Microbes Environ. Vol. 22, No. 4, 399–404, 2007
http://wwwsoc.nii.ac.jp/jsme2/

Microbes and Environments

Short Communication

Presence of *linA*-Homologous DNA Sequences in Different Types of Soil and Their Sequence Diversity

NORIKO KURAMOCHI[1], SHIGETO OTSUKA[1], MASAYA NISHIYAMA[1,†], and KEISHI SENOO[1]*

[1] *Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan*

(Received April 19, 2007—Accepted June 29, 2007)

linA is a gene encoding γ-hexachlorocyclohexane dehydrochlorinase identified in a γ-HCH-degrading *Sphingobium japonicum* UT26 that had been isolated from soil of an experimental field treated with γ-HCH. The presence of *linA*-homologous nucleotide sequences and their sequence diversity was investigated by *linA*-targeted PCR amplification of DNA obtained from different soils in the field and in Japan. *linA*-homologous sequences were obtained from almost all the soils some of which had never been treated with γ-HCH. Deduced amino acid sequences suggested that they can be regarded as fragments of *linA* that encode LinA. Some common, natural function of LinA in soil was suggested.

Key words: γ-HCH, *Sphingobium japonicum*, *linA*, soil DNA, PCR

Gamma-hexachlorocyclohexane (γ-HCH) is a recalcitrant organochlorine insecticide widely used since the 1940s.

In 1973, we established a long-term experimental upland field that is divided into plots. γ-HCH has been applied every year to the soil of some of the plots. The rate of degradation of the γ-HCH was low following the first application, but increased with the number of applications[28]. In 1986, from the soil of this field, we isolated an aerobic γ-HCH-degrading bacterium, *Sphingobium japonicum* SS86, which utilizes γ-HCH as a sole carbon and energy source[24]. The γ-HCH-degrading *Sphingobium* was present in the γ-HCH-plot as an indigenous soil microorganism after multiple applications of γ-HCH, whereas it was completely absent in the control plot where γ-HCH had never been applied[24,25]. Genetic and biochemical analyses revealed the genes (*linA*, *B*, *C*, *D*, *E*, *F*, and *R*)[4,5,9–11,15,17,19,21] and enzymes[13,14,18,20] responsible for the degradation of γ-HCH in *S. japonicum* UT26, which is a nalidixic acid-resistant mutant derived from SS86. *linA* encodes dehydrochlorinase (LinA) which eliminates hydrochlorine from γ-HCH[4,5,15,20]. *linA* is speculated to be a gene of foreign origin due to its low GC content compared with that of other *lin* genes in UT26[5,15]. Several other strains of γ-HCH-degrading *Sphingobium* isolated from soils in India[6,8,23], France[2,27], Spain[1,12] and Germany[1] harbor a *linA* gene nearly identical to that of UT26[1,2,7,8,22]. The presence of IS*6100* near *linA* in some strains[3,8] suggests its mobility, horizontal transfer, and wide distribution among soil microorganisms. However, no gene or nucleotide sequence highly homologous to *linA* has so far been included in DNA databases except those from γ-HCH-degrading *Sphingobium* strains.

These findings have raised questions over the sequence diversity of *linA*, distribution of *linA* among different types of soils, and original function of LinA in soil. We therefore attempted to obtain and analyze DNA clones with *linA*-homologous sequences from soils of different plots in the long-term experimental field, and different types of soils located in geographically distant places in Japan.

* Corresponding author. E-mail address: asenoo@mail.ecc.u-tokyo.ac.jp; Tel.: +81–3–5841–5139; Fax: +81–3–5841–8042.
† Present address: Faculty of Environmental Studies, Nagasaki University, 1–14, Bunkyo-chyo, Nagasaki 852–8521, Japan.

Ten different soil samples were used in this study, including one each from seven plots in the long-term experimental upland field[28]; the control plot (plot 1), a plot treated with manure (plot 2), a plot treated with the fungicide 2,4,5,6-tetrachloro-1, 3-isophtalonitrile (TPN) (plot 3), a plot treated with γ-HCH (plot 4), a plot treated with TPN and manure (plot 5), a plot treated with TPN and γ-HCH (plot 6), and a plot treated with TPN, γ-HCH, and manure (plot 7). The experimental field was set up in 1973 inside the campus of the University of Tokyo, Tokyo, Japan[28]. Since then, γ-HCH (10 mg kg^{-1} soil), TPN (40 mg kg^{-1} soil), and manure (3 kg m^2) have been applied once a year in May or June to the surface soil (10 cm) of the plots, solely (plots 2, 3, and 4), or by mixture (plots 5, 6, and 7). Soil of the field is classified as volcanic ash soil, Andosol. Properties of the soil were reported previously[28]. Fresh moist soil samples were collected from the surface layer of each plot, passed through a 2 mm screen, and used for the experiment without air-drying.

Another three samples of different soil types were collected from geographically distant places in Japan: gray lowland soil collected from an upland field at the Saitama Prefectural Experimental Station of Agriculture and Forestry, Kumagaya, Saitama prefecture (Saitama soil), brown lowland soil collected from Hyogo Prefectural Technology Center for Agriculture, Forestry and Fishery, Kasai, Hyogo prefecture (Hyogo soil), and yellow soil collected from Wakayama Prefectural Technology Center for Agriculture, Forestry and Fishery, Arita, Wakayama prefecture (Wakayama soil). These fields have been used for upland crop cultivation. γ-HCH has not been applied to the soils at least since 1967, when the use of γ-HCH in agricultural fields was prohibited in Japan. The chemical properties of the soils[28] are as follows: Saitama soil, pH(H_2O), 5.0; total carbon, 1.18%; total nitrogen, 0.13%; Hyogo soil, pH(H_2O), 7.8; total carbon, 1.44%; total nitrogen, 0.16%; and Wakayama soil, pH(H_2O), 5.9; total carbon, 2.02%; total nitrogen, 0.18%. The soil samples were collected, passed through a 2 mm screen, and kept in an air-dried state until used in this study.

All the ten soil samples mentioned above were incubated for three weeks at 30°C and 60% of maximum water holding capacity without the application of chemicals. As to the seven soils from the long-term experimental upland field (plots 1–7), dibenzofuran (DF)-treated samples were also prepared. Forty mg kg^{-1} of DF was applied to each soil by the Celite method[28], and incubated in the same way. In addition, for the soil of the γ-HCH-plot, a γ-HCH-treated sample (10 mg kg^{-1} soil by the Celite method) was prepared and incubated. For the incubation, 50 g of each soil sample was placed in a glass beaker, covered with aluminum foil, and incubated in the dark.

After the incubation, a 0.3 g portion of each sample was subjected to DNA extraction using a soil DNA extraction kit, ISOIL for beads beating (Nippon Gene, Tokyo, Japan). Using the soil DNA as template, PCR or nested PCR was performed trying to amplify *linA*-homologous sequences. Several forward and reverse primers were designed based on the nucleotide sequence of the ORF region of *linA*[5]: linA-F11, 5'-TAGACAGACTTGCAAGCCGG-3'; linA-F33, 5'-CGCGATTCAGGACCTCTACT-3'; linA-F42, 5'-GGACCTCTACTCTGACAAGC-3'; linA-R396, 5'-TCTCTAAGCGCAACGCATGC-3'; linA-R418, 5'-CCAGCGGGGTGAAATAGTTC-3'. The PCR was carried out in a 20 μl reaction mixture, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each of the forward and reverse primers, 8 μg of BSA, 2 units of Taq DNA polymerase (Sigma-Aldrich, MO, US), and 1–10 ng of template DNA. For the single PCR, the primer set linA-F33 and linA-R418 and the following conditions were used: initial denaturation at 94°C for 5 min; 30 or 35 cycles at 94°C for 1 min, at 55°C for 1 min, at 72°C for 1 min; final extension at 72°C for 7 min. For the nested PCR, the first reaction was performed in the same conditions using the primers linA-F11 and linA-R418. After the first PCR, 0.5 μl of the product was subjected to a second PCR, in which the primers linA-F42 and linA-R396 were used. Thirty cycles were employed for both the first and the second PCR. After the reaction, 5 μl of the PCR products was separated by electrophoresis in a 1.5% agarose gel, and visualized by ethidium bromide staining to check for the presence of amplified DNA.

Amplification products of expected size were obtained by single or nested PCR from DNA derived from all the samples except for the TPN-plot and DF-treated manure-plot. From the γ-HCH-treated γ-HCH-plot, and Saitama, Hyogo and Wakayama soils, the amplified products were generated by a single PCR. PCR products were passed through MicroSpin™ S-400 HR Columns (Amersham Biosciences, Buckinghamshire, England) to remove remaining dNTPs and primers. The purified PCR products were cloned with the pGEM®-T Easy System (Promega, WI, USA) according to the manufacturer's instructions. The sequences of five clones each from one PCR were determined with the DTCS Quick Start Kit for Dye Terminator Cycle Sequence (Beckman Coulter, CA, USA) and Genetic Analysis System CEQ 8000 (Beckman Coulter, CA, USA). The sequence data were compared with sequences available in the GenBank

Table 1. Position of nucleic substitutions in *linA*-homologous DNA sequences[a] from soil DNA and *linA* in γ-HCH-degrading *Sphingobium*

clone/strain[b] (accession number)	plot/soil (treatment if any[c])	position/nucleotide base[d,e] 58 63 68 70 88 99 103 105 117 121 130 174 194 203 210 211 212 228 229 238 246 284 286 287 299 327 328 331 332 337 374 389
*linA*1-1 (AB331608)	plot 1	· C · · · · · · · · · ·
*linA*1-2 (AB331609)		· G* · ·
*linA*1-4 (AB331610)		· · · · · · · · · · G* ·
*linA*1-3,5		· ·
*linA*2-1 (AB331611)	plot 1 (DF)	· · · A* ·
*linA*2-2 (AB331612)		· · G* · · G* ·
*linA*2-3 (AB331613)		· · G* ·
*linA*2-4 (AB331614)		· · · A* · · G* ·
*linA*2-5 (AB331615)		· · G* · A* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*3-1 (AB331616)	plot 2	· T · · · · · · · · · · · A · · · · · · · · · · · · · · · · ·
*linA*3-4 (AB331618)		· T · · · C · · C · · · · · G · · · · · · · · · · · · · · · ·
*linA*3-2,3,5 (AB331617)		· T · · · C · · · · · · · · G · · · · · · · · · · · · · · · ·
*linA*4-1,3 (AB331619)	plot 3 (DF)	· · G* · · G* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*4-2,4 (identical to *linA*2-2)		· · G* · · G* ·
*linA*5-1,2,3 (identical to *linA*4-1)	plot 4	· · G* · · G* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*5-4 (AB331620)		· T* G* · · · · · ·
*linA*6-1 (AB331621)	plot 4 (DF)	· · · · · · · · · · · · · · · · · T · · · · · · · · · · · · · ·
*linA*6-2,3,4,5		· ·
*linA*7-1,3,4 (AB331622)	plot 4 (γ-HCH)	· · G* · · G* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*7-2 (AB331623)		· · G* · · G* · · · · · · · · · · · · · C* · · · T* G* · · · · · ·
*linA*8-1,2,3,4	plot 5	· ·
*linA*8-5 (AB331624)		· C* · · · · · ·
*linA*9-1,2,3,5 (identical to *linA*2-1)	plot 5 (DF)	· · · A* ·
*linA*9-4 (identical to *linA*4-1)		· · G* · · G* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*10-1	plot 6	· ·
*linA*10-2 (identical to *linA*2-1)		· · · A* ·
*linA*10-3 (AB331625)		· · · · · · G* ·
*linA*10-4 (AB331626)		· · · A* · · · C* ·
*linA*11-1,4	plot 6 (DF)	· ·
*linA*11-2 (identical to *linA*2-1)		· · · A* ·
*linA*11-3 (identical to *linA*2-3)		· · G* ·
*linA*11-5 (AB331627)		· C* · · · · · · · ·
*linA*12-1	plot 7	· ·
*linA*12-2 (identical to *linA*2-4)		· · · A* · · G* ·
*linA*12-3 (identical to *linA*2-2)		· · G* · · · G* ·
*linA*12-4 (AB331628)		· · G* · · · G* · · · · · · · · · · · · · C · · T* G* · · · · · ·
*linA*12-5 (identical to *linA*2-1)		· · · A* ·
*linA*13-1,4 (identical to *linA*4-1)	plot 7 (DF)	· · G* · · · G* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*13-2 (AB331629)		· T* G* · T · · · · ·
*linA*13-3 (AB331630)		· · G* · T* G* · · · · · ·
*linA*13-5 (AB331631)		· · G* · · · G* · · A · · · · · · · · · · · · · T* G* · · · · · ·
*linA*14-1 (AB331632)	Saitama soil	· · · · · · · · · · · C ·
*linA*14-2 (AB331633)		· · G* · · · G* · · · · · · · · · · · · · · · · T* G* · · · · · G* ·
*linA*15-1 (AB331634)	Hyogo soil	· · G* · · · G* · · · · · · · · · · · · · · · · T* G* · · · · · ·
*linA*16-1 (AB331635)	Wakayama soil	· ·
*linA*16-2 (AB331636)		· · G* · · · G* · · · · · A · · · · · · · · · · T* G* · · · · · · G
Sphingobium japonicum UT26		A C C G G T A T T G A T G T A T G C A T T T C T T C G G C T A A
Sphingobium indicum B90A (*linA*1)		C* · G* · · · · · · · · · · · · A* C* · · · · · T* G* · · · · · ·
Sphingobium sp. Alpha4-2		C* · G* · · · · · · · · · · A* · A* C* · · · · · T* G* · · · · · ·
Sphingobium sp. DS3-1		· ·
Sphingobium sp. DS2		· ·
Sphingobium francense Sp+		· ·
Sphingobium sp. Gamma1-7		· A* T* G* · · ·
Sphingobium sp. Gamma12-7		· ·
Sphingobium sp. Alpha1-2		· ·
Sphingobium sp. Gamma16-1		· ·
Sphingobium sp. DS2-2		· ·

[a] DNA sequences of positions 42–396 with UT26 *linA* numbering were determined, except for *linA*7 (11–408), *linA*14 (33–408), *linA*15 (33–381) and *linA*16 (33–408).

[b] Clones *linA*1-*linA*16 were obtained from soils and determined in this study. The last 11 sequences were obtained from GenBank database for reference. The accession numbers of the reference were as follows; *Sphingobium japonicum* UT26, D90355; *Sphingobium indicum* B90A (*linA*1), AY331258; *Sphingobium* sp. Alpha4-2, AJ871384; *Sphingobium* sp. DS3-1, AJ871385; *Sphingobium* sp. DS2, AJ871378; *Sphingobium francense* Sp+, AY690622; *Sphingobium* sp. Gamma1-7, AJ871380; *Sphingobium* sp. Gamma12-7, AJ871381; *Sphingobium* sp. Alpha1-2, AJ871383; *Sphingobium* sp. Gamma16-1, AJ871382; *Sphingobium* sp. DS2-2, AJ871379.

[c] Dibenzofuran or γ-HCH was added to the soil samples at the incubation.

[d] The positions are according to UT26 *linA* numbering. Nucleotide bases denoted as "·" are the same as those of UT26 *linA*, while those denoted as "–" were not determined. The bases with * are nonsynonymous substitutions.

[e] *linA* of *Sphingobium indicum* B90A (*linA*1) and *Sphingobium* sp. Alpha4-2 are shorter than the others and positions 374 and 389 do not exist in them.

Table 2. Deduced amino acid sequences of *linA*-homologous DNA sequences from soil DNA and amino acid sequences of LinA in γ-HCH-degrading *Sphingobium*

clone/strain[a]	position/amino acid[b]																			
	20	23	24	30	35	44	55	65	68	71	77	80	86	95	96	100	110	111	113	125
*linA*1,3,5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*1-2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	V	·
*linA*1-4	·	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*2-1	·	·	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*2-2	·	G	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*2-3	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*2-4	·	·	I	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*2-5	·	G	·	S	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*3-1,2,3,4,5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*4-1,3	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*4-2,4	·	G	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*5-1,2,3	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*5-4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*6-1,2,3,4,5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*7-1,3,4	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*7-2	·	G	·	·	V	·	·	·	·	·	H	·	·	·	C	·	·	·	·	·
*linA*8-1,2,3,4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*8-5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	P	·	·	·	·
*linA*9-1,2,3,5	·	·	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*9-4	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*10-1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*10-2,4	·	·	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*10-3	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*11-1,4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*11-2	·	·	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*11-3	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*11-5	·	·	·	·	·	·	·	·	·	·	·	·	·	P	·	·	·	·	·	·
*linA*12-1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*12-2	·	·	I	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*12-3	·	G	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*12-4	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*12-5	·	·	I	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*13-1,4,5	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*13-2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*13-3	·	G	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·
*linA*14-1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*14-2	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	R
*linA*15-1	·	G	·	·	V	·	·	·	·	·	·	·	·	·	C	·	·	·	·	
*linA*16-1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
*linA*16-2	·	G	·	·	V	·	·	(·)	·	·	·	·	·	·	·	·	·	·	·	·
Sphingobium japonicum UT26	K	A	V	G	I	I	G	W	F	C	N	L	D	L	L	L	A	A	F	K
Sphingobium indicum B90A (*linA*1)	Q	G	·	·	·	·	·	·	·	T	·	·	·	·	C	·	·	·		
Sphingobium sp. Alpha4-2	Q	G	·	·	·	·	·	·	Y	T	·	·	·	·	C	·	·	·		
Sphingobium sp. DS3-1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sphingobium sp. DS2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sphingobium francense Sp+	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sphingobium sp. Gamma1-7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	C	·	·
Sphingobium sp. Gamma12-7	·	·	·	·	·	·	·	·	·	·	·	·	X	·	·	·	·	·	·	·
Sphingobium sp. Alpha1-2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sphingobium sp. Gamma16-1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sphingobium sp. DS2-2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	X	·	·	·

[a] Clones *linA*1-*linA*16 were obtained from soils and determined in this study. The last 11 sequences were obtained from GenBank database for reference. The accession numbers of the reference are shown in Table 1 (footnote).

[b] The positions are according to UT26 LinA numbering. Amino acids denoted as "·" are the same as those of UT26 LinA. "(·)" is a stop codon.

database.

Sixty-six clones were obtained from the PCR products and all of them had a nucleotide sequence nearly identical to that of *linA* of UT26 (Table 1). Fifteen clones had the same nucleotide sequence as that of *linA* of UT26 within the 350-base region cloned and sequenced in this study. The other clones showed different sequences with one- to six-base substitutions from that of *linA* of UT26. The base substitutions were at limited sites, especially positions 68, 103, 286, and 287 according to UT26 *linA* numbering. In addition, some of the positions are the same as those in the corresponding sequences of the *linA* of γ-HCH-decomposing *Sphingobium* isolated in India and Spain (e.g. positions 68, 286, and 287)[1,7]. In all, twenty-seven different sequences were obtained in this study. *Taq* DNA polymerase is known to have a certain error rate and produce mutated PCR products in a reaction. However, since the base substitutions do not distribute in a random manner, most of the sequences that have base substitutions at common positions would exist in the nature.

The nucleotide sequences were converted to amino acid sequences (Table 2). Most of the nucleotide substitutions were nonsynonymous, resulting in the substitution of at most four amino acids. The catalytic dyad H73-D25 and residue R129, which are essential for LinA activity[16], were conserved in all the deduced amino acid sequences derived from the twenty-seven nucleotide sequences. The cloned PCR products, therefore, can be regarded as fragments of *linA* that encode proteins showing LinA activity, although entire ORF sequences were not examined. The one exception is *linA*16-2, which contained a stop codon. It remains to be elucidated whether the substitutions influence LinA activity.

Of the twenty-seven nucleotide sequences, fifteen were obtained from the soils in the γ-HCH-treated plots (plots 4, 6, and 7) where γ-HCH-degrading *S. japonicum* is enriched. They include not only the sequence identical to that of UT26 or SS86, but several different sequences. From the plots, several strains of *linA*-harboring *S. japonicum* including SS86 (UT26) are the only members that have so far been isolated as γ-HCH-degrading microorganisms, in spite of many screening. The information suggests the presence in the plots of as yet unisolated *linA*-harboring γ-HCH-degrading *S. japonicum*. However, the presence of (i) yet to be isolated *linA*-harboring γ-HCH-degrading microorganisms other than *S. japonicum*, and (ii) *linA*-harboring but γ-HCH-non-degrading microorganisms, cannot be ruled out.

It was striking that *linA*-homologous sequences were detected from the soil of the control plot (plot 1) where γ-HCH-degrading microorganisms have never been isolated. Included among them was the *linA* sequence identical to that of UT26 (SS86). This suggests the presence in the plot of *linA*-harboring microorganisms including *S. japonicum* that lack some of the *lin* genes necessary for complete degradation of γ-HCH.

From the TPN-, manure- and/or DF-treated soils, *linA*-homologous sequences were also obtained. No remarkable characteristics specific to these sequences were observed. The same possibility of *linA*-harboring microorganisms as discussed for the control plot (plot 1) was suggested.

Surprisingly, *linA*-homologous sequences were amplified from DNA of different types of soils located at distant places in Japan, i.e. Saitama, Hyogo, and Wakayama prefecture. This means the presence of *linA*-harboring microorganisms in a wide variety of soils with different properties. Further study is necessary to elucidate the characteristics of the *linA*-harboring microorganisms.

The existence of *linA* in a wide variety of soils, even in soils not treated with γ-HCH, indicates a common, natural function of LinA in soil other than dehydrochlorination. The distribution of *linA*-harboring and γ-HCH-degrading *Sphingobium* in various types of soil in the world[1,7,12,27] supports this supposition.

Acknowledgements

Special thanks to the Saitama Prefectural Experimental Station of Agriculture and Forestry, Hyogo Prefectural Technology Center for Agriculture, Forestry and Fishery and Wakayama Prefectural Technology Center for Agriculture, Forestry and Fishery, for generously providing soil samples. We are grateful to Dr. Hiroki Rai, The University of Tokyo, Japan, for his help in collecting soil samples. This work was supported in part by a grant-in-aid for scientific research (No. 16380047) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Steel Industry Foundation for the Advancement of Environmental Protection Technology.

References

1) Böltner, D., S. Moreno-Morillas, and J.-L. Ramos. 2005. 16S rDNA phylogeny and distribution of *lin* genes in novel hexachlorocyclohexane-degrading *Sphingomonas* strains. Environ. Microbiol. **7**:1329–1338.
2) Cérémonie, H., H. Boubakri, P. Mavingui, P. Simonet, and T.M. Vogel. 2006. Plasmid-encoded γ-hexachlorocyclohexane degradation genes and insertion sequences in *Sphingobium francense* (ex-*Sphingomonas paucimobilis* Sp1). FEMS Microbiol. Lett. **257**:243–252.

3) Dogra, C., V. Raina, R. Pal, M. Suar, S. Lal, K.-H. Gartemann, C. Holliger, J.R. van der Meer, and R. Lal. 2004. Organization of *lin* genes and IS*6100* among different strains of hexachlorocyclohexane-degrading *Sphingomonas paucimobilis*: Evidence for horizontal gene transfer. J. Bacteriol. **186**:2225–2235.
4) Imai, R., Y. Nagata, K. Senoo, H. Wada, M. Fukuda, and K. Yano. 1989. Dehydrochlorination of γ-hexachlorocyclohexane (γ-BHC) by γ-BHC-assimilating *Pseudomonas paucimobilis*. Agric. Biol. Chem. **53**:2015–2017.
5) Imai, R., Y. Nagata, M. Fukuda, M. Takagi, and K. Yano. 1991. Molecular cloning of a *Pseudomonas paucimobilis* gene encoding a 17-kilodalton polypeptide that eliminates HCl molecules from γ-hexachlorocyclohexane. J. Bacteriol. **173**:6811–6819.
6) Johri, A.K., M. Dua, D. Tuteja, R. Saxena, D.M. Saxena, and R. Lal. 1998. Degradation of alpha, beta, gamma and delta-hexachlorocyclohexanes by *Sphingomonas paucimobilis*. Biotechnol. Lett. **20**:885–887.
7) Kumari, R., S. Subudhi, M. Suar, G. Dhingra, V. Raina, C. Dorga, S. Lal, J.R. van der Meer, C. Holliger, and R. Lal. 2002. Cloning and characterization of *lin* genes responsible for the degradation of hexachlorocyclohexane isomers by *Sphingomonas paucimobilis* strain B90. Appl. Environ. Microbiol. **68**:6021–6028.
8) Lal, R., C. Dogra, S. Malhotra, P. Sharma, and R. Pal. 2006. Diversity, distribution and divergence of *lin* genes in hexachlorocyclohexane-degrading sphingomonads. Trends Biotechnol. **24**:121–130.
9) Miyauchi, K., H.-S. Lee, M. Fukuda, M. Takagi, and Y. Nagata. 2002. Cloning and characterization of *linR*, involved in regulation of the downstream pathway for γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. Appl. Environ. Microbiol. **68**:1803–1807.
10) Miyauchi, K., S.-K. Suh, Y. Nagata, and M. Takagi. 1998. Clonig and sequencing of a 2,5-dichlorohydroquinone reductive dehydrogenase gene whose product is involved in degradation of γ-hexachlorocyclohexane by *Sphingomonas paucimobilis*. J. Bacteriol. **180**:1354–1359.
11) Miyauchi, K., Y. Adachi, Y. Nagata, and M. Takagi. 1999. Cloning and sequencing of a novel meta-cleavage dioxygenase gene whose product is involved in degradation of γ-hexachlorocyclohexane in *Sphingomonas paucimobilis*. J. Bacteriol. **181**:6712–6719.
12) Mohn, W.W., B. Mertens, J.D. Neufeld, W. Verstraete, and V. de Lorenzo. 2006. Distribution and phylogeny of hexachlorocyclohexane degrading bacteria in soils from Spain. Environ. Microbiol. **8**:60–68.
13) Nagata, Y., A. Futamura, K. Miyauchi, and M. Talagi. 2001. Two different types of dehalogenases, LinA and LinB, involved in γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26 are localized in the periplasmic space without molecular processing. J. Bacteriol. **181**:5409–5413.
14) Nagata, Y., K. Miyauchi, J. Damborský, K. Manova, A. Ansorgova, and M. Takagi. 1997. Purification and characterization of a haloalkane dehalogenase of a new substrate class from a γ-hexachlorocyclohexane-degrading bacterium, *Sphingomonas paucimobilis* UT26. Appl. Environ. Microbiol. **63**:3707–3710.
15) Nagata, Y., K. Miyauchi, and M. Takagi. 1999. Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. J. Ind. Microbiol. Biotechnol. **23**:380–390.
16) Nagata, Y., K. Mori, M. Takagi, A.G. Murzin, and J. Damborský. 2001. Identification of protein fold and catalytic residues of γ-hexachlorocyclohexane dehydrochlorinase LinA. PROTEINS: Structure, Function, and Genetics **45**:471–477.
17) Nagata, Y., M. Kamakura, R. Endo, R. Miyazaki, Y. Ohtsubo, and M. Tsuda. 2006. Distribution of γ-hexachlorocyclohexane-degrading genes on three replicons in *Sphingobium japonicum* UT26. FEMS Microbiol. Lett. **256**:112–118.
18) Nagata, Y., R. Imai, A. Sakai, M. Fukuda, K. Yano, and M. Takagi. 1993. Isolation and characterization of Tn*5*-induced mutants of *Pseudomonas paucimobilis* UT26 defective in γ-hexachlorocyclohexane dehydrochlorinase (LinA). Biosci. Biotech. Biochem. **57**:703–709.
19) Nagata, Y., R. Ohtomo, K. Miyauchi, M. Fukuda, K. Yano, and M. Takagi. 1994. Cloning and sequencing of a 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase gene involved in the degradation of γ-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J. Bacteriol. **176**:3117–3125.
20) Nagata, Y., T. Hatta, R. Imai, K. Kimbara, M. Fukuda, K. Yano, and M. Takagi. 1993. Purification and characterization of γ-hexachlorocyclohexane (γ-HCH) dehydrochlorinase (LinA) from *Pseudomonas paucimobilis*. Biosci. Biotech. Biochem. **57**:1582–1583.
21) Nagata, Y., T. Nariya, R. Ohtomo, M. Fukuda, K. Yano, and M. Takagi. 1993. Cloning and sequencing of a dehalogenase gene encoding an enzyme with hydrolase activity involved in the degradation of γ-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J. Bacteriol. **175**:6403–6410.
22) Pal, R., S. Bala, M. Dadhwal, M. Kumar, G. Dhingra, O. Prakash, S.R. Prabagaran, S. Shivaji, J. Cullum, C. Holliger, and R. Lal. 2005. Hexachlorocyclohexane-degrading bacterial strains *Sphingomonas paucimobilis* B90A, UT26 and Sp+, having similar *lin* genes, represent three distinct species, *Sphingobium indicum* sp. nov., *Sphingobium japonicum* sp. nov. and *Sphingobium francense* sp. nov., and reclassification of [*Sphingomonas*] *chungbukensis* as *Sphingobium chungbukense* comb. nov. Int. J. Syst. Evol. Microbiol. **55**:1965–1972.
23) Sahu, H.K., K.K. Patnaik, M. Sharmila, and N. Sethunathan. 1990. Degradation of alpha-, beta-, and gamma-hexachlorocyclohexane by a soil bacterium under aerobic conditions. Appl. Environ. Microbiol. **56**:3620–3622.
24) Senoo, K., and H. Wada. 1989. Isolation and identification of an aerobic γ-HCH-decomposing bacterium from soil. Soil Sci. Plant Nutr. **35**:79–87.
25) Senoo, K., M. Nishiyama, H. Wada, and S. Matsumoto. 1992. Differences in dynamics between indigenous and inoculated *Sphingomonas paucimobilis* strain SS86 in soils. FEMS Microbiol. Ecol. **86**:311–320.
26) Sparks, D.L., A.L. Page, P.A. Helmke, R.H. Loeppert, P.N. Soltanpour, M.A. Tabatabai, C.T. Johnston, and M.E. Sumnet. (ed.) 1996. Methods of Soil Analysis, Part 3, Chemical Methods. Soil Science Society of America, American Society of Agronomy, Madison, USA.
27) Thomas, J.-C., F. Berger, M. Jacquier, D. Bernillon, F. Baud-Grasset, N. Truffaut, P. Normand, T.M. Vogel, and P. Simonet. 1996. Isolation and characterization of a novel γ-hexachlorocyclohexane-degrading bacterium. J. Bacteriol. **178**:6049–6055.
28) Wada, H., K. Senoo, and Y. Takai. 1989. Rapid degradation of γ-HCH in upland soil after multiple applications. Soil Sci. Plant Nutr. **35**:71–77.