Journal of Biotechnology 146 (2010) 114–119

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Development of an autofluorescent *Pseudomonas nitroreducens* with dehydrochlorinase activity for efficient mineralization of γ-hexachlorocyclohexane (γ-HCH)

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ARTICLE INFO

Article history:
Received 28 August 2009
Received in revised form 19 January 2010
Accepted 25 January 2010

Keywords:
Hexachlorocyclohexane
Dehydrochlorinase
Green fluorescent protein
Biodegradation
Mineralization pathway

ABSTRACT

Biodegradation or bioremediation is a more efficient and environmental friendly method for detoxification of hexachlorocyclohexane (HCH) residues compared to physical and chemical methods. Here, we report the functional expression of dehydrochlorinase (LinA) and enhanced green fluorescent protein (EGFP) in *Pseudomonas nitroreducens* for efficient biodegradation of γ-HCH. The broad-host-range plasmid pVAG33, harboring dehydrochlorinase gene (*linA*) and enhanced green fluorescent protein gene (*egfp*), was constructed and transformed into the soil-isolated 1,2,4-trichlorobenzene (1,2,4-TCB)-degrading strain *P. nitroreducens* J5-1. Functional expression of LinA and EGFP was confirmed in the recombinant strain by Western-blotting analysis and by determining their enzymatic activities and fluorescence intensity. The recombinant strain could rapidly degrade 10 μg ml^{-1} γ-HCH in 28 h determined by GC-ECD analysis. It could completely mineralize γ-HCH via γ-HCH through 1,2,4-TCB and 3,4,6-trichlorocatechol and eventually entered the TCA cycle as determined by GC-MS analysis. The engineered strain can be applied in the form of a biocatalyst in a bioreactor for rapid degradation of HCH and chlorobenzene residues. Meanwhile, it can be easily monitored on-line by fluorescence of EGFP for its activity and fate.

1. Introduction

Hexachlorocyclohexane (HCH) is a broad-spectrum organochlorine insecticide, which has been used extensively worldwide for the control of agricultural pests and mosquitoes in malaria health programs prior to the 1990's. The technical formulation of HCH mainly consists of α- (60–70%), β- (5–12%), γ-(10–12%) and δ- (6–10%) isomers (Li et al., 2003). Among these four HCH isomers of technical-HCH (t-HCH), only γ-HCH (also called γ-BHC and lindane) has insecticidal activity. Today, the use of γ-HCH and t-HCH has been prohibited or restricted in most countries due to their toxicity and long persistence. However, large amounts of HCH still remain at the sites where they were produced or used and some countries are presently using γ-HCH, mainly for economic reasons. Therefore, many contaminated sites remain throughout the world and its contamination continues to be a global issue because HCH can travel by air to remote locations (Iwata et al., 1993; Nawab et al., 2003).

Dehalogenation is a key step in the degradation of halogenated compounds, such as HCH and DDT. Dehydrochlorinase LinA, first isolated from the γ-HCH-degrading bacterium *Sphingomonas paucimobilis* UT26, can catalyze the conversion of α- and γ-HCH to 1,2,4-trichlorobenzene (1,2,4-TCB) (Trantirek et al., 2001), which is an environmental pollutant included in the US EPA priority-pollutant list as a result of its toxicity and high persistence (den Besten et al., 1994). Moreover, 1,2,4-TCB is a dead-end product that cannot be further degraded by natural HCH-degrading bacteria (Nagasawa et al., 1993), resulting in inefficient HCH degradation with the accumulation of 1,2,4-TCB. Therefore, it may be a strategy to over-express LinA in natural 1,2,4-TCB degraders to get recombinant bacteria with LinA activity and 1,2,4-TCB mineralization capability for rapid biodegradation of HCH.

Enumerating and assessing of genetically engineered microorganisms (GEMs) in polluted soil and aquatic environments can be assisted by using stable marker systems with an easily detectable phenotype. The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is an ideal marker for monitoring the GEMs (Larrainzar et al., 2005). Fluorescence from GFP does not require

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doi:10.1016/j.jbiotec.2010.01.020

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

Strain or plasmid	Description	Source or literature
Strains		
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* (*lacZYA-argF*) Φ80*lacZ* ΔM15	Tiangen
Sphingobium japonicum UT26	Aerobic, motile, rod shaped; degrades α-, β-, γ- and δ-HCH	Nagata et al., 1999
Pseudomonas nitroreducens J5-1	Aerobic, motile, straight rod with polar flagellum; degrades 1,2,4-TCB; Wild type; G^-	Song et al., 2007
Plasmids		
pUC18	Amp^r; Source of *lac* promoter (P_{lac}-*egfp* and P_{lac}-*linA*)	Takara
pVLT33	Kan^r; *E. coli*/*Pseudomonas* shuttle vector, *oriT*, RSF1010, *oriV*, *lacIq*/*Ptac*	de Lorenzo et al., 1993
pEGFP-N3	Kan^r; cloning vector plasmid containing *egfp* gene	Clontech, USA
pVAG33	11.0-kb Kan^r encodes LinA and EGFP under the control of *lac* promoter, derivative of pVLT33	This study
Primers[a]		
P1	5′-GAATTCATGGTGAGCAAGGGCGAG-3′	This study
P2	5′-GGTACCTTACTTGTACAGCTCGTC-3′	This study
P3[b]	5′-GAGCTCGTTTCCCGACTGGAAAGCGG-3′	This study
P4	5′-GGATCCATGAGTGATCTAGACAGA-3′	This study
P5	5′-GGTACCTTATGCGCCGGACGGTGC-3′	This study
P6[b]	5′-CTGCAGGTTTCCCGACTGGAAAGCGG-3′	This study

E. coli: *Escherichia coli*; 1,2,4-TCB: 1,2,4-trichlorobenzene; EGFP: enhanced green fluorescent protein; δ-HCH: δ-hexachlorocyclohexane.
[a] Restriction sites in the sequences of the primers are underlined.
[b] P3 and P6 are the sequence of the upstream of *lac* promoter (−180bp to −160bp).

additional gene products, substrates, or other factors and can be detected non-invasively using fluorescence microscopy and flow cytometry (Chalfie et al., 1994). In field studies, GFP has been used as a marker to assess the fate and activity of specific degrading microorganisms (March et al., 2003). Enhanced GFP (enhanced green fluorescent protein [EGFP]; GenBank accession no. U57609), which is a redshifted 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).

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. *Pseudomonas nitroreducens* J5-1 (16S rRNA GenBank accession no. EF107515), isolated from chlorobenzene contaminated waste sites, was robust and ubiquitous in soils and capable of utilizing high concentrations 1,2,4-TCB (up to 400 μg ml^{-1}) as sole carbon source (Song et al., 2007). These desirable traits make this bacterium very attractive as a platform for *in situ* bioremediation. In this work, we demonstrated functional expression of LinA and EGFP in *P. nitroreducens* J5-1 to perform efficient biodegradation of γ-HCH.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *P. nitroreducens* J5-1 and *Sphingobium japonicum* UT26 were grown on 1/3 diluted Luria-Bertani (LB) medium (3.3 g of Bacto Tryptone per liter, 1.7 g of yeast extract per liter, 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 $MgSO_47H_2O$, 1.0 g NH_4Cl per liter, pH 7.0) supplemented with 20 μg ml^{-1} 1,2,4-TCB or 5 μg ml^{-1} γ-HCH at 30 °C. Antibiotics were used at final concentrations of 100 μg ml^{-1} for ampicillin, 50 μg ml^{-1} for kanamycin, and 25 μg ml^{-1} 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, mercuric thiocyanate and ferric ammonium sulfate were purchased from Sigma-Aldrich USA (J&K Chemical Ltd, Beijing, China). Analytical grade 1,2,4-TCB was purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China). T4 DNA ligase 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 Polymerase Chain Reaction (PCR) from plasmid pEGFP-N3 using primers P1 and P2. The PCR product was digested with *Eco*RI and *Kpn*I and inserted into similarly digested pUC18 generate pUG. Then, the *egfp* gene with a *lac* promoter was amplified by PCR from pUG using primers P3 and P2. The PCR product was digested with *Sac*I and *Kpn*I and inserted into similarly digested broad-host-range plasmid pVLT33 to generate pVG33. The *linA* gene (GenBank accession no. D90355) was PCR-amplified from the genomic DNA of strain UT26 with primers P4 and P5. The fragment was digested by *Bam*HI and *Hin*dIII and inserted into similarly digested pUC18 generate pUA. Then, the *linA* gene with the *lac* promoter was amplified by PCR from pUA using primers P6 and P5. The PCR product was digested with *Pst*I and *Hin*dIII and then ligated into similarly digested pVG33 to create the recombinant plasmid pVAG33. The correct sequence of the insert was confirmed by sequencing. Transformation of the recombinant plasmids into *P. nitroreducens* J5-1 was performed using the $CaCl_2/MgCl_2$ method (Sambrook et al., 2001; Shimazu et al., 2001). Expression of the recombinant plasmids was induced with 1 mM IPTG for 48 h at 30 °C when cells were grown to an $OD_{600\,nm}$=0.2.

2.4. SDS-PAGE and Western-blotting analysis

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 buffer. The resuspended buffer was incubated on ice for 1 h with addition of 10 μl of lysozyme (10 mg ml^{-1})

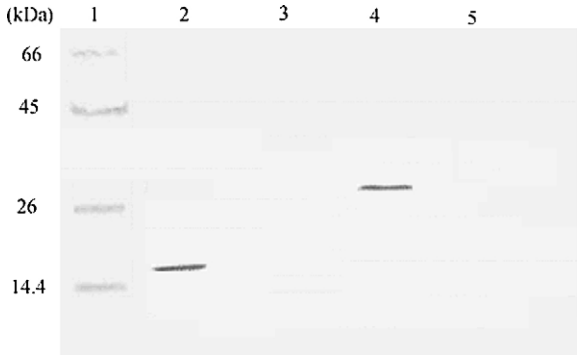

Fig. 1. Expression of pVAG33 in the recombinant *Pseudomonas nitroreducens* J5-1 as assessed by Western-blotting analysis. Lane 1, marker proteins; lane 2, cell lysates of J5-1 harboring pVAG33 incubated with polyclonal rabbit anti-LinA antibody; lane 3, cell lysates of J5-1 harboring pVLT33 incubated with polyclonal rabbit anti-LinA antibody (negative control); lane 4, cell lysates of J5-1 harboring pVAG33 incubated with polyclonal rabbit anti-GFP antibody; lane 5, cell lysates of J5-1 harboring pVLT33 incubated with polyclonal rabbit anti-GFP antibody (negative control). The molecular weight of marker proteins applied is indicated in kilodaltons.

and followed by sonication. Total cellular protein was determined according to the Bradford method (Bio-Rad) using bovine serum albumin as the standard. Each sample was mixed with SDS sample buffer, boiled for 7 min, and resolved by 10% (w/v) SDS-PAGE. For Western-blot analysis, the gel was transferred onto nitrocellulose membranes (Millipore) with a tank transfer system (Bio-Rad). Blotted membranes were placed in a blocking solution of 3% BSA in TTBS buffer (100 mM Tirs-Cl, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20) for 1 h. For immunodetection, primary GFP polyclonal antibody (1:1000) (Molecular Probes, Eugene, OR), primary LinA polyclonal antibody (1:2000) (Beijing Protein Institute Co. Ltd. Beijing, China) were diluted in TTBS buffer, respectively. The second antibody was an alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1:1500 (Zhongshan Biotechnology, Beijing, China). Reaction of alkaline phosphatase was developed by a solution containing 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP)/(Nitro blue tetrazolium chloride (NBT).

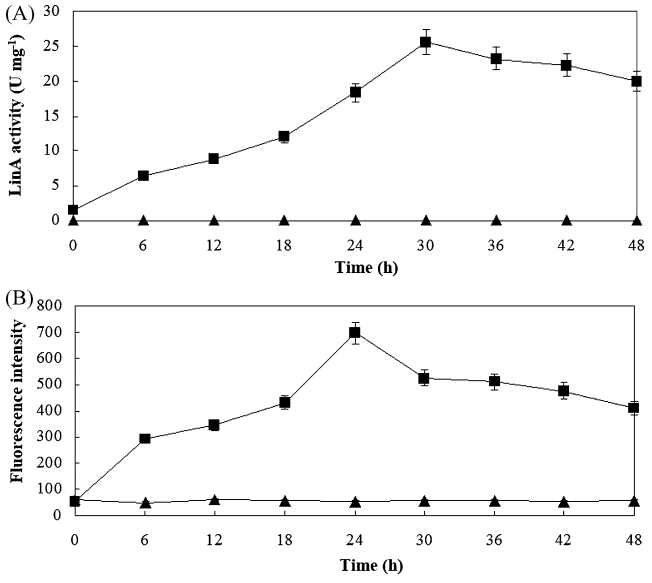

Fig. 2. Time course analysis of LinA activity (**A**) and fluorescence intensity (**B**) of the recombinant *Pseudomonas nitroreducens* J5-1 induced by IPTG for 48 h at 30 °C. (■, cell harboring pVAG33; ▲, cell harboring pVLT33). Error bars represent standard deviation from three independent experiments.

2.5. Determination of LinA activity and fluorescence intensity

Cell lysates of J5-1 harboring pVAG33 were prepared for analysis as above. LinA activity was assayed with γ-HCH as the substrate by the method of Nagata et al. (1993). Cell lysates harboring pVLT33 were used as background references. Cell lysates were 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 at 30 °C. One unit of LinA 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 (Iwasaki et al., 1952).

Whole-cells harboring pVAG33 were suspended in PBS buffer (pH 7.4) and diluted to OD_{600} = 1.0. The similarly diluted cells harboring pVLT33 were used as background references. GFP fluorescence intensity was determined using a fluorescence spectrophotometer (F-4500, HITACHI, Japan) with bandwidth of 5 nm, excitation wavelength of 488 nm and emission wavelength of 510 nm.

2.6. Biodegradation of γ-HCH by the recombinant Pseudomonas nitroreducens J5-1

The recombinant strain J5-1 harboring pVAG33 was precultured in 1/3 LB medium containing 50 μg ml^{-1} kanamycin at 30 °C and harvested during log phase. The cultures were centrifuged and the cell pellets were washed twice with fresh MSM medium used as inoculums. Then 10^6 cells ml^{-1} were used and inoculated into MSM supplemented with 0.1% glucose containing 10 μg ml^{-1} γ-HCH as the source of carbon and energy and 50 μg ml^{-1} of kanamycin. Cultures were maintained in 250 ml bottles at 30 °C on a shaker operated at 250 rpm. Appropriate controls containing medium plus γ-HCH were kept simultaneously. Cultures were run in triplicate to ensure accuracy.

Aliquots (1 ml) were taken out periodically and extracted twice with 4 ml of hexane. These extracts were dehydrated with sodium sulfate and were filtered (0.45 μm). The concentration of γ-HCH and 1,2,4-TCB was determined by GC-ECD analysis (Zhang et al., 2009). Under these conditions, the retention time of γ-HCH and 1,2,4-TCB was 4.70 min and 2.41 min, respectively. The metabolites of γ-HCH were identified by full-scan electron impact (EI) ionization of GC-MS analysis using an Agilent Technologies 6890N GC-5973N MSD (Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with a DB-5MS (95% polydimethyl siloxae 5% poly-1, 4-bis-dimethylsiloxae phenylene siloxae, 60 m × 0.25 mm ID, and film thickness 0.25 μm) capillary column and operated in splitless mode. The oven temperature was programmed as follows: hold

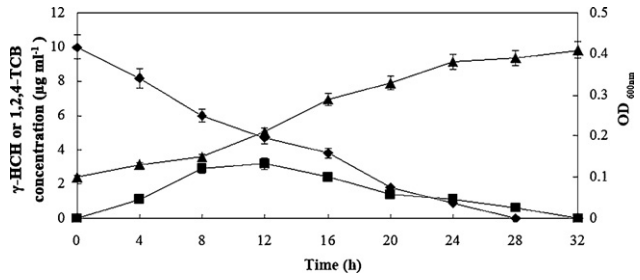

Fig. 3. Biodegradation of γ-HCH by the recombinant *Pseudomonas nitroreducens* J5-1 at an initial rate of 10^6 cells ml^{-1} in minimal salt medium supplemented with 0.1% glucose containing 10 μg ml^{-1} γ-HCH at 30 °C (♦, the concentration of γ-HCH; ■, the concentration of 1,2,4-TCB; ▲, $OD_{600\,nm}$). Bacterial growth was monitored by measuring OD at 600 nm. Error bars represent standard deviation from three independent experiments.

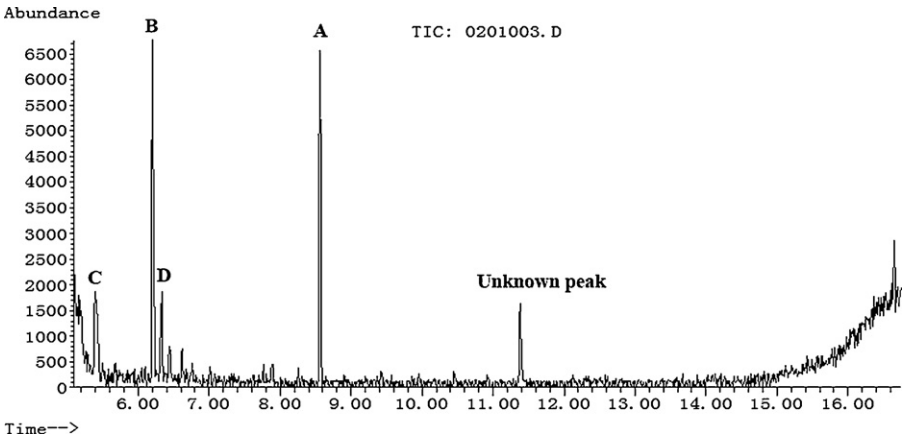

Fig. 4. GC-MS analysis of γ-HCH (A) and its metabolism products: γ-PCCH (B), 1,2,4-TCB (C) and 3,4,6-trichlorocatechol (D) by the recombinant *Pseudomonas nitroreducens* J5-1.

time at 100 °C, 5 min; ramp rate at 10 °C min^{-1} to 260 °C; hold time at 260 °C, 30 min. The temperatures corresponding to the transfer line and the ion trap were 280 °C and 220 °C, respectively, and the ionization energy was 70 eV. The injection volume was 1 μl via a splitless injection at 280 °C. Helium was used as a carrier at a flow rate of 1.0 ml min^{-1}.

3. Results

3.1. Confirmation of expression of LinA and enhanced green fluorescent protein in the recombinant J5-1

The *linA* and *egfp* genes were subcloned into a broad-host-range vector, pVLT33, to generate pVAG33. Western-blotting was performed to verify their synthesis using the rabbit anti-LinA antibody and rabbit anti-GFP antibody, respectively. The calculated molecular weights of LinA and EGFP are 16.5 and 27 kDa, respectively. As expected, two bands of the recombinant protein were detected in cell lysates carrying pVAG33, which matches well with that calculated from the protein sequences (Fig. 1, lanes 2 and 4). However, no signal was detected with the control cells carrying pVLT33 (Fig. 1, lanes 3 and 5). From the results, we concluded that recombinant LinA and EGFP were indeed expressed in the recombinant *P. nitroreducens* J5-1.

3.2. Determination of LinA and fluorescence intensity

Time-course studies of LinA activity and fluorescence intensity in J5-1 harboring pVAG33 were carried out at 30 °C at 6 h intervals and the corresponding enzyme activities and fluorescence intensities were determined, respectively. J5-1 cells harboring pVLT33 was used as background references. The LinA activity and fluorescence intensity could be detected at low level prior to induction as a result of *lac* promoter that weakly constitutively expressed. As shown in Fig. 2, the activity and fluorescence in the J5-1 cell lysate harboring pVAG33 increased gradually after induction with 1 mM IPTG and reached the maximum at 30 h and 24 h, respectively. This indicated both LinA and EGFP retained their activities under the control of *lac* promoter in the recombinant strain.

3.3. Biodegradation of γ-HCH by the recombinant J5-1

The recombinant J5-1 harboring pVAG33 was tested for its ability to degrade γ-HCH at 10 μg ml^{-1} in MSM containing 0.1% glucose and 50 μg ml^{-1} kanamycin. As shown in Fig. 3, 10 μg ml^{-1} γ-HCH could be rapidly degraded to an undetectable level within 28 h and the degradation was accompanied by bacterial growth ($OD_{600\,nm}$). Meanwhile, 1,2,4-TCB was produced continuously and accumulated during γ-HCH degradation. The concentration of

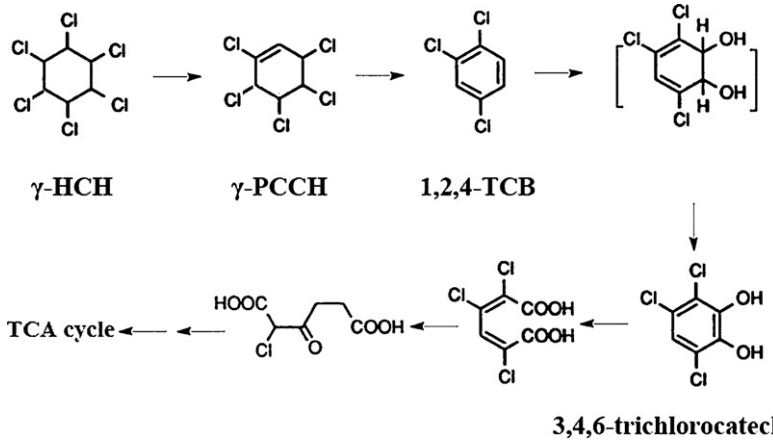

Fig. 5. Proposed pathway for the mineralization of γ-HCH by the recombinant *Pseudomonas nitroreducens* J5-1.

1,2,4-TCB reached the maximum at 12 h, which was subsequently degraded over the remainder of the experiment by the recombinant J5-1. Note that when γ-HCH was completely degraded at 28 h, 0.9 μg ml^{-1} 1,2,4-TCB still remained in the culture and was then completely degraded at 32 h.

The metabolites of γ-HCH degradation by the recombinant J5-1 were further analyzed by GC-MS analysis. The mass spectra were compared with respective mass spectra of authentic compounds, and also with the mass profile of the same compound available in the National Institute of Standard Technology (NIST) library, USA. As shown in Fig. 4, γ-pentachlorocyclohexene (γ-PCCH) and 1,2,4-TCB were detected in the culture, which were produced via dehydrochlorination reactions by enzymatic activities of LinA. Also, 3,4,6-trichlorocatechol could be detected, which was the main product from 1,2,4-TCB by the enzymatic activities of dioxygenase and dehydrogenase in J5-1 (Jiang et al., 2008). Therefore, our results demonstrated that γ-HCH could be completely mineralized via γ-HCH → γ-PCCH → 1,2,4-TCB → 3,4,6-trichlorocatechol→→ TCA cycle (Fig. 5). The novel pathway supports the conclusion that the process of mineralization of γ-HCH is initiated by dehydrochlorination leading to the generation of γ-PCCH and 1,2,4-TCB, and the latter degradation, then proceeds through formation of 3,4,6-trichlorocatechol, which is mineralized step by step by J5-1. However, further studies should be carried out to find whether there are other degradation ways by the recombinant J5-1 as there was one primary unexpected peak in Fig. 4.

4. Discussion

In the present study, the environmentally robust bacterium *P. nitroreducens* J5-1 with the capacity of rapidly degrading 1,2,4-TCB, 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 an RSF1010 derivative and therefore able to replicate in a wide variety of Gram-negative bacteria (de Lorenzo et al., 1993). The pVLT33-based vectors have been successfully used to express several proteins in various Gram-negative bacteria, such as *Moraxella* sp (Shimazu et al., 2001) and *Pseudomonas putida* JS444 (Lei et al., 2005). In this study, the middle-copy number plasmid pVAG33, derivative of pVLT33, was used for functional expression of LinA and EGFP in the recombinant J5-1 as determined by Western-blot analysis. Expression of *linA* and *egfp* in the recombinant J5-1 was under the control of relaxed-regulated *lac* promoter, respectively, which was weakly constitutively active even without IPTG or lactose induction. Overexpression of LinA and EGFP was obtained in the recombinant J5-1 by determining its enzymatic activities and fluorescence intensity. The results also demonstrated that the growth curves and degrading characteristics of the recombinant J5-1 were similar to those of the wild-type J5-1, showing that expression of LinA and EGFP in the recombinant strain did not affect cell viability and original degrading characteristics (data not shown).

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. Dehydrochlorinase LinA requires no cofactor for maintaining its activity, suggesting that LinA may be more suitable for field scale remediation. However, the dead-end product 1,2,4-TCB was produced by enzymatic activities of LinA and cannot be further degraded (Nagasawa et al., 1993). It will gradually accumulate and inhibit degradation of HCH, which becomes a rate-limiting step in the HCH biodegradation pathway (Nagata et al., 1999). In our study, 1,2,4-TCB was produced from the degradation of γ-HCH and then could be rapidly mineralized by the recombinant J5-1, which resulted in a novel and efficient γ-HCH mineralization pathway different from that of natural HCH-degrading bacteria. Besides, since LinA is also responsible for the initial dechlorination in the upstream biodegradation pathway of α- and δ-HCH (Nagata et al., 1999; Wu et al., 2007), the strain has a potential to degrade these two isomers.

GFP has been fused as a cell marker for expression of organophosphorus hydrolase (OPH) and methyl parathion hydrolase (MPH) in *E. coli* (Wu et al., 2000; Yang et al., 2008). However, in our previous study, we found that the fluorescence intensity and enzyme activity of LinA-EGFP fusion protein decreased significantly, which might be caused by changes of their active sites after being fused. In this work, since the expression of LinA and EGFP was under the control of the *lac* promoter respectively, both of them retained their activities in the recombinant bacterium. Also, time-course studies of LinA activities and fluorescence intensities showed they have similar variation trends, although they reached the maximum at 30 h and 24 h, respectively (Fig. 2). Therefore, our design provides a proper balance between the cell biocatalytic efficiency and on-line monitoring. EGFP could be utilized as a real-time monitoring tool in a bioreactor or future field-scale remediation.

Since HCH is a recalcitrant pollutant and some countries are presently using γ-HCH, there are large amounts of HCH remaining at the places where they were produced or used. Moreover, the majority of remaining sources have cited typical application rates of γ-HCH, from the 1960's to present, that range from 1 to 15 mg kg^{-1} soil in field (Bharati et al., 1998; Waliszewski, 1993). Bioremediation is a more efficient and environmental friendly method for detoxification of HCH residues compared to physical and chemical methods (Phillips et al., 2005). The laboratory strains such as *E. coli* are generally not suitable for *in situ* remediation since they are not adapted to these environments. Therefore, more effective and competitive strains, that are well-adapted to the fluctuating environmental conditions and competition from indigenous microbial populations, are required for *in situ* bioremediation of contaminated sites. In the present study, the soil-isolated *P. nitroreducens* J5-1 was successfully endowed with the capability of rapid mineralization of γ-HCH as a result of functional expression of dehydrochlorinase and the recombinant strain could rapidly degrade 10 μg ml^{-1} γ-HCH in 28 h. The strain may possess enormous potential to be utilized for the bioremediation of highly HCH-contaminated soil in industrial post-production places and waste dumping sites. Meanwhile, it could also potentially be applied in agriculture fields and water to reduce HCH residues though the concentration of HCH was lower in these places. Our present work provided a good basis for the application of the engineered strain for rapid biodegradation of γ-HCH and chlorobenzene residues in a bioreactor or in the environment and the strain could be easily monitored by fluorescence of EGFP for its activity and fate.

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 863 Hi-Tech Research and Development Program of the People's Republic of China (No. 2007AA06Z335) and the Innovation Program of the Chinese Academy of Sciences (No. KSCX2-YW-G-008).

References

Bharati, K., Padhy, S., Adhya, T.K., 1998. Accelerated biodegradation of γ-hexachlorocyclohexane (γ-HCH) in a flooded alluvial soil retreated with γ-HCH or its metabolite 1,2,4-trichlorobenzene (TCB). Bull. Environ. Contam. Toxicol. 60, 858–863.

Chalfie, M., Tu, Y., Euskirchen, G.W., Ward, W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263, 802–805.

Cormack, B.P., Valdivia, R.H., Falkow, S., 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173, 33–38.
den Besten, C., Brouwer, A., Rietjens, I.M.C.M., van Bladeren, P.J., 1994. Biotransformation and toxicity of halogenated benzenes. Hum. Exp. Toxicol. 13, 866–875.
de Lorenzo, V., Eltis, L., Kessler, B., Timmis, K.N., 1993. Analysis of *Pseudomonas* gene products using *lacIq*/*Pirp*-*lac* plasmids and transposons that confer conditional phenotypes. Gene 123, 17–24.
Iwasaki, I., Utsumi, S., Ozawa, T., 1952. New colorimetric determination of chloride using mercuric thiocyanate and ferric ion. Bull. Chem. Soc. Jpn. 25, 226.
Iwata, H., Tanabe, S., Sakai, N., Tatsukawa, R., 1993. Distribution of persistent organochlorines in the oceanic air and surface seawater and the role of ocean on their global transport and fate. Environ. Sci. Technol. 27, 1080–1098.
Janssen, D.B., 2004. Evolving haloalkane dehalogenases. Curr. Opin. Chem. Biol. 8, 150–159.
Jiang, J., Wang, H., Gao, J.S., Song, L., Ning, D.L., 2008. Cloning and sequence analysis of 1,2,4-trichlorobenzene dioxygenase and dehydrogenase genes. Huan Jing Ke Xue 29, 1655–1659.
Larrainzar, E., O'Gara, F., Morrissey, J.P., 2005. Applications of autofluorescent proteins for *in situ* studies in microbial ecology. Annu. Rev. Microbiol. 59, 257–277.
Lei, Y., Mulchandani, A., Chen, W., 2005. Improved degradation of organophosphorus nerve agents and *p*-nitrophenol by *Pseudomonas putida* JS444 with surface-expressed organophosphorus hydrolase. Biotechnol. Prog. 21, 678–681.
Li, Y.F., Scholtz, M.T., Van Heyst, B.J., 2003. Global gridded emission inventories of beta-hexachlorocyclohexane. Environ. Sci. Technol. 37, 3493–3498.
March, J.C., Rao, G., Bentley, W.E., 2003. Biotechnological applications of green fluorescent protein. Appl. Microbiol. Biotechnol. 62, 303–315.
Nagasawa, S., Kikuchi, R., Nagata, Y., Takagi, M., Matsuo, M., 1993. Aerobic mineralization of γ-HCH by *Pseudomonas paucimobilis* UT26. Chemosphere 26, 1719–1728.
Nagata, Y., Hatta, T., Imai, R., Kimbara, K., Fukuda, M., Yano, K., et al., 1993. Purification and characterization of γ-hexachlorocyclohexane (γ-HCH) dehydrochlorinase (LinA) from *Pseudomonas paucimobilis*. Biosci. Biotechnol. Biochem. 57, 1582–1583.
Nagata, Y., Miyauchi, K., Takagi, M., 1999. Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. J. Ind. Microbiol. Biotechnol. 23, 380–390.
Nawab, A., Aleem, A., Malik, A., 2003. Determination of organochlorine pesticides in agricultural soil with special reference to γ-HCH degradation by *Pseudomonas* strains. Bioresour. Technol. 88, 41–46.
Phillips, T.M., Seech, A.G., Lee, H., Trevors, J.T., 2005. Biodegradation of hexachlorocyclohexane (HCH) by microorganisms. Biodegradation 16, 363–392.
Sambrook, J., Fritsch, E.F., Maniatis, T., 2001. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York.
Shimazu, M., Mulchandani, A., Chen, W., 2001. Simultaneous degradation of organophosphorus pesticides and *p*-nitrophenol by a genetically engineered *Moraxella* sp. with surface-expressed organophosphorus hydrolase. Biotechnol. Bioeng. 76, 318–324.
Song, L., Wang, H., Jiang, J., Gao, J.S., Shi, H.C., 2007. Isolation, identification of 1,2,4-trichlorobenzene-degrading strain *Pseudomonas nitroreducens* J5-1 and cloning of chlorocatechol 1,2-dioxygenase gene. Huan Jing Ke Xue 28, 1878–1881.
Trantirek, L., Hynkova, K., Nagata, Y., Murzin, A., Ansorgova, A., Sklenar, V., et al., 2001. Reaction mechanism and stereochemistry of γ-hexachlorocyclohexane dehydrochlorinase LinA. J. Biol. Chem. 276, 7734–7740.
Waliszewski, S.M., 1993. Residues of lindane, HCH isomers and HCB in the soil after lindane application. Environ. Poll. 82, 289–293.
Wu, C.F., Cha, H.J., Rao, G., Valdes, J.J., Bentley, W.E., 2000. A green fluorescent protein fusion strategy for monitoring the expression, cellular location, and separation of biologically active organophosphorus hydrolase. Appl. Microbiol. Biotechnol. 54, 78–83.
Wu, J., Hong, Q., Sun, Y., Hong, Y., Yan, Q., Li, S., 2007. Analysis of the role of LinA and LinB in biodegradation of δ-hexachlorocyclohexane. Environ. Microbiol. 9, 2331–2340.
Yang, C., Zhao, Q., Liu, Z., Li, Q., Qiao, C., Mulchandani, A., et al., 2008. Cell surface display of functional macromolecule fusions on *Escherichia coli* for development of an autofluorescent whole-cell biocatalyst. Environ. Sci. Technol. 42, 6105–6110.
Zhang, H., Yang, C., Zhao, Q., Qiao, C., 2009. Development of an autofluorescent organophosphates-degrading *Stenotrophomonas* sp. with dehalogenase activity for the biodegradation of hexachlorocyclohexane (HCH). Bioresour. Technol. 100, 3199–3204.