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Research Article

Horizontal transfer of haloalkane dehalogenase (LinB) gene among novel hexachlorocyclohexane (HCH) degrading sphingomonads isolated from contaminated soil

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Abstract

Soil samples from a HCH dump site and agricultural fields were collected from northern part of India in order to determine residue levels of α-, β-, γ- and δ- HCH and distribution of HCH degrading sphingomonads. The concentration of HCH residues was found to be very high in dump site soil (upto 4252.47µg/g) but it was low in the soil collected from the agricultural lands (upto 7.7µg/g). Eight novel HCH degrading strains could be isolated from HCH dump site soil but none from agricultural soil. Southern blot hybridization studies revealed the presence of haloalkane dehalogenase (LinB) genes alongwith IS*6100* mobile genetic element in all of these strains. Further, 16S rDNA based phylogenetic analysis showed that these strains belong to the genus *Sphingobium* of family sphingomonadacae. An evidence for horizontal transfer of *linB* gene among these novel sphingomonads (only five were analysed) was derived by phylogenetic incongruency based approach. The sequence analysis of *linB* gene of novel isolates showed high sequence similarity (>98.9 to 100%) with each other indicating they were the homologs of same gene. Comparison of "species tree" with "*linB* gene tree" revealed incongruency among both the trees thus proving that this gene has disseminated among these novel sphingomonads in a recent HGT event. Presence of IS*6100* element in all the strains further supported the evidence. This study reveals that the members of family sphingomonadacae are acquiring *lin* genes by horizontal transfer under high selective pressure of HCH and such phenomenon at a contaminated site is quite significant for bioremediation of HCH.

Key words: Horizontal gene transfer (HGT), haloalkane dehalogenase (LinB) gene, hexachlorocyclohexane (HCH), sphingomonads, bioremediation.

Introduction

Bacteria have a tendency to adapt themselves for living in an environment contaminated with toxic xenobiotics. Horizontal gene transfer (HGT) enables bacteria to acquire the catabolic genes required for the degradation of such compounds [1, 2, 3, 4]. HGT has been shown in microcosm soil experiments in which soil was spiked with desired pollutant and was inoculated with donor and recipient /or indigenous bacteria [2, 4]. However, such studies do not reveal HGT events under natural conditions. To analyze *in situ* horizontal transfer of genes phylogenetic incongruency based approaches has been found most appropriate [5]. Such studies often infer horizontal transfer by showing that a highly conserved gene is shared by a group of taxonomically diverse hosts.

Driving force behind HGT is the presence of a suitable selective pressure of a xenobiotic and competence of bacteria for adaptation [2, 4]. Hexachlorocyclohexane (HCH) is a xenobiotic pesticide consisting mainly of 60-70% α-HCH, 10-15% γ-HCH, 5-12% β-HCH, 6-10% δ-HCH, 3-4% ε-HCH and 1% other isomers [6]. This mixture of isomers (technical-HCH) has been used against agricultural pests indiscriminately in the past and thus has led to serious environmental contamination. Besides the disposal of α–, β– and δ– isomers left after the purification of γ-HCH (lindane) has created dump sites [7]. Though all HCH isomers are recalcitrant but many bacteria have evolved so as to metabolize them thus ensuring their survival at contaminated sites. Although HCH degrading organisms fall in various genera and species, however members of five genera of family sphingomonadacae viz; *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella* [8, 9] has outnumbered others in this ability. Three archetypal sphingomonad strains-*Sphingobium indicum* B90A [10], *Sphingobium japonicum* UT26 [11] and *Sphingobium francense* Sp+ [12] isolated from HCH contaminated soil of India, Japan and France respectively are well known HCH degraders. Recently number of novel HCH degrading strains have been isolated from India [13, 14]. The HCH isomer degradation pathways have been well studied and characterized in these strains. For HCH degradation *linA* (dehydrochlorinase), *linB* (haloalkane dehalogenase), *linC* (dehydrogenase), *linD* (reductive dechlorinase), *linE* (ring-cleavage oxygenase), *linF* (reductase) *linGH* (succinyl-CoA: 3-oxoadipate CoA transferase), *linJ* (β-ketoadipyl CoA thiolase) and two regulatory genes *linI* and *linR* have been found essential [15, 16, 17, 18]. In addition *linK*, *linL*, *linM and linN* also play an indirect role in HCH degradation [19]. These genes encode permease, ATPase, periplasmic protein and lipoprotein, various parts of ABC type of transporter which facilitates the transport of HCH isomers into the cell.

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In recent years many more HCH degrading strains have been isolated from various parts of the world. All of them have majorly been found to be members of family sphingomonadacae [20, 21]. Surprisingly all these strains showed the presence of *lin* genes in them alongwith IS*6100* element which plays an important role in their organization [20, 21, 22]. From these studies it seems sphingomonads are more prone for adaptation towards HCH degradation. Though many HCH degrading sphingomonads have been isolated from HCH contaminated soil worldwide, however evidences for *in situ* HGT are lacking.

In this study we have isolated novel HCH degrading sphingomonads from a site heavily contaminated with HCH and characterized them phylogenetically. To prove that *linB* gene has horizontally transferred among these strains phylogenetic incongruency approach was utilized. The *linB* gene was chosen for phylogenetic analysis owing to its important role in initiating the pathway of most recalcitrant β-HCH isomer [23, 24]. This gene produces an enzyme haloalkane dehalogenase (LinB) which in the first and rate limiting step hydroxylates β-HCH to pentachlorocyclohexanol and tetrachlorocyclohexanediol [24]. Besides it is also involved in the degradation of α–, γ-and δ- HCH isomers.

Material and methods

Soil samples

Soil samples were obtained from two different sites. One site was an open dump located at Ummari, Lucknow and the second site was agricultural lands at Chittora near Delhi. Samples were collected from a depth of 20 cm, transported to laboratory and maintained at 4°C. Soil from dump site was found to have 27.6% sand, 28.4% slit, 44.0% clay, and 0.40% organic carbon, and the pH was 9.3 whereas agricultural land soil contained 67.6% sand, 20.0% slit, 12.4% clay, and 0.45% organic carbon, with a pH 7.6.

GC analysis for HCH residues

HCH isomers from contaminated soils were extracted according to procedure described by Prakash *et al.*, 2004 [25]. Soil samples were air dried, grinded and sieved. 10 g of this soil was taken in conical flask (250 ml volume) and mixed with 30ml of hexane: acetone (1:1). Flasks with soil sample and solvent were incubated on rotatory shaker at 250 rpm for 12 h. The solvent was taken out in a fresh tube and passed through saturated Na_2SO_4. The extract obtained after passing through column was dried in Buchi Rotavapor model R-114 (Buchi, Switzerland) and finally dissolved in 1 ml of hexane for further analysis. Residual levels of HCH isomers were measured by subjecting samples to gas liquid chromatograph (Shimadzu, GC-17A, Japan) equipped with a ^{63}Ni electron capture detector and a BPX50 capillary column (internal diameter 0.32 mm; SGE Australia Pty. Ltd., Australia). The oven, injector and detector temperatures were maintained at 200°C, 220°C and 250°C, respectively. The flow rate of nitrogen as a carrier gas was 27 ml per min.

Isolation of HCH degraders

One gram of soil was taken and serially diluted. Aliquots of 100 µl were plated on LB agar plates supplemented with streptomycin (200 µg/ml) for specific selection of sphingomonads [26] along with nystatin (30 µg/ml) to inhibit soil fungus. Yellowish to off-white colonies of presumptive sphingomonads were picked and patched again on the same media. Around 23 colonies were purified by repeatedly streaking. To identify HCH degrading strains, isolates were inoculated in 5 ml of LB medium supplemented with 5 µg/ml each of α–, β–, γ– and δ- HCH according to procedure earlier described [27]. *S. indicum* B90A, known HCH degrader was used as positive control and one uninoculated tube served as another control. All tubes were incubated at 28°C with constant shaking at 200 rpm. Aliquots of 200 µl were withdrawn from each sample at an interval of 2 hours and extracted with 500 µl of hexane twice; the extract was pooled and injected in GC under conditions described as above.

Resting cell assay for HCH isomers degradation

To compare the degradation ability of each strain towards α–, β–, γ– and δ- HCH isomers resting cell assay was performed as described earlier [24]. For this purpose, 250 ml culture in LB (optical density at 600 nm, 0.5) was raised and pelleted by centrifugation. Cell pellet (100 mg) of each strain was then washed twice with 0.1 M sodium phosphate buffer (pH 7) and suspended in 5 ml phosphate buffer. To this suspension α–, β–, γ– and δ- HCH isomers (5 µg/ml) were added separately. An aliquot of 0.2 ml of the reaction mixture was withdrawn from each flask periodically, extracted twice with 0.5 ml of hexane, pooled, and analyzed by gas chromatography as described previously.

Southern blot hybridization for linB gene

Genomic DNA of all strains was isolated as described elsewhere [27]. For the detection of *linB* gene, genomic DNA of isolated strains was digested with *HindIII* [4]. Digested DNA fragments were separated by gel electrophoreses and then transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, USA) and probed with (α-^{32}P) dATP (BRIT, Hyderabad, India)-labeled *linB*. DNA probes were made by random primed labeling method by using Megaprime™ DNA Labeling Systems (Amersham Biosciences, UK Limited). The (α-^{32}P) dATP labeled probe was allowed to hybridize to nylon membrane at 68°C overnight in rotating hybridization oven. Following hybridization washings of higher stringency solutions of 2 X SSC (1 X SSC, 150 mM NaCl and 15 mM sodium citrate, pH 7.0) plus 0.1% sodium dodecyl sulfate (SDS) at 68 °C and once in a solution containing 1 X SSC plus 0.1% SDS at room

temperature were given. The membranes were then exposed to X-ray films (Kodak India Ltd, Mumbai, India) overnight.

16S rRNA and *lin*B gene sequencing

For 16S rRNA gene sequence analysis PCR amplification was carried out by colony PCR. A single colony of each strain was boiled in 50 µl of water for about 10 min at 95 °C. The contents were centrifuged at 10,000 rpm for 5 min and 10 µl of supernatant after ten times dilution was used for DNA amplification. PCR amplification of 16S rRNA gene was carried out using universal primer set, 27F (5'GTTTGATCCTGGCTCAG3') and 1492R (5'TACGGTTACCTTGTTACGACTT3'). The PCR product was run in 3100 Avant™ Genetic Analyzer sequencer (Applied Biosystems, USA) at the Department of Zoology, University of Delhi, using MicroSeq® full gene 16S rRNA gene sequencing kit (Applied Biosystems, USA). Sequencing of *linB* was done from the amplified product itself. Genomic DNA from various strains was isolated as described earlier [27]. All PCR reactions were performed as described above. The primers used for *linB* gene amplification from various isolates were *linB* (forward) GC**GGTACC**CGATTCCTCGATTGA and *linB* (reverse) (GC**GGTACC**AAAATGAGCCGGTTC) derived from *linB* gene sequence of *S. indicum* B90A [22].

Phylogenetic analysis

The sequences of 16S rRNA gene sequences obtained were searched for homology in database by using BLAST (http://www.ncbi.nlm.nih.gov) and RDP (http://rdp.cme.msu.edu/html/). For phylogenetic analysis validated and published 16S rRNA gene sequences of *Sphingobium, Sphingomonas sensu stricto, Novosphingobium, Sphingopyxis* and *Sphingosinicella* [8, 9] showing close similarity with novel isolates were taken. *Zymomonas mobilis* ATCC 10988^T was selected as an outgroup. Multiple sequence alignment of 16S rRNA gene sequences was done by using ClustalX [28]. Common gaps in the sequences were removed. Phylogenetic tree was constructed using the neighbor-joining method [29], selecting bootstrap value of 100, using SEQBOOT and CONSENSE programs of the PHYLIP [30]. Phylogenetic tree was also constructed using parsimony method (DNAPARS). For phylogenetic incongruence comparison unrooted trees of 16S rRNA gene and *linB* gene sequences were also constructed similarly.

Nucleotide sequence accession number

The nucleotide sequence of 16S rRNA gene and *linB* gene in this study have been deposited in the Genbank database with accession numbers FJ966191 to FJ966197 and FJ966198 to FJ966202 respectively. Accession number for strain P25 which was deposited separately is EU781657

Results and discussions

HCH levels in soil samples

The soil samples showed variable levels of HCH contamination in both types of soils. The ΣHCH residue levels in agricultural soil were very less (upto 7.7µg/g) as compared to dump site (upto 4068.25µg/g) (Table.1). β-HCH was one of the predominant isomers (upto 4.7µg/g) followed by α-HCH (upto 2.9µg/g) in agricultural soil. The levels of γ-HCH and δ-HCH were very less (0.04µg/g and 0.03 µg/g respectively) as compared to the other two isomers. In contrast to agricultural soil α-HCH was the predominant HCH isomer (upto 4252.47µg/g) at dump site soil (Table.1). β-HCH was also present in higher concentrations (upto 119.64µg/g) but in lower amount as compared to α-HCH. The levels of γ- HCH and δ-HCH were up to 14.3µg/g and 50.28µg/g respectively. The characteristics of both types of soils were also measured. Agricultural soil was sandy with normal pH whereas dump site soil was normal loam with slight alkalinity (Table. 2). Although HCH has been banned now in most of the countries of the world however dump sites formed *a priori* to ban are still there [31]. The unusual process of lindane production is responsible behind the creation of dump sites. When lindane (γ-HCH), is purified from the technical HCH mixture α-, β-, and δ- isomers are treated as waste and are dumped in the open. High concentration of HCH at theses sites provides enough selective pressure for the evolution of bacteria towards HCH degradation.

Table 1. Levels of HCH residues in dump site soil and agricultural soil.

Sample	α-HCH (µg/g)	β–HCH (µg/g)	γ-HCH (µg/g)	δ-HCH (µg/g)	Total HCH (µg/g)
Dump site soil	4068.25±2.2	119.64±0.2	14.3±0	50.28±0	4252.47± 2.0
Agricultural soil	2.9±0.2	4.7±3	0.04	0.03	7.7±0.9

Table 2. Characteristics of soil samples. Soil analysis was carried out at IARI, PUSA, Delhi, India.

Sample	pH	E.C (dS/m)	Carbon (%)	P (Kg/Ha)	K (Kg/Ha)	Moisture (%)	N2 (Kg/Ha)	Sand (%)	Slit (%)	Clay (%)
Dump site soil	8.3	28	0.75H	80.6 H	274 M	14..99	91	49.6	50	0.4
Agricultural Soil	7.6	0.14	0.45 L	47.0 H	202 M	17.70	276 L	67.6	20	12.4

H, heavy; M, medium; L, low

Isolation of HCH degrading sphingomonads

Sphingomonads have shown a higher tendency towards HCH degradation [20, 21]. Therefore, the main focus was the isolation of HCH degrading sphingomonads in this study. Most of the sphingomonads are resistant to streptomycin [24] and hence the antibiotic was used in LB agar medium to specifically isolate them. From dump site twenty-three yellowish to off – white and morphologically different colonies of presumptive sphingomonads were isolated and analysed for HCH degradation. In this preliminary study, out of twenty-three strains only eight strains designated as P1, P3, P7, P8, P12, P18, P21 and P25 could degrade α–, β–, γ– and δ-HCH. Similarly, from agricultural soil sixteen sphingomonads strains were isolated and checked for HCH degradation. However, none of these strains could degrade HCH isomers and hence were not analyzed further.

To compare the individual rate of degradation of α–, β–, γ– and δ– HCH by strains P1, P3, P7, P8, P12, P18, P21 and P25 resting cell assays were performed. *S. indicum* B90A which is already known to degrade α–, β–, γ– and δ– HCH was used as reference strain in these assays [10]. It was found that β–, γ– and δ– HCH were degrade at comparable rates by all the strains as compared to *S. indicum* B90A (data not shown) except α-HCH which was degraded at a faster rate (Fig. 1).

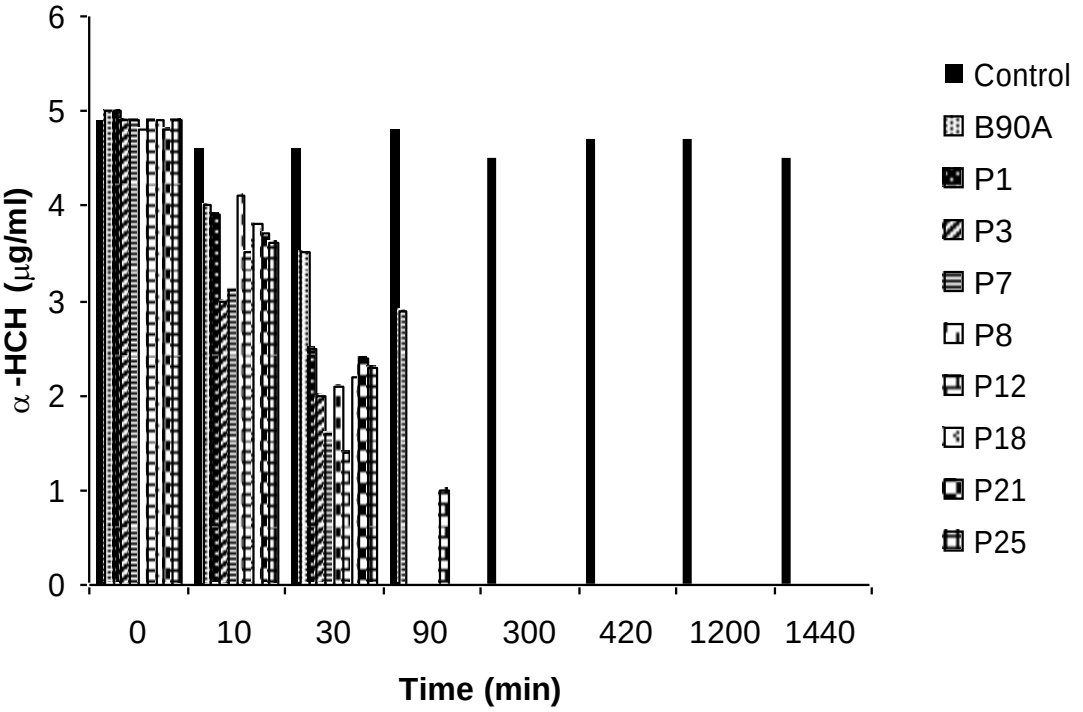


Figure 1: Degradation of HCH isomers α- HCH (A), β- HCH (B), γ- HCH (C) and δ-HCH (D) by strains P1, P3, P7, P8, P12, P18, P21 and P25 in phosphate buffer. An initial inoculum of 100mg cell pellet was suspended in 5 ml of phosphate buffer and each HCH isomer was added separately (5μg/ml). Samples were withdrawn periodically, extracted with hexane, and analyzed on a gas chromatograph equipped with an electron capture detector.

Success in isolating eight HCH degrading strains from highly contaminated dump site soil as compared to less contaminated agricultural soil, from which no HCH degrading strain could be isolated, indicates that high

levels of HCH contamination must have provided a sufficient level of selective pressure for these bacteria to get evolved at this site. The possibility that HCH degraders could have evolved after the removal of soil samples does not arise as bacteria were isolated immediately that too by direct serial dilution method. No enrichment of the soil sample was done at any stage of the experiment. Thus, it can be affirmed that the HCH degraders isolated in this study were indigenous to the soil and have evolved there only. Further all the eight strains isolated could degrade α–, β–, γ– and δ- HCH isomers like *S. indicum* B90A strain isolated in India [10]. So far, the strains isolated elsewhere could not show such a wide range of HCH degradation [20, 21]. Thus, the strains isolated in this study have a great bioremediation value owing to their ability to degrade all the four major HCH isomers.

Detection of *lin*B gene in novel HCH degraders

The *lin* genes are known to degrade HCH isomers. Since novel strains showed the degradation of HCH isomers therefore we analyzed the presence of these genes especially *linB* by Southern blot hybridization. Hybridization with *linB* gene probe gave single band in all of these strains (Fig. 2). In all of the strains IS*6100* element was also present and varying number of hybridization signals was obtained (data not shown). The *linB* shared approximately same position in genomic DNA in all of these strains which reflects that these genes may have conserved flanking regions also. In this analysis all HCH degrading strains showed the presence of *lin* genes and IS*6100* element in their genomes. Presence of *linB* genes in these novel isolates indicates that these isolates utilize the same pathway for HCH degradation as reported earlier [22, 17].

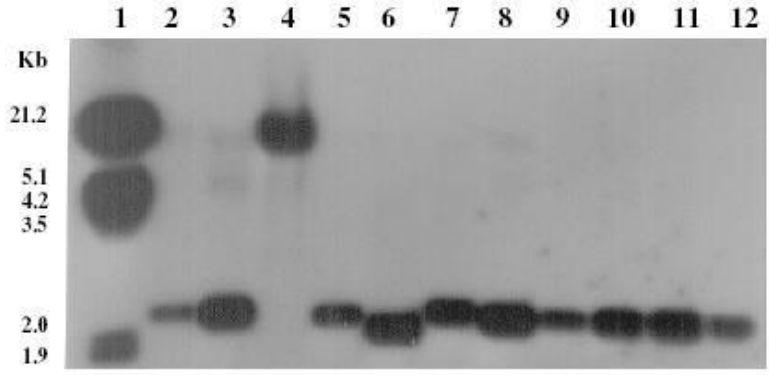


Figure 2. Southern blot hybridization of genomic DNAs of B90A, Sp+ and UT26, P1, P3, P7, P8, P12, P18, P21 and P25 digested with *HindIII* for *linB* gene. Lane1, Marker λ DNA *EcoR1/HindIII* digested; lane2, *Sphingobium indicum* B90A; lane 3, *Sphingobium francense* Sp+; lane4, *Sphingobium japonicum* UT26; lane5, P1; lane6, P3; lane7, P7; lane8, P8; lane 9, P12; lane10, P18; lane 11, P21; lane12, P25.

16S rRNA based phylogenetic analysis of novel HCH degraders

So far most of the HCH degrading strains isolated worldwide have been found to be member of family sphingomonadaceae [14, 21]. Therefore, to ensure whether the novel HCH degrading strains isolated in this study also belong to same family 16S rRNA phylogenetic characterization of these strains was done. From these studies it was revealed that the novel HCH degrading strains belong to *Sphingobium* genus of group Sphingomonads (Fig. 3). Strains P1, P18 and P21 showed 99.6%, 99.8% and 99.7% similarity to strain *Sphingobium herbicidovorans*. Strains P3, P7 and P8 were found 99.7%, 99.5% and 99.8% similar to *Sphingobium japonicum* UT26, a known HCH degrading strain. Strain P12 and strain P25 were respectively found 99.8% and 97.7% similar to *Sphingobium fuliginis* and *Sphingobium ammiense*. All of the novel isolates were found to cluster together among above mentioned members of genus S*phingobium* in the phylogenetic tree (Fig.3). It seems that sphingomonads have a stronger tendency to get adapted toward HCH utilization. Sphingolipids present in the membrane of sphingomonads [9] and ABC type of transporter [19] help in the easy transport of HCH isomers into the bacterial cell and may be aiding these bacteria towards HCH adaptation.

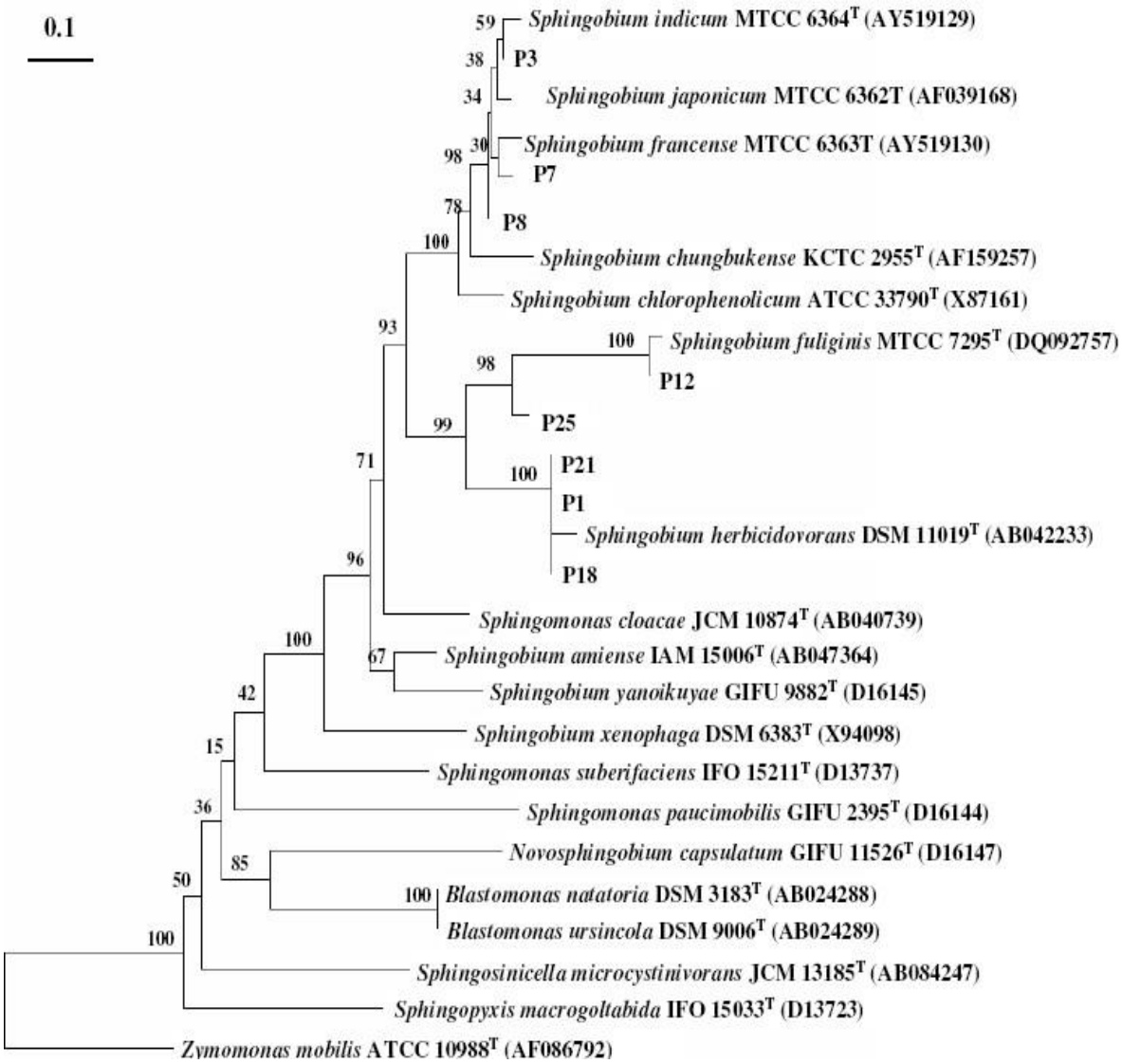


Figure 3. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing evolutionary relationship between strains P1, P3, P7, P8, P12, P18, P21 and P25 and members of representative genera *Sphingobium*, *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella*. Nucleotide substitution rates were calculated by Jukes and Cantor model and tree was constructed by using neighbour-joining method. Rooting was done by taking *Zymomonas mobilis* ATCC 10988^T (AF086792) as outgroup. Bootstrap values (based on 100 resamplings) have been given at branch points. Scale bar, 0.1 nucleotide substitution per nucleotide position.

Sequence similarity among *lin*B genes of novel strains and *S. indicum* B90A

In order to derive retrospective evidence of HGT of *lin* genes *linB* was selected as representative of HCH degradation pathway. This gene has been found to play a very important role in degradation of HCH isomers and involved in rate limiting step. The *linB* of these strains could be amplified using the primers designed for *linB* of B90A. Successful amplification of the *linB* gene indicates that there was sequence conservation among the genes at least within primer binding regions. If it is assumed that this gene has been acquired by these strains by HGT, the gene present in all isolates should be closely related.

The *linB* could be sequenced from five strains P1, P7, P8, P12 and P21. Sequence analysis of *linB* gene revealed that out of five strains, four strains (P1, P8, P12 and P21) have 100% identical *linB* gene (Fig. 4). The *linB* gene of strain P7 showed difference of one amino acid only. When these genes were compared to B90A it was found that four of these strains (P1, P8, P12 and P21) has *linB* virtually the allele of same gene with 99.8% identity differing only at one amino acid position (Fig. 4). In these strains a single non-aromatic amino acid, Threonine (T81) at position 81 in B90A has been replaced with Alanine (A) amino acid (Fig.4). However, *linB* of strain P7 revealed 99.3% identity with B90A and differs at four positions 4, 81,147 and 253 with respect to the other strains (Fig.4). Thus, the *linB* gene of all strains has been found to be the allele of same gene showing >99%

identity with that of B90A. Along with that the sequence alignments of the deduced amino acid sequences of haloalkane dehalogenase also revealed that putative active site residues (Asp-108, Glu-132, and His-272) which form the catalytic pocket were also conserved in all of these strains (Fig.4).

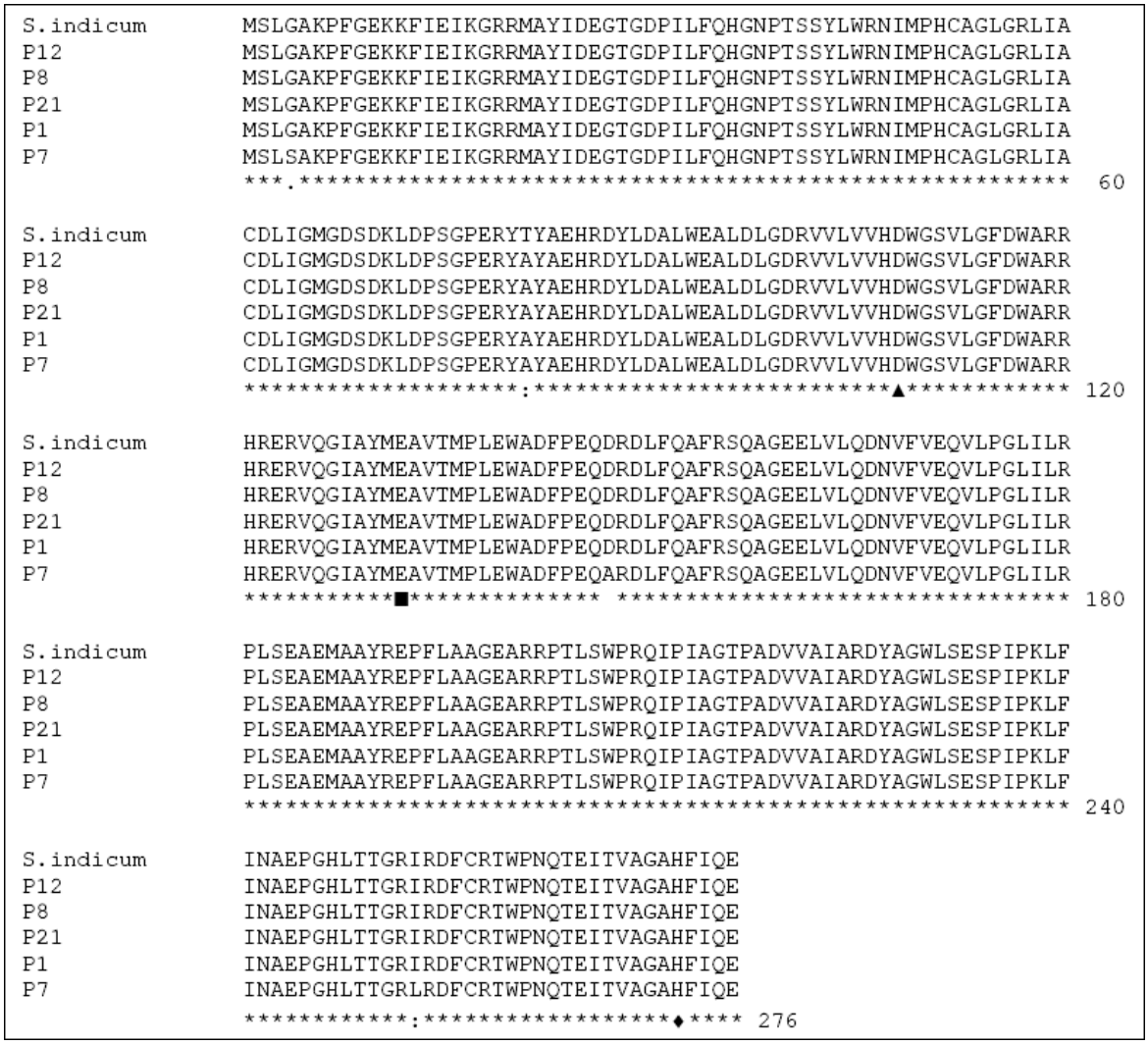
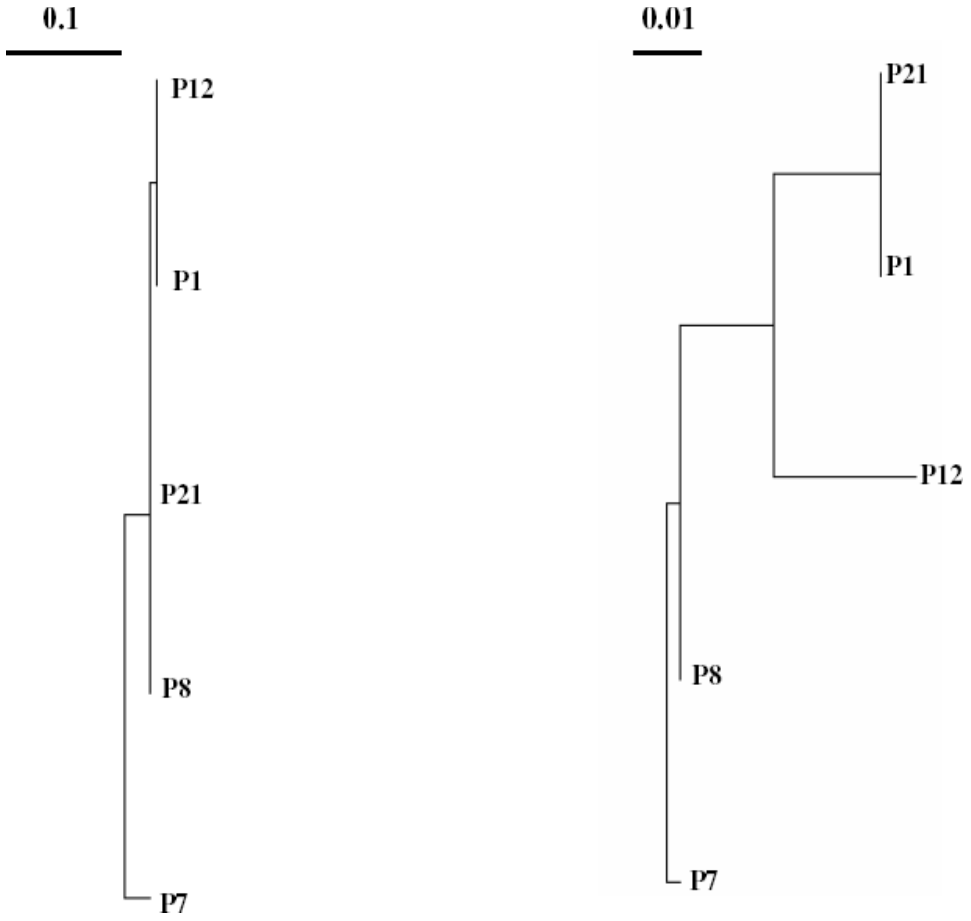


Figure 4: Alignment and comparison of the predicted amino acid sequences of *linB* for the hexachlorocyclohexane degrading sphingomonads with *S.indicum* B90A. Asp-108, Glu-132, and His-272 are putative active site residues of LinB and are similar in all the *linB* genes of novel isolates. (▲, Asp-108; ■, Glu-132; ♦, His-272).

Phylogenetic comparison of 16S rRNA and *lin*B genes

From comparative analysis of sequence divergence of 16S rRNA and *linB* gene it was revealed that *linB* found in these strains were homologs and 16S rRNA gene represents the phylogeny of different species. The phylogenetic trees that were based on the 16S rRNA and *linB* gene sequences were found to be incongruous and hence provide strong evidence of horizontal transfer of *linB* gene among these strains (Fig. 5A & 5B). In gene tree, *linB* of all strains clustered together with minor divergence in P7 while in species tree the strains showed important evolutionary distances (Fig. 5A). It indicates that *linB* gene has disseminated in these bacteria by independent events of horizontal transfer with little divergence as compared to 16S rRNA gene which is highly conserved. Presence of IS*6100* element in the genomes of novel isolates further provides supporting evidence for HGT of *linB* among theses strains [22].



(A) (B)

Figure 5: Unrooted phylogenetic trees of the *pcpB* gene (A) and 16S rRNA gene (B) of hexachlorocyclohexane degrading sphingomonads based on differences in the nucleotide sequences. Scale bars indicate 0.01 and 0.1 nucleotide substitutions per position.

Phylogenetic incongruency test has been regarded as the most rigorous criterion for proving horizontal transfers of biodegradative genes so far and the approach has been applied in various examples like horizontal transfer of *nah* gene [32] and *pcpB* gene [33]. Here in case of HCH for the first time we were able to deductively provide evidence for recent event of HGT of *linB* gene *in situ* among HCH degrading sphingomonads indigenous to a dump site.

It is clear from this study that the presence of HCH has created a selective pressure which plays a role in directing the event of recent HGT of *lin* genes. The ability of sphingomonads to get adapted to utilize HCH by acquiring these genes at a contaminated site is beneficial for enhanced natural bioremediation of harmful hexachlorocyclohexane. So far only *S. indicum* B90A has been capable of degrading all the HCH isomers in field conditions [23] and the strains isolated in this study can be used for bioremediation of HCH by bioaugmentation, biostimulation and monitored natural attenuation.

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Declaration

Authors declare no conflict of interest among themselves.

Authors' Contribution

AS: Experimental design, implementation and preparation of manuscript and RL: Conceiving the idea, supervision of experiments, proof-reading of the manuscript

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