Bioresource Technology 100 (2009) 3199–3204

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Development of an autofluorescent organophosphates-degrading *Stenotrophomonas* sp. with dehalogenase activity for the biodegradation of hexachlorocyclohexane (HCH)

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ARTICLE INFO

Article history:
Received 19 November 2008
Received in revised form 4 February 2009
Accepted 4 February 2009
Available online 9 March 2009

Keywords:
Hexachlorocyclohexane
Organophosphates
Dehalogenase
Green fluorescent protein
Bioremediation

ABSTRACT

Simultaneous biodegradation of hexachlorocyclohexane (HCH) and organophosphates (OPs) by a recombinant *Stenotrophomonas* sp. was studied in the study. The broad-host-range plasmid pVGAB, harboring enhanced green fluorescent protein gene (*egfp*) and dehalogenase genes (*linA* and *linB*), was constructed and transformed into the OP-degrading strain *Stenotrophomonas* YC-1 to get the recombinant strain YC-H. Over-expression of dehalogenase (LinA and LinB) and enhanced green fluorescent protein (EGFP) was obtained in YC-H by determining their enzymatic activities and fluorescence intensity. YC-H was capable of rapidly and simultaneously degrading 10 mg/l γ-HCH and 100 mg/l methyl parathion (MP) determined by GC–ECD analysis. A bioremediation assay with YC-H inoculated into fumigated and nonfumigated soil showed that both 10 mg/kg γ-HCH and 100 mg/kg MP could be completely degraded within 32 days. The novel EGFP-marked bacterium could be potentially applied in the field-scale decontamination of HCH and OPs residues in the environment.

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1. Introduction

The technical formulation of hexachlorocyclohexane (t-HCH), a recalcitrant pesticide, mainly consists of α-, β-, γ- and δ-isomers (Li et al., 2003). Among these four isomers, only γ-HCH (also called γ-BHC or lindane) has insecticidal activity. It has been used widely throughout the world for pest control in agriculture mosquito control in public health efforts prior to the 1990s. However, serious environmental problems have resulted from HCH usage, since all its isomers are toxic, highly persistent in the environment and tend to accumulate in biological tissues (Zou and Matsumura, 2003). Although, the use of HCH was banned or restricted in most countries, its residues still remain present in many agricultural soils (Nawab et al., 2003), and even in vegetables, grains and fruits (Sanghi and Tewari, 2001). Synthetic organophosphates (OPs) are a group of highly toxic chemicals that exhibit broad-spectrum activity against insects and are widely used against major agricultural pests, accounting for about 38% of total pesticides used globally (Singh and Walker, 2006). However, continuous and excessive use of OPs has also caused not only nerve and muscular diseases in human and animals but contamination of ecosystems in different parts of the world (Karalliede and Senanayake, 1999; Sogorb et al., 2004; Singh and Walker, 2006).

Bioremediation is the process by which living organisms degrade or transform hazardous organic contaminants. It is considered to be a more efficient, convenient, and environment friendly method for detoxification of HCH and OPs residues than more costly physical and chemical methods such as incineration in the environment (Phillips et al., 2006; Singh and Walker, 2006; Raina et al., 2008). Due to the presence of various types of pesticide residues in the environment, multifunctional genetically engineered microorganisms (GEMs) are potentially useful in cleaning up these pollutants (Carlos and Itziar, 1999; Chen et al., 1999). Bacteria with genetic material for the degradation of specific types of pesticides are known and their genes can potentially be transferred into bacteria with different degradation capabilities to generate GEMs with broader degradation spectrum. *Sphingobium japonicum* UT26 is a unique microorganism that utilizes γ-HCH as its sole source of carbon and energy under aerobic conditions (Imai et al., 1989). The enzymes and genes involved in the degradation pathway of γ-HCH in UT26 have been elucidated (Nagata et al., 1999, 2007). The chlorpyrifos-degrading bacterium *Stenotrophomonas* YC-1, isolated from the sludge of the wastewater treating system, is robust and ubiquitous in soils and capable of degrading a variety of OP pesticides (Yang et al., 2006). These desirable traits make this bacterium very attractive as a platform for *in situ* bioremediation.

Enumerating and assessing of GEMs in polluted soil and aquatic environments can be assisted by using stable marker systems with an easily detectable phenotype. The green fluorescent protein

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doi:10.1016/j.biortech.2009.02.008

(GFP) marker is preferred over other marker genes because it does not require any substrate, and no background activity is present in indigenous soil microorganisms and plants (Jansson, 2003). Enhanced GFP (EGFP; GenBank accession no. U57609), which is a red-shifted variant of GFP, assembles the chromophore more rapidly, shows much stronger fluorescence than wild-type GFP, and fluoresces after exposure to daylight (Cormack et al., 1996). GFP is an effective real-time monitoring tool for ecological studies of GEMs in the environment (Errampalli et al., 1999; Bhatia et al., 2002; Fuchslin et al., 2003; Zhang et al., 2008).

Methyl parathion (MP), a widely used OP pesticide, was classified by the US Environmental Protection Agency as a Toxicity Category I (most toxic) insecticide (Jaga and Dharmani, 2006). Therefore, MP and γ-HCH were chosen as the model compounds for this study. In this work, a broad-host recombinant plasmid pVGAB, harboring marker gene *egfp* and the dehalogenase genes *linA* and *linB*, was constructed and transformed into YC-1 resulting in strain YC-H. The degradation characteristics of strain YC-H in culture and soil samples were studied. Our results demonstrated that YC-H was endowed with the capability to rapidly degrade both HCH and OPs and could be monitored online easily, which should open up new avenues in treating recalcitrant environmental pollutants.

2. Methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Sphingobium japonicum* UT26 and *Stenotrophomonas* YC-1 or YC-H were grown on 1/3 Luria–Bertani (LB) medium (3.3 g of Bacto Tryptone, 1.7 g of yeast extract and 5 g of sodium chloride, per liter) or minimal salt medium (MSM) (2.0 g Na_2HPO_4, 0.75 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g NH_4Cl per liter, pH 7.0) supplemented with 100 mg/l MP or 5 mg/l γ-HCH added if needed at 30 °C. *Escherichia coli* strains were grown on LB medium at 37 °C. Antibiotics were used at final concentrations of 50 mg/l for ampicillin, 25 mg/l for kanamycin, and 25 mg/l for nalidixic acid.

2.2. Chemicals and enzymes

All organic solvents were glass-distilled grade or high-performance liquid chromatography grade (Tedia, USA). Analytical grade γ-HCH was purchased from Sigma–Aldrich USA (Genetimes Technology, China). Analytical grade 1-chlorobutane (1-CB) was purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China). T4 DNA ligase, isopropyl thiogalactoside (IPTG), and restriction enzymes for DNA manipulations were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China).

2.3. Construction of the recombinant expression plasmid

The *egfp* gene (GenBank accession no. U57609) was amplified by PCR from plasmid pEGFP-N3 using primers pUGf and pUGr. The PCR product was digested with *Sac*I and *Kpn*I and inserted into similarly digested pUC18 generate pUG. Then the *egfp* gene with *lac* promoter was amplified by PCR from pUG using primers pU18Gf and pUGr. The PCR product was digested with *Eco*RI and *Kpn*I and inserted into similarly digested broad-host-range plasmid pVLT33, resulting in pVG. The *linA* gene containing its constitutive expressed promoter (GenBank accession no. D90355) was amplified by PCR from the genomic DNA of strain UT26 with primers pUAf and pUAr. The PCR product was digested by *Kpn*I and *Bam*HI, and then ligated to a similarly digested pVG to get pVGA. Finally, the *linB* gene containing its constitutive expressed promoter (GenBank accession no. D14594) was amplified by PCR from the genomic DNA of strain UT26 with primers pUBf and pUBr. The PCR product was digested with *Pst*I and *Hin*dIII and inserted into similarly digested pVGA, resulting in the final recombinant plasmid designated as pVGAB. The correct sequence of the insert was confirmed by sequencing prior to characterization studies. Plasmid DNA was isolated using the alkaline lysis method, with the exception of plasmid pVLT33 and its derivatives which were isolated using the boiling lysis method (de Lorenzo et al., 1993). The structure of the recombinant plasmid pVGAB is shown in Fig. 1. Transformation of recombinant plasmid pVGAB into *Stenotrophomonas* YC-1 was performed using the $CaCl_2$ method (Sambrook et al., 2001).

2.4. Determination of LinA and LinB activity and fluorescence intensity in YC-H

Cell lysate was prepared for analysis as follows: cells were harvested, washed with 100 mM phosphate buffer (pH 7.4) and then resuspended in the same phosphate buffer. The resuspended buffer was incubated on ice for 1 h with addition of 10 μl of lysozyme

Table 1
Bacterial strains, plasmids, and primers used in this study.

Strain or plasmid	Relevant genotype or characteristics	Source or literature
Strains		
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1*_(*lacZYA-argF*) Φ80*lacZ* Δ*M15*	Tiangen
Sphingobium japonicum UT26	Aerobic, motile, rod shaped; degrades α-, γ- and δ-HCH	Nagata et al. (2007)
Stenotrophomonas YC-1	Aerobic, motile, straight rod with polar flagellum; organophosphates-degrading wild-type strain; G^-	Yang et al. (2006)
Plasmids[a]		
pUC18	Amp^r; cloning vector for construction of P_{lac}–*egfp*	Takara
pVLT33	Kan^r; RSF1010-*lacIq*/*Ptac* hybrid broad-host-range expression vector	de Lorenzo et al. (1993)
pEGFP-N3	Kan^r; cloning vector plasmid containing *egfp* gene	Clontech, USA
pVGAB	12.5-kb Kan^r encodes EGFP–LinA–LinB protein, derivative of pVLT33	This study
Primers[b]		
pUGf	5′-GAATTCATGGTGAGCAAGGGCGAGGAGCTG-3′	This study
pUGr	5′-GGTACCTTACTTGTACAGCTCGTCCAT-3′	This study
pUAf	5′-GGTACCCATGAAGACGCCGATGCA-3′	This study
pUAr	5′-GGATCCTTATGCGCCGGACGGTGC-3′	This study
pUBf	5′-CTGCAGATCGATGAGATTCTCGCG-3′	This study
pUBr	5′-AAGCTTTTATGCTGGGCGCAATCG-3′	This study
pU18Gf	5′-GAGCTCGTTTCCCGACTGGAAAGCGG-3′	This study

[a] Amp^r, ampicillin resistant; Kan^r, kanamycin resistant.
[b] Restriction sites in the sequences of the primers are underlined.

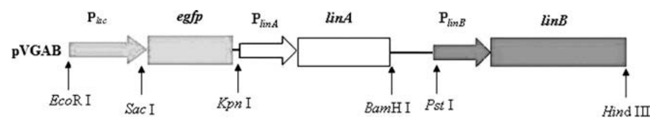

Fig. 1. Structure of the recombinant plasmid pVGAB. The *egfp* gene was amplified by PCR from pEGFP-N3. The *linA* and *linB* genes and their respective promoters were amplified by PCR from the genomic DNA of strain UT26. Expression of *egfp* is under the control of *lac* promoter from pUC18 while *linA* and *linB* were regulated by their own promoters.

(10 mg/ml), followed by sonication in three pulses of 10 s each. Total cellular protein was determined by the Bradford method with bovine serum albumin as a standard.

LinA and LinB activity were assayed with γ-HCH and 1-chlorobutane (1-CB) as the substrate (Nagata et al., 1993, 1997), respectively. Cell lysate was diluted with 100 mM phosphate buffer (pH 7.4) to yield a final volume of 1.0 ml in a microtube and incubated with 30 μmol of γ-HCH or 50 mM of 1-CB of at 30 °C. One unit of LinA and LinB activity was defined as the amount of enzyme required for the release of 1 μmol of chloride ion per min under these conditions. The amount of chloride ion released was measured spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulfate by the method of Iwasaki et al. (1952).

The determination of fluorescence intensity was carried as follows. YC-H cells were grown to an OD_{600} = 0.6 and induced with 1 mM IPTG for 36 h at 30 °C. The cells were collected, washed three times with PBS buffer (pH 7.4). Then they were resuspended in PBS buffer and diluted to OD_{600} = 1.0. YC-1 cells harboring pVLT33 was used as background references. EGFP fluorescence intensity was determined using a fluorescence spectrophotometer (F-4500, HITACHI, Japan) with band width of 5 nm, excitation wavelength of 488 nm and emission wavelength of 510 nm.

2.5. Biodegradation of γ-HCH and MP by Stenotrophomonas YC-H and Sphingobium japonicum UT26 in MSM

Unless stated otherwise, the pesticides biodegradation experiments in the study were carried out as below: the strains YC-H (or UT26) was precultured in 1/3 LB medium at 30 °C and harvested during log phase, respectively. The cultures were centrifuged and the cell pellets were washed twice with fresh MSM (pH 7.0) used as inoculums. Then 10^6 cells/ml were used and inoculated into MSM supplemented with 0.1% glucose containing 100 mg/l MP and 10 mg/l γ-HCH as the source of carbon and energy and 25 mg/l of kanamycin added for YC-H. Cultures were maintained in 250 ml bottles at 30 °C on a shaker operated at 200 rpm. Appropriate controls containing medium plus γ-HCH and MP were kept simultaneously. Measurements are the average of three separate cultures.

Aliquots (1 ml) were removed periodically and extracted twice with 4 ml of ethyl acetate for MP and hexane for γ-HCH. These extracts were dehydrated with sodium sulfate and filtered (0.45 μm). Samples of 1 μl (diluted if necessary) were analyzed using a Hewlett–Packard 5890 II GC (Hewlett–Packard, Wilmington, DE, USA) equipped with ECD detector and an OV-1701 gas chromatography column (polydimethylsiloxane 25 m × 0.32 mm × 0.25 μm). The column, injector, and detector temperatures were maintained at 210, 250, and 300 °C, respectively, with a flow rate of carrier gas (nitrogen) of 5.4 ml/min. The concentration was determined by comparing peak area of the samples to a standard curve. Under the GC–ECD analysis conditions, the retention time of γ-HCH and MP were 4.70 and 6.84 min, respectively and the concentration was determined by comparing peak area of the samples to a standard curve.

2.6. Simultaneous degradation of MP and γ-HCH by YC-H in soil

The soil samples were from the campus of Tsinghua University, Beijing, China and were never exposed to any HCH and OPs pesticides before. The soil had a pH of 7.12 and its organic carbon is 5.33 g/kg. The content of N, P, and K available in the soil were 35.26, 8.93, and 29.55 g/kg, respectively.

Soil samples (5 kg) were sterilized by fumigation with chloroform for 10 days at 30 °C (Singh et al., 2004). Subsamples (100 g) of the fumigated and nonfumigated soil were treated under aseptic condition with MP (100 mg/kg) and γ-HCH (10 mg/kg), respectively. Both fumigated and nonfumigated soils were inoculated with the strain YC-H at the rate of 10^6 cells/g, respectively. Meanwhile, fumigated and nonfumigated soils without inoculation were kept as controls. Soils were run in triplicate for each strain to ensure accuracy. The inoculums were thoroughly mixed into the soils under sterile condition. The soil moisture was adjusted by the addition of distilled water to 50% of its water-holding capacity. To prevent photodegradation of the pesticides in soil, the pots were kept covered with a black sheet. During incubation, subsamples were collected at periodic intervals from the replicated pots of each treatment and were analyzed immediately. Sterility of the fumigated soil was carefully checked by the plating method and no microorganism growth was found. MP and γ-HCH were extracted from the soil by the methods of Singh et al. (2002) and Bidlan et al. (2004), respectively. Analytical methods of MP and γ-HCH were the same as those in Section 2.5. In order to study whether the fluorescence in YC-H was stable in soil, 1.5 g of both fumigated and nonfumigated soil samples were taken out periodically and suspended with PBS buffer (pH 7.4). The cells in the suspension were collected by centrifugation, washed three times with PBS buffer and fixed in 2% formaldehyde for 10 min. Cells were centrifuged and resuspended in 1 ml PBS buffer. Finally, cells were examined by a Nikon Optiophot fluorescence microscope.

3. Results and discussion

3.1. Construction of the recombinant strain YC-H

Construction of the broad-host recombinant plasmid pVGAB was demonstrated in Fig. 1. Expression of marker gene *egfp* was under the control of relaxed-regulated *lac* promoter from pUC18, which was weakly constitutively active even without IPTG or lactose induction. Expression of the dehydrochlorinase gene *linA* and haloalkane dehalogenase gene *linB* was regulated by its own constitutive expressed promoters and SD sequences (Fig. 1). Therefore, these genes could be expressed in the recombinant strain even without induction by substrates. Finally, pVGAB was successfully transformed into the soil-dwelling OPs-degrader *Stenotrophomonas* YC-1 to obtain YC-H. Expression of *linA*, *linB* and *egfp* was certified by RT-PCR analysis.

To test whether expression of EGFP–LinA–LinB inhibits cell growth, we studied the growth kinetics and degrading abilities of YC-H in MSM supplemented with 200 mg/l MP and compared with that of the wild-type YC-1. The result showed that the growth curve of YC-H was nearly identical with that of YC-1 during 48 h of incubation (Fig. 2), which demonstrated that expression of pVGAB in the recombinant strain did not affect cell viability and original degrading characteristics.

In order to check the stability of YC-H, the GEM cells were grown in selective (5 mg/l kanamycin) and non-selective 1/3 LB cultures at 30 °C and then transferred to the next culture under the same conditions every 4 days. Meanwhile, the rest cultures were centrifuged and 10^6 cells/ml were inoculated into MSM supplemented with 0.1% glucose containing 50 mg/l MP and 5 mg/l γ-

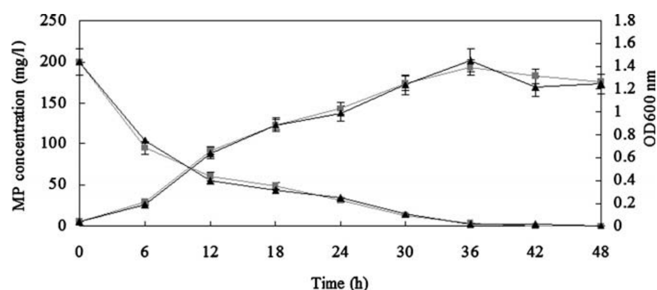

Fig. 2. Cell growth kinetic and degrading abilities of *Sphingomonas* sp. YC-H (▲) and YC-1 (■) in MSM medium containing 200 mg/l MP at 30 °C. Error bars represent standard deviation from three independent experiments.

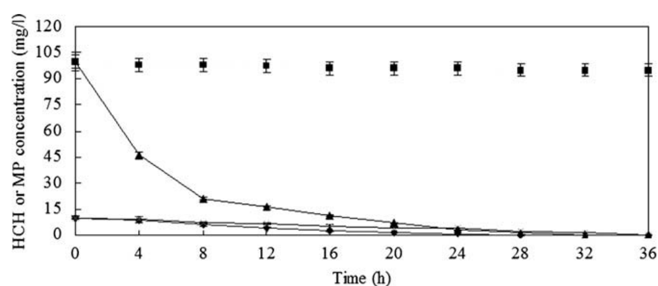

Fig. 3. Biodegradation of γ-HCH and MP by YC-H and UT26 at an initial rate of 10^6 cells/ml in MSM supplemented with 0.1% glucose containing 10 mg/l γ-HCH and 100 mg/l MP at 30 °C (×-the degradation of γ-HCH by YC-H; ▲-the degradation of MP by YC-H; ♦-the degradation of γ-HCH by UT26; ■-the degradation of MP by UT26). Error bars represent standard deviation from three independent experiments.

HCH to determine the degradation capacity for γ-HCH and fluorescence intensity of YC-H. The procedure was repeated a sixth time. The results showed that in selective and non-selective medium, the degradation capacity and fluorescence intensity of YC-H at different time points was almost the same over the 24-d period, which indicated that no loss of *egfp*, *linA* and *linB* genes occurred after growth at least 150 generations and showed that YC-H was genetically stable.

3.2. Determination of LinA and LinB activity and fluorescence intensity

Time-course studies of LinA and LinB activities were carried out at 30 °C, with the same amount of YC-H cells (OD_{600} = 1.0) being collected at 6 h intervals, and the corresponding enzyme activities determined. As shown in Table 2, LinA and LinB activity was not detected at 0 h and enzymatic activities remained almost the same during the course, which confirmed that under the regulation of their own constitutive expressed promoters, both *linA* and *linB* could express without induction of γ-HCH.

Time-course studies of fluorescence intensity (Table 2) were also carried out with 1 mM IPTG at 30 °C with the same amount of cells (OD_{600} = 1.0). EGFP fluorescence at 0 h (post-induction) was higher than the original background level (YC-1 cells harboring pVLT33) as a result of P_{lac} that weakly constitutively expressed without IPTG induction. After 1 mM IPTG induction, the fluorescence intensity was rapidly and dramatically promoted in the following 36 h.

3.3. Biodegradation of γ-HCH and MP by YC-H in MSM

In order to determine the degradation rate of MP and γ-HCH, aliquots (1 ml) were removed periodically from the MSM medium inoculated with *Stenotrophomonas* YC-H (or *Sphingobium japonicum* UT26) and containing 100 mg/l MP and 10 mg/l γ-HCH and were analyzed by GC–ECD. As shown in Fig. 3, the rates of degradation of γ-HCH by YC-H were higher than those for UT26. The γ-HCH at an initial concentration of 10 mg/l was degraded to an undetectable level by YC-H within the first 28 h while the same amount of γ-HCH was degraded within 36 h by UT26. The rates became much slower from 20 to 32 h perhaps because of the accumulation of intermediate of γ-HCH. MP (100 mg/l) was completely degraded via hydrolysis of the phosphotriester bond in 28 h by YC-H and its hydrolysis product *p*-nitrophenol (PNP) accumulated, however, the concentration of MP remained almost unchanged in the culture of U26, which indicated that U26 grew mainly on γ-HCH and glucose added as carbon sources.

Table 2
Time-course analysis of LinA and LinB activity and fluorescence intensity (induced by 1 mM IPTG) of YC-H for 36 h at 30 °C. Error bars represent standard deviation from three independent experiments.

Time (h)	LinA activity (U/mg)	LinB activity (U/mg)	Fluorescence intensity
0	ND	ND	112 ± 6
6	9.62 ± 0.85	1.44 ± 0.88	289 ± 22
18	10.22 ± 0.66	1.32 ± 1.31	391 ± 63
24	9.97 ± 1.13	1.18 ± 0.71	498 ± 29
30	10.35 ± 0.76	1.50 ± 0.19	555 ± 29
36	9.59 ± 0.91	1.21 ± 0.42	529 ± 16

3.4. Simultaneous degradation of MP and γ-HCH by YC-H in soil

In order to determine whether YC-H cells degraded MP and γ-HCH in soil and to examine the growth of the YC-H cells when in competition with indigenous bacteria and in the presence of alternative carbon sources, fumigated and nonfumigated soil were inoculated with YC-H cells at the rate of 10^6 cells/g (Fig. 4). Only 4% of γ-HCH was degraded in both control soil samples; however, about 14% of MP was degraded in 32 days in nonfumigated soil most likely due to indigenous microbial transformation. Bacterial counts on Km and MP plates showed that the initial inoculation of YC-H

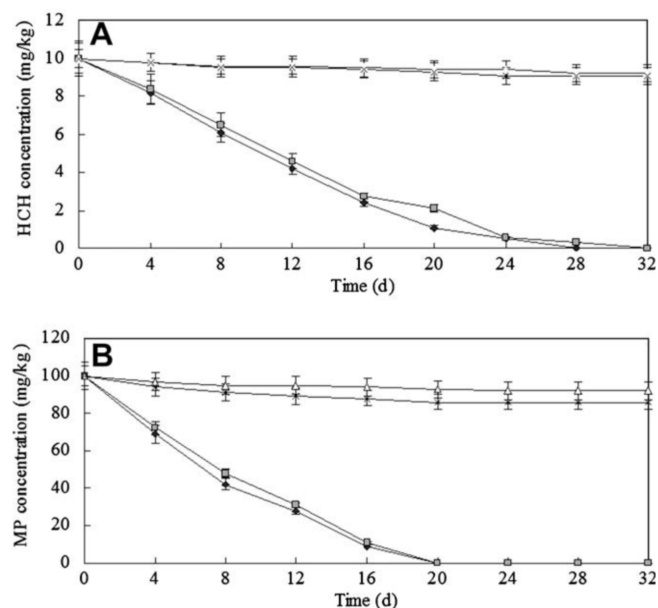

Fig. 4. Simultaneous degradation of γ-HCH (A) and MP (B) by YC-H at an initial rate of 10^6 cells/g in soil containing 10 mg/kg γ-HCH and 100 mg/kg MP at 30 °C (♦-fumigated soil inoculated; ■-nonfumigated soil inoculated; △-fumigated soil uninoculated; ×-nonfumigated soil uninoculated). Error bars represent standard deviation from three independent experiments.

cells in both soils was 4.2 ± 0.85 × 10^7 CFU/g dry soils. In the present study, both MP (100 mg/kg) and γ-HCH (10 mg/kg) could be degraded within 32 days in fumigated and nonfumigated soil samples. In fumigated soil, MP and γ-HCH were degraded more rapidly by YC-H cells and the numbers of the cells decreased slower than those in nonfumigated soil, suggesting that there was competition between the indigenous population and the inoculated strain. After incubation for 25 days, strain YC-H could be isolated from the nonfumigated soils using plate counting technology, which indicated that the organism could be responsible for the observed degradation. Moreover, strain YC-H still could be identified by its fluorescence when γ-HCH was completely degraded after 32 days in both soil samples (data not shown). These results showed that YC-H with stable fluorescence might possess potential to be utilized for bioremediation of HCH residues in the environment.

Some studies on actual field-scale treatment of HCH-contaminated soil mainly focused on the bioaugmentation or optimization of environmental conditions to enhance degradation by indigenous microorganisms (Phillips et al., 2005). However, the slow rate of degradation of soil-dwelling microorganisms may limit their bioremediation of sites contaminated with HCH. Therefore, it may be a possible strategy to create robust strains which degrade HCH more effectively by introducing key genes for HCH degradation. In this study, the soil bacterium *Stenotrophomonas* YC-1, which had rapid degrading capacity and broad-spectrum for a variety of OPs, was chosen as our host strain and platform for the construction of a HCH-degrading microorganism. The broad-host-range vector, pVLT33, used in this study is a RSF1010 derivative and therefore able to replicate in a wide variety of Gram-negative bacteria (de Lorenzo et al., 1993). Over-expression of LinA and LinB and EGFP was obtained in YC-H by determining their enzymatic activities and fluorescence intensity. More importantly, expression of *egfp*, *linA* and *linB* in the broad-host recombinant plasmid pVGAB was regulated by constitutive expressed promoters without induction of substrates, which made it more suitable for future field-scale bioremediation. Our results demonstrated that YC-H was capable of rapidly degrading both HCH and OPs and degraded γ-HCH more rapidly than the natural strain UT26. More importantly, the biodegradation characteristic for γ-HCH and fluorescence intensity were genetically stable after successive plating on non-selective media.

Dehalogenases are key enzymes in the degradation of halogenated compounds such as HCH that occur as soil pollutants (Janssen, 2004). Because HCH has six chlorine atoms per molecule, dechlorination is a very significant step in its degradation. The dehalogenase LinA is responsible for the initial dechlorination of α-, γ-, and δ-HCH while LinB is responsible for the dechlorination of β-HCH (Nagata et al., 2005, 2007). Our results demonstrated that YC-H was successfully endowed with the capability to rapidly degrade γ-HCH as a result of enzymatic activities of LinA and LinB. Although, HCH cannot be completely mineralized by YC-H, the resulting products such as pentachlorocyclohexanols (PCCH) and dichlorocyclohexadienediols (DDOL) have lower hydrophobicity or lower chemical stability than HCH, and the bacteria that degrade and utilize them may exist in the polluted environment, allowing the complete degradation of HCH by a combination of biological pathways. Besides, since LinB is a member of haloalkane dehalogenases with relatively broad substrate specificity, the strain has a great potential to degrade various environmental pollutants such as synthetic haloalkanes that occur as soil pollutants (Nagata et al., 1997; Janssen, 2004; Kmunicek et al., 2005).

In our studies on lab-scale soil bioremediation by YC-H, both γ-HCH and MP could be completely degraded within 32 days in both soil samples, indicating that YC-H has a great potential for the bioremediation of HCH and OPs residues in natural environment even in the presence of indigenous microbial competition and other carbon sources. However, in actual field-scale bioremediation, bioavailability of contaminants is affected mostly by many physical, chemical and structural properties of both the contaminants and the soil matrix. Therefore, the effectiveness depends both on the degrading microorganisms and the contaminants bioavailability. Moreover, since our construction was based on a broad-host-range and conjugative plasmid, the recombinant plasmid might be transferred to representatives of the indigenous microflora and a high transfer rate of the plasmid to indigenous microflora could help to increase HCH degradation, which could be seen as part of the bioremediation treatment. The strain could be identified in soil samples on the basis of green fluorescence after 32 days, which showed GFP is a powerful real-time monitoring tool for the visualization of YC-H. This work will be useful for further study of the environment behavior of the strain by its fluorescence in the environment.

We report a novel EGFP-marked strain with the capability to rapidly degrade both HCH and OPs, which offers a strong platform toward the successful utilization of this environmentally robust bacterium for a wide range of biocatalysis applications. Our present work provided a good basis for optimism to reduce the impact of HCH and OPs to clean-up environmental sites from their contamination. Further studies should be conducted to examine the interactions of the soil environment with the strain and the effects of multiple soil parameters on degradation. This will be great useful for the application of the strain in the field-scale bioremediation of highly HCH-and OPs-contaminated sites in the future.

Acknowledgements

We especially thank Y. Nagata, Department of Environmental Life Sciences, Tohoku University, for providing *Sphingobium japonicum* UT26. This work was supported by grants from the Innovation Program of the Chinese Academy of Sciences (No. KSCX2-YW-G-008) and the 863 Hi-Tech Research and Development Program of the People's Republic of China (No. 2007AA06Z335).

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