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Presence of Multiple Molecular Forms of 'Haloalkane Dehalogenase LinB' in the Metagenome of Hexachlorocyclohexane Contaminated Soils

Ashish Srivastava[1,2*] Sunil G,.Babu[2] and Ashwani Kumar [1]

1. Environmental Biotechnology Division, CSIR-Indian Institute of Toxicology Research Lucknow, India
2. Department of Biotechnology, Babasaheb Bhimrao Ambedkar University Lucknow, India.

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Email:aashishbiotech@gmail.com

ABSTRACT

Enzyme Haloalkane dehalogenase LinB mediates the metabolism of several HCH-isomers and also their PCCHs, which are formed by the activity of LinA. Two prototype LinB variants, type1 and type2, that differ by seven residues, and exhibit differences in their activity and substrate range, have been characterized earlier. In this report, we isolated a new variant, LinB-type3 from metagenome of contaminated soil, which differs from -type1 and -type2 by ten and six residues, respectively. Evaluation of its activity with six substrates revealed that it mediates the metabolism of β- and -PCCH, but was not active for -HCH, β-HCH, -HCH and γ-PCCH. The report highlights the power of metagenomic approaches for the identification of novel gene variants..

INTRODUCTION

Technical hexachlorocyclohexane (t-HCH) is a chlorinated insecticide that consists predominantly of α- (60-70%), β- (5-12%), γ- (10-12%) and δ-isomers (6-10%). All the isomers persist in the environment, and soils contaminated with these are present all around the world (Willett et al. 1998; Nagata et al. 2007; Lal et al. 2010). Several organisms that mediate the biodegradation of either one or more of these isomers have been characterized (Nagata et al. 2007; Lal et al. 2010). The pathway for aerobic degradation of γ-HCH has been studied in detail. The first enzymatic step is its metabolism by an enzyme 'HCH dehydrochlorinase (LinA)' which metabolizes it by a two step reaction i.e. its conversion into γ-pentachlorocyclohexene (γ-PCCH). This gets further metabolized into 1,3,4,6-tetrachloro-1,4-cyclohexadiene (TCDN). The formed TCDN is metabolized by enzyme 'haloalkanedehalogenase-LinB' in two steps into 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (Nagata et al. 1993). While LinB was initially identified as second enzyme of the γ-HCH degradation pathway, subsequently its activity for the metabolism of α-HCH into 2,5,6-trichloro-2-cyclohexene-1,4-diol (A4), and for β- & δ-HCH in two steps into the corresponding PCHL (2,3,4,5,6-pentachlorocyclohexanol) and TCDL (2,3,5,6-tetrachlorocyclohexanediol) was also reported (Raina et al. 2007, Wu et al. 2007). In addition, LinB also mediates the metabolism of β-, γ- & δ-PCCH into the corresponding tetra- and tri-products, respectively (Raina et al 2008).

Variant forms of LinB with improved activity for the transformation of persistent HCH-isomers will be useful for remediation of contaminated sites. These can either be generated by protein engineering or sourced from natural sites. In this study, several HCH-contaminated soils were evaluated for the presence of novel variants of linB, and activities of their expression products on β-HCH and δ-HCH were determined.LinB variants i.e LinB-type1, identified initially from the bacterium Sphingobium japonicum UT26, and another LinB-type2, from S. indicum B90, and subsequently from several other strains, have been characterized. They belong to the haloalkane dehalogenases (EC 3.8.1.5) family, which are α/β-hydrolases that mediate the cleavage of carbon-halogen bonds. Both LinBs are 296 residues long, differ from each other by seven residues, and exhibit several differences in their enzyme activity. Thus, while -type2 catalyses the two-step dehalogenation of β- or δ-HCH into the corresponding 2,3,4,5,6-pentachlorocyclohexanol (PCHL) and 2,3,5,6-tetrachlorocyclohexane-1,4-diol (TCDL), -type1 mediates only the first step i.e., formation of PCHL. Further, the activity of LinB-type2 for the conversion of β-HCH into PCHL is 10-20 folds higher than -type1 (Sharma et al. 2006; Ito et al. 2007).The dynamics simulation analysis of the two LinBs revealed that the entrance of the substrate access tunnel of LinB-type1 is more flexible than that of -type2, and could be responsible for the observed differences in their activities.

MATERIALS & METHODS

Isolation of soil metagenomic DNA and amplification of linB genes

Six soil samples that were collected from the surroundings of an HCH-manufacturing unit 'India Pesticide Limited', located at Chinhat industrial area, near Lucknow city in India, were used in the study. These had varying levels of contamination by different HCH-isomers (Table 1). Metagenomes from these soils were isolated by using 'Fast DNA Spin Kit for soil' (Qbiogene, Heidelberg, Germany) as per manufacturer's instructions. Amplification of linB was done by using a forward and reverse primer F1 & R1, respectively (Table 2), and the enzyme Pfu DNA Polymerase (Fermentas, Hanover, MD, USA), as described earlier (Macwan et al., 2011).

Table 1. Contamination level of HCH-isomers in soil samples used in this study.

HCH-isomer (mg g^{-1} soil)	Soils					
	S1	S2	S3	S4	S5	S6
α-	124.0	13.60	2.8	0.03	0.01	0.01
β-	27.0	19.50	13.5	0.18	0.29	0.86
γ-	4.6	0.15	0.1	BDL#	BDL	BDL
δ-	4.0	BDL	BDL	BDL	BDL	BDL
Σ - HCH*	159.6	33.25	16.4	0.21	0.30	0.87

* Σ-HCH is the sum of α-, β-, γ- and δ-HCH; # Detection limit was 0.005mg g-1 soil

Molecular cloning, nucleotide sequencing and Expression of various LinB proteins

The amplified products were cloned in E.coli-DH5 cells, as described earlier (Macwan et al., 2011). Plasmid DNA was prepared by from the randomly selected transformants, and both strands of the inserts were sequenced on ABI PRISM-3100 sequencer (Applied Biosystems, Foster city, CA, USA) by using universal forward and reverse M13 primers. Nucleotide sequences were aligned by Clustal W algorithm (DNAStar Inc. WI, USA) and compared with prototype linB-UT26. Genes for selected LinBs were used for the transformation of competent E.coli BL21 (DE3) cells and the expressed protein were purified as described earlier (Macwan et al. 2011). Aliquots of 0.2 ml (1.0 mg protein ml-1), in 50 mM sodium phosphate buffer (pH 8.0) containing 15% glycerol, were stored at -20°C.

Table 2. Primers used in the study*

Primers	Sequence	Characteristics
F1	GCGGATCCGCATGAGCCTCGGCGCAAAGCCA	1-21 b p, preceded by the site for Bam HI (underlined)
R1	GTCCGGCGATTGCGCCCAGCATAACTCGAGGC	868-891 bp preceded by the site for XhoI (underlined)
F2	CATATGAGCCTCGGCGCAAAGCC	1-20 bp preceded by the site for NdeI (underlined)
R2	GTCCGGCGATTGCGCCCAGCACTCGAG	868-888 bp preceded by the site for XhoI (underlined)

* were based on the sequence of LinB-UT26 (Accession no. D14594)

Enzyme activity

Dehalogenase activity of different LinB proteins was determined as described earlier (Ito et al. 2007; Wu et al. 2007b). Briefly, 100μl reaction medium, contained 68 μM of α-/β-/δ-HCH (stock solution 1mg/ml in DMSO) or 340μM of β-/γ-/δ-PCCH, 50 mM potassium phosphate buffer (pH 7.5), 10% glycerol. Enzyme concentrations for all the assays were, as described in the results section. After incubation at 30°C for specified time, samples were acidified with HCl, extracted with ethyl acetate and analyzed by gas chromatography (Clarus-500, Perkin Elmer, Waltham, Massachusetts, USA) (Kumar et al. 2005).

RESULTS AND DISCUSSION

Presence of multiple variants of linB in soil metagenomes

Nucleotide sequencing of the cloned PCR products identified the presence of three linB gene variants in the metagenome of HCH-contaminated soils (Table 3). While the two of these were >99% identical to previously described LinB-type1 and LinB-type2, the third was a newly identified variant LinB-type3. Presence of these varied substantially in different soils (Table 3). Thus, in soils S2 and S5, LinB-type2 was the only variant present. Also, it was the dominant variant in S3 (70%). In soils S4 and S6, variant LinB-type1 was dominantly present (70%). On the other hand, the variant LinB-type3 was most abundant (90%) in S1.

While LinB-type3 had a difference of ten residues with -type1, it differed by six residues with -type2 (Figure 1). Thus, residue 81 is A in -type1, changed to T in -type2 but is A again in -type3. Residues A112, I134, A135, I138 and A247were changed to V112, V134, T135, L138 and H247 in -type2, and same changes were also seen in LinB-type3. Residues V173, L177, A224 and L248 were same in -type1 and -type2, but were changed to F173, A177, V224 and V248 in LinB-type3. Residue 253 was different in all three LinBs i.e. it was M, I & L in -type1, -type2 and -type3, respectively.

Table 3. Relative abundance of three major linBs in metagenome different soil samples

Soils	Relative abundance of linBs (%)		
	-type1	-type2	-type3
S1	0	10	90
S2	0	100	0
S3	5	70	25
S4	72	28	0
S5	0	100	0
S6	71	29	0

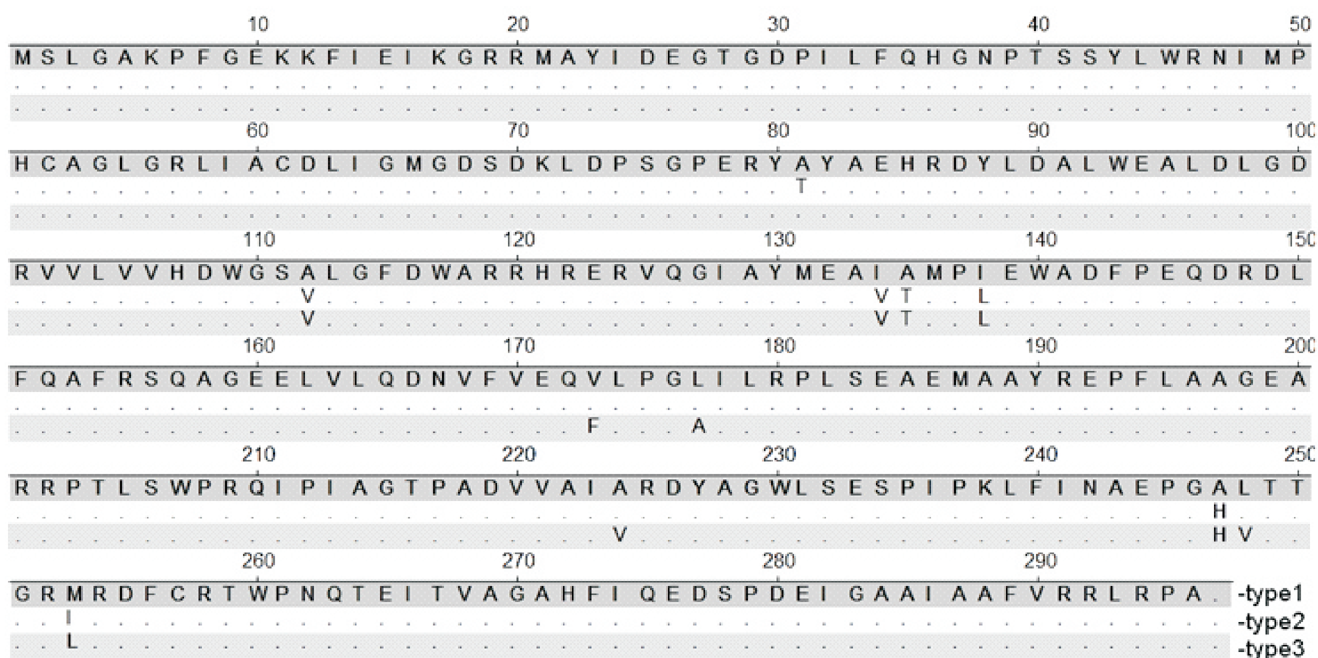

Fig.1. Amino acid sequence of major LinB variants obtained from the HCH-contaminated soil metagenomes. In LinB-type2 and type3, only the residues that are different than -type1 are shown.

Table 4. Summary of the amino acid residues, differing in three LinBs

LinB	81	112	134	135	138	173	177	224	247	248	253
-type1	A	A	I	A	I	V	L	A	A	L	M
-type2	T	V	V	T	L	V	L	A	H	L	I
-type3	A	V	V	T	L	F	A	V	H	V	L

Transformation of α-, β- and δ-HCH by different LinB variants

Incubation of LinB-type1 and -type2 with α-HCH led to its metabolism to a metabolite 2,5,6-trichloro-2-cyclohexene-1,4-diol (A4) (Figure 2). The activity of -type1, however, was 40 fold lower than LinB-type2 (Table 5). No discernable activity of -type3 was observed with α-HCH. Incubation of LinB-type1 and -type2 led to the metabolism of β-HCH also, but the reaction with -type2 was 25 fold faster than -type1 (Table 5). Also, while the formed metabolite PCHL (B1) was further metabolized to TCDL by LinB-type2, no such conversion was observed with -type1 (Figure 2). With LinB-type3, on the other hand, no discernable activity was observed. Similar to β-HCH, LinB-type1 and -type2 mediated the metabolism to δ-HCH, and again the activity with -type2 was 18 fold faster than -type1. Also, while the formed δ-PCHL was metabolized further to TCDL by -type2, no such reaction was observed with -type1. Again, no discernable activity of LinB-type3 with δ-HCH was observed.

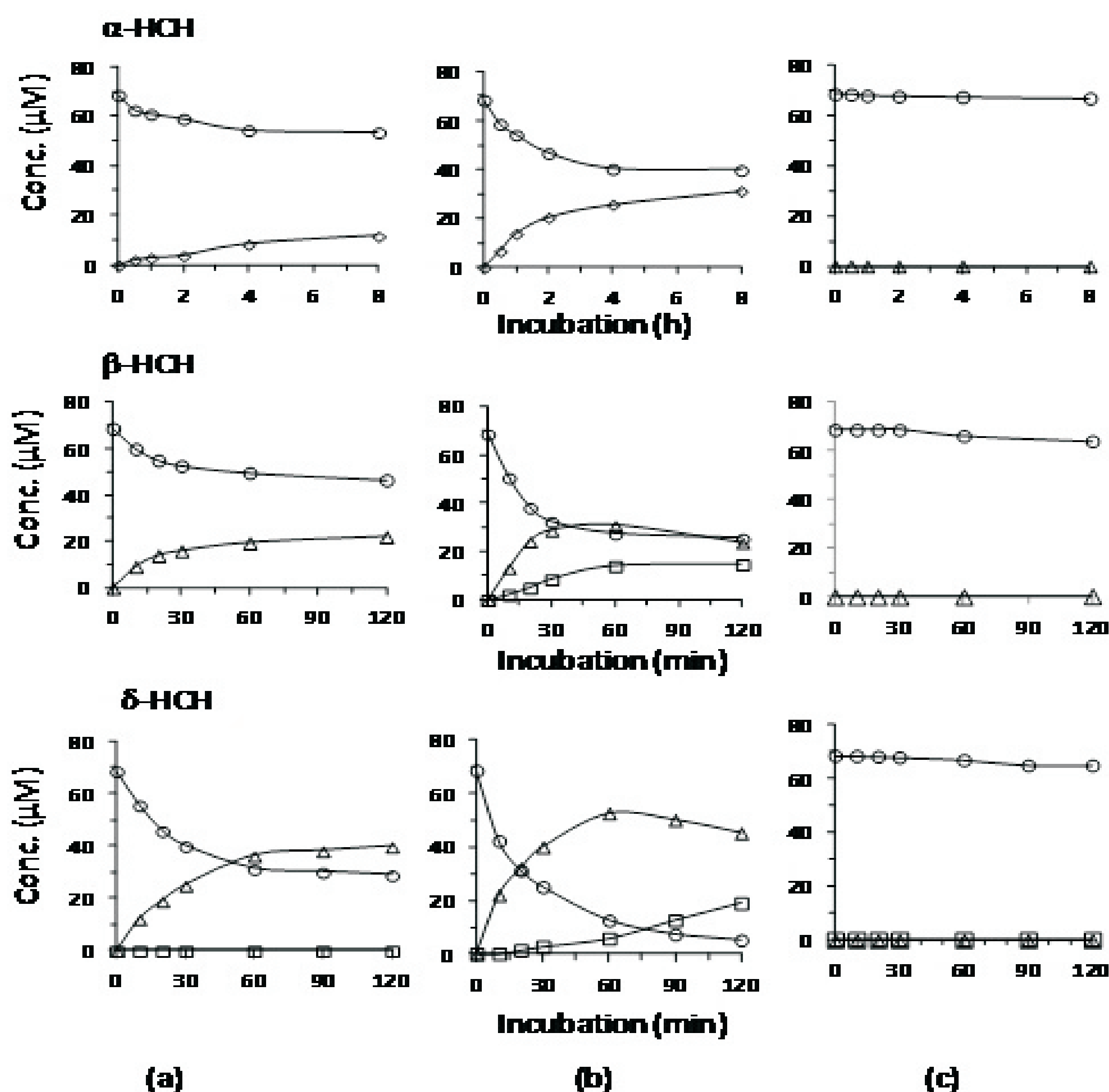

Fig 2. Activity of purified LinB-type1(a), -type2 (b) and -type3 (c) for α-, β-, and δ-HCH. Amounts of residual HCH (circle), and formed metabolites A4 (diamond), PCHL (triangle) and TCDL (square) are shown. While the enzyme concentration of LinB-type1in reaction with -, β and -HCH was 400, 50 and 50 µg ml-1, respectively, concentration of LinB-type2 was 100, 5 and 5 µg ml-1, respectively. Concentration of LinB-type3, however, was 400 µg ml-1for all the isomers.

Table 5. Specific activity of different LinBs with different HCH and PCCH-isomers

LinB	Specific activity (nmoles min^{-1} mg^{-1} protein)					
	α-HCH	β - HCH	α-HCH	β-PCCH	γ-PCCH	α-PCCH
-type1	0.04	3.66	6.66	13833	7	5433
-type2	1.50	71.66	105	14000	14	5660
-type3	0	0	0	105	0	160

Transformation of β- , g- and d-PCCH by different LinB variants

Incubation of all the three LinBs i.e. -type1, -type2 or -type3 caused the metabolism of β-PCCH (Figure 3). The reaction rates of -type1 and -type2 were comparable, but the activity of -type3 was nearly 100 fold lesser (Table 5). While all the LinBs caused the formation of metabolite A3, but only -type2 caused its further metabolism to A4. Similarly, while LinB-type1 and -type2 are able to mediate the metabolism of γ-PCCH, no such reaction was observed with -type3 (Figure 3). Also, while the metabolites G3 were formed by the activity of both LinB-type1 and -type2, only -type2 could further metabolize it to G4. The three LinBs were also able to mediate the metabolism of δ-PCCH into D3. The rates of reaction by -type1 and -type2 were comparable, the activity of -type3 was 35 fold lesser (Table 5). Furthermore, while LinB-type2 could also mediate the transformation of the formed D3 to D4, no such activity was observed with LinB-type1 and -type3.

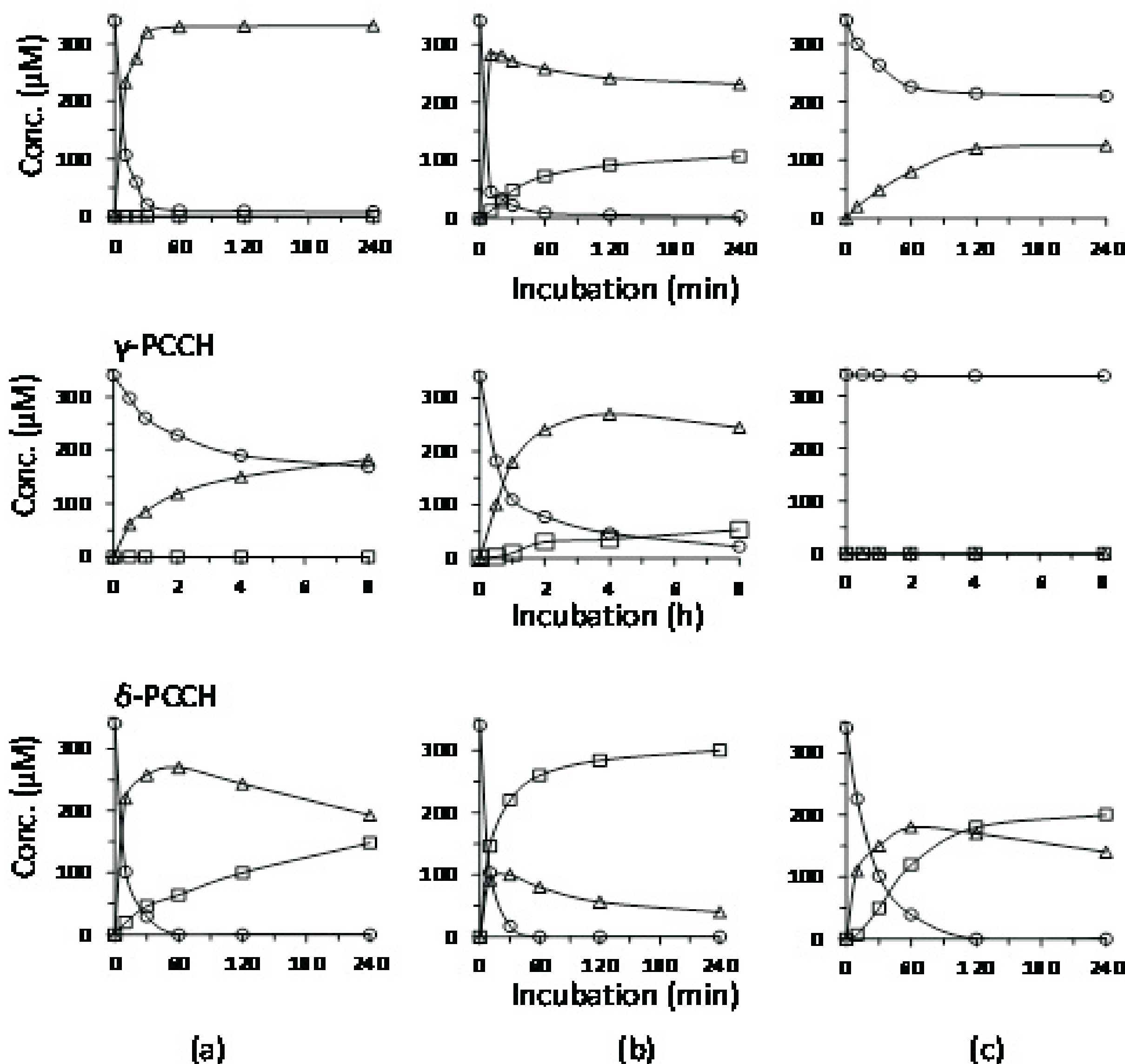

Fig.3. Activity of purified LinB-type1(a), -type2 (b) and -type3 (c) for β-, γ- and (b) δ-PCCH. Amounts of residual PCCH (circle), and the formed metabolites A3/G3/D3 (triangle) and A4/G4/D4 (square) have shown. The enzyme concentrations of LinB-type1and -type2 in the reaction with β- γ- and δ-PCCH were 0.1, 50, 1 µg ml-1, respectively. Concentration of LinB-type3, however, was 50 µg ml-1for all the isomers.

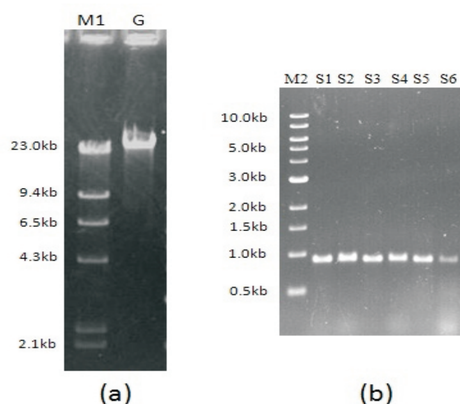

Fig. 4. Agarose gel electrophoresis of (a): isolated metagenomic DNA, and (b): PCR product obtained by using linB primers. M1: λ HindIII digested DNA, M2: 1kb DNA ladder.

Enzyme kinetics confirmed the observations of LinB-type1 and type2 with various substrates, reported earlier (Lal et al 2010). In the event of extremely low activities of LinB-type3 with some substrates, and no activity with some other substrates, the biological role of this variant in HCH-contaminated soils is not clear. LinBs have earlier been shown to have a broad substrate range (Janssen, 2004). It is, therefore, a possibility that a true substrate for this enzyme has not been deciphered thus far.

CONCLUSION

Presence of three linB gene variants was identified in the metagenome of HCH-contaminated soils, one of which, LinB-type3 was novel. It was different from previously described -type1 and -type2 by ten and six residues, respectively. The relative abundance of these variants in different soils varied substantially, and did not show any apparent correlation with the contamination of different HCH-isomers. The activity of LinB-type3 has been reported here for the first time. This enzyme was active for the metabolism of β- and δ-PCCH, but the activities were substantially lower than -type1 and -type2. The report highlights the power of metagenomic approaches for the identification of novel gene variants.

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