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Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26

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γ-Hexachlorocyclohexane (γ-HCH; also called BHC or lindane) is one of the highly chlorinated pesticides which can cause serious environmental problems. *Sphingomonas paucimobilis* UT26 degrades γ-HCH under aerobic conditions. The unique degradation pathway of γ-HCH in UT26 is revealed. In the upstream pathway, γ-HCH is transformed to 2,5-dichlorohydroquinone (2,5-DCHQ) by two different dehalogenases (LinA and LinB) and one dehydrogenase (LinC) which are expressed constitutively. In the downstream pathway, 2,5-DCHQ is reductively dehalogenated, and then ring-cleaved by enzymes (LinD and LinE, respectively) whose expressions are regulated. We have cloned and sequenced five structural genes (*linA*, *linB*, *linC*, *linD*, and *linE*) directly involved in this degradation pathway. The *linD* and *linE* genes form an operon, and its expression is positively regulated by the LysR-type transcriptional regulator (LinR). The genes *linA*, *linB*, and *linC* are constitutively expressed, and are present separately from each other in the UT26 genome. Cell fractionation analysis, Western blotting, and immuno electron microscopy revealed that LinA and LinB are localized in the periplasmic space of UT26.

Keywords: *Sphingomonas*; γ-hexachlorocyclohexane; biodegradation; dehalogenase

Introduction

γ-Hexachlorocyclohexane (γ-HCH; also called BHC or lindane) is a halogenated organic insecticide which has been used worldwide. Because of its toxicity and persistence in soil, many countries have prohibited the use of γ-HCH. However, many contaminated sites still remain throughout the world. Moreover, some countries are presently using γ-HCH for economic reasons, and thus new sites are continuously being contaminated by γ-HCH and its stereoisomers (a technical mixture of HCH consists of α, β, γ, and δ isomers) [4,20].

γ-HCH is degraded rapidly under anaerobic conditions, but it is considered extremely persistent under aerobic conditions. *Sphingomonas* (formerly *Pseudomonas*) *paucimobilis* SS86 was isolated from an experimental field to which γ-HCH had been applied once a year for 12 years [68]. We isolated a mutant of *S. paucimobilis* SS86, named UT26, that has nalidixic acid resistance as a genetic marker [15]. Strains SS86 and UT26 utilize γ-HCH as a sole source of carbon and energy under aerobic conditions.

The degradation pathways of γ-HCH are of interest because of its structural features: a cyclohexane skeleton and a highly chlorinated state. General information for degradation of HCH by microorganisms was reviewed in another article [23]. In this article, we focus on the enzymes and genes involved in the degradation pathway of γ-HCH in *S. paucimobilis* UT26. Some parts of this article were previously reviewed [47].

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Degradation pathway of γ-HCH in UT26

The degradation pathway of γ-HCH in strain UT26 is shown in Figure 1. Because γ-HCH has six chlorine atoms per molecule, dechlorination is a very significant step in its degradation. In fact, we have shown that three different types of dechlorination reactions are sequentially involved in the degradation of γ-HCH by *S. paucimobilis* UT26. The first reaction is dehydrochlorination of γ-HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via γ-pentachlorocyclohexene (γ-PCCH) [16]. The second reaction is hydrolytic dechlorination of 1,4-TCDN to 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) [43]. The third reaction is reductive dechlorination of 2,5-dichlorohydroquinone (2,5-DCHQ), which is produced from 2,5-DDOL by a dehydrogenase (LinC) [45], to hydroquinone (HQ) via chlorohydroquinone (CHQ) [37].

Because 2,5-DCHQ is mineralized by strain UT26 [41], the pathway via 2,5-DCHQ is considered to be the assimilation pathway of γ-HCH in UT26. Recently, we reported the degradation pathway of 2,5-DCHQ in strain UT26 (K Miyauchi, Y Nagata and M Takagi, unpublished data). HQ, which is produced from 2,5-DCHQ by LinD is ring-cleaved by a novel type of dioxygenase (LinE) to γ-hydroxymuconic semialdehyde (γ-HMSA). However, the pathway from CHQ to HQ seems not to be the major pathway in UT26, since CHQ is a better substrate for LinE than for LinD. As a result, most of the CHQ is directly ring-cleaved by LinE to acylchloride, although we have directly detected only maleylacetate and β-ketoadipate. β-ketoadipate is expected to be easily utilized in strain UT26.

Through this series of reactions, two dead-end products, 1,2,4-trichlorobenzene (1,2,4-TCB) and 2,5-dichlorophenol

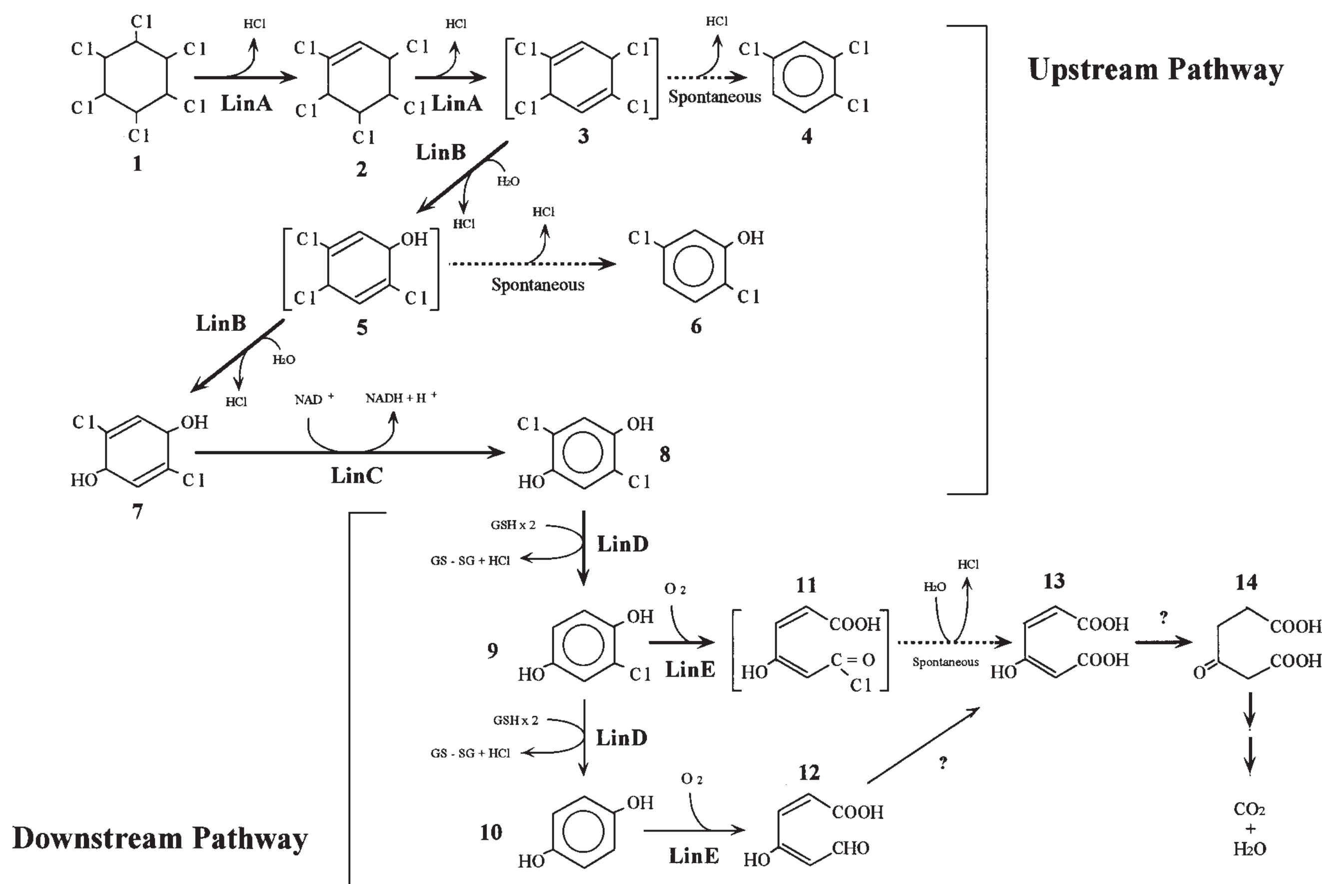

Figure 1 Proposed degradation pathway of γ-HCH in *Sphingomonas paucimobilis* UT26. Compounds: 1, γ-hexachlorocyclohexane (γ-HCH, γ-BHC, lindane); 2, γ-pentachlorocyclohexene; 3, 1,3,4,6-tetrachloro-1,4-cyclohexadiene; 4, 1,2,4-trichlorobenzene; 5, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 6, 2,5-dichlorophenol; 7, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 8, 2,5-dichlorohydroquinone; 9, chlorohydroquinone; 10, hydroquinone; 11, acylchloride; 12, γ-hydroxymuconic semialdehyde (γ-HMSA); 13, maleylacetate; 14, β-ketoadipate.

(2,5-DCP), are produced from the unstable intermediates 1,4-TCDN and 2,4,5-DNOL, respectively [40–42]. We have not yet detected the degradation activity of γ-HMSA by strain UT26. γ-HMSA may also be a dead-end product.

In strain UT26, the activities of LinA, LinB, and LinC are constitutively expressed [47], while those of LinD and LinE are inducibly expressed [46]. The degradation pathway of γ-HCH to 2,5-DCHQ was named the upstream pathway, and that of 2,5-DCHQ was named the downstream pathway (Figure 1).

Genes involved in γ-HCH degradation

The broad-host-range cosmid vector pKS13 [28] was used for the construction of a strain UT26 gene library in *Escherichia coli* HB101. Each clone of the *E. coli* library was mobilized into *Pseudomonas putida* PpY101 by triparental mating [28]. The resultant library in *P. putida* was screened for γ-HCH and its degradation metabolites by gas chromatography with an electron capture detector.

We have cloned five structural genes and one regulatory gene involved in degradation of γ-HCH in strain UT26 (Table 1). The *linA* gene encodes γ-HCH dehydrochlorinase (LinA), which is responsible for the conversion of γ-HCH to 1,4-TCDN via γ-PCCH [16,17]. The *linB* gene encodes 1,4-TCDN halidohydrolase (LinB), which is responsible for the conversion of 1,4-TCDN to 2,5-DDOL [43]. The *linC* gene encodes 2,5-DDOL dehydrogenase (LinC), which is responsible for the conversion of 2,5-DDOL to 2,5-DCHQ [45]. The *linD* gene encodes 2,5-DCHQ reductive dechlorinase (LinD), which is responsible for conversion of 2,5-DCHQ to HQ [37]. Recently, we cloned and sequenced a new gene (*linE*), which encodes the ring-cleavage oxygenase (LinE) which is responsible for conversion of CHQ to acylchloride and of HQ to γ-HMSA (K Miyauchi, Y Nagata and M Takagi, unpublished data). The *linA*, *linB*, and *linC* genes are constitutively expressed [47], while the *linD* and *linE* genes are inducible in strain UT26 ([37]; K Miyauchi, Y Nagata and M Takagi, unpublished data). The *linD* and *linE* genes form an operon with other open reading frames (Figure 2) whose functions are still unknown. Expression of *linD* and *linE* is regulated by the LysR-type transcriptional regulator (LinR) whose gene is located in the upstream region of the *linE* gene (Figure 2).

Four plasmids were detected in the CsCl-purified preparation from strain UT26 [16]. None of six genes for γ-HCH degradation hybridized with these four plasmids, suggesting that all of these genes are located on chromosomal DNA. However, the possibility of locating them on an unrecoverable plasmid, such as a megaplasmid or linear plasmid, cannot be excluded. The spontaneous mutants lacking

Table 1 Six genes involved in the γ-HCH degradation in *Sphingomonas paucimobilis* UT26

Gene	Nucleotide (bp)	Amino acid residue	Molecular mass (kD)	G+C content (%)	Function	Expression in UT26	Reference
linA	468	156	17.3	53.9	Dehydrochlorinase	Constitutive	[16]
linB	888	296	33.1	62.5	Halidohydrolase	Constitutive	[43]
linC	750	250	25.6	64.3	Dehydrogenase	Constitutive	[45]
linD	1038	346	38.4	61.0	Reductive dechlorinase	Inducible	[37]
linE	963	321	36.0	60.1	Ring-cleavage oxygenase	Inducible	Miyauchi, Nagata and Takagi,
linR	909	303	33.6	61.3	LysR-type transcriptional regulator for *linD* and *linE*	?	unpublished data

Name	Number of nucleotide (bp)	Number of amino acid	Molecular weight (kDa)	G + C content (%)	Function / Homologue (acc. no [a]) (identity / similarity)
linD	1038	346	38.4	60.8	**2,5-dichlorohydroquinone reductive dehalogenase**
linE	963	321	36.0	60.1	**(chloro) hydroquinone 1,2-dioxygenase**
linR	909	303	33.6	61.3	**LysR-type transcriptional regulator**
orf2	798	266	27.6	62.0	unknown / β-ketoadipate enol-lactone hydrolase (AF009224-17) (22% / 42%)
orf3	714	238	25.5	61.9	unknown / carboxyl esterase (D90904-70) (21% / 37%)
orf4	639	213	22.9	60.9	unknown / 5-chloro-1,2,4-THB dechlorinase (U19883-5) (13% / 29%)
orf5	963	321	35.1	59.8	unknown / vanillate demethylase reductase subunit (M22077-2) (53% / 70%)
orf6	(816) [b]	(272) [b]	—	(61.0) [b]	unknown / AraC / XylS family transcriptional regulator (L02356-2) (19% / 34%) [b]
orf7	915	305	34.3	57.9	unknown / vanillate demethylase oxygenase subunit (Y11521-1) (28% / 46%)
orf8	2544	848	91.1	59.5	unknown / pesticin receptor (Z35106-1) (20% / 35%)

[a] Accession number of DAD database.
[b] Note that the open reading frame does not terminate within the sequenced region.

Figure 2 Gene organization of the operon containing *linD*, *linE*, and their flanking regions. Size and direction of open reading frames are shown by arrows.

the whole *linA* or *linC* gene are easily isolated, indicating that these two genes are located on the relatively unstable DNA region.

The G+C content of the *linA* gene is considerably lower than that of the other five genes (*linB*, *linC*, *linD*, *linE* and *linR*) (Table 1) and the genomic DNA of the *S. paucimobilis* strains (65%) [57], suggesting that the *linA* gene may have originated from another organism with a lower G+C content. As described above, *linD* and *linE* form an operon. The *linA*, *linB*, and *linC* appear to locate separately from each other in the strain UT26 genome; at least these three genes are not organized as an operon. Considering the fact that genes involved in catabolic pathways usually form operons in prokaryotic cells [73], the genes involved in the upstream pathway of γ-HCH degradation in strain UT26 seem to be an exception. Similar cases were reported with genes for 2,4-dinitrotoluene degradation by *Pseudomonas* sp DNT [70] and for pentachlorophenol degradation by *Sphingomonas chlorophenolica* strain ATCC 39723 [55,56]. In these cases (including that of strain UT26), it is suggested that genes which take part in other function(s) may be involved. Recently, it was revealed that the genes in *Sphingomonas* necessary for degradation of one type of aromatic compound are distributed into multiple operons that also possess genes for the degradation of other aromatic compounds [1,61,82]. It is proposed that members of the genus *Sphingomonas* have a less well evolved and regulated but more dynamic genetic organization than organisms such as *Pseudomonas* species [1].

In the following sections, properties of the genes and enzymes which are involved in γ-HCH degradation by strain UT26 are discussed in detail.

Enzymes for the upstream pathway

γ-HCH dehydrochlorinase (LinA)

A database search failed to find any significantly homologous sequences to the *linA* gene. Southern blot analyses

using the *linA* gene as a probe against total DNAs of several related strains revealed no homologous sequence [16]. The origin of the *linA* gene is of great interest, but is still unknown. Recently, genes homologous to *linA* were cloned independently by two groups in France and India. Thomas *et al* [71] isolated a *linA*-like gene by the PCR technique from a newly isolated γ-HCH-degrading bacterium. The gene they isolated was identical to the *linA* gene that we cloned. However, the gene that Lal *et al* cloned from *Sphingomonas paucimobilis*, which is the same strain reported by Sahu *et al* [64], is partly different from ours (R Lal, personal communication). The deduced amino acid sequences of our LinA and Lal's exhibit 90.4% identity. Most of the differences are found at the C-terminal region. The evolutionary relationship between these genes remains to be elucidated.

The *linA* gene was highly expressed in recombinant *E. coli* cells, and the gene product (LinA) was purified to homogeneity [44]. In addition to γ-HCH and γ-PCCH, α- and δ-isomers of HCH and α-PCCH were also dehydrochlorinated by LinA; however, β-HCH was not [44]. These results are consistent with those obtained using resting cells of strain UT26 [40]. It is suggested that dehydrochlorination by LinA occurs stereoselectively at a *trans* and diaxial pair of hydrogen and chlorine [40]. The other chlorinated compounds tested were not dehydrochlorinated by the purified enzyme [44], indicating that the substrate specificity of LinA is narrow.

Dehydrochlorinase is an enzyme which eliminates HCl from the substrate molecule, leading to the formation of a double bond [10,22]. The properties of three dehydrochlorinases (including LinA) have so far been reported. A eukaryotic dehydrochlorinase isolated from *Musca domestica* catalyzes the monodehydrochlorination of 1,1,1-trichloro-2,2-bis (*p*-chloro-phenyl) ethane (DDT) [7,34]. 3-Chloro-D-alanine dehydrochlorinase was isolated from *P. putida* [39]. LinA is, however, very different from these two other dehydrochlorinases. DDT dehydrochlorinase and 3-chloro-D-alanine dehydrochlorinase require glutathione (GSH) and pyridoxal 5′-phosphate (PLP), respectively, for their activities, while LinA does not require any cofactors. Purified LinA did not show glutathione *S*-transferase (GST) or DDT dehydrochlorinase activity in the presence of glutathione. Thus, it seems most likely that LinA is not a GST-type enzyme. LinA is thought to be a unique dehydrochlorinase, and its mechanism of dehydrochlorination is of great interest. Recently Murzin proposed that LinA is a member of a novel structural superfamily of enzymes, and predicted a 3D structure (AG Murzin, personal communication). This superfamily [38] contains four proteins, scytalone dehydratase, steroid Δ-isomerase, nuclear transport factor-2, and the β-subunit of naphthalene dioxygenase, known structures with different enzymatic activities. We are now trying to elucidate the reaction mechanism of LinA according to his model.

The LinA activity is expressed in strain UT26 grown with no inducer [15]. A sequence (5′-CAGAC-GAAGCTAAATAT-3′) partially homologous to the consensus sequence of the constitutive promoters in *P. putida* (5′-AA-AAATG-TAAATAT-3′) [18] was located between 112 and 127 bp upstream of the initiation codon of the *linA* gene. Northern blotting analysis of total RNA of strain UT26 using the *linA* gene as a probe revealed one major band of around 690 nucleotides (nt) (Y Nagata, R Imai and M Takagi, unpublished data). The size of the mRNA estimated from the positions of the putative promoter and the terminator sequences was approximately 630 nt [16], and was similar to that of the observed transcript. Primer extension analysis revealed that the adenine at −97 and the thymine at −93 were the initiation sites of the transcription (Y Nagata, R Imai and M Takagi, unpublished data). These results strongly suggest that the sequence homologous to the consensus sequence of the constitutive promoters in *P. putida* is functional for constitutive expression of the *linA* gene in *S. paucimobilis* UT26, and that the *linA* gene is transcribed in a mono-cistronic manner. Furthermore, the G+C contents of the regions upstream and downstream of the putative *linA* promoter and terminator, respectively, are higher than those of the *linA* gene and its flanking regions (Figure 3). It is speculated that the *linA* gene including its promoter and terminator was introduced into the genome of strain UT26 by transfer from another organism with a low G+C content during evolution of this organism in soil.

1,4-TCDN halidohydrolase (LinB)

The deduced amino acid sequence of LinB shows significant similarity to three types of α/β-hydrolase fold enzymes [54], haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 [21], haloacetate dehalogenase (DehH1) from *Moraxella* sp B [25], and serine hydrolases represented by 2-hydroxymuconic semialdehyde hydrolase (DmpD) from *Pseudomonas* sp CF600 [51]. The identities between these enzymes and LinB were 29.3%, 22.6%, and 20.5%, respectively. Renilla-luciferin 2-monooxygenase (LUCI) from *Renilla reniformis* [35] showed the highest identity to LinB (41.2%), but both seem not to be monooxygenases. Conversely, LUCI is considered to be a member of the α/β-hydrolase family, judging from the deduced amino acid sequence. LinB also showed significant similarity with part of the epoxide hydrolases from eukaryotic cells [2,12,30]. A novel human serine hydrolase, which showed similarity with bacterial serine hydrolase (BphD, DmpD, TodF, and XylF) has been reported [59]. Evolutionary and functional relationships of these bacterial and eukaryotic hydrolases require further study.

The deduced amino acid sequence of LinB showed the highest level of similarity to haloalkane dehalogenase (DhlA) from *X. autotrophicus* GJ10 [21], suggesting that LinB belongs to the family of haloalkane dehalogenase enzymes catalyzing dehalogenation by a hydrolytic mechanism [43]. We have shown that LinB has broader specificity than Dh1A. For example, 1-chlorodecane and 2-chlorobutane, which are poor substrates for DhlA [27], were good substrates for resting recombinant *E. coli* cells overproducing LinB [43]. Furthermore, the *linB* gene from *S. paucimobilis* UT26 was highly expressed in *E. coli*, and the LinB was purified to homogeneity and characterized [48]. Principal component analysis of substrate activities of various haloalkane dehalogenases suggested that LinB probably constitutes a new substrate specificity class within this group of enzymes [48]. It would be useful to analyze the reaction mechanism of LinB in order to better under-

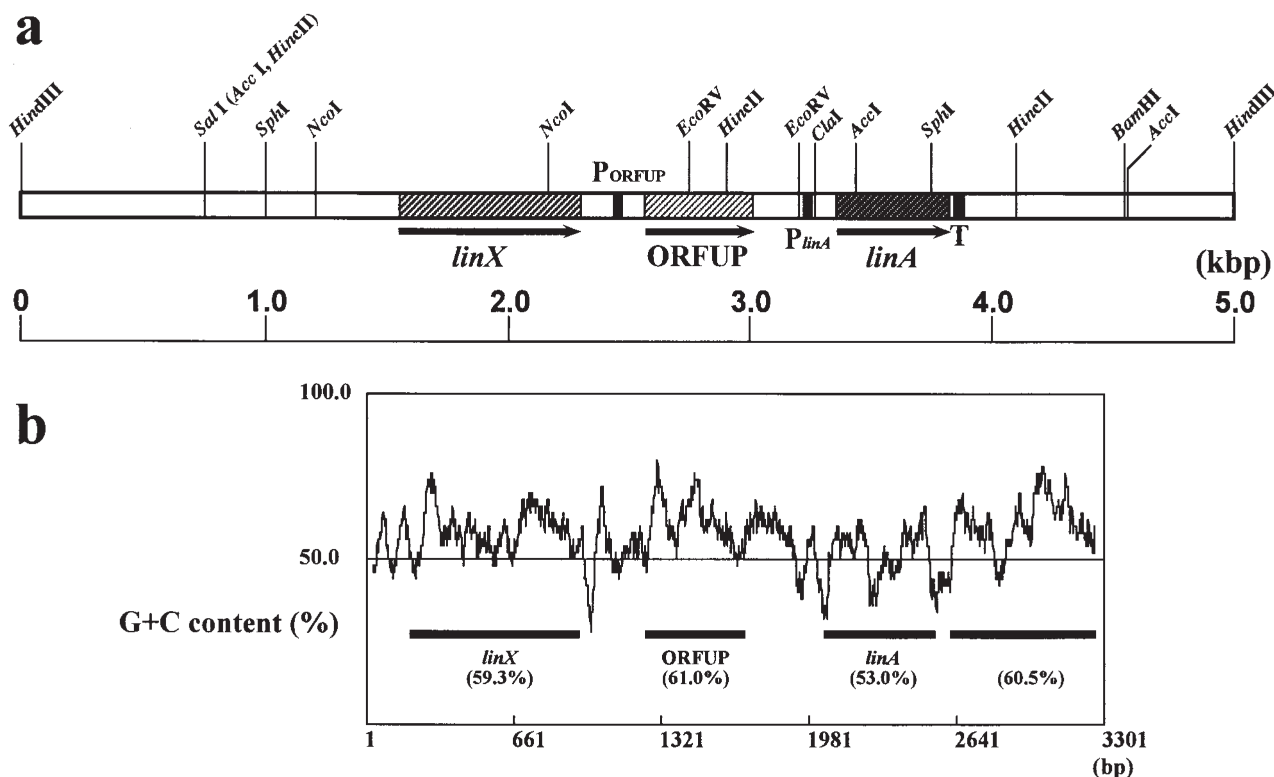

Figure 3 Restriction map and G+C content of the *linA* gene and its flanking regions. (a) Restriction map of the 5-kb *Hin*dIII fragment containing the *linA* gene. The location and direction of *linX*, ORFUP, and *linA* are indicated by the arrows under the restriction map. P_{ORFUP} and P_{linA} indicate the putative promoter sequences (Y Nagata, R Imai and M Takagi, unpublished data). T at the end of the *linA* gene indicates the proposed terminator nucleotide [16]. (b) G+C content distribution diagram of *linA* and its flanking regions in *S. paucimobilis* UT26. The G+C content distribution was visualized using GENETYX-MAC software (version 8.0; Software Development Co, Tokyo, Japan). Average span was 50. The G+C content of the *linA* gene is obviously lower than that of its neighboring regions.

stand the structure-function relationships within halidohydrolases. As described above, LinB is believed to belong to the family of α/β-hydrolases which employ a catalytic triad, ie nucleophile-histidine-acid, during the catalytic reaction [54]. The position of the catalytic triad within the sequence of LinB was probed by site-directed mutagenesis. The catalytic triad residues of the haloalkane dehalogenase LinB are proposed to be D108, H272 and E132 [14]. For further analysis, we determined the three-dimensional structure of LinB. The purified LinB was crystallized using the hanging-drop vapour-diffusion method [69]. The crystals diffract to at least 1.60 using synchrotron X-ray under cryogenic (100 K) conditions [69]. Recently, the structure of LinB was solved (J Marek, J Vevodov, I Smatanov, LA Svensson, J Newman, Y Nagata, J Damborsky and M Takagi, unpublished data) by molecular replacement with dehalogenase (DhaA) from *Rhodococcus* [32].

2,5-DDOL dehydrogenase (LinC)

The deduced amino acid sequence of LinC showed significant similarity to members of the short-chain alcohol dehydrogenase superfamily [24,50,58]. It is thought that there are two highly conserved regions of these enzymes [50,58]. The first conserved region is located at the N-terminus. The predicted structures of several dehydrogenases indicate that this region consists of alternating regions of β-sheet and α-helix (β-α-β) and that this region has been proposed to be part of the NAD^+ binding site. The second homologous region can be seen around positions 150 and 154 of the consensus sequence [50]. Two amino acids, tyrosine at position 150 and lysine at position 154, are highly conserved among the members of the short-chain alcohol dehydrogenase family. The tyrosine residue is conserved in all of them, while the lysine residue is not. Ensor and Tai demonstrated that substitution of the tyrosine by alanine abolished the 15-hydroxyprostaglandin dehydrogenase activity [9]. In the deduced amino acid sequence of LinC, the amino acid residues mentioned above are highly conserved, suggesting that LinC has the activity of NAD^+-dependent dehydrogenation.

Members of the short-chain alcohol dehydrogenase family are distributed from bacteria to humans. The question then arises concerning the substrate specificities of these enzymes. In preliminary studies, we tested whether another short-chain dehydrogenase enzyme has LinC activity (= 2,5-DDOL dehydrogenase activity). Recombinant *E. coli* cells expressing BphB (biphenyl-*cis*-diol dehydrogenase from *Pseudomonas* sp KKS102; [11]), or BnzB (*cis*-benzene glycol dehydrogenase from *Pseudomonas putida* BE-81; [19]), which is also known as TodD (*cis*-toluene dihydrodiol dehydrogenase from *Pseudomonas putida* F1; [81]) did not show the activity.

We showed that there is a gene in the vicinity of the *linA* gene, named *linX*, encoding a protein which has LinC (2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase) activity (Figure 3) [45]. The deduced amino acid sequence

of LinX shows 33.1% identity with that of LinC, and LinX is also a member of the short-chain alcohol dehydrogenase family. The region between *linX* and *linA* was homologous to the *linX* gene at the nucleotide level (69%). An open reading frame of 294 nucleotides was found in this region, and was named ORFUP. ORFUP encodes a 10.1-kDa polypeptide consisting of 98 amino acid residues. The deduced amino acid sequence of ORFUP showed 70.7% identity with that of LinX for the 58 N-terminal amino acid residues. It is interesting that there is a sequence near the *linA* gene which has the potential to convert one of the intermediate metabolites of γ-HCH. It is possible that strain UT26 is in the process of forming a kind of operon for γ-HCH degradation. This sequence, however, is thought to be non-functional, judging from the following two results (Y Nagata, R Imai and M Takagi, unpublished data). First, the ORFUP is much shorter than LinX (Figure 3). Second, *E. coli* harboring the plasmid overexpressing this region showed no LinC activity and produced a short protein similar in size to the estimated size of the protein product of ORFUP. Only a few point mutations would be necessary to make this sequence functional as a gene for dehydrogenase.

Enzymes for the downstream pathway

2,5-DCHQ reductive dehalogenase (LinD)

The deduced amino acid sequence of LinD shows some similarity to class theta glutathione *S*-transferases (GSTs). GSTs are categorized into four classes, alpha, beta, pi, and theta, and all known bacterial GSTs are placed in class theta [75]. Unlike other classes of GSTs, there is little information on class theta GSTs concerning their three-dimensional structures and residues necessary for activity except for some cases [60,62,74,76]. PcpC of *Sphingomonas chlorophenolica* ATCC 39723, which is responsible for the conversion of tetrachlorohydroquinone to 2,6-DCHQ [56], is the enzyme most similar to LinD.

Recombinant *E. coli* cells overproducing LinD convert 2,5-DCHQ to HQ via CHQ, although the conversion rate from CHQ to HQ is much lower than that from 2,5-DCHQ to CHQ [37]. The LinD activity of the cell-free crude extract of *E. coli* overproducing LinD rose in the presence of glutathione [37]. Northern blot analysis revealed that the expression of the *linD* gene is induced by 2,5-DCHQ [37].

(Chloro) hydroquinone 1,2-dioxygenase (LinE)

The *linE* gene, whose product (LinE) is responsible for CHQ degradation, is located 3 kb upstream from the *linD* gene (Figure 2). The deduced amino acid sequence of LinE showed significant similarity to PcpA (51% identity and 72% similarity), which is involved in pentachlorophenol degradation in *S. chlorophenolica* ATCC 39723, although its function is still unknown ([77]; note that PcpA appears as 2,6-dichloro-*p*-hydroxyquinone chlorohydrolase in the data bank; see below), and to some ORFs in *Bacillus subtilis* (YkcA, YodE, and YdfO) whose function is also unknown. In addition to these open reading frames, LinE showed a very low level of similarity to *meta*-cleavage dioxygenases. The alignment between LinE and *meta*-cleavage dioxygenases is shown in Figure 4. All three amino acids for Fe^{2+} binding [8], which were revealed by the crystallographic analyses of BphC (2,3-dihydroxy biphenyl 1,2-dioxygenases) from *Pseudomonas* sp KKS102 [67] and *Burkholderia cepacia* LB400 [13], are conserved in LinE, suggesting that LinE is a member of this type of dioxygenase.

CHQ and HQ were converted to maleylacetate and γ-HMSA (a slightly yellow compound [46]), respectively by *E. coli* overproducing LinE (K Miyauchi, Y Nagata and M Takagi, unpublished data), indicating that LinE has ring-cleavage dioxygenase activity for CHQ and HQ. Acylchloride, which is proposed to be a direct metabolite of CHQ, is easily converted to maleylacetate in the presence of water. Because the activity of LinE on CHQ seems to be much stronger than that of LinD, it is most likely that CHQ is mainly degraded by LinE and that the degradation pathway via HQ is a minor pathway in strain UT26.

As far as we know, LinE is the first enzyme reported which prefers HQ as its substrate over catechol, which is one of major substrates for *meta*-cleavage dioxygenases. The crude extract of *E. coli* overproducing LinE consumed oxygen when CHQ or HQ, but not catechol, was added (K Miyauchi, Y Nagata and M Takagi, unpublished data). In contrast to LinE, *E. coli* overproducing a typical ring-cleavage dioxygenase, XylE (catechol 2,3-dioxygenase) [29], did not show activity toward CHQ or HQ. These results show that LinE is a novel type of *meta*-cleavage dioxygenase, which cleaves the aromatic ring with two hydroxy groups in the *para*-position. Although PcpA, which shows the highest level of similarity to LinE, appears as 2,6-dichloro-*p*-hydroxyquinone chlorohydrolase in the data bank (accession number M55159), there is no direct evidence about it. Conversely we have some direct evidence that PcpA has ring-cleavage dioxygenase activity toward HQ, CHQ, and 2,6-DCHQ (Y Ohtsubo, K Miyauchi, K Kanda, T Hatta, H Kiyohara, T Senda, Y Nagata, Y Mitsui, and M Takagi, unpublished data).

A regulatory factor for the linD and linE gene expression (LinR)

The deduced amino acid sequence of LinR shows similarity to LysR-type transcriptional regulators (LTTRs) [66]. Some LTTRs are involved in the degradation pathways of aromatic compounds such as catechol [63], naphthalene [78], and chlorocatechol [33,36]. The palindromic $TN_{11}A$ sequence is known as a recognition sequence for LTTRs. We found the palindromic sequence immediately upstream of *linE* (K Miyauchi, Y Nagata and M Takagi, unpublished data). The fragment containing the sequence was ligated with the reporter gene, *lacZ*, and was inserted into the plasmid expressing LinR under the control of a *lac* promoter. When the resultant plasmid was introduced into *E. coli*, the LacZ activity rose in the presence of 2,5-DCHQ or CHQ in the medium (K Miyauchi, Y Nagata and M Takagi, unpublished data). Furthermore, Northern blot analysis for total RNAs of strains UT26 and UT102, a mutant of *linR* [46], revealed that the expression of *linD* and *linE* was induced in the presence of 2,5-DCHQ, CHQ or HQ in strain UT26, but not in strain UT102 (K Miyauchi, Y Nagata and M Takagi, unpublished data). These results indicate that LinR is a positive transcriptional regulator for expression of *linD* and *linE*.

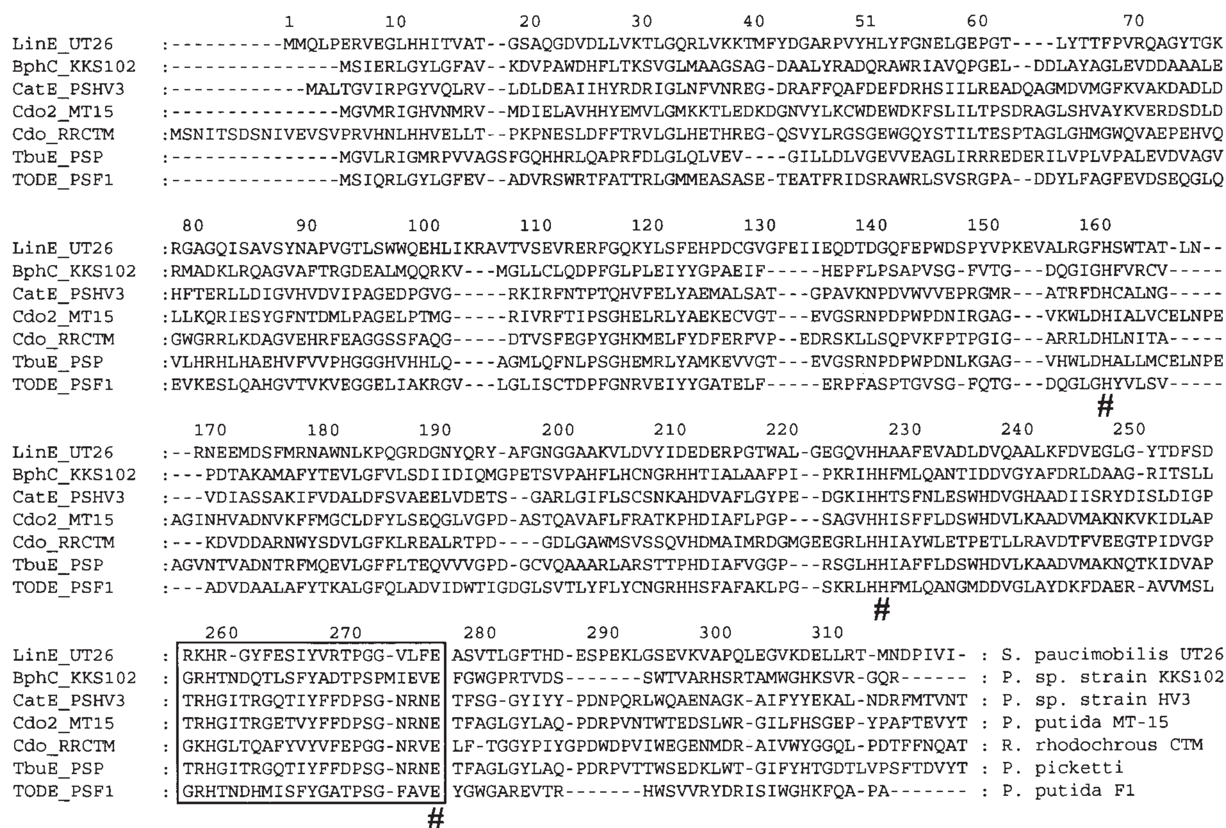

Figure 4 Alignment between LinE and *meta*-cleavage dioxygenases. The residues involved in Fe^{2+} binding in BphC of *Pseudomonas* sp KKS102 are marked with a sharp. The alignment was generated by using the ClustalX sequence alignment program. Abbreviations: BphC__KKS102, 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp strain KKS102 [28]; CatE__PSHV3, catechol 2,3-dioxygenase from *Sphingomonas* sp strain HV3 [79]; Cdo2__MT15, catechol 2,3-dioxygenase from *P. putida* MT-15 [26]; Cdo__RRCTM, catechol 2,3-dioxygenase from *Rhodococcus rhodochrous* [6]; TbuE__PSP, catechol 2,3-dioxygenase from *Ralstonia pickettii* [31]; TODE__PSF, 3-methylcatechol 2,3-dioxygenase from *P. putida* F1 [81].

Subcellular localization of enzymes for γ-HCH degradation

In order to explore the localization of enzymes for γ-HCH degradation in strain UT26, the cellular proteins were separated into periplasmic, cytosolic and membrane fractions after osmotic shock [49]. Most of the LinA and LinB activities (71% and 83%, respectively) were detected in the periplasmic fraction. For further analysis, antibodies were raised against LinA and LinB using purified enzymes produced in recombinant *E. coli* cells [49]. Western blot analysis of each cell fraction revealed that most of LinA and LinB was present in the periplasmic fraction. LinA and LinB were not detected in the extracellular fraction, indicating that LinA and LinB are not secreted extracellularly [49]. The periplasmic localization of LinA and LinB was confirmed by immunoelectron microscopy [49] (Figure 5). LinA and LinB were almost exclusively detected in the periphery of the cells.

The periplasmic space lies between the inner and outer membranes of Gram-negative bacteria, and is the location of many functions [3,52,53]. For example, some proteins residing in the periplasmic space have important functions in detection and processing of essential nutrients and their transport into the cell. Enzymes that detoxify antibiotics, such as β-lactamase, also exist in the periplasmic space [53]. Our findings have added another to the list of reactions that occur in the periplasmic space: degradation of halogenated xenobiotics. Elimination of halogens from halogenated xenobiotic molecules is a key step in their degradation because the carbon-halogen bond is relatively stable [10]. These compounds may enter the periplasm through non-specific porins in the outer membrane. Since dehalogenases degrade complex halogenated molecules into simpler ones for utilization and possibly for detoxification, the localization of dehalogenases in the periplasmic space seems reasonable.

In general, the signal peptide of periplasmic proteins functions in the translocation process [52]. We were surprised to find that these dehalogenases are not subject to N-terminal processing during translocation to the periplasmic space [49]. We suggest that dehalogenases of *S. paucimobilis* UT26 are exported by a secretion mechanism that differs from the signal peptide-based secretion mechanism that is common in prokaryotes. Two mechanisms have been reported by which proteins that lack a typical N-terminal signal peptide and that are not processed during translocation are secreted [65]. However, in both cases the proteins cross both cell membranes whereas the dehalogenases of *S. paucimobilis* UT26 are simply exported to the periplasm and are not secreted into the culture medium. A similar finding was made in a study of chitinase produced by *Serratia marcescens* [5]. A novel mechanism for protein

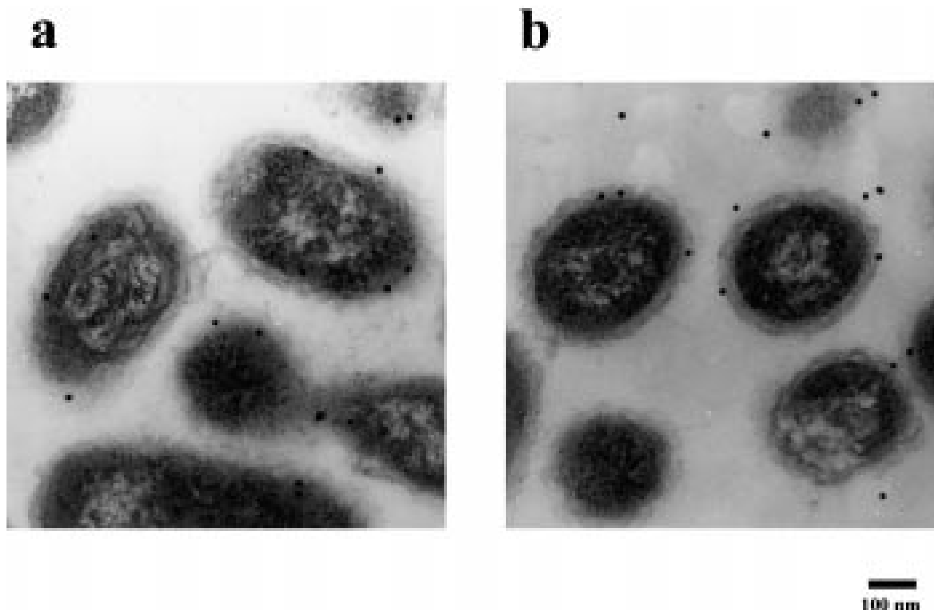

Figure 5 Immunogold-labeling electron microscopy. See Ref [49] for details. (a) Strain UT26 with anti-LinA antibody. (b) Strain UT26 with anti-LinB antibody.

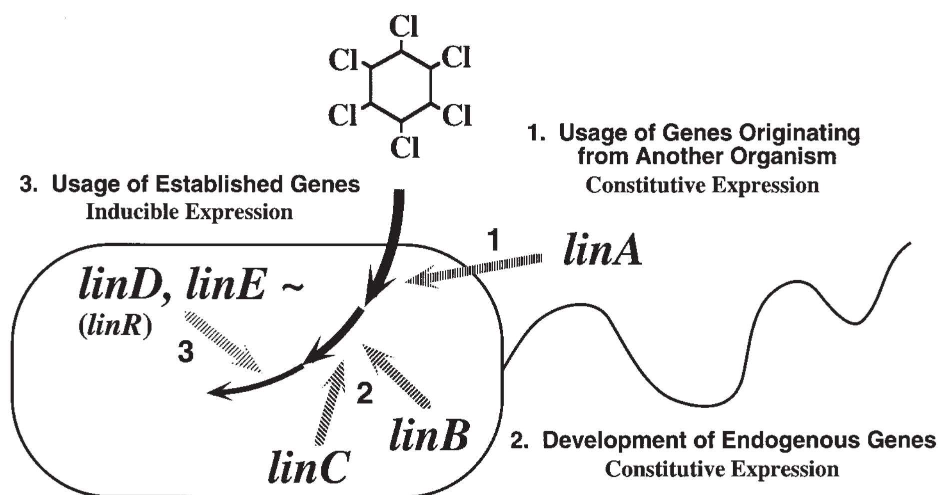

Figure 6 Proposed mechanism of adaptive aquisition of assimilation ability for γ-HCH in *S. paucimobilis* UT26. See text for details.

accumulation in the periplasmic space may be one of several protein translocation pathways that operate in Gram-negative bacteria.

Conclusions and prospects

We have demonstrated a unique degradation pathway of γ-HCH in *S. paucimobilis* UT26 and have cloned genes involved in this pathway. It seems that the pathway from γ-HCH to 2,5-DCHQ (we named it the upstream pathway) is especially specific for γ-HCH degradation in strain UT26. The 2,5-DCHQ degradation pathway (we named it the downstream pathway) seems to be relatively widely distributed in nature. There might be other aerobic γ-HCH degradation pathways which are not yet identified. For example, *Burkholderia cepacia* AC1100 degrades 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) via 2,5-DCHQ. However, the 2,5-DCHQ degradation pathway in AC1100 is different from that in strain UT26 [80]. Comparison of the γ-HCH degradation pathway in strain UT26 with such other pathways in other organisms will be of interest.

We propose a model for adaptive acquisition of the γ-HCH assimilation ability in *S. paucimobilis* UT26 (Figure 6). For the first attack on γ-HCH, strain UT26 seems to use an enzyme coded by a gene that may have originated from another organism (*linA*). Then, strain UT26 degrades the intermediate metabolites, which seem to be relatively common substrates, by developing variants of endogenous genes (*linB* and *linC*). Finally, for the common substrate (2,5-DCHQ), strain UT26 uses established genes which are expressed inducibly.

Our current understanding can be applied for bioremediation of contaminated environments. Strain UT26 grows slowly when γ-HCH is supplied as sole source of carbon

and energy. Because the genes for γ-HCH degradation which we cloned could be overproduced in other organisms, it may be possible to create strains which degrade γ-HCH more effectively. The introduction of genes for γ-HCH degradation into plants is one possible strategy. In preliminary studies, we introduced the *linA* gene with a CaMV 35S promoter into tobacco by using *Agrobacterium tumefaciens*, and succeeded in expressing LinA activity in the transgenic plant.

The γ-HCH degradation pathway in strain UT26, however, is inefficient because two dead-end products, 1,2,4-TCB and 2,5-DCP are produced (Figure 1). Enhancement of LinB activity in strain UT26 or introduction of the *linA* gene into a strain which can assimilate 1,2,4-TCB, such as *Pseudomonas* sp strain P51 [72], are strategies for creating strains which degrade γ-HCH more effectively than strain UT26 does.

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