

Biochemistry of Microbial Degradation of Hexachlorocyclohexane and Prospects for Bioremediation

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INTRODUCTION

The γ -isomer of hexachlorocyclohexane (HCH), formerly known as benzene hexachloride (BHC), is one of the now notorious organochlorine group of insecticides. About 600,000 tons were used throughout the world between the 1940s and the 1990s to control a wide range of agricultural, horticultural, and public health pests (80, 182, 183, 186, 187). Mounting concerns about its nontarget toxicity and persistence have since caused it to be deregistered in most countries (183, 189), but it is very stable in the environment (23, 192), and it is still being manufactured in India for local and export uses (187), so residue problems will continue for many decades.

HCH is commercially manufactured by the photochemical chlorination of benzene in the presence of UV light. The technical-grade HCH produced in this way comprises mainly five variously stable isomers: α (60 to 70%), β (5 to 12%), γ (10 to 12%), δ (6 to 10%), and ϵ (3 to 4%) (183). These isomers differ in the spatial orientations of the chlorine atoms around the cyclohexane ring (Fig. 1). Although only the γ -isomer is insecticidal (163), technical HCH was used widely as a cheap but effective insecticide in developing countries. It is possible to extract and purify the γ -HCH isomer from technical HCH, and the purified γ -isomer, commonly known as lindane, was also commonly used as an insecticide, particularly in developed countries.

The production and use of HCH/lindane has led to two major residue problems. One involves the point source contamination of very high concentrations due to the open-air stockpiling of waste isomers during lindane production (183, 187); the production of 1 ton of lindane generates 8 to 12 tons of “HCH muck,” consisting primarily of the α -, β -, and δ -HCH isomers. The α -, β -, and δ -isomers are in many ways more

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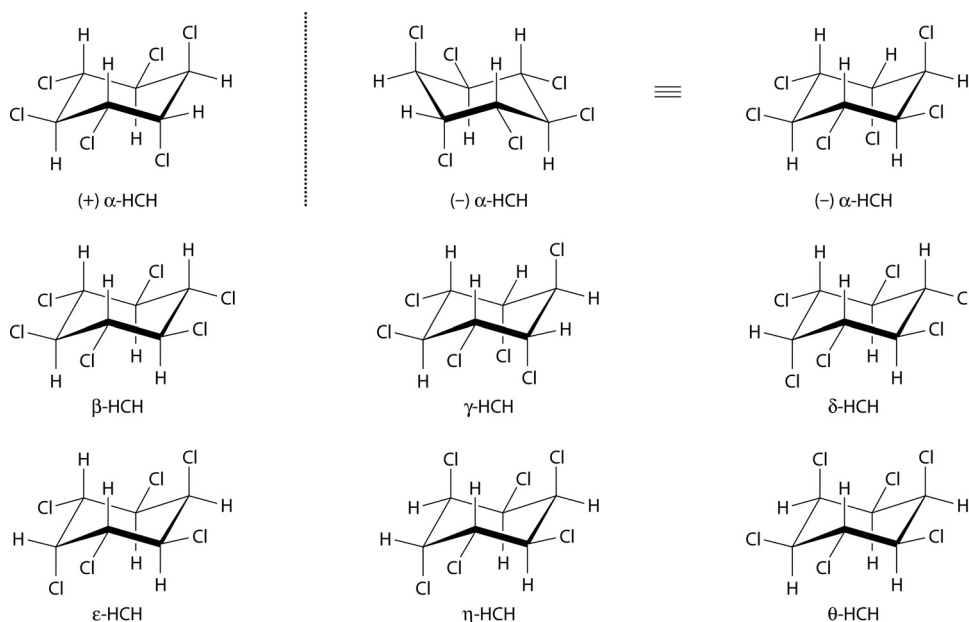


FIG. 1. Axial versus equatorial arrangements of chlorine atoms in the five major isomers of HCH plus the less common η - and θ -isomers. Note that α -HCH also exists in two enantiomeric (+ and -) forms.

problematic than lindane itself; α -HCH forms the major portion of technical HCH, and the β - and δ -isomers are significantly more stable than γ -HCH (181). Sites heavily contaminated with HCH have been reported from The Netherlands (180), Brazil (129), Germany (68, 159), Spain (29, 148), China (198), Greece (49), Canada (135), the United States (135), and India (139, 162) (Fig. 2). The 1998 Food and Agriculture Organization (FAO) inventory of obsolete, unwanted, and/or banned pesticides also found unused stockpiles of both technical

HCH and lindane (2,785 tons of technical HCH, 304 tons of lindane, and 45 tons of unspecified HCH material) scattered in dump sites in Africa and the Near East (<http://www.fao.org>). Weber et al. (187) estimated that four to six million tons of various HCH materials have been dumped worldwide, which is similar in scale to the combined totals of dumped materials for all other persistent organic pollutants (POPs) defined by the Stockholm Convention (187).

The second problem involves the diffuse contamination of



FIG. 2. Known locations of HCH dumps in excess of 50,000 tons in Brazil (38), Canada (135), Germany (159, 183), Spain (26, 29, 181), India (30, 139), and the United States (135). HCH-degrading sphingomonads have been recovered in six countries in Europe and Asia, although precise locations are often not reported (see text).

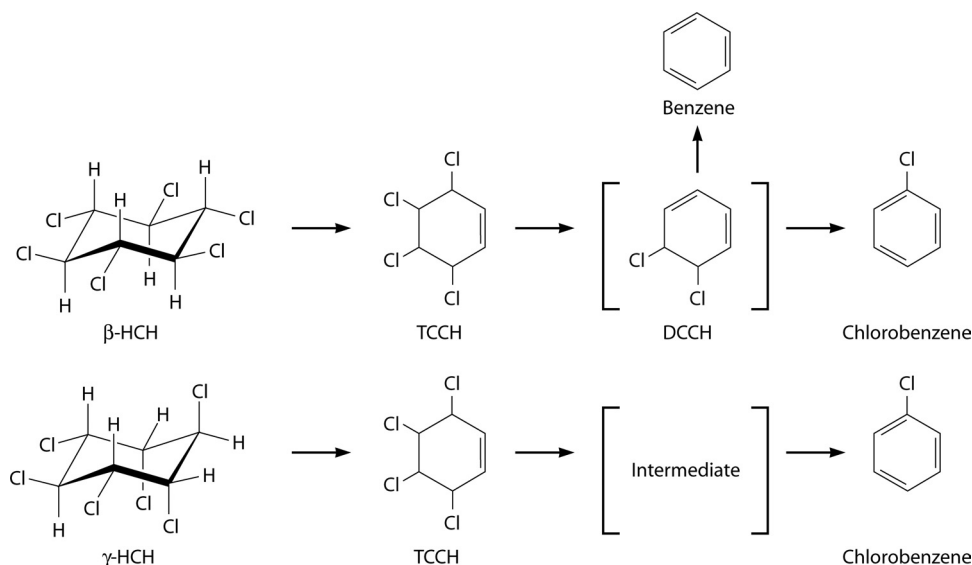


FIG. 3. Consensus anaerobic degradation pathway of γ - and β -HCH. Note that two intermediates that have been proposed but not yet observed empirically are shown in square brackets. The structures of TCCH and DCCH are shown in the planar format because their stereochemistries have not been established.

the environment with lower HCH concentrations due either to dispersal from stockpiles or to the use of the insecticide. The majority of HCH waste has been discarded in the open or stored at various levels of containment near the production sites, although in some cases, the top layer of soil at such sites has been removed for use in filling material for the construction of roads and buildings (97, 145, 181). HCH residues at many of the sites have percolated into the soil and thence contaminated groundwater (145). Residues of HCH isomers have now been reported for many countries in samples of air (76, 79, 138), water (8, 71, 139), soil (29, 50, 71, 139, 144), food commodities (7, 17, 175, 184), milk (153, 197), fish (44, 87), and mammals (48) and even from human blood samples (15, 169) and adipose tissue (14, 73). Environmental residues from sites as remote from the major regions of production and use as the Arctic, Antarctic, and Pacific Ocean have been reported (63, 185). Despite its widespread deregistration, HCH thus continues to generate serious residue problems in a variety of circumstances around the world.

Reports on the anaerobic microbial degradation of HCH isomers started appearing in the 1960s, and subsequently, aerobic bacteria capable of degrading HCH isomers were also found. Genes involved in degradation (*lin* genes) have been isolated from aerobic bacteria and heterologously expressed, and the gene products have been characterized. We now have a reasonable understanding of the diversity, organization, and distribution of HCH catabolic genes in certain sphingomonads in particular (75), and there is growing interest from a variety of government, industry, and science agencies in using them to develop bioremediation technologies for the decontamination of HCH-contaminated sites (16, 19, 96, 144). This article outlines our knowledge of the microbial degradation of HCH and discusses the issues surrounding the potential uses of the system for the bioremediation of HCH-contaminated sites.

ANAEROBIC DEGRADATION

It was initially believed that HCH biodegradation is largely an anaerobic process, and variable levels of anaerobic degradation of α -, β -, γ -, and δ -HCH have indeed been observed. There are no reports as yet on the anaerobic degradation of the ϵ -isomer, but it is relatively unstable anyway. Isolates capable of degrading one or more of the other four HCH isomers under anaerobic conditions include *Clostridium rectum* (125), *Clostridium sphenoides* (58, 84), *Clostridium butyricum*, *Clostridium pasteurianum* (64), *Citrobacter freundii* (64), *Desulfovibrio gigas*, *Desulfovibrio africanus*, *Desulfococcus multivorans* (21), and a *Dehalobacter* sp. (178). The γ - and α -isomers are degraded more rapidly than δ - and β -HCH (34, 66, 85, 91, 141).

Several studies reported the production of chlorobenzene during the anaerobic degradation of γ -HCH (Fig. 3) (11, 21, 98, 126, 137). Ohisa et al. (126) proposed that the degradation proceeds through two dichloroeliminations, resulting in the formation of γ -3,4,5,6-tetrachloro-1-cyclohexene (γ -TCCH) and, subsequently, 5,6-dichlorocyclohexa-1,2-diene. Chlorobenzene is then produced by a dehydrochlorination reaction. Several reports confirmed the presence of γ -TCCH as an intermediate in the anaerobic degradation of γ -HCH (11, 58, 64, 126). The degradation of γ -HCH also results in the formation of small amounts of trichlorinated benzenes and benzene itself (64).

The available data suggest that the anaerobic degradation of α -, β -, and δ -HCH also proceeds at least in part through successive dichloroeliminations and then dehydrochlorination to produce chlorobenzenes. Heritage and MacRae (58) originally proposed that the anaerobic degradation of α -HCH proceeds via δ -3,4,5,6-tetrachloro-1-cyclohexene (δ -TCCH), although the end product could not be identified. However, Middeldorp et al. (90) subsequently found that

the anaerobic degradation of both α -HCH and δ -HCH produces chlorobenzene. Middeldorp et al. (98) also found that the anaerobic degradation of β -HCH (Fig. 3) results in the formation of dichlorocyclohexadiene via δ -TCCH by successive dichloroeliminations, some of which is further converted to chlorobenzene by dehydrochlorination. Interestingly, however, those authors also found that some of the dichlorocyclohexadiene was converted to benzene by an additional dichloroelimination reaction. Recent studies have confirmed that β -HCH can be converted to both benzene and chlorobenzene under anaerobic conditions (97, 178).

Quintero et al. (140) recently suggested that another anaerobic pathway could also generate chlorobenzene from all four of the major HCH isomers. They studied anaerobic liquid and soil slurry systems of undefined microbial contents and reported the production of pentachlorocyclohexane, followed by 1,2- and 1,3-dichlorobenzene and then chlorobenzene. For the α - and γ -isomers, those authors also observed the intermediate products tetrachlorocyclohexene and trichlorobenzene. How these findings relate to the consensus pathway from the other studies described above remains to be demonstrated, but it may well relate to the use of the slurry systems reported by Quintero et al. (141). One major factor restricting the microbial degradation of HCH isomers under anaerobic conditions is the strong adsorption of the isomers onto soil (146); degradation is much faster under liquid or slurry conditions (39, 141, 146). Significantly, the pentachlorocyclohexane and tetrachlorocyclohexene seen by Quintero et al. (141) in their laboratory slurry systems were recently seen in groundwater from HCH-contaminated sites but not from sediment samples from the same sites (145).

Most studies of anaerobic HCH degradation reported the accumulation of chlorobenzene and benzene (24, 98, 179), although Quintero et al. (140) detected only traces of these metabolites, and Jagnow et al. (64) reported the complete degradation of γ -HCH at least to chloride and chloride-free metabolites in mixed and pure cultures of *Clostridium* species. There have been other reports of the anaerobic mineralization of benzene (41, 147), but we are unaware of other reports of the anaerobic degradation of chlorobenzene. Both chlorobenzene and benzene, however, can be readily mineralized under aerobic conditions (36, 37, 45, 93; see below).

To the best of our knowledge, nothing has yet been reported for specific genes and enzymes involved in anaerobic HCH degradation.

AEROBIC DEGRADATION

Several studies have reported various levels of degradation of the four major HCH isomers under aerobic conditions (5, 107, 150–152) (Table 1). As with anaerobic degradation, essentially no work has yet been done on the biodegradation of the relatively unstable ϵ -HCH. Most of the HCH-degrading aerobes known to date are members of the family *Sphingomonadaceae* (74, 75); so far, 30 HCH-degrading sphingomonads from different parts of the globe have been reported (Table 1). It is not clear how soon after the first use of HCH these strains emerged; the first strain

TABLE 1. Aerobic HCH-degrading microorganisms

Organism(s)	Reference(s)
<i>Alcaligenes faecalis</i> S-1 and <i>A. faecalis</i> S-2.....	52
<i>Arthrobacter citreus</i> BI-100	34
<i>Bacillus circulans</i> and <i>Bacillus brevis</i>	51
<i>Bacillus</i> sp.....	196
<i>Escherichia coli</i>	46
<i>Microbacterium</i> sp. ITRC1	88
<i>Pandoraea</i> sp.....	128
<i>Pseudomonas aeruginosa</i>	82
<i>Pseudomonas aeruginosa</i> ITRC-5	70
<i>Pseudomonas putida</i>	12
<i>Pseudomonas</i> sp.....	121
<i>Pseudomonas</i> sp.....	177
<i>Rhodanobacter lindaniclasticus</i> RP5557	120, 173
<i>Sphingobium chinhatense</i> IP26.....	30
<i>Sphingobium indicum</i> B90A.....	152
<i>Sphingobium japonicum</i> UT26.....	155
<i>Sphingobium francense</i> Sp+	27
<i>Sphingobium quisquiliarum</i> P25.....	9
<i>Sphingobium ummariense</i> RL-3	160
<i>Sphingobium</i> sp. BHC-A	62
<i>Sphingobium</i> sp. MI1205	83
<i>Sphingobium</i> sp. SS04-1	193
<i>Sphingobium</i> sp. SS04-2	193
<i>Sphingobium</i> sp. SS04-3	193
<i>Sphingobium</i> sp. SS04-4	193
<i>Sphingobium</i> sp. SS04-5	193
<i>Sphingobium</i> sp. UM2.....	31
<i>Sphingobium</i> sp. HDU05.....	31
<i>Sphingobium</i> sp. HDIP04	31
<i>Sphingobium</i> sp. F2	31
<i>Sphingomonas</i> sp. DS2.....	20
<i>Sphingomonas</i> sp. DS2-2	20
<i>Sphingomonas</i> sp. DS3-1	20
<i>Sphingomonas</i> sp. UM1	31
<i>Sphingomonas</i> sp. α 1-2	105
<i>Sphingomonas</i> sp. α 4-2	105
<i>Sphingomonas</i> sp. α 4-5	105
<i>Sphingomonas</i> sp. α 16-10	105
<i>Sphingomonas</i> sp. α 16-12	105
<i>Sphingomonas</i> sp. γ 1-7.....	105
<i>Sphingomonas</i> sp. γ 12-7.....	105
<i>Sphingomonas</i> sp. γ 16-1.....	105
<i>Sphingomonas</i> sp. γ 16-9.....	105
<i>Streptomyces</i> sp. M7	13
<i>Xanthomonas</i> sp. ICH12.....	89

was reported in the late 1980s from Japan (155), but reports from other countries quickly followed. Most research has focused on 3 of the 30 strains, initially named *Sphingomonas paucimobilis*, UT26, B90A, and Sp+, isolated from HCH-contaminated soils in Japan (155), India (152), and France (27), respectively. A polyphasic approach including 16S rRNA gene analysis subsequently revealed that these strains are actually three distinct species of *Sphingobium*: *Sphingobium japonicum* UT26, *Sphingobium indicum* B90A, and *Sphingobium francense* Sp+, respectively (132). Hereafter, however, we simply call them UT26, B90A, and Sp+. The other 27 HCH-degrading sphingomonads were isolated from HCH-contaminated sites in Germany (20), Spain (105), China (83), Japan (62), and India (30). Although all these strains degrade HCH, there are some isomer-specific differences between strains in the early steps of the degradation pathway (20, 109), which are summarized in Fig. 4 and 5.

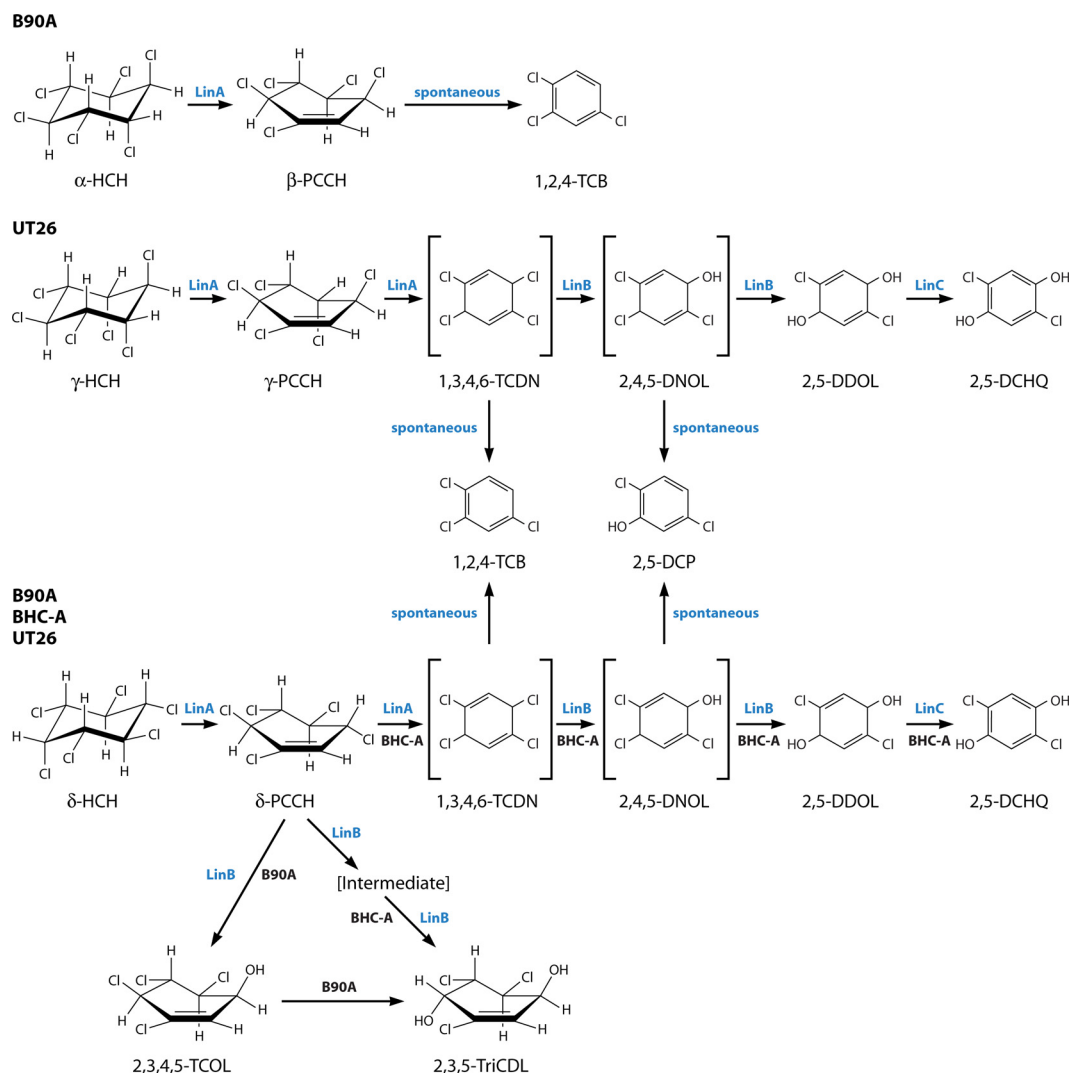


FIG. 4. Upstream pathway for the aerobic degradation of α -, γ -, and δ -HCH initiated by two successive dehydrochlorination reactions. 1,3,4,6-TCDN and 2,4,5-DNOL are shown in square brackets because they have not been demonstrated empirically. A further intermediate in δ -HCH degradation that has not been demonstrated empirically in the BHC-A strain is also shown in square brackets. This intermediate may be 3,4,5,6-TCOL, as was demonstrated for B90A. Note that the LinA, LinB, and LinC enzymes believed to catalyze several of the reactions are indicated, although direct evidence for the role of these enzymes in the strains indicated is not always available (see text).

γ -HCH

The aerobic degradation pathway of γ -HCH has been studied in some detail for UT26 (109, 115) (Fig. 4). It was suggested that two initial dehydrochlorination reactions produce the putative product 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,3,4,6-TCDN) via the observed intermediate γ -pentachlorocyclohexene (γ -PCCH) (61). Subsequently 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) is generated by two rounds of hydrolytic dechlorinations via a second putative metabolite, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL) (117). 2,5-DDOL is then converted by a dehydrogenation reaction to 2,5-dichlorohydroquinone (2,5-DCHQ) (118). The formation of 2,5-DCHQ completes what is known as the upstream degradation pathway.

It was suggested that the major upstream pathway reactions described above are enzymatically catalyzed, but two other,

minor products, 1,2,4-trichlorobenzene (1,2,4-TCB) and 2,5-dichlorophenol (2,5-DCP), are produced, presumptively by spontaneous dehydrochlorinations of the two putative metabolites, 1,3,4,6-TCDN and 2,4,5-DNOL (106–108). Both 1,2,4-TCB and 2,5-DCP appear to be dead-end products in this strain.

The first step in the subsequent, downstream degradation pathway is a reductive dechlorination of 2,5-DCHQ to chlorohydroquinone (CHQ) (102). The pathway then bifurcates, with the minor route being a further reductive dechlorination to produce hydroquinone (HQ), which is then ring cleaved to γ -hydroxymuconic semialdehyde (γ -HMSA). The major route involves the direct ring cleavage of CHQ to an acylchloride, which is further transformed to maleylacetate (MA) (100). MA is converted to β -keto adipate (42) and then to succinyl coenzyme A (CoA) and acetyl-CoA, which are both metabolized in the citric acid cycle (109). These reactions are summarized in Fig. 6.

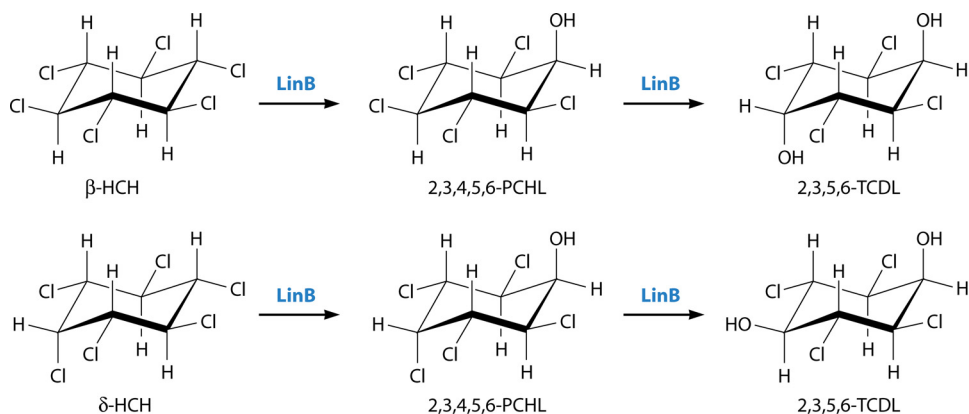


FIG. 5. Upstream pathway for the aerobic degradation of β - and δ -HCH involving two successive hydrolytic dechlorination reactions. LinB has been directly implicated in these reactions in some although not all of the strains shown.

Work on the aerobic degradation pathways of the α - and β -HCH isomers has so far focused on the initial steps in the upstream pathway (119, 143, 158). However, some differences from that observed for γ -HCH by UT26 described above are already emerging.

α -HCH

α -HCH degradation has been studied with the B90A strain (Fig. 4), and the first step is again dehydrochlorination (167, 168). α -HCH actually exists in two enantiomeric forms (Fig. 1), each of which is converted to its respective β -PCCH enantiomer. (+)- α -HCH is converted to β -(3*S*,4*S*,5*R*,6*S*)-1,3,4,5,6-PCCH, and (–)- α -HCH becomes β -(3*R*,4*R*,5*S*,6*R*)-1,3,4,5,6-PCCH (167).

Interestingly, these two β -PCCHs are then metabolized to 1,2,4-TCB (167). Although not established empirically, extrapolation from observations of γ -HCH (and δ -HCH [see below]) degradation suggests that it would also occur via TCDN and two further rounds of dehydrochlorination. TCDN would spontaneously be converted to 1,2,4-TCB, which again would be a dead-end product in B90A, as it was in UT26. Further empirical work on the route from β -PCCH to 1,2,4-TCB in B90A (which mineralizes α -HCH [R. Lal et al., unpublished data]) is needed because recent work with resting *Escherichia coli* cells heterologously expressing *lin* genes suggests the possibility of several routes (see LinB Haloalkane Dehalogenase below).

β -HCH

β -HCH is the most recalcitrant of all the major HCH isomers and does not undergo mineralization easily (6). Its relative stability is attributed to the fact that it is the only fully equatorially substituted HCH isomer (Fig. 1). This appears to be a barrier to the dehydrochlorination reactions, which are the first steps in the degradation of γ - and α -HCH described above and which apparently require axial chlorine atoms (see below). Instead, it seems that hydrolytic dechlorination reactions now become more feasible (Fig. 5). The transformation of β -HCH to 2,3,4,5,6-pentachlorocyclohexanol (PCHL) has been observed for all five strains tested. PCHL appears to be a terminal product in UT26 and Sp+ (119, 158), whereas in B90A, *Sphingobium* sp. MI1205, and *Sphingobium* sp. BHC-A

(hereafter simply MI1205 and BHC-A), it is further converted to 2,3,5,6-tetrachlorocyclohexanediol (2,3,5,6-TCDL) (62, 142, 190). Thus, there is both a difference between strains in the metabolism of β -HCH and a difference within strains such as B90A and UT26 in the ways in which they metabolize β - versus γ - or α -HCH.

δ -HCH

Two upstream pathways have been proposed for the degradation of δ -HCH (Fig. 4 and 5). One, outlined by Wu et al. (191) for BHC-A at least, is similar to the dehydrochlorinase-initiated route by which γ -HCH is degraded in UT26. The other pathway, also seen in these strains plus some others, is essentially the same as the hydrolytic dechlorination-led route by which β -HCH is degraded as described above, together with the same strain-specific variations described above (142, 191). This duality of degradative pathways is most likely a consequence of δ -HCH possessing a single axial chlorine in the cyclohexane ring. The axial chlorine is evidently amenable to dehydrochlorination via the same mechanism as that of the axial chlorines in γ -HCH, whereas the equatorial chlorines are amenable to hydrolytic dechlorination via the same mechanism as that of the equatorial chlorines in β -HCH.

In the case of the hydrolytic dechlorination-led route, δ -HCH is converted to PCHL in UT26 and Sp+ but with no further metabolism of the PCHL evident in these strains (158). On the other hand, in B90A and BHC-A, PCHL is again generated but then further converted to TCDL (142, 158, 191). These strain differences are thus perfectly correlated with the pattern seen for β -HCH, implying a common enzymatic cause.

In the case of the dehydrochlorinase-initiated route, for both strains BHC-A and B90A, δ -HCH is initially converted to δ -PCCH (142, 191) and, as Wu et al. (191) suggested, for BHC-A at least, then on to the putative metabolite 1,3,4,6-TCDN in reactions analogous to those for γ -HCH. The metabolism of the putative 1,3,4,6-TCDN is then proposed to continue down a pathway that is precisely analogous to that suggested for γ -HCH by strain UT26 (191). This would involve two successive hydrolytic dechlorinations to produce first the presumptive 2,4,5-DNOL metabolite and then the empirically validated 2,5-DDOL. The dehydrogenase reaction to produce

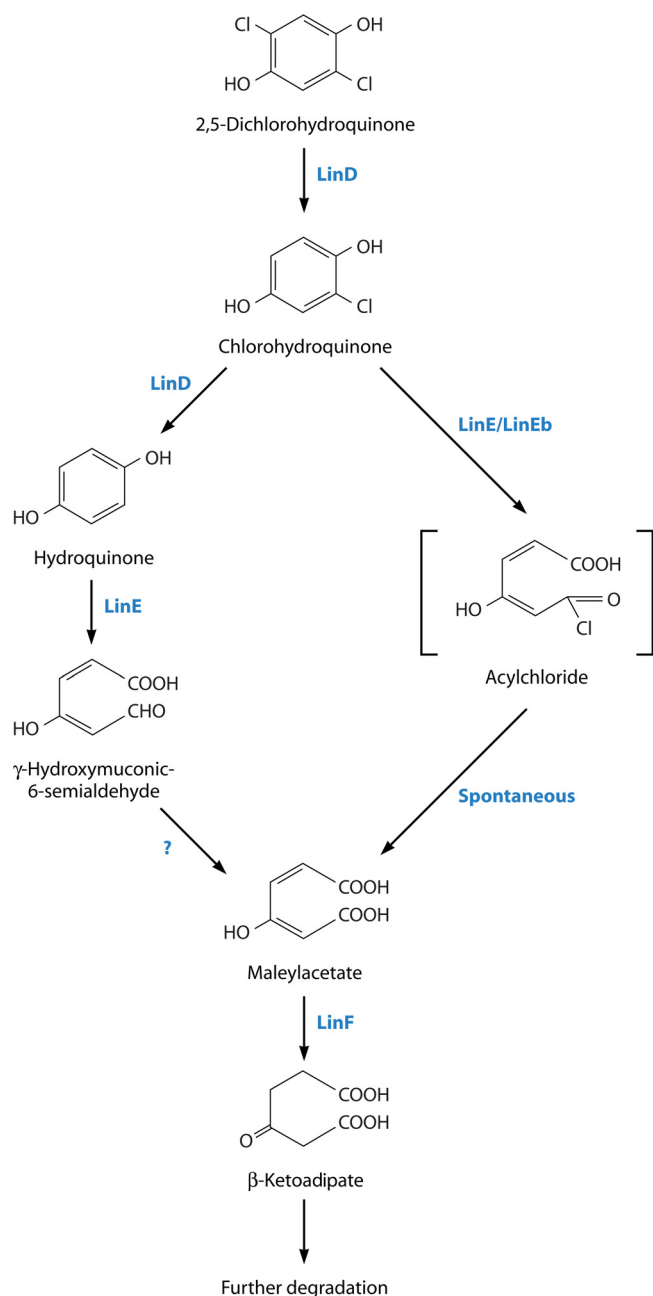


FIG. 6. Downstream pathway for the aerobic degradation of the γ -HCH isomer in strain UT26 together with the Lin enzymes catalyzing various steps. The intermediate shown in the square brackets (acylchloride) is hypothetical, and the reaction shown by a question mark has not been demonstrated empirically. The enzyme catalyzing the conversion of γ -hydroxymuconic-6-semialdehyde to maleylacetate is not known.

2,5-DCHQ then follows. There are also, again, the minor, dead-end products 1,2,4-TCB and 2,5-DCP. Interestingly, however, BHC-A is also able to take δ -PCCH through two successive hydrolytic dechlorinations to generate 2,3,5-trichloro-5-cyclohexene-1,4-diol (2,3,5-TriCDL), probably via 2,3,4,5-tetrachloro-5-cyclohexene-1-ol (2,3,4,5-TCOL), as was also shown in the case of strain B90A (see above).

It is, in fact, anomalous for δ -HCH to go through two dehydrochlorination reactions in this strain when it has only one axial Cl. This might be due to a peculiarity of the strain, or it might indicate that the proposed requirement for axial chlorine arrangements is not the full explanation for the isomer specificity of the dehydrochlorination reaction. An empirical demonstration of the putative 1,2,4,5-TCDN metabolite will be an important priority from here. This issue is considered again in the section on LinA below.

THE *lin* GENES

Genes necessary for the aerobic degradation of γ -HCH (called *lin* genes) were initially identified and characterized for UT26 (115) and were subsequently recovered from B90A (40, 72) as well. Very similar *lin* genes have also been identified for all the other HCH-degrading sphingomonads tested (20, 27, 62, 75, 190, 193). In UT26, where the system is best characterized (109) (Table 2), the pathway is comprised as follows: *linA*, encoding a dehydrochlorinase (61); *linB*, encoding a haloalkane dehalogenase (117); *linC*, encoding a dehydrogenase (118); *linD*, encoding a reductive dechlorinase (102); *linE/linEb*, encoding a ring cleavage oxygenase (42, 100); *linF*, encoding a maleylacetate reductase (42); *linGH*, encoding an acyl-CoA transferase (109); and *linJ*, encoding a thiolase (109), plus *linR/linI*, which are regulatory genes (101, 109).

Thus, *linA* to *linC* encode the enzymes responsible for the upper pathway, and *linD* to *linJ* encode those enzymes for the lower pathway. The organization of these genes in operons will be covered briefly below.

Evidence across a variety of strains indicates that the *linA*-encoded HCH dehydrochlorinase (LinA) (142, 167, 191) and the *linB*-encoded haloalkane dehalogenase (LinB) (62, 119, 142, 158, 190, 191) catalyze the dehydrochlorinase and hydrolytic dechlorinase reactions, respectively, in the upper pathway. There is unequivocal experimental evidence that LinA dehydrochlorinates γ -, α -, and δ -HCH, while LinB hydrolytically dechlorinates β - and δ -HCH in all strains examined. However, an interpretation of the roles of LinA and LinB in some of the subsequent reactions is not so straightforward because certain key metabolites have not been recovered experimentally for the dehydrochlorinase-led pathway for the γ -, α -, and δ -isomers, and there are strain differences with respect to the hydrolytic dechlorinase-led pathway for β - and δ -HCH.

LinA HCH DEHYDROCHLORINASE

Biochemistry

LinA is a homotetrameric protein with a 16.5-kDa molecular mass (111, 112), which appears to be located in the periplasm of sphingomonads (110), although a homologue in *Rhodanobacter lindaniclasticus* appears to be secreted extracellularly (120, 173). LinA is believed to be a unique type of dehydrogenase. It has no close relatives but shows low-level sequence similarity to a small group of proteins of diverse function and an α/β -crystallin-like structural fold (116, 176). Homology models based on these known structures suggest a catalytic mechanism similar to that of sctalone dehydratase, and the behaviors of mutants of key resi-

TABLE 2. HCH-degradative (*lin*) genes in HCH-degrading sphingomonads^a

Gene	Amino acid residue(s)	Function	Strains (GenBank accession no.)
<i>linA1/linA/linA2</i>	154/156	Dehydrochlorinase	UT26 (D90355), B90/B90A (AY331258, AY150580), Sp+ (AY903217, AY690622), DS2 (AJ871378), DS2-2 (AJ871379), γ 1-7 (AJ871380), δ 12-7 (AJ871381), γ 16-1 (AJ871382), α 1-2 (AJ871383), α 4-2 (AJ871384), DS3-1 (AJ871385), BHC-A (DQ372106)
<i>linB</i>	296	Haloalkane dehalogenase	UT26 (D14594), B90A (AY331259), Sp+ (AY903216), BHC-A (DQ246619), MI1205 (AB278602), SS04-1 (AB304077), SS04-2 (AB304078), SS04-3 (AB304079), SS04-4 (AB304080), SS04-5 (AB304081)
<i>linC</i>	250	Dehydrogenase	UT26 (D14595), B90A (AY331258), Sp+ (DQ111068), BHC-A (DQ462464)
<i>linD</i>	346	Reductive dechlorinase	UT26 (D89733), B90A (AY334273), BHC-A (DQ480725)
<i>linE</i>	321	Ring cleavage oxygenase	UT26 (AB021867), B90A (AY334273), BHC-A (DQ399709)
<i>linR</i>	303	LysR family transcriptional regulator	UT26 (AB021863), B90A (AY334273), BHC-A (DQ399711)
<i>linF</i>	352	Maleylacetate reductase	UT26 (AB177985), BHC-A (DQ399710)
<i>linEb</i>	319	Ring cleavage oxygenase	UT26 (AB177985)
<i>linG</i>	156	Acyl-CoA transferase	
<i>linH</i>	212	Acyl-CoA transferase	
<i>linI</i>	267	IcIR family transcriptional regulator	
<i>linJ</i>	403	Thiolase	
<i>linK</i>	316	Putative ABC transporter system, inner membrane protein	UT26 (AB267475)
<i>linL</i>	282	Putative ABC transporter, ATPase	UT26 (AB267475)
<i>linM</i>	320	Putative ABC transporter system, periplasmic protein	UT26 (AB267475)
<i>linN</i>	202	Putative ABC transporter system, lipoprotein	UT26 (AB267475)
<i>linX^b</i>	250	Dehydrogenase	UT26 (D23722), B90/B90A (AY331258, AY150580), BHC-A (DQ486136)

^a Data from Nagata et al. (115), Kumari et al. (72), Dogra et al. (40), Böltner et al. (20), C eremonie et al. (27), Lal et al. (75), Ito et al. (62), Nagata et al. (109), Wu et al. (191), and Yamamoto et al. (193).

^b *linX* is apparently not required for the γ -HCH degradation pathway of *S. japonicum* UT26 (118).

dues in LinA are consistent with this mechanism and the assignment of at least some parts of the substrate binding pocket (116). It is proposed that H73 and D25 form a catalytic dyad; H73 abstracts a proton from the HCH ring, and the resulting positively charged H73 residue is stabilized through its interaction with D25. Upon the abstraction of the proton and the formation of the double bond, the leaving-group chloride is expelled in a bimolecular elimination reaction.

Only qualitative work on the substrate range of LinA has been performed so far (111), and this suggests that its substrate range may be restricted to α -, γ -, and δ -HCH and their corresponding PCCH products. Since β -HCH, which is not hydrolyzed by LinA, is the only major isomer that lacks at least one adjacent *trans*-diaxial H/Cl pair (Fig. 1), it was proposed and subsequently confirmed that the dehydrochlorination of the other HCH isomers by LinA occurs stereoselectively at this hydrogen/chlorine pair (108, 111, 176). A similar reaction mechanism was proposed for the PCCHs, yielding TCDNs (111, 176, 190). Significantly, however, Wu et al. (191) reported that LinA catalyzes the dehydrochlorination of both δ -HCH and its product, δ -PCCH, even though δ -PCCH does not possess a *trans*-diaxial H/Cl pair. Additional work is clearly required to fully understand the molecular basis for the LinA reaction and its isomer specificities.

Variants of LinA

Most strains tested have one copy of the *linA* gene, but B90A and *Pseudomonas aeruginosa* ITRC-5 are known to have two,

denoted *linA1/linA2* and *linAa/linAb* (40, 72, 75, 161). There is also evidence from DNA hybridization studies that some other strains may also have two copies of the *linA* gene (31). The LinA1 and LinA2 enzymes from B90A are known to differ by about 10% in their amino acid sequences. The *linA* genes of UT26 and B90A were shown to be constitutively expressed (115, 168), although there is evidence for some inducible expression in *Rhodanobacter lindaniclasticus* (120, 173).

Figure 7 shows the differences among the sequences of LinA enzymes from known HCH-degrading bacteria, including sphingomonads (20, 27, 40, 61, 72, 109, 191), *Pseudomonas aeruginosa* ITRC-5 (161), *Rhodanobacter lindaniclasticus* (120, 173), and *Xanthomonas* sp. ICH12 (89). The sequences fall into four major groups.

The first and largest group (10 sequences) includes the single LinA sequences from the well-characterized strains UT26 and BHC-A plus one (LinA2) of the two sequences from the well-characterized strain B90A. These sequences are in fact identical, although three other members of this group, found in *P. aeruginosa* LinAb, *Sphingomonas* sp. NM05, and *Sphingomonas* sp. γ 1-7, differ by two to four residues.

Sequences in the second group show 12 other single-amino-acid differences. Four of these differences cluster around the catalytic dyad residues D25 and H73 (although D25 and H73 themselves are conserved), and there is another cluster of five changes around, and including, the R129 residue, which has also been implicated in substrate

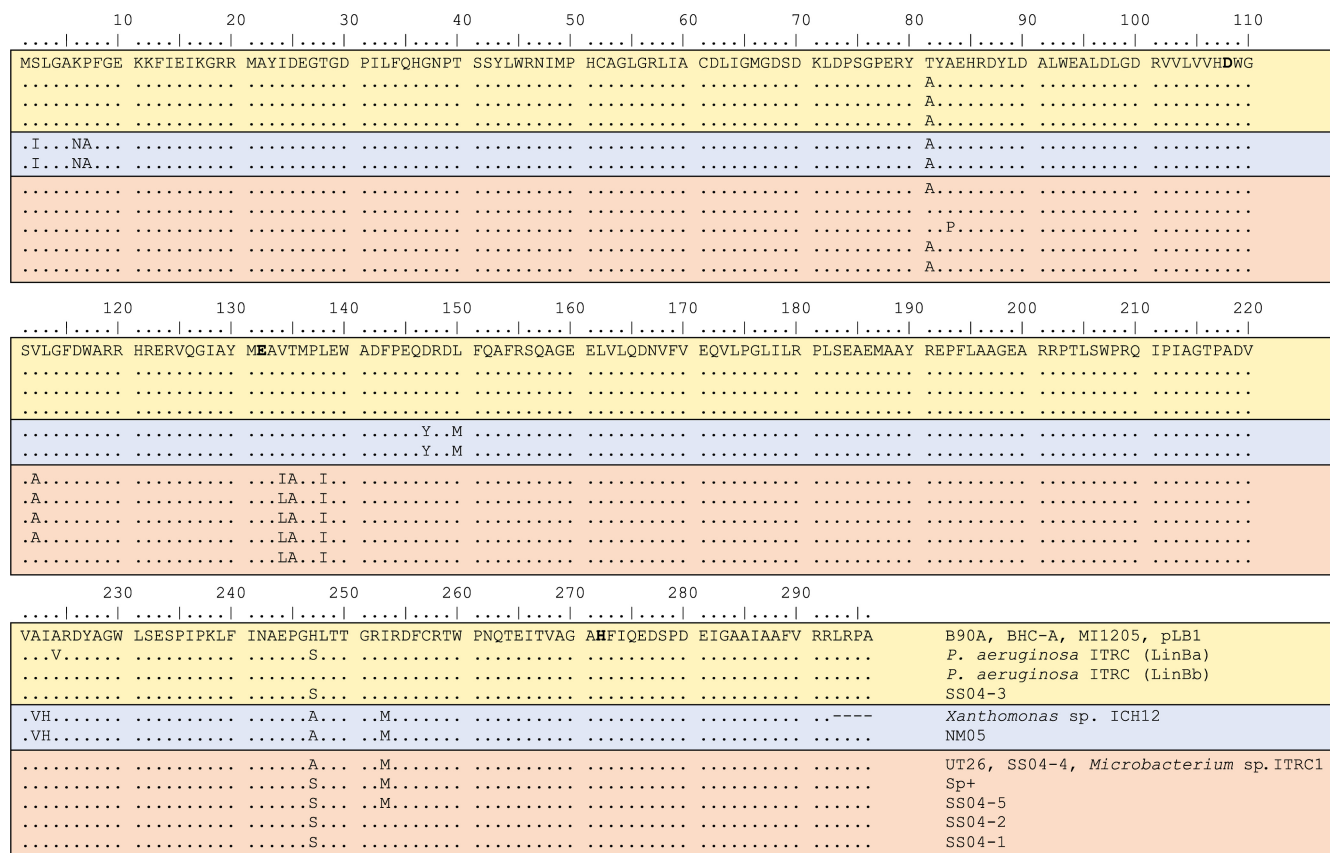


FIG. 8. Sequence differences among LinB variants from known HCH-degrading bacteria. Residues D108, E132, and H272 that form the catalytic triad are shown in boldface type. The three groups of sequences described in the text are separated by solid lines. Note that the C terminus of the *Xanthomonas* sp. ICH12 LinB enzyme has not been determined.

(65), the catalytic mechanism of LinB is proposed to involve an interaction of the substrate with the catalytic triad (D108, H272, and E132, i.e., nucleophile-histidine-acid) and a group of halide-stabilizing residues (principally N38 and W109) (18, 32, 60, 130). It was suggested that nucleophilic attack by D108 at the carbon atom of the substrate results in C-Cl bond cleavage by an S_N2 displacement mechanism, resulting in the formation of an acyl-enzyme intermediate. E132 functions to orient and increase the basicity of H272, which in turn activates a water molecule for the hydrolysis of the acyl-enzyme and regeneration of the active site. The NH groups of N38 and W109 stabilize the developing negative charge on the halide in the transition state of the S_N2 displacement reaction.

The specificity of LinB for different HCH isomers has not been studied in any detail. However, data for purified, heterologously expressed LinB (69) reveal a very broad substrate preference for halogenated compounds up to a chain length of eight carbon atoms. The maximum catalytic efficiency (k_{cat}/K_m) was found to be $233 \text{ s}^{-1} \text{ mM}^{-1}$ for 1-iodohexane. LinB was also shown to catalyze the hydrolytic dechlorination of β - and δ -HCH although at considerably lower efficiencies ($k_{cat}/K_m = 1 \text{ s}^{-1} \text{ mM}^{-1}$ for β -HCH) (119). As expected, heterologously expressed LinB does not catalyze the hydrolytic dechlorination of γ -HCH, although surprisingly, some low-level activity against the α -isomer was recently reported for LinB from B90A (143). Significantly,

LinB from B90A and MI1205 is an order of magnitude more efficient than LinB from UT26 at catalyzing the dechlorination of β -HCH, and it also catalyzes the dechlorination of the product PCHL at the 4-position, resulting in the dihydroxylated product 2,3,5,6-TCDL (62, 158). The biochemical differences between LinB of B90A and LinB of UT26 provide an elegant explanation for the differences between these two strains in their performance of the hydrolytic dechlorination-led degradation of β -HCH.

Interestingly, while most HCH-degrading strains have only a single *linB* gene, *Sphingobium* sp. MI1205 and *P. aeruginosa* ITRC-5 were reported to contain an IS6100-flanked duplication of a *linB* variant similar to the *linB* sequences found in strains B90A and BHC-A (62, 161). This cluster is also identical to the one found for plasmid pLB1, isolated from soil, suggesting a horizontal transfer of *linB* genes (103). The *linB* gene is known to be constitutively expressed in UT26 and B90A at least (115, 168).

Variants of LinB

Figure 8 summarizes the amino acid differences among sequenced LinB proteins from known HCH-degrading sphingomonads (20, 27, 40, 62, 109, 117, 190, 191) as well as the HCH-degrading *Microbacterium* sp. ITRC1 (88), *Xanthomonas* sp. strain ICH12 (89), and *Pseudomonas aeruginosa* ITRC-5

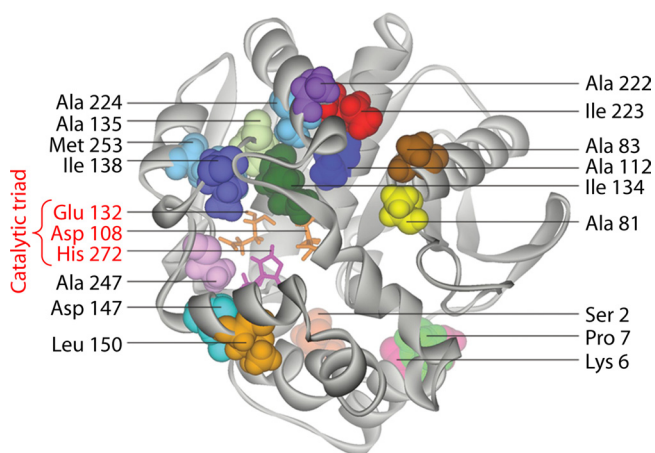


FIG. 9. Variant positions in the structure of LinB. The active-site residues D108, E132, and H272 are marked with red text. The coordinates used were those reported under Protein Data Bank accession number 1MJ5 (166).

(161). Figure 8 also contains a sequence from plasmid pLB1, also obtained from HCH-contaminated soils (103). These sequences fall into three major groups. One group, comprising seven sequences, includes sequences from the well-characterized strains B90A, BHC-A, and MI1205 plus one from another sphingomonad, one from a plasmid (pLB1) isolated directly from soil, and the two genes from *P. aeruginosa* strain ITRC-5. A second group (containing just two sequences from the sphingomonad NM05 and *Xanthomonas* sp. ICH12) is quite closely related to the first but contains seven unique substitutions and two others shared with the third group. The third group, including sequences from the well-characterized strains UT26 and Sp+ plus four from other sphingomonads and *Microbacterium* sp. ITRC1, is characterized by a cluster of three unique changes near E132 of the catalytic triad plus a small number of changes elsewhere in the sequence.

The sequence differences between the first and third groups described above are particularly interesting because the well-characterized members of the first group (B90A, BHC-A, and MI1205) have an ability to degrade PCHL, which the well-characterized members of the third group (UT26 and Sp+) lack (see above). Site-directed mutagenesis and structural modeling suggest that seven amino acid differences between B90A/BHC-A/MI1205 and UT26/Sp+ explain this difference, with V134I and H247A (B90A/BHC-A/MI1205 amino acid first) of particular importance (62) (Fig. 9). Specifically, V134 and H247 are essential for the correct orientation of PCHL for S_N2 attack. The other five differences, T81, V112, T135, L138, and I253, also lie in the active site (T135 and L138 close to V134 and the catalytic triad E132) and contribute to the activity difference in a more subtle fashion.

Unresolved Substrate Specificity Issues

Very recently, Raina et al. (143) showed that resting cells of *E. coli* heterologously expressing the *linB* gene from B90A can act on α -HCH and on β - and γ -PCCH, which are the products of LinA action on α - and γ -HCH (see above). Two stereoisomers of 3,4,5,6-tetrachloro-2-cyclohexene-1-ol and 2,5,6-tri-

chloro-2-cyclohexene-1,4-diol were identified as being products of these reactions (Fig. 10). These metabolites had not been reported for HCH-degrading strains (see above), and α -HCH had not previously been reported as a substrate for any LinB variant. Moreover, the kinetics of these reactions were not reported by Raina et al. (143). Nevertheless, given that the two key metabolites in the pathway of LinA/dehydrochlorinase-led degradation of the γ -, α -, and δ -isomers of HCH proposed by Wu et al. (190) have not been observed either, some aspects of this pathway currently remain open to question. Detailed comparative kinetics of purified, heterologously expressed LinA and LinB enzymes from key strains with several metabolites will be crucial to the full elucidation of the pathway.

LinC DEHYDROGENASE

Less is currently known about the third upstream pathway enzyme, LinC, or any of the downstream pathway proteins.

LinC is a 2,5-DDOL dehydrogenase in the short-chain alcohol dehydrogenase family (118, 133). A general catalytic mechanism was proposed for these enzymes (67), involving the participation of a conserved Ser-Tyr-Lys catalytic triad and an NAD^+ cofactor. Specifically, the tyrosine hydroxyl is stabilized in a deprotonated state by the amino group of the lysine. The deprotonated tyrosine then participates in proton abstraction from the hydroxyl group of the substrate, which is further activated through a hydrogen-bonding interaction with the serine. Finally, hydride transfer from the substrate to NAD^+ forms NADPH and the reduced product, i.e., the conversion of 2,5-DDOL to 2,5-DCHQ.

linC genes have been recovered from several HCH-degrading sphingomonads (20, 27, 40, 118, 190), as a single copy in both UT26 and B90A (40, 118) but in two copies in Sp+ (75). Variants of LinC have also been amplified directly from HCH-contaminated soils (47). Amino acid identities among the available sphingomonad LinC sequences are 98 to 100%. Like *linA* and *linB*, *linC* was found to be constitutively expressed in strains UT26 and B90A (115, 168). More divergent (97 to 98% amino acid identities) *linC* genes have also been recovered from HCH-degrading *Pseudomonas aeruginosa* ITRC-5 (161) and *Microbacterium* sp. ITRC1 (88).

Nagata et al. (118) also discovered a *linX* gene in the vicinity of the *linA* gene of UT26 that encodes a protein with 33% amino acid identity to LinC and also some limited 2,5-DDOL dehydrogenase activity (Table 2). *linX* genes have now been found in most of the HCH-degrading sphingomonads, generally in multiple copies (20, 40). Three copies are found in the vicinity of the *linA1/linA2* genes in B90A; *linX1* and *linX3* are identical, and *linX2* is 67% different at the amino acid level (40). Nagata et al. (118) showed that LinX is not necessary for the degradation of γ -HCH *in vivo*, but its real physiological role is not clear.

THE DOWNSTREAM PATHWAY

Most of the HCH-degrading sphingomonads tested, with the notable exception of *Sphingomonas* sp. γ 12-7, contain the genes *linD* and *linE*, which encode the first two (reductive dechlorinase and ring cleavage oxygenase, respectively) steps

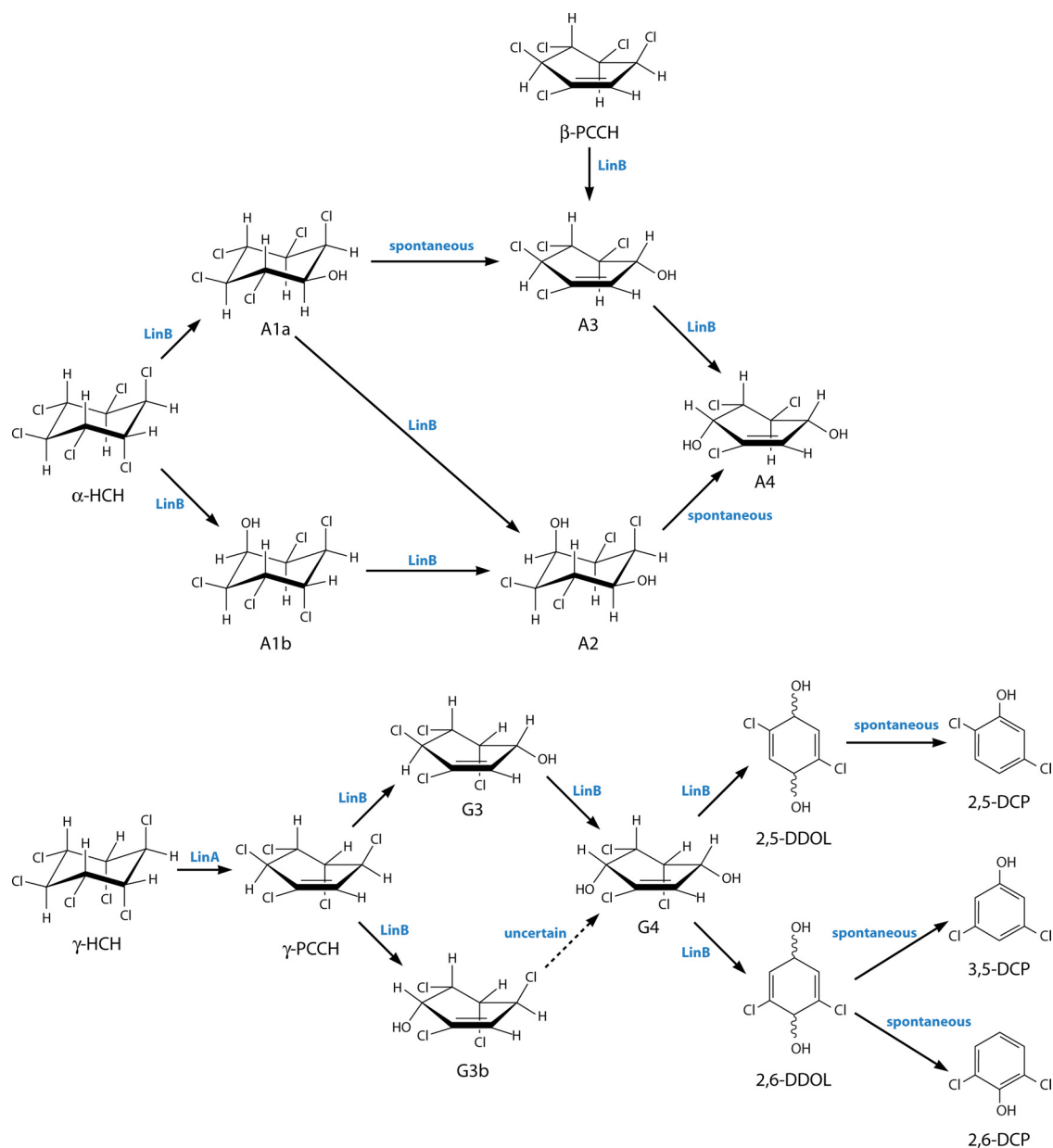


FIG. 10. Reactions of α -HCH and β - and γ -PCCH catalyzed by heterologously expressed LinB from B90A observed by Raina et al. (143). Product nomenclature was described previously by Raina et al. (143).

in the downstream pathway, as well as the regulatory gene *linR*, which encodes the LysR-type transcriptional regulator LinR (20, 27, 40, 115, 191). These three genes as well as *linF* (encoding maleylacetate reductase) are also present in the HCH-degrading *Pseudomonas aeruginosa* ITRC-5 (161). In strain UT26, the *linD* and *linE* genes are located near each other and may form an operon (100), and their transcription is induced by LinR in the presence of their respective substrates 2,5-DCHQ (LinD), CHQ (LinD), and HQ (LinE) (101) (Fig. 6). Intriguingly, Suar et al. (168) reported that the *linD* and *linE* genes can also be induced with α - and γ - but not β - and δ -HCH in strain B90A; as noted above, this strain will produce the 2,5-DCHQ, CHQ, and HQ substrates for LinD and LinE from γ - and δ -HCH. Further work is needed to understand how these patterns

of gene induction relate to metabolite flow through the pathways and the operon arrangements of the *lin* genes.

Strain UT26 also has a second ring cleavage oxygenase, LinEb, which is 53% identical to LinE and also contributes to the degradation of CHQ (42). The *linEb* gene is located close to the *linF* gene, and an open reading frame located between the two encodes another LysR-type regulator, which is implicated in their regulation (42).

The *linGH* and *linJ* genes, which encode the acyl-CoA transferase and thiolase required for the conversion of β -ketoacid to succinyl-CoA and acetyl-CoA, were recently identified in UT26, as was the *linI* gene, which encodes an IclR-type transcriptional regulator that may regulate their expression (109).

The *linK*, *linL*, *linM*, and *linN* genes recently identified in UT26 encode a permease, ATPase, periplasmic protein, and lipoprotein, respectively, which together form a putative ABC-type transporter system (43). This system is required for the utilization of γ -HCH, probably by conferring tolerance to toxic dead-end metabolites such as 2,5-DCP (43). Homologues showing high levels of similarity to the *linKLMN* genes have been found only in sphingomonads, suggesting that they might be important for the high metabolic activity of sphingomonads toward a range of xenobiotic compounds.

EVOLUTIONARY ISSUES AND SCOPE FOR IMPROVEMENT

There are some striking similarities in the mechanisms by which at least the aerobic HCH-degradative pathways have evolved and those that have been found for other xenobiotics (174). Notable here are the roles of horizontal gene transfer across species and insertion sequence-mediated transposition within species in the rapid assembly (<50 years) of a novel catabolic pathway (75). However, there are also some very distinctive aspects to the evolution of the *lin* system, and these suggest that there is substantial scope for further improvement in various key aspects of the pathway.

One line of evidence indicating that the *lin* system has been disseminated across sphingomonads and other species by horizontal gene transfer is the strong similarity in the sequences of the characterized *lin* genes from different species. Most notable here is the finding of closely similar *linA*, *linB*, and *linC* genes in the Gram-positive *Microbacterium* sp. ITRC1 (88), which is phylogenetically well removed from the sphingomonads and other Gram-negative organisms that account for all the other reported *lin* gene occurrences. The inferred LinB sequence of ITRC1 was identical to that of UT26, and its inferred LinC differed from the UT26 counterpart by only 2 of 141 residues. Similarly, although the organizations of the genes are quite variable among species (see below), this variation bears no relationship to the phylogeny of the organisms. For example, *linA*, *linB*, and *linDER* from *Pseudomonas aeruginosa* ITRC-5 show microsyntenous arrangements that are strikingly similar to those of their counterparts in UT26 and B90A (161).

Interestingly, codon usage and GC content are very different in *linA* than they are in other *lin* genes or, indeed, most other sphingomonad genes (40, 115). This suggests that *linA*, at least, may have been recruited from a different source organism than other *lin* genes, although the others could have sphingomonad origins.

A second line of evidence for the role of horizontal gene transfer events in the spread of the *lin* genes across species is the location of many of them in plasmids. Several other genes encoding xenobiotic degradation have been found on plasmids in sphingomonads (10, 124), and *lin* genes have been recovered from plasmids in UT26 (113), Sp+ (27), and B90A (86). At least some of the *lin* gene occurrences outside the sphingomonads are also associated with plasmids (173). Evidence for the conjugative nature of several of these plasmids (103, 113, 173) supports their possible role in the spread of *lin* genes among species.

However, not all *lin* genes are located on plasmids. In UT26, for example, they are dispersed across at least three replicons:

linA to *linC* are on chromosome I, *linF* is on chromosome II, and *linDER* is on plasmid pCHQ1 (113). Some of the spread of *lin* genes across species, and in particular within species, is instead attributed to their association with IS6100 insertion sequences. Most of the *lin* genes found to date are in fact associated with IS6100, both within (20, 27, 31, 40, 62, 103, 109) and outside (161) (Table 3) the *Sphingomonadaceae*. IS6100 sequences have also been associated with the spread of genes for degrading antibiotics (170, 172) and other pesticides (59, 81). The data for the *lin* genes implicate a role of IS6100 sequences in the dispersion of individual *lin* genes both among different hosts and among replicons within species.

The latter movements within species in turn lead to some of the changes in *lin* gene copy numbers and the coalescence of certain *lin* genes into operons. For example, the duplication of *linA* genes and the deletion of *linDER* genes in B90A and the deletion of one copy of *linA* in Sp+ have all been attributed to IS6100-mediated recombination events (40). Similarly, the colocalization of *linX*, *linX2*, and *linA* in strains B90A, Sp+, DS3-1, α 1-2, and α 4-2 has been attributed to recombination events mediated by IS6100 (20, 40, 75).

While the prominent roles of insertion sequences (including IS6100) and conjugative plasmids in assembling and dispersing novel catabolic pathways are common features of many microbial xenobiotic detoxification systems, there are also some other, unusual aspects of the HCH/Lin system that are informative for both fundamental biochemical and applied bioremediation perspectives.

One unusual feature of aerobic HCH degradation is that essentially only one pathway and one genetic system have as yet been found. Multiple systems have been reported for several other pesticides and toxins. For example, at least three and often more have been described for organophosphates, carbamates, glyphosate, triazines, 2,4,6-trinitrotoluene, and even other organochlorines like endosulfan and trichloroethylene (3, 28, 81, 154, 156, 157, 164, 188; C. J. Hartley, S. J. Dorrian, L. J. Briggs, M. R. Williams, R. J. Russell, and J. G. Oakeshott, international patent application PCT/Au2007/000640). This unusual feature of the *lin* system may reflect the relative recalcitrance of HCH to degradation, as is also suggested by the length of the *lin* pathway. All the examples described above involve catabolic pathways that are shorter than the *lin* pathway.

Another unusual feature of the *lin* system is that it has been found mainly in a single genus. Seventeen of just over 20 well-characterized *lin* isolates are sphingomonads, and most of the others are also from relatively closely related Gram-negative organisms. To our knowledge, there is just a single report of the *lin* system in a Gram-positive organism (see above). In contrast, some of the other systems described above are widely distributed across diverse Gram-positive and -negative genera (59, 156), and a couple that are more restricted, like the *esd/ese* system for endosulfan degradation, tend to be restricted largely to the Gram-positive *Actinomycetes*, which are well known for the versatility of their secondary metabolisms (188). It was suggested that the concentration of HCH metabolic capabilities in the sphingomonads may be because much of the downstream pathway and perhaps also some of the progenitors for the missing upstream steps preexisted for other reasons in certain sphingomonads (40, 115).

TABLE 3. Association of *lin* genes with IS6100 in HCH-degrading sphingomonads

Organism	Association ^a					Reference(s)
	<i>linA</i> /IS6100	<i>linB</i> /IS6100	<i>linC</i> /IS6100	<i>linDER</i> /IS6100	<i>linF</i> /IS6100	
<i>S. japonicum</i> UT26	+/+	+/-	+/+	+/+	+/+	109
<i>S. indicum</i> B90A	+/+	+/+	+/+	+/+	+/ND	40, 75
<i>S. francense</i> Sp+	+/+	+/+	+/ND	+/+	+/ND	27, 75
<i>S. ummariense</i> RL-3	+/+	+/+	+/+	+/+	ND/ND	31, 160
<i>S. chinhatense</i> IP26	+/+	+/+	+/+	+/+	ND/ND	30, 31
<i>Sphingomonas</i> sp. DS2	+/-	+/+	+/-	+/ND	ND/ND	20
<i>Sphingomonas</i> sp. DS2-2	+/-	+/+	+/-	+/ND	ND/ND	20
<i>Sphingomonas</i> sp. DS3-1	+/+	+/+	+/+	+/ND	ND/ND	20
<i>Sphingomonas</i> sp. α 1-2	+/+	+/+	+/+	+/ND	ND/ND	20
<i>Sphingomonas</i> sp. α 4-2	+/+	+/+	+/+	+/ND	ND/ND	20
<i>Sphingomonas</i> sp. γ 1-7	+/-	+/+	+/+	+/ND	ND/ND	20
<i>Sphingomonas</i> sp. γ 12-7	+/-	+/+	+/-	-/ND	ND/ND	20
<i>Sphingomonas</i> sp. γ 16-1	+/-	+/+	+/-	+/ND	ND/ND	20
<i>Sphingobium</i> sp. MI1205	ND/ND	+/+	ND/ND	ND/ND	ND/ND	62
<i>Sphingobium</i> sp. BHC-A	+/ND	+/ND	+/ND	+/ND	+/ND	191
<i>Sphingomonas</i> sp. UM1	+/+	+/+	+/+	+/+	ND/ND	31
<i>Sphingobium</i> sp. UM2	+/+	+/+	+/+	+/+	ND/ND	31
<i>Sphingobium</i> sp. HDU05	+/+	+/+	+/+	+/+	ND/ND	31
<i>Sphingobium</i> sp. HDIP04	+/+	+/+	+/+	+/+	ND/ND	31
<i>Sphingobium</i> sp. F2	+/+	+/+	+/+	+/+	ND/ND	31
<i>Sphingobium</i> sp. SS04-1	+/ND	+/+	+/ND	+/ND	ND/ND	193
<i>Sphingobium</i> sp. SS04-2	+/ND	+/+	+/ND	+/ND	ND/ND	193
<i>Sphingobium</i> sp. SS04-3	+/ND	+/+	+/ND	+/ND	ND/ND	193
<i>Sphingobium</i> sp. SS04-4	+/ND	+/+	+/ND	+/ND	ND/ND	193
<i>Sphingobium</i> sp. SS04-5	+/ND	+/+	+/ND	+/ND	ND/ND	193

^a ND, not determined.

The highly variable organization of *lin* genes across species is also a distinctive feature of this system. Catabolic genes for many other xenobiotics are generally organized into operons and coordinately regulated (53, 55, 99, 156, 157, 195). Individual *lin* genes vary in copy number and genetic localization, and while there is some condensation into clusters and operons, the constitutions of these clusters and operons again vary across strains (27, 86, 113). Interestingly, dispersed distributions of catabolic genes have been found for a few other xenobiotics among the sphingomonads, for example, for the genes involved in the degradation of pentachlorophenol in *Sphingobium chlorophenolicum* ATCC 39723 (25), dibenzo-*p*-dioxin by *Sphingomonas* sp. RW1 (2), and protocatechuate by *Sphingomonas paucimobilis* SYK-6 (94). The scattered and variable arrangement of *lin* genes may thus partly reflect some unusual feature of sphingomonad genetics. However, given both the refractory nature of HCH and its isomeric complexity, we suggest that it also could reflect a degradative system still evolving toward an optimal state(s).

Part of our suspicion that the *lin* system is still evolving quite rapidly comes from a consideration of its enzymatic efficiency. The first detoxification step in other xenobiotic catabolic systems, for example, the Oph/OpdA organophosphate-degrading enzyme, approach diffusion-limited kinetics (194), and some of those for glyphosate, carbamates, and atrazine also have specificity constants in excess of $10^4 \text{ s}^{-1} \text{ M}^{-1}$ (28, 154). However, the specificity constants of LinB for its preferred HCH isomer are nearly 3 orders of magnitude lower, and the specificity constant of LinA has not been estimated at this time. The isomeric complexity of the HCHs and their early-step metabolites also present a challenge to the reaction biology of LinA and LinB, and we are already seeing divergent copies of

both enzymes emerge that have distinct isomer preferences, albeit still very modest enzyme efficiencies (see above).

HCH BIOREMEDIATION

Bioremediation technologies potentially have four types of applications involving the cleanup of contaminated soils, liquid wastes, stockpiles, and commodities (149, 171). All four are relevant to HCH, even though it is no longer in widespread use as an insecticide. Its persistence and the processes used in its production mean that contaminated soils and stockpiles are a particular priority, with contamination of liquid wastes (e.g., agricultural runoff) and commodities also still being problematic because of diffusion from heavily contaminated soils and stockpiles. Essentially all the work on HCH bioremediation to date involves contaminated soils.

The two major approaches taken to soil bioremediation to this point have been biostimulation and bioaugmentation. Biostimulation involves the addition of oxygen and/or inorganic nutrients to stimulate the growth of resident bacteria that have some capacity to break down the contaminant in question. Bioaugmentation involves the introduction of additional bacteria that have the capacity, acquired naturally or by genetic modification in the laboratory, to break down the contaminant. The two approaches are not mutually exclusive. Both have been applied to HCH-contaminated sites, with various levels of success.

Biostimulation

The first attempt at full-scale *in situ* HCH bioremediation was carried out at an industrial site contaminated with low levels ($<1 \text{ mg kg}^{-1}$) of HCH muck in The Netherlands (77,

180). The biostimulation of an anaerobic zone with an electron donor degraded all HCH isomers to benzene or chlorobenzene, and this plume was then extracted to a wastewater treatment facility where the benzene and chlorobenzene were mineralized aerobically. Costs of over a million euros and time frames of years were nevertheless justified because of the high value of the land once remediated.

Phillips et al. (135) used proprietary biostimulation agents known as Daramend products derived from natural plant fibers to treat 1,100 tons of soil contaminated with high HCH concentrations ($\sim 5,000 \text{ mg kg}^{-1}$) in the United States. Based on developmental work in laboratory microcosms (134), two Daramend treatment approaches (cycled anaerobic/aerobic treatment and a strictly aerobic treatment) using two different Daramend products (D6390 and D6386, respectively) were used. Some tillage to enhance aerobic degradation was also used, and reductions averaging just under 50% were achieved over a year of treatment.

Rubinos et al. (148) reported the use of another, less elaborate biostimulation-plus-tillage trial of a soil with high HCH concentrations ($> 5,000 \text{ mg kg}^{-1}$) in Spain. The process, which they called land farming, involved the mixing of nitrogen and phosphate nutrients and lime (to correct otherwise acidic pH) plus regular irrigation and tillage over an 11-month period. Reductions in α - and γ -HCH concentrations of over 80% were achieved, although no decrease in the β -HCH concentration was observed. Interestingly, the major metabolites were PCCH and TCCH, suggesting a mix of aerobic and anaerobic degradation.

Most recently, an *ex situ* biostimulation process has also shown promise for HCH bioremediation (31). In this experiment, soil with indigenous microflora from a dump site for HCH muck was mixed with pristine garden soil, and aeration, moisture, and nutrients were provided intermittently. Reductions in total HCH concentrations (starting at 75 mg kg^{-1} , summed over the α -, β -, and δ -isomers) were found to be $\sim 3\%$ and 30% at the end of 24 and 240 days, respectively. A comparison of genetic markers over time showed changes in microbial community structure and stimulation of the indigenous sphingomonad population during the experiment. More elaborate studies are now needed to clarify the role of culturable and unculturable microbes during the course of biostimulation-mediated degradation of HCH.

Thus, the consensus from the four biostimulation trials described above is that significant reductions in HCH concentrations can be achieved, even with relatively inexpensive protocols. However, time frames of many months are required, and for the more heavily contaminated sites, only partial reductions were achieved, even for the less stable isomers, hence the interest in bioaugmentation.

Bioaugmentation

The first report of a bioaugmentation trial with HCH, by Bidlan et al. (16), demonstrated the removal of all four major HCH isomers from spiked soils in the laboratory after the addition of an HCH-degrading microbial consortium. Starting concentrations were very low (0.25 mg kg^{-1}), but no detectable HCH remained 5 days after the addition of an HCH-degrading microbial consortium. Mertens et al. (96) then demonstrated

the removal of around half of the γ -HCH added (50 mg liter^{-1} added every few days) to either liquid or soil microcosms by the inoculation of a single HCH-degrading isolate, *Sphingomonas* sp. γ 1-7, encapsulated in open-ended silicone tubes.

Most recently, Raina et al. (144) demonstrated the removal of most of a mix of HCH isomers by the inoculation of strain B90A (at $3 \times 10^{10} \text{ cells kg}^{-1}$ soil, immobilized on corncob powder) both into pits of transplanted contaminated soil (60 to 70 mg kg^{-1} HCH in 100 kg soil) and *in situ* at an HCH-contaminated agricultural site ($\sim 4 \text{ mg kg}^{-1}$ HCH in 3 by 3 m^2 up to a depth of 20 cm soil). The viability of the added cells was about 20% after 8 days, after which more cells were inoculated. Degradation was uneven across isomers, with over 80% of the α - and γ -HCH removed but only about half of the β - and δ -HCH removed. Nevertheless, this study represents an important proof of concept in achieving a significant cleanup of all the major HCH isomers in the field by bioaugmentation under conditions approaching economic viability.

One refinement of the bioaugmentation process is to use degradative strains that are good colonizers of the rhizosphere and to inoculate them into the root zone of compatible crops in contaminated soil, a process known as rhizoremediation. The potential benefit of the method is the development and maintenance of high levels of degradative bacteria in the soil without the need for high-titer inoculations initially or ongoing supplementation. Böltner et al. (19) proposed the potential of this method for the treatment of various organic chemicals. The rhizoremediation of laboratory soils spiked with 500 mg kg^{-1} γ -HCH by the incubation of pregerminated corn seeds covered with two HCH-degrading sphingomonads (strains GOF-203 and Ans-PL0) resulted in about a 30% reduction in γ -HCH levels after 25 days (19). Field studies are now needed to demonstrate the full potential of this approach.

Free-Enzyme Bioremediation

While we could find no reports of HCH bioremediation in stockpiles, liquid wastes, or commodities, there is considerable work on the cleanup of other pollutants from such environments. Some of the most promising advances come not from the use of live microbes but from the use of free enzymes (22, 171). The concept of enzymatic bioremediation centers on the use of enzymes that can catalyze the substantive detoxification of the pollutant(s) in question in a single step with reasonable kinetics ($> 10^4 \text{ s}^{-1} \text{ M}^{-1}$) and without the need for expensive cofactors. These enzymes are heterologously expressed in commercial microbial expression vectors, minimally purified, and formulated in various ways to address the particular stability requirements pertaining to the environments of their use.

Although genetic manipulation (GM) technology is used in the production of free-enzyme bioremediants, no live organisms remain in the formulated products, which therefore avoids any potentially contentious issues around the release of live genetically modified organisms (GMOs). Moreover, free-enzyme bioremediation does not require microbial growth for activity. Hence, it can be effective in short time frames (less than a day, depending on the application) and in circumstances where microbial growth might be either problematic technically (e.g., very concentrated point source contamination) or

unacceptable (e.g., potable water supplies). The technology does depend on a liquid, or at least very moist, environment to allow the enzyme access to the substrate. Nevertheless, enzymatic bioremediants are now commercially available for the cleanup of certain pesticides in large-scale liquid wastes (1, 15, 28, 154) and show promise for the treatment of various stockpiles, soils, and, in some cases, external surfaces of commodities (22, 35). The good results for the enzymatic treatment of stockpiles are of particular interest with respect to very concentrated HCH deposits like dump sites, where microbial growth is severely limited by the availability of nutrients.

Since neither LinA nor LinB requires cofactors and each removal of a chlorine atom from the cyclohexane ring reduces toxicity by severalfold, it is well worth considering their potential as starting resources for the development of an enzymatic bioremediation technology for HCH.

FUTURE PROSPECTS

Although scientific interest in HCH degradation was initially motivated by environmental and health concerns due to HCH residues, its aerobic degradation is now proving an excellent model for investigating fundamental issues in microbial and molecular evolution. Because HCH is such a chemically refractory molecule, the evolution of an effective detoxification and utilization pathway has required the acquisition of qualitatively new functions for certain individual enzymes plus the assembly of these and other enzymes into a coordinately regulated pathway. The isomer complexity of HCH adds a further degree of difficulty, with major differences between isomers in the reactions by which their breakdown can be catalyzed, at least under aerobic conditions. These have apparently necessitated the recruitment of alternative enzymes for different isomers in certain upstream steps in the aerobic pathway. Work to date indicates that several microbes, often but not always sphingomonads, are assembling the requisite capabilities, although the phenotype remains variable and probably not optimized in either its genetics or its biochemical efficacy.

Although evidence for the anaerobic degradation of HCH was reported nearly 2 decades earlier than evidence for its aerobic degradation, work on the latter has advanced far further since. Even for the latter, however, there remain several crucial gaps in our knowledge.

One fundamental gap concerns the reactions in the upper pathway, where the most biochemically problematic detoxification steps occur. Two sets of observations indicate that certain key elements of the widely accepted scheme for this pathway may be incorrect. One disconcerting observation has been that the presumptive dehydrochlorination of the putative δ -HCH metabolite δ -PCCH by LinA is inexplicable in terms of the bimolecular elimination reaction characteristic of enzymes from the family in which LinA sits (142). The second recent surprise has been the finding that heterologously expressed LinB (from B90A at least) has a wider substrate specificity across HCH and PCCH isomers *in vitro* (albeit with unknown kinetics) than the accepted pathway assumed, and moreover, it produces metabolites from some of these substrates that do not sit in the accepted pathway either (143). There is now considerable doubt as to the validity of certain steps in the

pathway that have hitherto been inferred rather than empirically demonstrated.

The resolution of these uncertainties about the upper metabolic pathway clearly requires more detailed metabolite analyses of HCH degradation in HCH-degrading bacteria. Another important goal will be to better understand the biochemistry of the LinA and LinB enzymes. Particular priorities in the latter are detailed kinetic analyses of heterologously expressed LinA and LinB with a range of resolved isomers of putative substrates within the pathway. LinA has recently been crystallized (127), so its structure should soon be published. This would also be an invaluable aid to understand its mechanism and the structure-function relationships underpinning that mechanism.

There are also other important aspects of the Lin pathway that we currently do not understand. One of these involves the mysterious LinX enzymes, which are distantly related to LinC and show some LinC function *in vitro* but are apparently not essential for γ -HCH degradation, at least *in vivo*. One possibility is that LinX catalyzes the transformation of γ -hydroxybutyric-6-semialdehyde to maleylacetate in the downstream pathway. This, like the upstream step catalyzed by LinC, requires a dehydrogenation step. This step is not essential for HCH degradation *in vivo* because a bifurcation in the downstream pathway two steps prior to the production of γ -hydroxybutyric-6-semialdehyde provides another option for the catabolic process.

Another major gap in our current knowledge of the system concerns the extent and biological significance of genetic variation both in the organization of the various *lin* genes and in the coding sequences of the key upstream *linA* and *linB* genes. Although some condensation of *lin* genes into operons has been reported, the complements and structures of the operons reported are still highly variable, and some *lin* genes remain apparently unlinked to the operons, either on plasmids or elsewhere in the various genomes. Such variation is unusual and suggests that the evolution of *lin* operons is still a work in progress. Several studies have found transposable elements, in particular IS6100 elements, in the vicinity of the *lin* genes. This is not unusual for recently assembled catabolic operons, but it is certainly consistent with a dynamic, ongoing process of the organization of *lin* genes into arrangements suited to coordinate control. Time course studies of the genetics of HCH degradation in highly contaminated sites could prove very informative in this context.

Evidence to date indicates high levels of polymorphism in the amino acid sequences of both the LinA and LinB enzymes, with convincing if indirect evidence that at least some of it affects function. The LinB proteins of UT26 and B90A in particular seem to differ qualitatively in their HCH isomer preferences. Similarly profound differences appear to exist between the α -HCH enantiomer preferences of LinA1 and LinA2. However, there could well be many other qualitative and quantitative differences in the activities of other LinA and LinB variants. Further work is needed to elucidate the extent of the variation, the molecular basis of isomer-specific differences in the functions of some of the variants, and the role of these differences in generating the metabolic diversity needed to deal with the plethora of isomers involved in the first four steps of the upstream pathway.

The anaerobic pathway for HCH degradation remains

poorly understood. Few metabolites have been demonstrated empirically, differences in the pathway between isomers remain unresolved possibilities, and essentially nothing is known about the enzymology or genetics of the process. Upstream dichloroelimination reactions are implicated, with hydrolytic dechlorination likely further downstream. However, enzymes to catalyze these reactions could come from a variety of protein families (165). Much basic biochemical work is needed, and genome sequencing could well prove rewarding for some of the anaerobes involved.

Although the Lin pathway has now been recovered from many different sphingomonads and a few other bacteria isolated from HCH-contaminated sites, there is still a great deal that we do not know about the function of the pathway in this ecosystem. We cannot even yet conclude that the sphingomonads are the major hosts for the pathway, since many recent studies have selected primarily for sphingomonads (by a facile antibiotic screen) and then secondarily tested them for their HCH-degradative abilities. More importantly, we do not know to what extent the evolution of HCH-degrading organisms at contaminated sites is accelerating the rate at which the HCH load is being reduced. This must be an urgent priority for further work. Equally, however, unless the rate has been massively accelerated, the size of the stockpiles and the stability of the compounds are such that many environments heavily contaminated with HCH will remain with us for decades, if not centuries, hence the priority to develop effective bioremediation technologies.

Both the biostimulation and bioaugmentation approaches taken thus far for the remediation of HCH-contaminated soils have met with some success, for certain isomers at least. Both technologies are still in early developmental stages, and substantial further improvements should still be quite readily achievable. While some cleanup situations involving land of potentially high value could withstand high costs, there are many more situations, particularly in the developing world, where costs will need to be low if treatment is to be a realistic option. Further work on application protocols and, for bioaugmentation at least, on strain development is clearly needed. The sphingomonads are clearly the group of first choice for further work on strain development. Their niche in the rhizosphere also predisposes them to many bioremediation applications, and strains with improved growth and viability properties under conditions of use should be quite readily selectable. Many sphingomonads are now quite tractable to isolation, laboratory culture, and selection. Importantly, the remarkable diversity of secondary metabolism seen across the sphingomonads is now attracting the interest of the biotechnology industry for a range of different applications (4), so the infrastructure of knowledge and resources for their genetic analysis and manipulation and commercial fermentation is also rapidly expanding.

The Lin pathway is clearly the most accessible (and possibly the only) biochemical system of the sphingomonads, or other bacteria, for aerobic HCH detoxification. The substrate and isomer coverage and kinetics of LinA and LinB will be critical properties to consider both for the development of improved strains to be used in soil bioremediation and for the development of enzymatic bioremediation strategies for stockpile and liquid contamination.

The enzymes should be amenable to various modern research technologies for obtaining improved variants either by the isolation of new variants from uncultured environmental samples/bacteria or by the improvement of existing variants by techniques of *in vitro* evolution. Early work on both approaches has yielded promising new variants of LinA and/or LinB (95). The availability of high-throughput screening assays, for some of the reactions at least (90, 104, 136), plus the crystal structures of the enzymes will help these efforts enormously.

We believe that there are good prospects for developing economically viable HCH bioremediation technologies based on the sphingomonad/Lin systems: soil bioremediation through various biostimulation/bioaugmentation approaches and stockpile and, as necessary, liquid remediation through direct enzymatic approaches. Significant work on enzyme characterization, particularly for LinA, and strain and enzyme improvement is still needed. However, the promise of the system evident thus far and the potential of modern microbial and enzyme research technologies to make radical improvements give us confidence that the development of successful technologies for a most pernicious pollutant is quite achievable.

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