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REVIEW

Pseudomonas sp. to *Sphingobium indicum*: a journey of microbial degradation and bioremediation of Hexachlorocyclohexane

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Abstract The unusual process of production of hexachlorocyclohexane (HCH) and extensive use of technical HCH and lindane has created a very serious problem of HCH contamination. While the use of technical HCH and lindane has been banned all over the world, India still continues producing lindane. Bacteria, especially Sphingomonads have been isolated that can degrade HCH isomers. Among all the bacterial strains isolated so far, *Sphingobium indicum* B90A that was isolated form HCH treated rhizosphere soil appears to have a better potential for HCH degradation. This conclusion is based on studies on the organization of *lin* genes and degradation ability of B90A. This strain perhaps can be used for HCH decontamination through bioaugmentation.

Keywords HCH · *Sphingobium indicum* B90A · Bioremediation

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Introduction

HCH (1, 2, 3, 4, 5, 6- hexachlorocyclohexane) is an effective insecticide that has been used to protect standing crop against grasshoppers, cohort insects, rice insects, wireworms, and other agricultural pests; in warehouses and in public health programs for the control of vector-borne diseases (malaria, scabies etc.). HCH as an insecticide proved to be so effective that it partly replaced DDT in many countries. Its extensive use has accrued enormous benefit but on the other hand created a serious problem of contamination of the environment. Several studies conducted in the past years have indicated contamination of water, soil, vegetables and other food commodities by HCH isomers [1–6]. Even though HCH was recognized as a problematic organochlorine compound in the 1970s, the practice to analyze HCH residues from different components of environment and isolate HCH degrading microbes from contaminated soils gained momentum only in late 1980s and early 1990s. However, not many HCH degrading bacterial strains were isolated at that time. The first bacterial strain found to degrade four isomers of HCH (α, β, γ, and δ) was *Pseudomonas* sp. (now *Sphingobium indicum* B90A), which was isolated by Sethunathan and coworkers in 1990 from sugarcane fields in India [7]. This was the first report on microbial degradation of the most recalcitrant β isomer of HCH. Gradually, the strain was extensively studied and has formed the basis of several novel discoveries such as the association of mobile genetic element (IS*6100*) with the catabolic *lin* genes [8]; the elucidation of HCH degradation pathway intermediates [9] as well as application-based bioremediation studies [10]. In this article we describe the alarming situation of environmental contamination by HCH, the function and genetic

organization of *lin* genes in *S. indicum* B90A and the possibilities of decontamination of HCH through bioremediation by bioaugmentation.

HCH as a contaminant

Commercially, HCH is synthesized by the chlorination of benzene in the presence of UV (IARC, 1973). The process results in the production of technical grade HCH which is a mixture of all its isomeric forms in varying proportions. Technical HCH thus basically consists of five stable isomers *viz.*, α- (60–70%), β- (5–12%), γ- (10–12%), δ- (6–10%) and ε- (3–4%) [11, 12]. All HCH isomers are stereochemically different from each other (Fig. 1) and only γ-HCH (lindane) is endowed with insecticidal property [13, 14]. In developing countries, use of technical grade HCH has been preferred over the purified lindane due to its low manufacturing cost. These two forms have been commercially sold under the names Isogam, Gammexane®, Benexane, BoreKil, Lindafor, Ben-Hex, Lindane etc.

The orientation of chlorine atoms around the cyclohexane ring in HCH decides the differences in physico-chemical properties such as solubility, sorption, volatilization and persistence of its isomers in the environment [15] (Table 1). These differences are attributed to the axial or equatorial position of chlorine atoms, such that there are four axial chlorines in case of α-HCH, three for γ-HCH, two for ε- one for δ, and none for β-HCH (Fig. 1). The β isomer that contains all the six chlorines atoms in equatorial position is the most stable and persistent of all HCH isomers and is recalcitrant to microbial degradation.

Both technical HCH and lindane have been used extensively in the past. Total usage of technical HCH in China, India, Japan and United States is given in Table 2. The extensive and indiscriminate use of HCH over the past decades has led to spread of its isomers to various components of the environment creating a serious problem

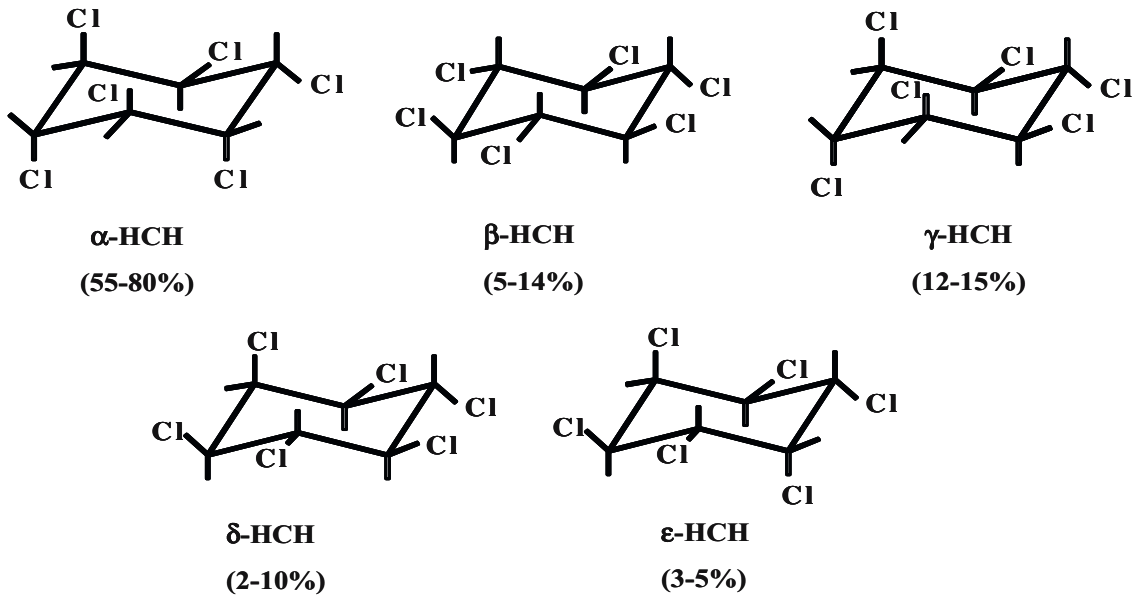

Fig. 1 Chair conformations of HCH isomers. The axial (a) and equatorial (e) positions of the chlorine atoms are as follows: α-HCH: aaaaee, β-HCH: eeeeee, γ-HCH: aaaeee, δ-HCH: aeeeee, ε-HCH: aeeaee.

Table 1 Properties of various HCH isomers (Philips *et al.* 2005)

Property	α-HCH	β-HCH	γ-HCH	δ-HCH	ε-HCH
Melting point (^{0}C)	159-160	309-310	112-113	138-139	219-220
Boiling point (^{0}C)	288 at 760 mm Hga	60 at 0.5 mm Hga	323.4 at 760 mm Hga	60 at 0.36 mm Hga	-
Vapor pressure(mmHg)	4.5 x10 $^{-5}$ at 25 ^{0}C	3.6 x10 $^{-7}$ at 20 ^{0}C	3.1 x10 $^{-5}$ at 25 ^{0}C	3.5x10 $^{-5}$ at 25 ^{0}C	-
Solubility in water (mg/l)	10	5	Insoluble 7.52 at 25 ^{0}C	10	-
Solubility in 100 g ethanol (mg)	1.8	1.1	6.4	24.4	-

±: Values are standard errors, BCF: Bioconcentration factor, -: Not available.

Table 2 Total technical HCH usage in China, India, Japan and the United States.

Country	Production Ban	Total Usage (kt)	Arable Land (kha)	Avg. usage Density (t/kha/yr)	Total Usage Density (t/kha)
China [74]	1983	4460	100,523	1.34	44.37
India [75]	1997	1000	168,260	0.1	5.94
Japan [76,77]	1972	400	4,953	3.65	83.85
United States [20, 78]	1976	350	188,293	0.06	1.86

Table 3 Maximum HCH residues reported in HCH dumpsites/ industrial waste site in some countries of the world

Country	Maximum HCH residues (mg/kg of soil)						Ref.
	α	β	γ	δ	ε	Total-HCH	
Brazil	6200	7320	140	530	n.d	14190	23
Canada	18000	1800	4000	1300	n.d	25100	15
Germany	1.33	15.43	0.02	0.24	n.d	17.02	21
Spain	25	15	2.2	0.5		42.7	25
Spain	45815	34830	47.6	343	n.d	81036	24
Spain	15500	140	447	73.7	77.2	16238	26
India	79940	44850	990	n.d	n.d	125280	29

n.d. not determined

of contamination. The severity of the problem is reflected from the fact that HCH residues continue to be detected in air, soil and water and even in some pristine locations like Arctic region, Antarctica, Pacific Ocean etc. [11, 16, 17, 18, 19]. The HCH isomers are among the most abundant organochlorine contaminants in the Arctic Ocean (USEPA, 2008) [20].

Production of one ton of lindane generates a waste of around 6-10 tons of other isomers [21]. A massive 10 million tons of the technical HCH has been used world over from 1948 to 1997 [16, 22]. This non-insecticidal waste called 'HCH muck' generated by lindane manufacturing units has been discarded either in sealed concrete containers or in open areas. The unregulated disposal of HCH muck has led to the creation of a large number of dumpsites at and near the production site and is a cause of concern. These sites serve as reservoirs from where HCH residues are spreading to far off regions due to leaching and aerial transport. Such HCH dumpsites are present all over the world and have been reported from Rio de Janeiro (Brazil) [23] , Pontevedra (Spain) [24,25] , Bilbao (Spain) [26], Chemnitz (Germany) [27], North Carolina (US) [http://www.earthfax.com/WhiteRot/PCP.htm] and Bitterfield (Germany) [28], Lucknow (India) [29]. These reports are just a small proportion of dumpsites brought to public notice. On the other hand, for a number of cases illegal disposal of HCH muck remains usually unreported. Maximum HCH residues in the HCH dumpsite/industrial waste sites are listed in Table 3.

All HCH isomers are toxic, carcinogenic, endocrine disrupters and are known to exert damaging effects on the reproductive and nervous systems in mammals [16, 30, 31]. Due to its toxic and carcinogenic properties, the use of HCH was banned in most of the developed countries during the 1970s and 1980s. Gradually, some of the developing countries also banned or restricted the use of technical HCH and lindane [22]. But this ban has not reduced the problem posed by HCH. The α-HCH has been released in large quantities in the environment and is carcinogenic, β-HCH even though present in lesser amounts is highly persistent and reported to be estrogenic (EPA, 2003). Moreover, lax environmental laws in a number of countries have failed to prevent illegal disposal of HCH waste.

India started the production of technical HCH in 1952 [32] and perhaps was the largest user of technical HCH and DDT in the world. Technical HCH and DDT amounted to 70% of total insecticide production in the 1980's. From 1948 to 1995 around one million tons of technical HCH was used in India. Residues of HCH have been reported in soil [6, 33, 34, 35], drinking water [1, 2] and food products [3, 4, 5]and even from soft drinks [6]. Realizing the widespread contamination by HCH and toxic nature of HCH isomers the use of technical HCH was banned in India in 1997 but restricted use of lindane is still permitted. India has produced 6,353 ton of lindane for export and indigenous use during 1997 to 2006 (Department of Chemicals and Petrochemicals, India) that would mean a production of nearly 60,000 tons of HCH muck consisting of α-, β and

δ-HCH. Out of the total, 603.58 tons of lindane has been exported to various countries (Director General of Foreign Trade, New Delhi, India). 5–8% of lindane is sold to pharmaceutical companies in India, 25–30% is used for formulation and 15–20% is exported annually. No systematic survey has been carried out to locate the dumpsites created during this period of lindane production. We located an industry producing lindane since 1997 in Northern India and also the HCH dumpsites that have been created during this period.

From *Pseudomonas* sp. to *Sphingobium indicum* B90A

The first report of an aerobic bacterial strain *Pseudomonas paucimobilis* SS86 that degraded HCH appeared around 1990, this strain was isolated from an upland experimental field in Japan where γ-HCH had been applied [36]. *Sphingomonas paucimobilis* UT26 is a nalidixic acid resistant strain of *Pseudomonas paucimobilis* SS86 that degraded α-, γ- and δ-HCH aerobically [37]. Another HCH degrading *Pseudomo-*

Table 4 HCH degrading aerobic bacteria

Bacteria	Degradation	References
Pseudomonas putida	γ-HCH	79
Escherichia coli	γ-HCH	80
Pseudomonas sp.	γ-HCH	81
Pseudomonas vesicularis P59	Mineralisation of α-HCH	82
Sphingobium japonicum UT26	α-, β-, γ- and δ-HCH	36,83,38
Sphingobium indicum B90A	α-, β-, γ- and δ-HCH	7,46,38
Rhodanobacter lindaniclasticus	α- and γ-HCH	39
Pandoraea sp.	α- and γ-HCH	84
Pseudomonas sp.	γ-HCH	53
Sphingobium francense Sp+	α-, β-, γ- and δ-HCH	41,38
Pseudomonas aeruginosa ITRC-5	α-, β-, γ- and δ-HCH	54
Sphingomonas sp. DS2	α-, β-, γ- and δ-HCH	27
Sphingomonas sp. DS2-2	α-, β-, γ- and δ-HCH	27
Sphingomonas sp. DS3-1	α-, β-, γ- and δ-HCH	27
Sphingomonas sp. $\gamma^{1\text{-}7}$	α-, β-, γ- and δ-HCH	27
Sphingomonas sp. $\gamma^{16\text{-}1}$	α- and γ-HCH	27
Sphingomonas sp. $\gamma^{16\text{-}9}$	α- and γ-HCH	26
Sphingomonas sp. $\gamma^{12\text{-}7}$	α- and γ-HCH	27
Sphingomonas sp. $\gamma^{1\text{-}2}$	α- and γ-HCH	27
Sphingomonas sp. $\gamma^{4\text{-}2}$	α-, β-, γ- and δ-HCH	27
Sphingomonas sp. $\gamma^{4\text{-}5}$	α- and γ-HCH	26
Sphingomonas sp. $\gamma^{16\text{-}10}$	α- and γ-HCH	26
Sphingomonas sp. $\gamma^{16\text{-}12}$	α- and γ-HCH	26
Microbacterium sp. ITRC-1	α-, β-, γ- and δ-HCH	55
Sphingomonas sp. BHC-A	α-, β-, γ- and δ-HCH	65
Sphingobium sp. MI1205	α-, β-, γ- and δ-HCH	85
Microbial Consortium *Pseudomonas flurescens* biovarII *Pseudomonas diminuta* *Pseudomonas flurescens* biovarI *Burkholderia pseudomallei* *Pseudomonas putida* *Flavobacterium sp.* *Vibrio aginolyticus* *Pseudomonas aeruginosa* *Pseudomonas stutzeri* *Pseudomonas flurescens* biovar	α-, β-, γ- and δ-HCH	57

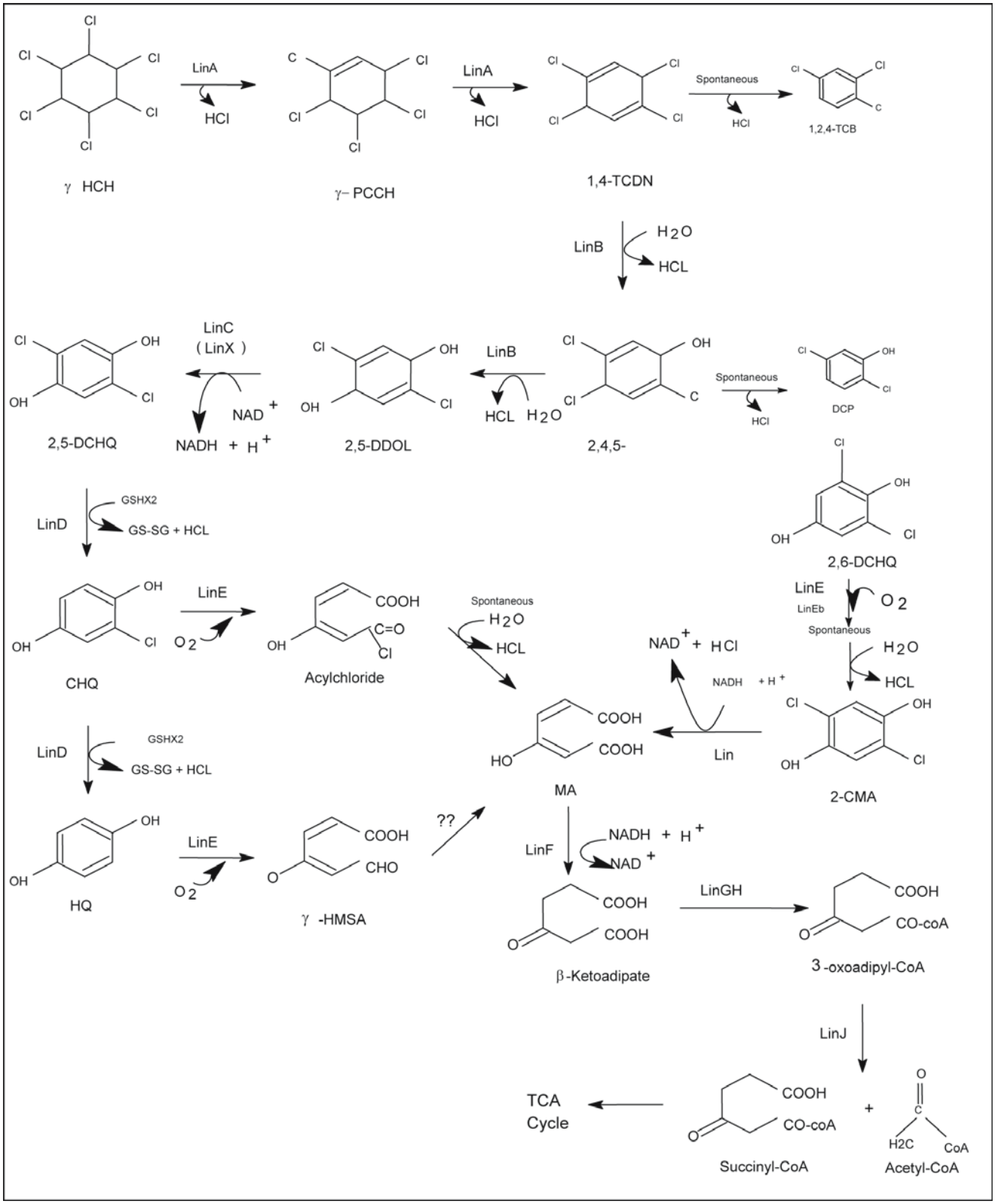

Fig. 2 γ-HCH degradation pathway in *Sphingobium japonicum* UT26 (Nagata *et al*., 2007). γ-HCH; γ-hexachlorocyclohexane**,** γ-PCCH: gamma-pentachlorocyclohexene, 1,4-TCDN: 1,3,4,6-tetrachloro-1,4-cyclohexadiene, 1,2,4-TCB: 1,2,4 trichloro-benzene, 2,4,5-DNOL: 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 2,5-DCP: 2,5-dichlorophenol, 2,5-DDOL: 2,5-dichloro-2, 5-cyclohexadiene-1,4-diol, 2,5-DCHQ: 2,5-dichlorohydroquinone, 2-CHQ: 2-chlorohydroquinone, HQ: hydroquinone, γ−HMSA: gamma-hydroxymuconic semialdehyde, MA: maleylacetate, 2-CMA: 2 chloro maleylacetate,TCA: tricarboxylic acid cycle.

nas sp. was isolated from sugarcane fields in India [7] and this was the first report of aerobic degradation of even β-HCH by a bacterium. Later, both these strains were named as *Sphingobium francense* UT26 and *Sphingobium indicum* B90A [38]. Additionally bacterial strains, *Rhodanobacter lindaniclasticus* [39, 40] and *Sphingomonas paucimobilis* Sp+ [41] were isolated from HCH contaminated soil in France. The three *Sphingomonas* strains were later reclassified as distinct species of genus *Sphingobium* namely *Sphingobium indicum* B90A, *Sphingobium japonicum* UT26 and *Sphingobium francense* Sp^+ by using polyphasic taxonomical approach [38]. Some Gram-positive HCH degrading bacteria like *Bacillus circulans* and *Bacillus brevis* have also been reported in literature that degrade all the HCH isomers including β-HCH [42]. Recently, several new HCH degrading bacterial strains have been isolated from dumpsites of Germany and Spain and all of them belong to the family *Sphingomonadaceae* [26,27]. The list of HCH degrading strains is increasing and new bacterial species that degrade HCH isomers are being added up (Table 4). But sphingomonads still continue to emerge as one predominant group among HCH degrading organisms.

Among all these strains reported, the genetics and biochemistry of degradation of HCH isomers have been worked out in *Sphingobium japonicum* UT26 and *Sphingobium indicum* B90A. The degradation pathway of γ-HCH has been worked out in detail [43, 44]. However, studies have just begun to explore the degradation pathway of α-, β- and δ-HCH.

Unfolding of HCH degradation pathway and *lin* genes in *Sphingobium indicum* B90A

By 2000, studies on *Sphingomonas paucimobilis* B90A isolated by Sethunathan and coworkers made it apparent that it has a better potential for HCH degradation [45] as compared to the then known HCH degrading strains. Sethunathan and his colleagues had indicated this in their pioneering study [45] at a time when molecular genetics involved in the degradation of even γ-HCH was not understood very clearly. Until 2004 reports that appeared on HCH degradation by Nagata and coworkers [44] suggested that UT26 degrades only α-, δ-, and γ-HCH (till then it was not known that UT26 also degrades β-HCH) and contains *lin*A, *lin*B, *lin*C and *lin*DER genes that encode HCH dehydrochlorinase, haloalkane dehalogenase, dehalogenase and ring cleavage dioxygenase leading to the conversion of γ-HCH to pentachlorocyclohexene, dichlorocyclohexadiene, dichlorohydroquinone, chlorohydroquinone, hydroquinone, acyclchloride, γ-hydroxymuconic acid and maleylacetate. In this pathway γ-HCH was found to degrade through a central intermediate chlorohydroquinone (CHQ) (Fig. 2) [44]. Until then it was believed that α-HCH is also degraded through a similar pathway as reported for γ-HCH.

In an attempt to investigate the presence of similar catabolic *lin* genes in B90A, the genomic DNA of *Sphingomonas paucimobilis* B90A was hybridized using P^{32} ATP labeled *lin*A probe from UT26. *Sphingomonas paucimobilis* B90A was found to contain two copies of *lin*A gene [46]. A thorough analysis of two copies of *lin*A revealed 88% amino acids similarity between them. The C-terminal region of one of the *lin*A gene in B90A was found to be replaced by 22 nucleotides of adjoining IS*6100* element [8]. The two copies were named as *lin*A1 and *lin*A2 and while *lin*A1 was found only in *S. paucimobilis* B90A, *lin*A2 was 100% identical to that of *S. paucimobilis* UT26 and *S. paucimobilis* Sp+ [46]. Cloning and expression of these two gene revealed that *lin*A1 re-

Table 5 Comparison of *lin* genes & IS*6100* in B90A, Sp+ and UT26

GENE	No. of nucleotides(aa)			Function	Stability in		
	STRAIN	STRAIN	STRAIN		STRAIN	STRAIN	STRAIN
	B90A	UT26	Sp+		B90A	UT26	SP+
linA2/linA	468(156)	468(156)	ND	*Dehydrochlorinase*	++	+	ND
linB	888(296)	888(296)	888(296)	*Halidohydrolase*	++	+	-
linC	750(250)	750(250)	750(250)	*Dehydrogenase*	++	+	-
linD	1038(346)	1038(346)	1038(346)	*Reductive dechlorinase*	+	+	++
linE	963(321)	963(321)	963(321)	*Ring cleavage dioxygenase*	+	+	++
linR	909(303)	909(303)	909(303)	*Transcriptional regulator*	+	+	+
linX1	750(250)	750(250)	750 (250)	*Dehydrogenase*	++	+	+
linX2	750(250)	ND	ND	*Dehydrogenase*	++	ND	ND
linX3	750(250)	ND	ND	*Dehydrogenase*	++	ND	ND
tnpA	792(264)	792(264)	792(264)	*Transposase*	+	ND	-

*** ++ highly stable, + stable, - unstable.

tained the dehydrochlorinase activity even with the 22-nucleotide variation. The association of *lin* genes with IS*6100* further prompted us to investigate the organization of *lin* genes in B90A. DNA-DNA hybridization data revealed that *lin* genes were nearly identical in B90A, Sp+ and UT26 and were found to be associated with IS*6100* [8]. Although, the copy number of genes other than *lin*A was same in all the three species, the number of *lin*A genes and IS*6100* differed among them (Table 5). This study provided evidence that genetic organization of *lin* genes and their stability is strongly associated with IS*6100* [8]. IS*6100* was initially isolated from *Mycobacterium fortuitum* and copies of IS*6100* that were sequenced from B90A, Sp+ and UT26 were found to be 100% identical to that of *Mycobacterium fortuitum* [8]. This perhaps became the first report that proposed the concept of horizontal transfer of *lin* genes among sphingomonads.

Localization and Genetic Organization of *lin* genes in *S. indicum* B90A

The *lin* genes in B90A are either scattered or organized in different operons (Fig. 3). At least five different transcriptional units, *lin*XA, *lin*B, *lin*C and *lin*DER encode the lindane degradation pathway [47]. *lin*DER form an operon with *lin*R as its positive transcriptional regulator [48]. Attempt was also made to investigate the expression of

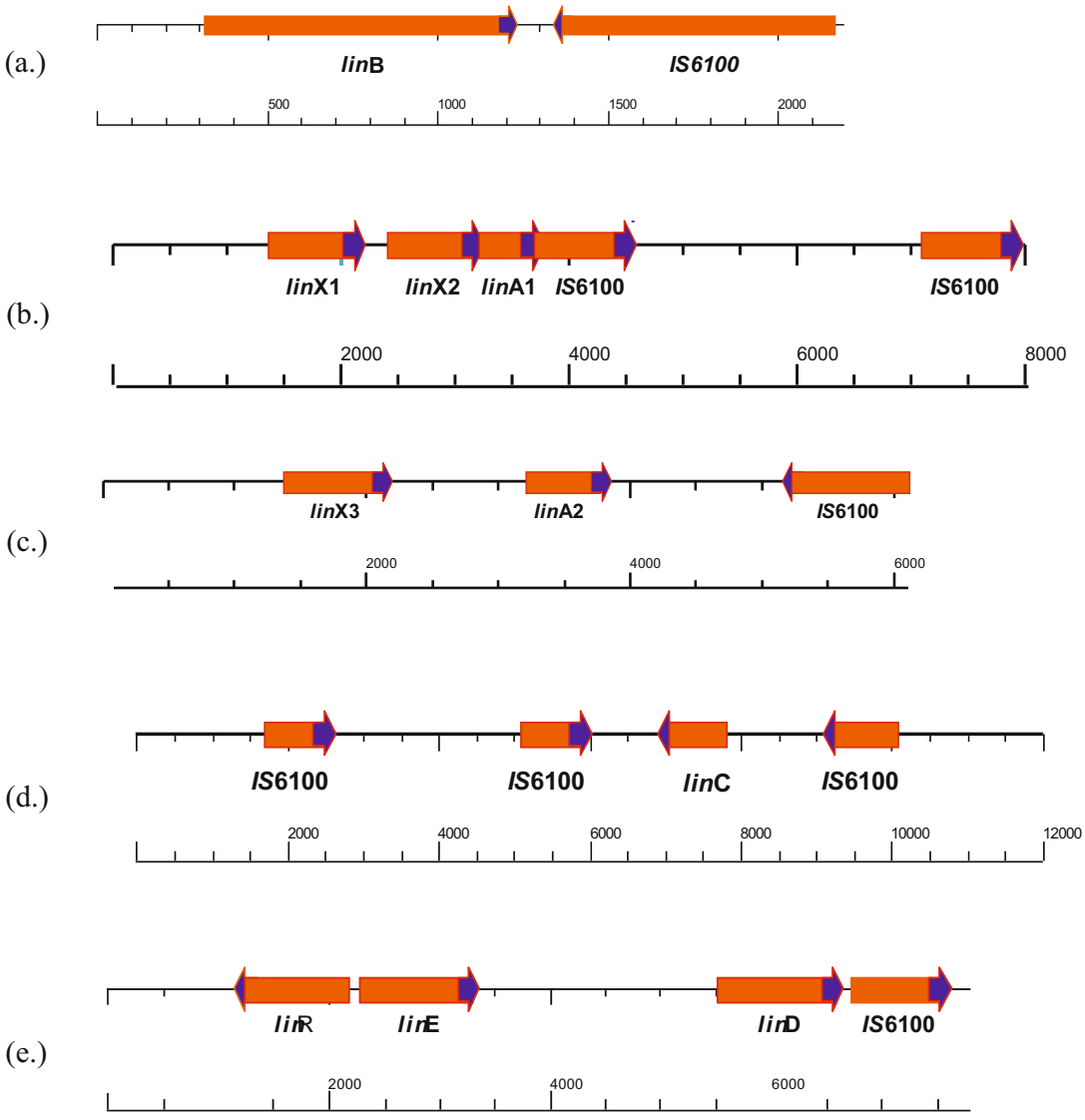

Fig. 3 Organization of *lin* genes in B90A in five different transcriptional units are and their association with the IS6100 element (a.) *lin*B (b.) *lin*X1*lin*X2 *lin*A1 (c.) *lin*X3 *lin*A2 (d.) *lin*C (e.) *lin*DE and *lin*R . (Arrows denote the direction of transcription.)

lin genes in B90A [47]. Irrespective of the addition of any of HCH isomers *lin*A1, *lin*A2, *lin*B and *lin*C were constitutively expressed. On the contrary *lin*D and *lin*E were induced when γ- and α-HCH were added to the medium but not by the addition of β- and δ-HCH. These studies indicated that the pathway for degradation of β- and δ-HCH is perhaps different from γ- and α-HCH. In addition, these studies raised several questions concerning the evolution of sphingomonads especially from high dose point contaminated sites [26, 27]. Although, γ- and perhaps α-HCH seem to be completely mineralized by sphingomonads, it is not known whether they are used by these organisms as sources of carbon and energy [44].

Systematics of HCH-degraders:

As mentioned earlier, Sahu *et al*. (1990) [7] were the first to isolate *Pseudomonas* sp., a HCH-degrader from an Indian sugarcane field which was later classified as *Sphingobium indicum* B90A by polyphasic approach [38]. The genus *Sphingomonas* was created by Yabuuchi *et al*. (1990) [49] to accommodate strictly aerobic, chemoheterotrophic, yellow-pigmented, Gram-negative, rod-shaped bacteria that contain glycosphingolipids as the cell envelope. Based on phylogenetic, chemotaxonomic and physiological analysis, Family *Sphingomonadaceae* has been divided into five genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, *Sphingopyxis, Sphingosinicella* [50, 51]. We isolated seven HCH-degraders from different HCH-contaminated sites in India and on the basis of 16S rRNA gene sequencing; it was found that all these strains belong to the family *Sphingomonadacae*. Recently several reports of isolation of HCH degraders have come up from India. Nawab *et al*. (2003) [52] isolated *Pseudomonas* sp. from agricultural field which can degrade only γ-HCH. Whereas Kumar *et al*. (2005) [53] reported *Pseudomonas aeruginosa* ITRC-5 from HCH-dump site that degraded all the four isomers. Manickam *et. al*. (2006 and 2007) [54, 55] reported HCH-degrader *Microbacterium* sp. ITRC1 and *Xanthomonas* sp. ICH12 from rhizosphere soil and waste water from a HCH-manufacturing unit. Murthy *et. al*. (2007) [56] made an HCH-degrading consortium, which degrades all the four isomers. The consortium includes *Pseudomonas flurescens* biovar II, *Pseudomonas diminuta*, *Pseudomonas flurescens* biovar I, *Burkholderia pseudomallai*, *Pseudomonas putida*, *Flavobacterium* sp., *Vibrio alginolyticus, Pseudomonas aeruginosa, Pseudomonas stutzeri and Pseudomonas flurescens* biovar.

Evolution of *lin* genes to perform newer function in B90A

At the time of discovery of two copies of *lin*A or HCH dehydrochlorinase gene, perhaps it was not realized that *lin* gene are evolving at a rate much faster than scientific imagination. The identification of two copies of *lin*A in B90A was initially thought to be accidental. However, while looking for separate functions for *lin*A1 and *lin*A2 it turned out that these genes preferentially act on the two enantiomeric forms [57]. This became possibly the first example to demonstrate enantiomer specific evolution of genes. LinA1 and LinA2 differ from each other by 18 amino acids, 6 of which are located at the C-terminal region alone. In addition, *lin*A1 encodes 154 amino acids whereas *lin*A2 encodes 156 amino acids (Fig 4) However, it remains to be seen which amino acid residues confer this enantiomeric specificity. The analysis of *lin* genes among three different strains of sphingomonads B90A, Sp+ and UT26 revealed that these contain nearly identical *lin* genes although they were isolated from different geographical locations. This finding raised three crucial questions:

1) do *lin* genes originally belong to sphingomonads?
2) did *lin* genes move from some other original host to sphingomonads?

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LinA2-B90A  MSDLDRLASRAAIQDLYSDKLIAVDKRQEGRLASIWWDDAEWTIEGIGTYKGPEGALD 58
LinA-UT26   MSDLDRLASRAAIQDLYSDKLIAVDKRQEGRLASIWWDDAEWTIEGIGTYKGPEGALD 58
LinA1-B90A  MSDLDRLASRAAIQDLYSDQLIGVDKRQESRPASIWWDDAEWTIEGIGTYKGPEGALD 58

LinA2-B90A LANNVLWPMFHECIHYGTNLRLEFVSADKVNGIGDVLLLGNLVEGNQSILIAAVFTDEYE 118
LinA-UT26  LANNVLWPMFHECIHYGTNLRLEFVSADKVNGIGDVLLLGNLVEGNQSILIAAVFTDEYE 118
LinA1-B90A LANNVLWPMYHETIHYGTNLRLEFVSADKVNGIGDVLCLGNLVEGNQSILIAAVYTNEYE 118

LinA2-B90A RRDGVWKFSKRNACTNYFTPLAGIHFAPPG IHFAPSGA 156
LinA-UT26  RRDGVWKFSKRNACTNYFTPLAGIHFAPPG IHFAPSGA 156
LinA1-B90A RRDGVWKLSKLNGCMNYFTPLAGIHFAPP GALLQKS-- 154
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Fig. 4 Amino acid sequence comparison LinA of UT26 and, LinA1 and LinA2 of B90A.

3) do these three strains B90A, Sp+ and UT26 represent three different strains of *Sphingomonas paucimobilis* or are they three different species having acquired *lin* genes independently?

Research during subsequent years provided some answers to these questions that need to be further explored. Studies on the organization of *lin* genes in these three strains [8] made it clear that *lin* genes have entered in sphingomonads from outside sources and do not originally belong to sphingomonads. All these *lin* genes were subsequently reported to be either present on plasmids or chromosomes [41, 58, 59]. Additionally, polyphasic approach based taxonomical characterization revealed that these three strains are infact three different species of the genus *Sphingobium* [38]. Thus B90A, UT26 and Sp+ were named as *Sphingobium indicum*, *Sphingobium japonicum* and *Sphingobium francense* respectively. This led to the conclusion that under similar stress of HCH, these three strains acquired *lin* genes independently in spite of being present at three different geographical locations.

Further studies on *lin* genes of B90A and their comparison with that of UT26 and Sp+ revealed that *lin* genes in B90A have diversified very quickly and carry out several additional functions that are not performed by *lin* genes isolated from UT26 and Sp+. A closer investigation revealed that this difference might be due to isolation of B90A from sugarcane fields treated with technical HCH [7] whereas Sp+ [41] and UT26 [36] were isolated from soils that were treated with γ-HCH alone.

Until 2005, only *S. indicum* B90A was known to degrade β-HCH. However, it became clear from subsequent studies [60] that UT26 also degrades β-HCH albeit at lower rates. This was perhaps one of the reasons that the degradation of β-HCH by UT26 and Sp+ could not be initially noticed. It was the Japanese group [60] who for the first time reported that LinB (haloalkane dehalogenase) encoded by *lin*B of UT26 is responsible for the initial transformation of β-HCH to pentachlorocyclohexanol (PCCH), a product that was not degraded further. Although purified preparation of LinB of strain UT26 transformed β-HCH to PCCH, crude cell incubation with strain UT26 had no affect on β-HCH transformation [60]. However, this was in contrast to B90A that was repeatedly found to degrade β-HCH [7, 8, 46, 61]. This prompted us to look into the degradation of β-HCH by using purified LinB of B90A as well as whole cell preparation of *Sphingobium indicum* B90A [62]. The studies turned out to be very interesting. Purified preparation of LinB of B90A not only transformed β-HCH in the first step almost 50 times faster than that of UT26 but it also transformed δ-HCH to corresponding mono and dihydroxylated metabolites that were identified as respective pentachlorocyclohexanols and tetrachlorocyclohexandiols [62]. This is in contrast to LinB of UT26 [60] and Sp+ [62] that did not transform pentachlorocyclohenaols. Until this time it was not clear why the β- and δ-HCH degradation differed so markedly among B90A, Sp+ and UT26. LinB enzyme of B90A differs from Sp+ and UT26 by seven amino acids (Fig. 5) and though these differences are located outside the catalytic domain, they seem to play a major role in determining the efficiency of β-HCH degradation by LinB [62] .

Sphingobium indicum B90A thus emerges as a model for studying the degradation pathways of HCH isomers and different functions of *lin* genes. The following conclusions can be safely drawn from studies conducted on B90A:

1) *lin*A1 and *lin*A2 diverged to perform enantiomer specific degradation of HCH.
2) *lin* genes form a comprehensive network that act on several substrates. Studies on the degradation of β- and δ-HCH with purified LinB from *E. coli* revealed that these isomers are hydroxylated to form pentachlorocyclohexanols (B1 and D1) and tetrachlorocyclohexandiols (B2 and D2) [62]. Subsequent studies have confirmed the formation of B1 and B2 from β-HCH and D1 and D2 from δ-HCH in a resting cell assay of strain B90A [9] (Figs. 6, 7). In addition, this assay further revealed the formation of D3 and D4 when incubated with δ-HCH. However, these metabolites were not formed when δ-HCH was incubated in presence of LinB. Further studies revealed that D3 and D4 were not formed in δ-HCH degradation via D1 and D2 respectively but from δ-PCCH (formed by dehydrochlorination of δ-HCH by LinA) as a result of hydrochlorination reaction by LinB [9] (Fig. 7).
3) In *Sphingobium indicum* B90A, *lin* genes were found to be located either on plasmids (*lin*A1, *lin*C, *lin*DER, *lin*X1 and *lin*X2) or on chromosomes (*lin*A2, *lin*B and *lin*X3) [59]. On the contrary in *S. japonicum* UT26, *lin* genes were found to be dispersed on three circular replicons (*lin*A, *lin*B and *lin*C) on chromosome I and *lin*DER on the conjugative plasmid pCHQ1 [58]. However, in *S. francense* Sp+, genes *lin*A, *lin*B, *lin*C and *lin*X were shown to be located on three different plasmids [41]. All these studies point out that *lin* genes are still evolving and are perhaps passed on to sphingomonads through plasmids. Till date only one plasmid that was trapped from soil has been found to contain *lin* genes [63] and has been characterized. However, these plasmids in B90A and other strains are yet to be characterized.

```
linB_Ut26       MSLGAKPFGE KKFIEIKGRR MAYIDEGTGD PILFQHGNPT SSYLWRNIMP
linB_Sp+        MSLGAKPFGE KKFIEIKGRR MAYIDEGTGD PILFQHGNPT SSYLWRNIMP
lin_B_B90A      MSLGAKPFGE KKFIEIKGRR MAYIDEGTGD PILFQHGNPT SSYLWRNIMP

linB_Ut26       HCAGLGRLIA CDLIGMGDSD KLDPSGPERY AYAEHRDYLD ALWEALDLGD
linB_Sp+        HCAGLGRLIA CDLIGMGDSD KLDPSGPERY TYAEHRDYLD ALWEALDLGD
lin_B_B90A      HCAGLGRLIA CDLIGMGDSD KLDPSGPERY TYAEHRDYLD ALWEALDLGD

linB_Ut26       RVVLVVHDWG SALGFDWARR HRERVQGIAY MEAIAMPIEW ADFPEQDRDL
linB_Sp+        RVVLVVHDWG SALGFDWARR HRERVQGIAY MEALAMPIEW ADFPEQDRDL
lin_B_B90A      RVVLVVHDWG SVLGFDWARR HRERVQGIAY MEAVTMPLEW ADFPEQDRDL

linB_Ut26       FQAFRSQAGE ELVLQDNVFV EQVLPGLILR PLSEAEMAAY REPFLAAGEA
linB_Sp+        FQAFRSQAGE ELVLQDNVFV EQVLPGLILR PLSEAEMAAY REPFLAAGEA
lin_B_B90A      FQAFRSQAGE ELVLQDNVFV EQVLPGLILR PLSEAEMAAY REPFLAAGEA

linB_Ut26       RRPTLSWPRQ IPIAGTPADV VAIARDYAGW LSESPIPKLF INAEPGALTT
linB_Sp+        RRPTLSWPRQ IPIAGTPADV VAIARDYAGW LSESPIPKLF INAEPGSLTT
lin_B_B90A      RRPTLSWPRQ IPIAGTPADV VAIARDYAGW LSESPIPKLF INAEPGHLTT

linB_Ut26       GRMRDFCRTW PNQTEITVAG AHFIQEDSPD EIGAAIAAFV RRLRPA
linB_Sp+        GRMRDFCRTW PNQTEITVAG AHFIQEDSPD EIGAAIAAFV RRLRPA
lin_B_B90A      GRIRDFCRTW PNQTEITVAG AHFIQEDSPD EIGAA
```

Fig. 5 Amino Acid differences found in LinB of B90A , Sp+ and UT26

Distribution of *lin* genes among HCH degrading bacteria

Family *Sphingomonadaceae* has a dominant role in the degradation of HCH isomers. All these sphingomonads have similar *lin* genes for HCH degradation. *S. indicum* B90A, *S. francense* Sp+ and S. *japonicum* UT26 have similar *lin*A, *lin*B, *lin*C, *lin*D, *lin*E, *lin*R and *lin*F genes for γ-HCH degradation [8, 46]. The rate of HCH degradation in *S. francense* Sp+ is similar to *S. japonicum* UT26 but all HCH isomers are degraded much more efficiently by *S. indicum* B90A.

Phylogenetic analysis of newly isolated strains and already reported *S. indicum* B90A, *S. francense* Sp+ and *S. japonicum* UT26 clearly showed that *S. indicum* B90A, *S. francense* Sp+ and *S .japonicum* UT26 form a common cluster and none of the newly isolated strain come under this cluster except *Sphingobium* sp. BHC-A. They not only formed a separate cluster but also significantly diverged from each other. Some of the strains like, *Sphingomonas* sp. DS2 and *Sphingomonas* sp. α16-10 were isolated from Germany and Spain, respectively but they were phylogenetically close to each other (Fig. 8). In a similar manner, strains *Sphingomonas* sp. DS2-2 and *Sphingomonas* sp. DS3-1 were isolated from Germany but they diverged phylogenetically from each other. One of the HCH degrading Chinese strain BHC-A [64] made common cluster with *S. francense* Sp+, *S. japonicum* UT26 and *S. indicum* B90A (Fig. 8). All these strains have been reported to contain similar *lin*A, *lin*B, *lin*C, *lin*D, *lin*E, *lin*R and *lin*F genes for HCH degradation (Table 6). All the *lin* genes in these strains were also associated with IS*6100* as already reported in *S. indicum* B90A, *S. francense* Sp+ and *S. japonicum* UT26 [8, 27] (Table 7). Loss of *lin* genes is also associated with loss of IS*6100*. The association of IS*6100* with *lin* genes indicates an important role played by IS*6100* in mobilization of *lin* genes. The reason behind the adaptability of sphingomonads to such distinct and heavily contaminated sites is still

not known. However, surface of sphingomonads are highly hydrophobic due to the presence of sphingolipids that may facilitate the assimilation of hydrophobic compounds like HCH [65]. Recently, Aso *et al* (2006) [66] reported that sphingomonads contain superchannels in their cell membrane that might permit the transport of macromolecules such as HCH and presence of *lin* genes in sphingomonads would facilitate HCH degradation. The presence of IS*6100* in these newly isolated strains also signifies the horizontal gene transfer of *lin* genes among sphingomonads [27]. Singh *et. al* (2007b) [67]reported the selective loss of *lin* genes of *Pseudomonas aeruginosa* ITRC-5 under different growth conditions. *Pseudomonas aeruginosa* ITRC-5 was isolated by selective enrichment on technical HCH and that had the potential to degrade all isomers of HCH efficiently [68]. All *lin* genes were however lost in *Pseudomonas aeruginosa* ITRC-5 during growth in LB medium and loss of one or two copies of *lin* genes during growth in mineral salt medium containing only γ-HCH as source of carbon and energy. The loss of *lin* genes is also associated with loss or rearrangement of IS*6100*. The unstable nature of *lin* genes in *Pseudomonas* and stable nature in sphingomonads has not been investigated. However, there is a need to understand the diversity, distribution and nutritional requirements of these HCH degrading microorganisms to develop a successful bioremediation technology [69].

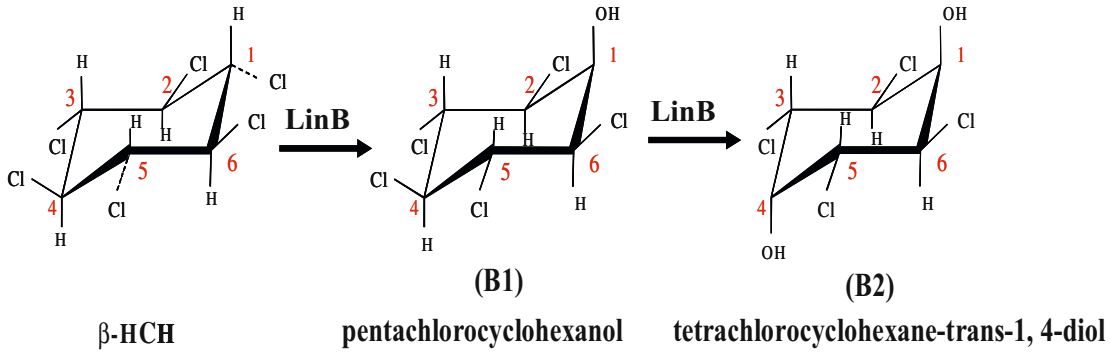

Fig. 6 Degradation pathway of β-HCH in *Sphingobium indicum* B90A.

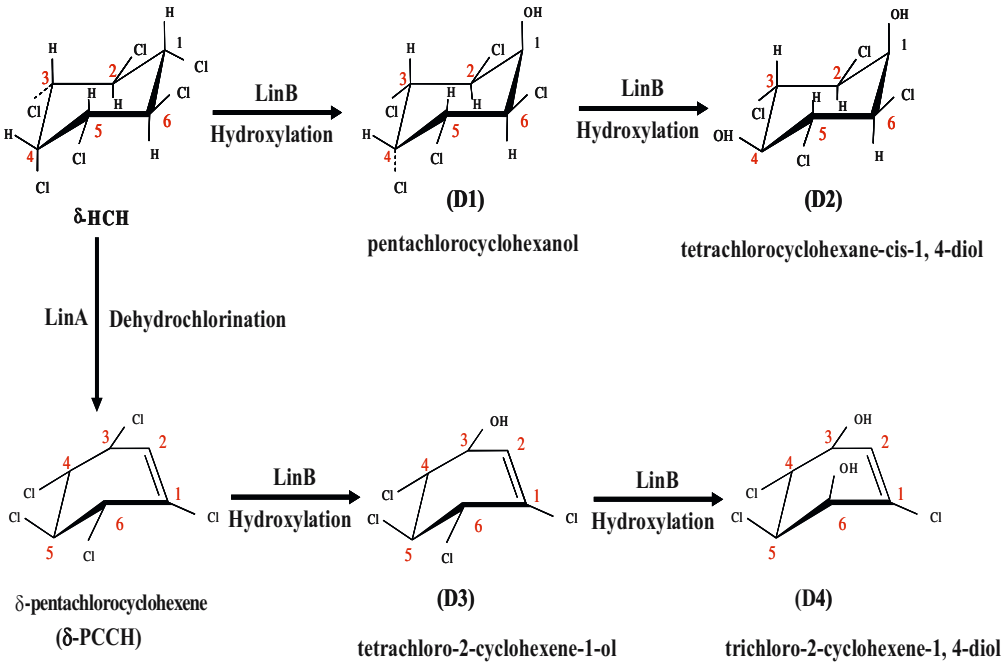

Fig. 7 Degradation pathway of δ-HCH in *Sphingobium indicum* B90A (Raina *et al.,* 2007).

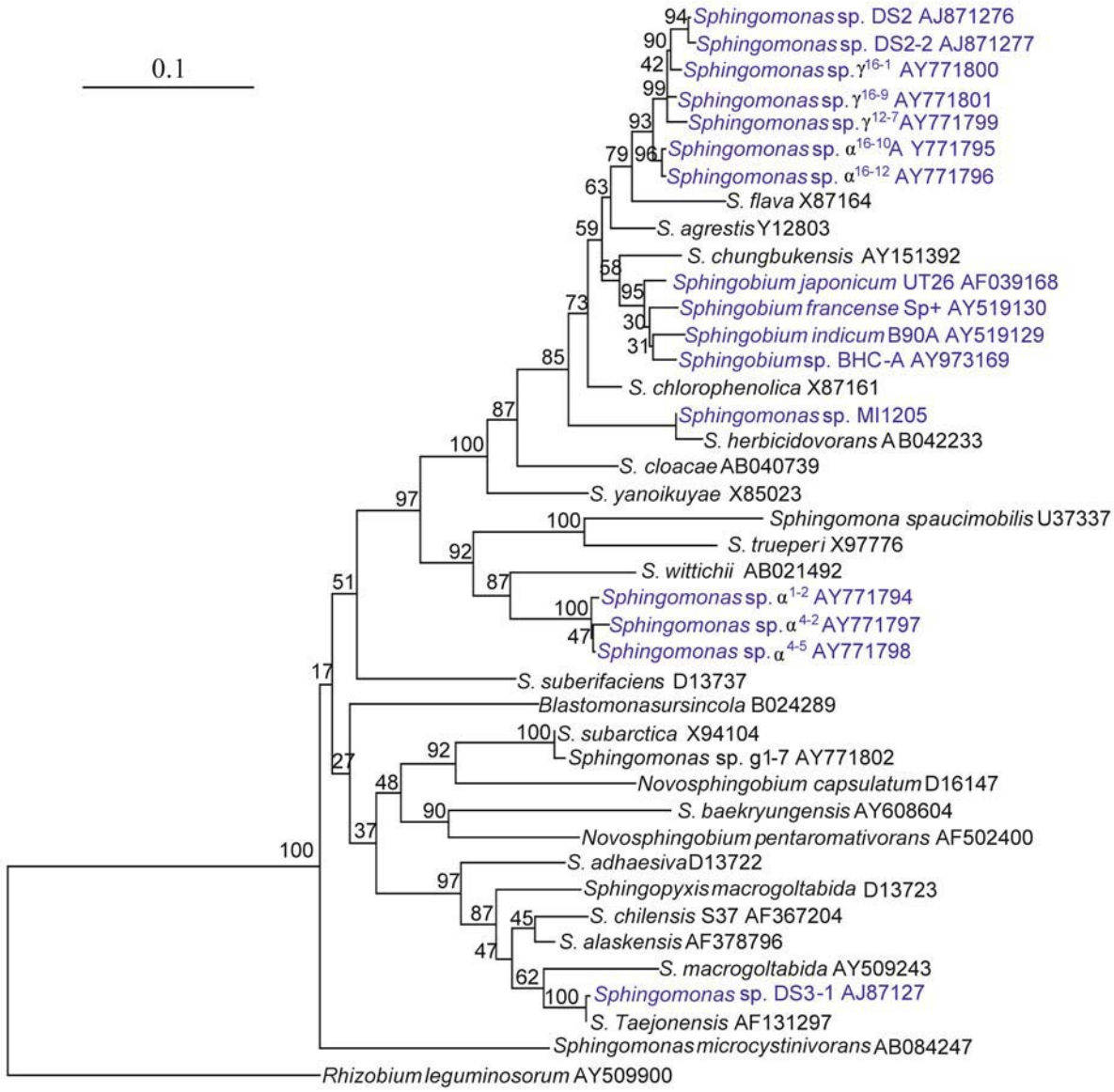

Fig. 8 Phylogenetic tree (Neighbor joining method) of HCH degrading sphingomonads (blue color) and non-HCH degrading sphingomonads (black color) was constructed by using 16S rRNA gene sequences. *Rhizobium leguminosarum* was used as outgroup. Bootstrap values (in percentage of 100 replicates) and DNA database accession numbers are indicated. The scale bar indicates substitution per site.

Bioremediation of HCH

Bioremediation has been proposed as an apt method for decontamination of HCH as chemical and physical methods are not only costly but also ineffective [70, 71, 72]. Since *S. indicum* B90A has now been reported to have better potential for HCH degradation, this strain can be used for this purpose. In recent studies where B90A was used for decontamination, it was found that:

1. B90A does not survive very well when added to soil [10]. There is more than 60% mortality after 8days and thus repeated inoculum is needed.
2. A cell number of 10^6 cells/g soil is good enough to obtain degradation of HCH isomers upto >90%.
3. The survival of B90A and degradation depends on the soil type.
4. Bioaugmentation is successful only in soils having low level of HCH contamination but not at high dose point contaminated soils.

Thus further studies are needed to develop bioremediation technique especially for the decontamination of HCH residues from dump sites.

Table 6 Presence of *lin* genes and IS*6100* in HCH degrading bacterial strains [40,66,75,76,78,87]. (+ and – indicate presence and absence respectively)

lin genes/ *IS*6100	DS2	DS2-2	DS3-1	α1-2	α4−2	γ1-7	γ12-7	γ16-1	UT26	B90	B90 A	ITRC-1	BHC-A	ITRC-5	MI120-5
*lin*A	+	+	+	+	+	+	+	+	+	+	+	-	+	nd	nd
*lin*B	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	+
*lin*C	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	nd
*Lin*X1	+	+	+	+	+	+	+	+	+	+	+	nd	nd	nd	nd
*Lin*X2	+	+	+	+	+	+	+	+	+	+	+	nd	+	nd	nd
*lin*D	+	+	+	+	+	+	-	+	+	-	+	nd	+	nd	nd
*lin*E	+	+	+	+	+	+	-	+	+	-	+	nd	+	nd	nd
*lin*R	+	+	+	+	+	+	-	+	+	-	+	nd	+	nd	nd
*IS*6100	+	+	+	+	+	+	+	+	+	+	+	nd	nd	nd	nd

nd: Not Determined.

Table 7 Genetic organization of *lin* genes and IS*6100* in HCH degrading bacterial strains [40,66,75,76,78,87]

lin genes/ *IS*6100	DS2	DS2-2	DS3-1	α1-2	α4−2	γ1-7	γ12-7	γ16-1	UT26	B90	B90 A	ITRC-1	BHC-A	ITRC-5	MI120-5
*Lin*X,X2,A/ IS6100	+/-	+/-	+/+	+/+	+/+	+/-	+/-	+/-	+/-	+/+	+/+	nd	nd	nd	nd
*IS6100/ lin*B/is6100	./+/+	./+/+	+/+/+	./+/+	+/+/+	./+/+	+/+/+	+/+/+	./+/+	./+/+	+/+/+	nd	nd	nd	nd
*IS6100/ lin*C/IS6100	./+/.	./+/.	+/+/+	./+/+	./+/+	./+/+	./+/.	./+/.	./+/+	+/+/+	+/+/+	nd	nd	nd	nd
*Lin*R/E	+/+	+/+	+/+	+/+	+/+	+/+	./.	+/+	+/+	+/+	+/+	nd	nd	nd	nd

Conclusion from the review

HCH isomers are the most debatable pollutants due to their toxic, carcinogenic and persistent nature. Although, HCH use and production has been banned all over the world, already existing huge stockpiles of HCH and creation of new HCH dumpsites due to lindane production in India are the major sources of HCH contamination. There is a need to decontaminate these HCH dumpsites and to develop a decontamination strategy. Because physical and chemical methods are costly and not very effective, bioremediation can be one of the affordable methods for decontamination of HCH from dumpsite. No successful HCH bioremediation technique has been developed till now. Several laboratory scale microcosm studies are available but main reasons for the failure of *in situ* bioremediation of HCH contaminated site could be that the strategies worked out in laboratory conditions do not work in the field. Therefore, a deeper knowledge of soil micro- or macro-environment is required. Additionally there is a need to understand the degradative pathways of β- and δ-HCH. Perhaps use of bacterial consortia instead of single organism could be a better approach for HCH degradation through bioaugmentation.

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