the (–) enantiomer. Concurrent formation and subsequent dissipation of  $\beta$ -pentachlorocyclohexene enantiomers was also observed in these experiments, indicating that there was enantioselective formation and/or dissipation of these enantiomers. LinA1 preferentially formed (3*S*,4*S*,5*R*,6*R*)-1,3,4,5,6-pentachlorocyclohexene, and LinA2 preferentially formed (3*R*,4*R*,5*S*,6*S*)-1,3,4,5,6-pentachlorocyclohexene. Because enantioselectivity was not observed in incubations with whole cells of *S. paucimobilis* B90A, we concluded that LinA1 and LinA2 are equally active in this organism. The enantioselective transformation of chiral  $\alpha$ -HCH by LinA1 and LinA2 provides the first evidence of the molecular basis for the changed enantiomer composition of  $\alpha$ -HCH in many natural environments. Enantioselective degradation may be one of the key processes determining enantiomer composition, especially when strains that contain only one of the *linA* genes, such as *S. paucimobilis* UT26, prevail.

The insecticidal properties of hexachlorocyclohexane (HCH) were discovered independently by Dupire and Thomas in the early 1940s (3, 21). As early as 1942, it was known that only the  $\gamma$  isomer of HCH was active insecticidally (21). Nevertheless, technical HCH, which typically consisted of 60 to 70%  $\alpha$ -HCH, 5 to 12%  $\beta$ -HCH, 10 to 15%  $\gamma$ -HCH, 6 to 10%  $\delta$ -HCH, and smaller amounts of other isomers and congeners (1, 7), became one of the most important insecticides. Although HCH is banned in most industrialized countries, the use of pure  $\gamma$ -HCH (lindane) continues in some developing countries.

The application of approximately 10 million tons of technical HCH between 1948 and 1997 (12) led to the release of approximately 6.5 million tons of  $\alpha$ -HCH into the environment. Thus,  $\alpha$ -HCH has become an important environmental contaminant that is frequently detected in environmental and biological samples (12, 13). As the only HCH isomer,  $\alpha$ -HCH is chiral and exists in two enantiomeric forms (optical isomers) (Fig. 1). In technical HCH,  $\alpha$ -HCH is present as a racemic (1:1) mixture of the two enantiomers (1). Enantiomer-specific analyses have revealed various enantiomer

compositions of  $\alpha$ -HCH in environmental and biological samples (5, 9, 10, 16). Molecular details of enantioselectivity are known for a limited selection of chiral pollutants, but not very much is known about the mechanisms of the enantioselective metabolism of  $\alpha$ -HCH or about the organisms and enzymes involved (17).

Two bacterial strains (*Sphingomonas paucimobilis* UT26 and *S. paucimobilis* B90A) which are able to degrade  $\gamma$ -HCH also degrade  $\alpha$ -HCH (6, 8, 20). Likewise, purified LinA from *S. paucimobilis* UT26, a dehydrochlorinase catalyzing the first step in the degradation pathway of  $\gamma$ -HCH, dehydrochlorinates not only  $\gamma$ -HCH but also  $\alpha$ -HCH,  $\delta$ -HCH,  $\gamma$ -pentachlorocyclohexene ( $\gamma$ -PCCH), and  $\alpha$ -PCCH, whereas  $\beta$ -HCH is not dehydrochlorinated (18). For  $\gamma$ -HCH, Trantirek et al. (23) elucidated the mechanism and the stereochemistry of the reactions catalyzed by LinA. In the first step,  $\gamma$ -HCH is enantioselectively transformed to (3*R*,4*S*,5*S*,6*R*)-1,3,4,5,6-PCCH, which is then converted to 1,2,4-trichlorobenzene (1,2,4-TCB) via the presumed but unstable intermediate (3*R*,6*R*)-1,3,4,6-tetrachlorocyclohexa-1,4-diene. When synthetic, racemic  $\gamma$ -PCCH was incubated with LinA, both enantiomers were converted, at different rates, giving rise to 1,2,4-TCB, as well as 1,2,3-TCB (23).

A closer look at the organization of the *lin* genes in strains UT26 and B90A revealed that these genes are present in both strains. In contrast to UT26, there are two copies of *linA* (*linA1* and *linA2*) and three copies of *linX* (*linX1*, *linX2*, and *linX3*) in

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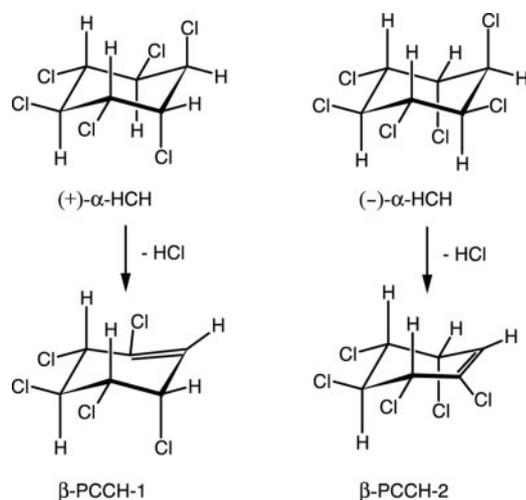


FIG. 1. Structures of  $\alpha$ -HCH and  $\beta$ -PCCH enantiomers. The thermodynamically most stable conformers (1) are shown. The absolute configuration of (+)- $\alpha$ -HCH is (+)- $\alpha$ -1*S*,2*R*,3*R*,4*S*,5*S*,6*S*-HCH (14).  $\beta$ -PCCH-1 and  $\beta$ -PCCH-2 are  $\beta$ -(3*S*,4*S*,5*R*6*R*)-1,3,4,5,6-PCCH and  $\beta$ -(3*R*,4*R*,5*S*,6*S*)-1,3,4,5,6-PCCH, respectively, and the number at the end of each designation indicates the order of elution on the enantioselective high-resolution gas chromatography column (see Fig. 2).

strain B90A (2, 22). The amino acid sequences of LinA1 and LinA2 are 88% identical to each other and 88% and 100% identical, respectively, to the amino acid sequence of LinA of *S. paucimobilis* strain UT26. Due to their similarity, *linA1* cannot be distinguished from *linA2* by hybridization (22). However, expression of *linA1* and *linA2* in *Escherichia coli* BL21 led to functional *S*-glutathione transferase (GST) fusion proteins that dehydrochlorinated  $\alpha$ -,  $\gamma$ -, and  $\delta$ -HCH but not  $\beta$ -HCH (11). Here we report on the enantioselective transformation of  $\alpha$ -HCH by LinA1 and LinA2 expressed as GST fusion proteins in *E. coli* BL21. Interestingly, LinA1 and LinA2 exhibited opposite stereoselectivities.

#### MATERIALS AND METHODS

**Source of HCH isomers.**  $\gamma$ -[ $^{13}\text{C}$ ]HCH, which was used as an internal standard, was obtained from Cambridge Isotope Laboratory (Cambridge, MA), courtesy of P. Schmid, EMPA Dübendorf, Dübendorf, Switzerland. Analytical grade  $\alpha$ -,  $\gamma$ -, and  $\delta$ -HCHs were obtained from Ehrenstorfer GmbH (Augsburg, Germany).

**Bacterial strains and plasmids.** *S. paucimobilis* B90A and *S. paucimobilis* UT26 were obtained from N. Sethunathan, Central Rice Research Institute, Cuttack, India (20), and Y. Nagata, Department of Biotechnology, University of Tokyo, Tokyo, Japan, respectively. *E. coli* BL21, a general host strain for overexpression of GST-fused plasmid vector pGEX-5X-3, was purchased from Amersham Pharmacia (United States).

**Construction of pLINEA1 and pLINEA2.** Specific expression plasmids were constructed with the *linA1* and *linA2* genes of *S. paucimobilis* B90A. The *linA1* and *linA2* genes were amplified from the B90A genome by PCR with primers 1

to 3 (Table 1). PCR amplification was performed with a Nugene thermocycler (Progene, Cambridge, United Kingdom) as described previously (11, 22). The amplified *linA1* and *linA2* genes were cloned into pGEX-5X-3 as described previously (11), and the resulting plasmids were designated pLINEA1 and pLINEA2, respectively.

**Selection of pLINEA1 and pLINEA2 and determination of their activities.** *E. coli* clones containing pLINEA1, and pLINEA2 were grown in LB medium containing 50  $\mu\text{g}/\text{ml}$  of ampicillin and 5  $\mu\text{g}/\text{ml}$  of  $\gamma$ -HCH or 5  $\mu\text{g}/\text{ml}$  of  $\alpha$ -HCH. Aliquots (200  $\mu\text{l}$ ) of each sample were removed, extracted with 400  $\mu\text{l}$  of hexane, and analyzed by gas chromatography (GC) as described previously (11). The clones that degraded  $\gamma$ -HCH and  $\alpha$ -HCH were selected, and sequences of cloned *linA* open reading frames were determined with an automated DNA sequencer (ABI PRISM model 377, version 3; Applied Biosystems, United States) in the Department of Biochemistry, South Campus, University of Delhi, Delhi, India.

**$\alpha$ -HCH transformation studies with pLINEA1, pLINEA2, and strain B90A.** For transformation studies with pLINEA1, pLINEA2, and strain B90A, cultures of each clone or strain were grown in 5 ml medium (for B90A, mineral salts medium with glucose at 30°C [11]; and for pLINEA1 and pLINEA2 in *E. coli* BL21, LB medium containing 50  $\mu\text{g}/\text{ml}$  of ampicillin at 37°C). From a culture grown overnight a 1-ml inoculum was transferred to 20 ml of either mineral salts medium containing 0.1% (wt/vol) glucose or LB medium containing  $\alpha$ - or  $\gamma$ -HCH (5  $\mu\text{g}/\text{ml}$ ). The cultures were incubated on a shaker at 180 rpm and 37°C. At regular 1-h intervals, 1 ml of culture was withdrawn from each sample, extracted with 1 ml of *n*-hexane, and fortified with 10  $\mu\text{l}$  of  $\gamma$ -[ $^{13}\text{C}$ ]HCH (6 ng/ $\mu\text{l}$  in nonane). Extraction of the sample was repeated with an additional 1 ml of hexane, and the combined extracts were dried over anhydrous sodium sulfate and reduced under a gentle stream of nitrogen to obtain 0.5 ml (final volume) for analysis.

**Enantioselective high-resolution gas chromatography analyses.** Aliquots (2  $\mu\text{l}$ ) of the extracts were analyzed by enantioselective gas chromatography-mass spectrometry (GC-MS), using a Finnigan Voyager quadrupole MS with electron impact ionization (70 eV, 200°C) and full-scan ( $m/z$  35 to 300; 0.4 s/scan; nominal mass resolution) or selected-ion monitoring conditions. The analytes were quantified in the selected-ion monitoring mode using the  $m/z$  180.9 ion for HCH and PCCH isomers,  $m/z$  186.9 for  $\gamma$ -[ $^{13}\text{C}$ ]HCH, and  $m/z$  179.9 for TCB. A 16-m PS086-permethylated  $\beta$ -cyclodextrin (PMCD) fused silica column (inside diameter, 0.35 mm; relative amount of PMCD, 10%; film thickness, 0.25  $\mu\text{m}$ ) was used. The chiral selector (PMCD) and the polysiloxane cophase were the same as those in previous studies (1, 15), and therefore, the same elution order for  $\alpha$ -HCH and  $\beta$ -PCCH enantiomers was expected for this column. The GC conditions were as follows: split/splitless injection (250°C, 80-s splitless time) and a temperature program consisting of 50°C for 2 min isothermally, an increase in the temperature at a rate of 20°C/min to 120°C, an increase at a rate of 3°C/min to 220°C, and 3 min isothermally at 220°C.

The amounts of HCH and PCCH isomers were determined from peak area ratios relative to  $\gamma$ -[ $^{13}\text{C}$ ]HCH. Due to a lack of quantitative standards for PCCH, quantitation of PCCH isomers was done by assuming that the response was the same as that for HCH at  $m/z$  180.9, which is reasonable considering the similarity of the mass spectra (see Fig. 3).

#### RESULTS AND DISCUSSION

**Structural considerations.** The absolute configurations of the  $\alpha$ -HCH enantiomers are known from X-ray crystallography (14) and are shown in Fig. 1 in their thermodynamically most stable conformations (chair forms, maximum number of chlorines in equatorial position). All HCH isomers may form chiral metabolites, such as tetra- and pentachlorocyclohexenes. The first step in the metabolism of HCH isomers is generally con-

TABLE 1. PCR primers used in this study

Primer	Sequence (5'-3') <sup>a</sup>	Added restriction site	Designation	GenBank accession no. of target gene
1	<u>GCGGATCCGCATGAGTGATCTAGACAGACTT</u>	BamHI	<i>linA1</i> and <i>linA2</i> sense	AY150579, AY150580
2	<u>GCCTCGAGTCACGATTTTTGCAACAGAGC</u>	XhoI	<i>linA1</i> antisense	AY150579
3	<u>GCCTCGAGTTATGCGCCGGACGGTGCGAAATG</u>	XhoI	<i>linA2</i> antisense	AY150580

<sup>a</sup> Added restriction sites are underlined.

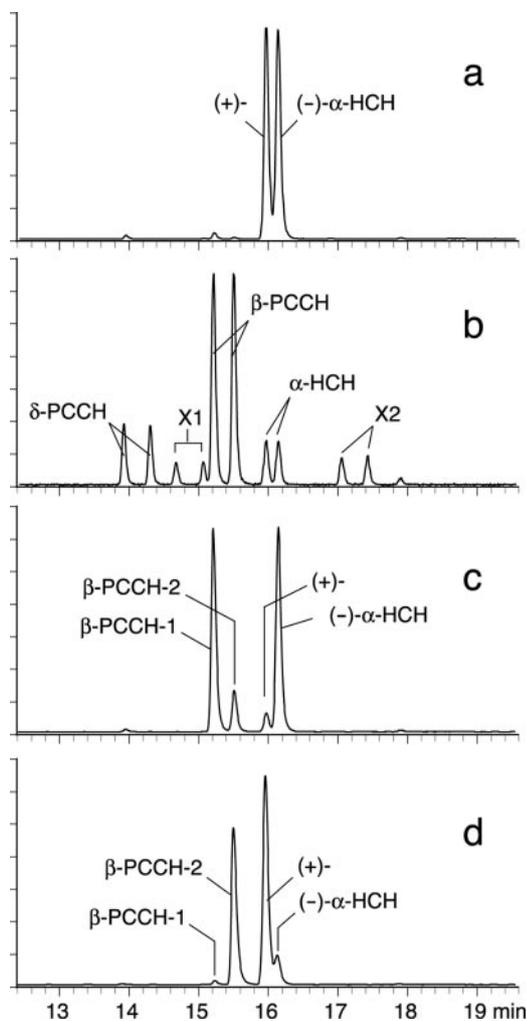


FIG. 2. Electron impact selected-ion monitoring chromatograms ( $m/z$  181). Separation was performed with an enantioselective PS086-PMCD high-resolution GC column, and detection was performed by monitoring the selected ion ( $m/z$  181) current normalized to the highest peak. (a) *rac*- $\alpha$ -HCH; (b) mixture of synthetic PCCHs prepared from *rac*- $\alpha$ -HCH as described by Buser and Müller (1); (c and d) *rac*- $\alpha$ -HCH incubated for 5 h with LinA1 and LinA2, respectively. Note the nonracemic composition of residual  $\alpha$ -HCH and of  $\beta$ -PCCH formed during incubation (c and d). For an explanation of  $\beta$ -PCCH-1 and  $\beta$ -PCCH-2 see the legend to Fig. 1.

sidered to be an HCl elimination (dehydrochlorination), which preferentially occurs with H and Cl atoms in axial positions at neighboring (vicinal) carbon atoms (antiperiplanar HCl elimination), leading to PCCHs. For each  $\alpha$ -HCH enantiomer there are theoretically two ways to eliminate HCl; both eliminations lead to the same  $\beta$ -PCCH enantiomer. In this way, HCl elimination from (+)- $\alpha$ -HCH leads to  $\beta$ -(3*S*,4*S*,5*R*,6*R*)-1,3,4,5,6-PCCH, and HCl elimination from (–)- $\alpha$ -HCH leads to  $\beta$ -(3*R*,4*R*,5*S*,6*S*)-1,3,4,5,6-PCCH (Fig. 1).

**Isomer and enantioselective analyses of  $\alpha$ -HCH and its metabolites.** The analytical conditions used were very similar to those used in a previous study (1). In particular, the same stationary phase (PS086) and the same chiral selector (PMCD) were used for enantioselective GC-MS. In this way similar selectivities with respect to elution sequence and enantiomer

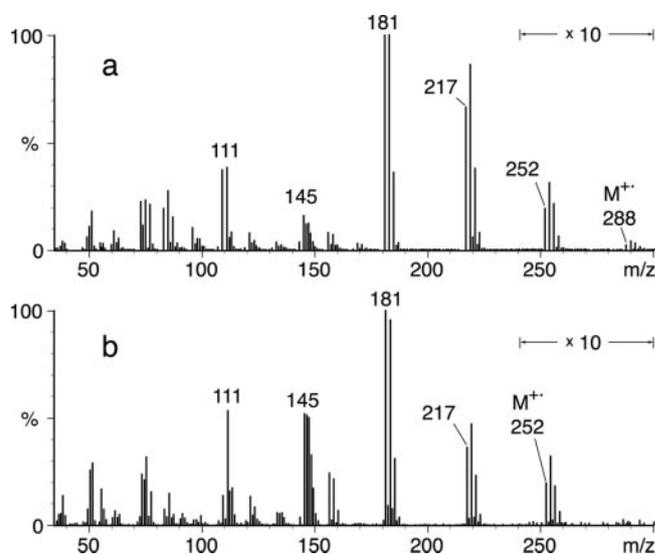


FIG. 3. Electron impact mass spectra of  $\alpha$ -HCH (a) and  $\beta$ -PCCH (b).

resolution of  $\alpha$ -HCH and PCCH enantiomers were obtained (Fig. 2 shows representative chromatograms). The resolution of  $\alpha$ -HCH and PCCHs is particularly important, because the electron impact mass spectra of these compounds are similar (Fig. 3).

Synthetic PCCHs, prepared from racemic  $\alpha$ -HCH in pyridine, were available for reference purposes from a previous study (1). In the mixture, the predominant PCCH was  $\beta$ -PCCH formed through preferred HCl elimination with H and Cl in axial positions. Besides  $\beta$ -PCCH, there were also smaller amounts of  $\delta$ -PCCH and two PCCHs with unknown configurations (X1 and X2), which were apparently formed through *cis* elimination and/or isomerization reactions (Fig. 2b) (1).

Figures 2c and d show representative chromatograms from incubations of *rac*- $\alpha$ -HCH with *E. coli* BL21 containing pLINEA1 and pLINEA2, respectively, after 5 h of incubation.  $\beta$ -PCCHs were formed as intermediates, whereas the other PCCHs present in the synthetic PCCH mixture were not formed. In addition, it is evident that the two  $\alpha$ -HCH enantiomers were depleted to different extents, indicating that there was an enantioselective degradation process. Interestingly, the enantioselectivities with LinA1 and LinA2 were opposite. As shown by the chromatograms in Fig. 2, incubation of *rac*- $\alpha$ -HCH with LinA1 (Fig. 2c) resulted in selective elimination of (+)- $\alpha$ -HCH and selective formation of (3*S*,4*S*,5*R*,6*R*)-1,3,4,5,6-PCCH, which eluted earlier and was designated “ $\beta$ -PCCH-1.” In contrast, the incubation with LinA2 (Fig. 2d) showed that there was selective elimination of (–)- $\alpha$ -HCH with selective formation of  $\beta$ -(3*R*,4*R*,5*S*,6*S*)-1,3,4,5,6-PCCH, which eluted second and was designated “ $\beta$ -PCCH-2.” Dissipation of *rac*- $\alpha$ -HCH and concurrent formation and dissipation of  $\beta$ -PCCH in incubations with *S. paucimobilis* B90A, on the other hand, showed no apparent enantioselectivity (chromatograms not shown).

1,2,4-TCB was found to be a further metabolite of  $\alpha$ -HCH in the incubation experiments (chromatograms not shown). As the experiments were carried out in open vessels, some loss

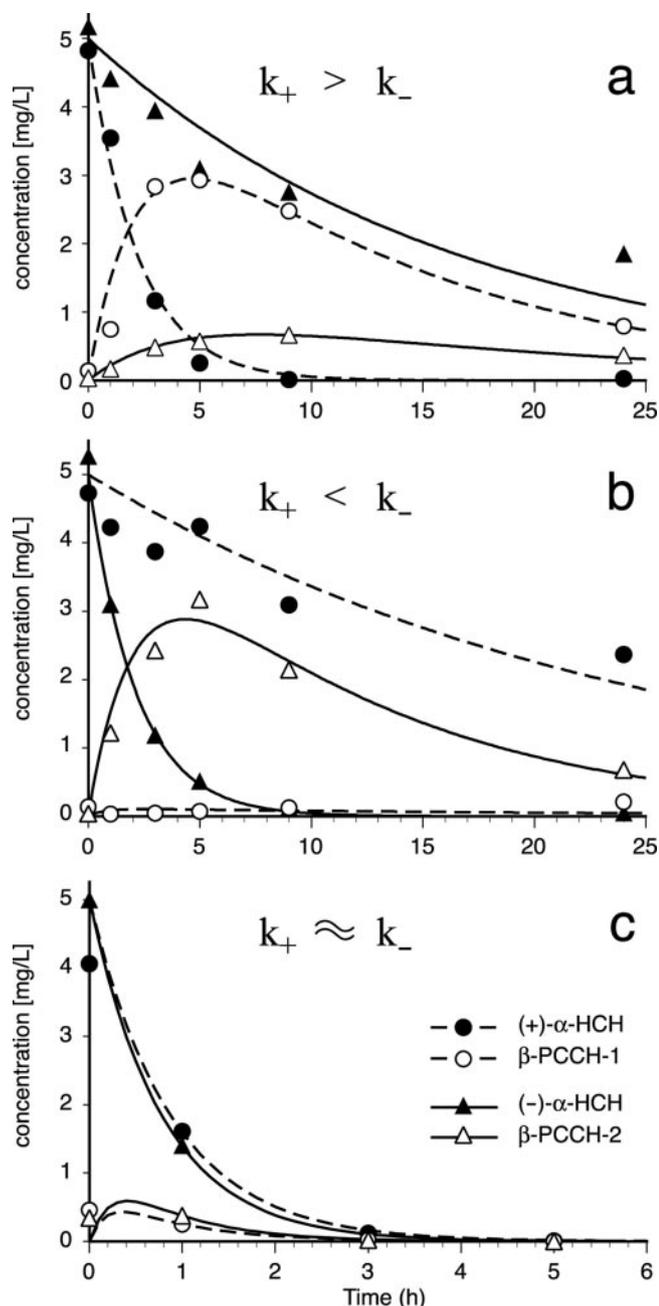


FIG. 4. Degradation of racemic  $\alpha$ -HCH incubated with *E. coli* BL21 overproducing GST-fused LinA1 (a) and LinA2 (b) and with *S. paucimobilis* B90A (c). Note the opposite enantioselectivities for the incubations with LinA1 and LinA2 and the lack of enantioselectivity for the incubation with *S. paucimobilis* B90A. The curves were modeled by using the best-fit rate constants listed in Table 2.

of the volatile compound TCB, in contrast to the nonvolatile compounds HCCHs and PCCHs, could not be prevented. Therefore, the concentration of TCB was not quantitatively evaluated. Other products, such as additional PCCHs and tetrachlorocyclohexadienes, were not detected by GC-MS with the experimental setup used in this study.

**Kinetics of  $\alpha$ -HCH degradation.** Degradation of  $\alpha$ -HCH proceeded fairly rapidly under the conditions selected in the

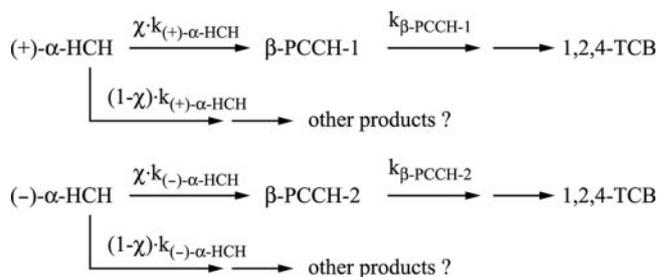


FIG. 5. Degradation scheme for setting up the first-order reaction model with the AQUASIM software (19).

presence of LinA1, LinA2, and *S. paucimobilis* B90A, and there was extensive dissipation of the parent compound within 24 h (Fig. 4). The kinetic plots also clearly indicate that dissipation of  $\alpha$ -HCH in the presence of LinA1 and LinA2 was enantioselective, whereas in the presence of *S. paucimobilis* B90A no apparent enantioselectivity was observed. Concurrent formation and subsequent dissipation of  $\beta$ -PCCH were also observed in all experiments, although in the incubations with *S. paucimobilis* B90A the levels of both  $\beta$ -PCCH enantiomers remained low.

We evaluated the kinetics of  $\alpha$ -HCH dissipation and  $\beta$ -PCCH formation and dissipation with the modeling software AQUASIM (19), assuming first-order reactions and setting up the model as shown in Fig. 5. The corresponding rate coefficients are shown in Table 2, and the fitted curves are plotted in Fig. 4 along with measured concentrations. In principle, HCH degradation products other than  $\beta$ -PCCH are possible from degradation of  $\alpha$ -HCH, even though such products were not evident from the full-scan GC-MS chromatograms. Therefore, a factor ( $\chi$ ) correcting for the unknown stoichiometry was introduced into the fitting procedure. The best fit of the experimental data was obtained with a  $\chi$  value 85%, suggesting that  $\beta$ -PCCH is indeed the predominant product of primary degradation of  $\alpha$ -HCH.

**Enantioselectivity.** The excess of the rate for the (+) enantiomer over the (-) enantiomer in a particular experiment can be defined as a measure of the enantioselectivity (ES) of the reaction (15) according to the following reac-

TABLE 2. Rate coefficients and enantioselectivity of transformation derived from the incubation experiments with *E. coli* BL21 expressing GST-fused LinA1 and LinA2 and with whole cells of *S. paucimobilis* B90A<sup>a</sup>

Rate coefficient or ES	Incubation with:		
	<i>E. coli</i> BL21 (pLINEA1)	<i>E. coli</i> BL21 (pLINEA2)	<i>S. paucimobilis</i> B90A
Transformation of $\alpha$ -HCH			
$k_+$ ( $\text{h}^{-1}$ )	$0.45 \pm 0.03$	$0.040 \pm 0.006$	$1.1 \pm 0.2$
$k_-$ ( $\text{h}^{-1}$ )	$0.060 \pm 0.010$	$0.47 \pm 0.06$	$1.2 \pm 0.2$
ES	$0.76 \pm 0.08$	$-0.84 \pm 0.15$	$-0.09 \pm 0.13$
Transformation of $\beta$ -PCCH			
$k_{\beta\text{-PCCH-1}}$ ( $\text{h}^{-1}$ )	$0.078 \pm 0.009$	$1.4 \pm 0.9$	$4.5 \pm 2.8$
$k_{\beta\text{-PCCH-2}}$ ( $\text{h}^{-1}$ )	$0.24 \pm 0.08$	$0.089 \pm 0.008$	$3.2 \pm 1.5$

<sup>a</sup> The rate constants were obtained by fitting the data to the reaction scheme shown in Fig. 5.

tion:  $ES = (k_+ - k_-)/(k_+ + k_-)$ , where  $k_+$  and  $k_-$  are the first-order rate constants for the transformation of the (+) and (-) enantiomers of  $\alpha$ -HCH, respectively. The equation for ES is analogous to the equation used to calculate enantiomeric excess (4). The ES values defined in this way range from -1 to 1, and they are zero if a reaction is nonenantioselective ( $k_+ = k_-$ ). An ES value of 1 means that the reaction is fully enantioselective with respect to the (+) enantiomer ( $k_- = 0$ ), whereas an ES value of -1 means that the reaction is fully enantioselective with respect to the (-) enantiomer ( $k_+ = 0$ ). Analysis of the fitted rate constants showed that although whole-cell incubations with strain B90A did not show significant enantioselective transformation of  $\alpha$ -HCH, incubations with overexpressed LinA1 and LinA2 GST fusion proteins showed strong opposite enantioselectivities for  $\alpha$ -HCH with an ES value of 0.76 for LinA1 and an ES value of -0.81 for LinA2, respectively (Table 2).

Enantioselective transformation of  $\gamma$ -HCH and  $\gamma$ -PCCH by LinA, which is identical to LinA2 at the amino acid level, has been reported (23). Apparently, LinA is able to specifically differentiate the enantiotopic faces formed by the H and Cl in axial positions and to form a single PCCH enantiomer during enzymatic dehydrochlorination of  $\gamma$ -HCH (23). Here we show for the first time that LinA1 and LinA2/LinA exhibit stereoselectivity toward  $\alpha$ -HCH enantiomers. Furthermore, each enzyme preferentially converting one of the  $\alpha$ -HCH enantiomers formed only one of the  $\beta$ -PCCH enantiomers (Table 2).

LinA1 differs from LinA2 by 18 amino acids, 6 of which are located in the C-terminal region. Additionally, *linA1* encodes 154 amino acids, whereas *linA2* encodes 156 amino acids. At the moment, it is not known which differences in amino acid composition are responsible for the opposite stereoselectivities of the two enzymes, but future work will aim at elucidation of the amino acids responsible. Since both the *linA1* and *linA2* genes were present and expressed in *S. paucimobilis* B90A, nonenantioselective transformations were not a surprise for incubations with whole cells of strain B90A. Although there has been no other report yet, it might be expected that many bacterial strains have evolved with *linA1*- or *linA2*-like genes. Such strains may eventually be responsible for causing the changed enantiomer compositions of  $\alpha$ -HCH in different environmental compartments (5). Future work will also aim at elucidation of structure activity-relationships with LinA1 and LinA2.

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