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Characterization of a Newly Isolated *cis*-1,2-Dichloroethylene and Aliphatic Compound-Degrading Bacterium, *Clostridium* sp. Strain KYT-1

Eun-Sook Kim[1], Izumi Nomura[2], Yuki Hasegawa[2], and Kazuhiro Takamizawa[1,2]*

[1] United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[2] Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

Abstract A *cis*-1,2-dichloroethylene (*cis*-DCE)-degrading anaerobic bacterium, *Clostridium* sp. strain KYT-1, was isolated from a sediment sample collected from a landfill site in Nanji-do, Seoul, Korea. The KYT-1 strain is a gram-positive, endospore-forming, motile, rod-shaped anaerobic bacterium, of approximately 2.5~3.0 μm in length. The degradation of *cis*-DCE is closely related with the growth of the KYT-1 strain, and it was stopped when the growth of the KYT-1 strain became constant. Although the pathway of *cis*-DCE degradation by strain KYT-1 remains to be further elucidated, no accumulation of the harmful intermediate, vinyl chloride (VC), was observed during anaerobic *cis*-DCE degradation. Strain KYT-1 proved able to degrade a variety of volatile organic compounds, including VC, isomers of DCE (1,1-dichloroethylene, *trans*-1,2-dichloroethylene, and *cis*-DCE), trichloroethylene, tetrachloroethylene, 1,2-dichloroethane, 1,1,1-trichloroethane, and 1,1,2-trichloroethane. Strain KYT-1 degraded *cis*-DCE at a range of temperatures from 15 to 37°C, with an optimum at 30°C, and at a pH range of 5.5 to 8.5, with an optimum at 7.0.

Keywords: *cis*-1,2-dichloroethylene, anaerobic degradation, halogenated aliphatic compounds, strain KYT-1

Volatile organic compounds including tetrachloroethylene (PCE) and trichloroethylene (TCE) are the most frequently detected organic pollutants in the environment. They are well known for their excellent solubilities, and are used extensively in various fields including the manufacture of semiconductors, metallic instrument processing, and in dry cleaning as cleaning agents or solvents. However, leakage from machines or inappropriate handling procedures, careless disposal of used solvents, and the exposure of these solvents to subsurface water has resulted in serious problems in both soil and subsurface waters. The regular method for the treatment of pollution caused by such organic compounds is to draw out the subsurface water, and subject it to aeration followed by adsorption with active carbon. However, such chemical and physical treatment methods cannot be applied to all polluted areas [1,2]. Under such circumstances, biological treatment methods (bioremediation) can prove a useful soil pollution countermeasure technology. In *in situ* bioremediation, in which microorganisms are introduced as a treatment, information regarding the microorganism's characteristics and environmental conditions are vitally important [3]. Currently, a great deal of research has been conducted regarding microorganisms that degrade chlorinated compounds such as PCE and TCE. However, the majority of the organisms have been determined to generate *cis*-DCE (*cis*-1,2-dichloroethylene) and VC (vinyl chloride) as by-products of PCE and TCE [4-8]. The accumulation of *cis*-DCE and VC in the environment is considered rather undesirable, as *cis*-DCE is a suspected carcinogen, whereas VC is the most toxic compound. *Dehalococcoides* sp. strain BAV1 is the only isolate known to respire all DCE isomers and VC [9]. Only a few organisms including *Dehalococcoides ethenogens* 195 are able to completely dechlorinate PCE and TCE [10-12]. Thus, this research has spurred interest in the discovery and identification of an organism which can degrade *cis*-DCE under anaerobic conditions, and has also generated interest in the degradation characteristics and mechanism pathways relevant to this organism.

Anaerobic mixed cultures evidencing *cis*-DCE-degradation abilities were obtained from a landfill site in Nanji-do, Seoul, Korea. Degradation experiments under different culture conditions, and in particular, under a variety of electron-accepting conditions [13-15], were conducted in order to gain insight into the microorganism compositions in the mixed culture, and to determine the appropriate electron-accepting conditions. The total number of microorganisms in the environmental sample was directly determined via fluorescence microscopy, in

***Corresponding author**
Tel: +81-58-293-2906 Fax: +81-58-293-2906
e-mail: tak2003@gifu-u.ac.jp

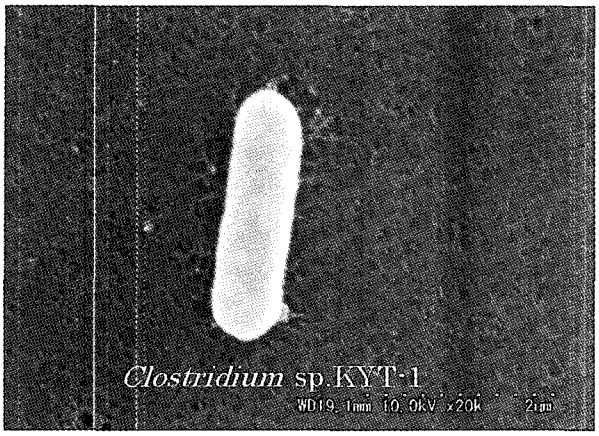

Fig. 1. Scanning electron micrograph of strain KYT-1.

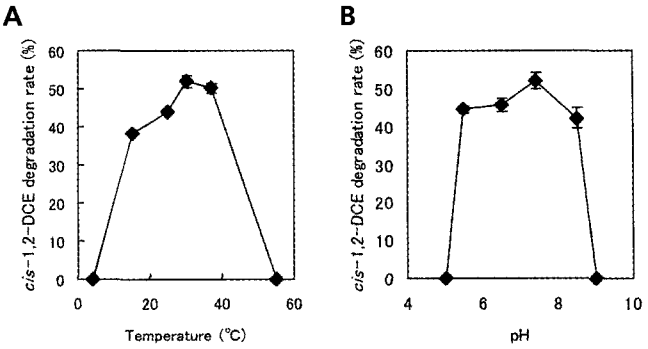

Fig. 2. Effect of (A) temperature and (B) pH on *cis*-DCE degradation. Percentage of degradation compared with controls (without cells) was calculated. Initial concentration of *cis*-DCE was 51.6 μM. The temperature-dependence determinations were conducted at pH 7.2, and pH-dependence was determined at 30°C. Cultivation was performed for 7 days. The results are expressed as the means of duplicate experiments.

accordance with the methods of Hobbie *et al.* [16]. Methanogens [14], sulfate reducers [15], and Fe (III) reducers [13,17] were counted via the most probable (MPN) method. The mixed culture contained (per milliliter) 6.8×10^5 of microbial cells, which contained Fe (III)-reducers (in MPN 1.3×10^4), methanogens (9.3×10^3), and sulfate reducers (2.3). The highest rate of *cis*-DCE degradation was observed under Fe (III)-reducing conditions (data not shown). In order to maintain the degrading activity of the culture via weekly subculture, a Fe (III)-reducing culture medium was employed. The *cis*-DCE-degrading bacterium was then isolated using Fe (III)-reducing culture medium and agar (plate method). The agar medium contained the following (per liter): K_2HPO_4, 3.0 g; KH_2PO_4, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; L-asparagine, 5 g; D-glucose, 10 g; ferric citrate, 0.6 g; *p*-aminobenzoic acid, 0.01 g; biotin, 0.01 mg; agar, 20 g at pH 7.2. Nine milliliters of the medium were then autoclaved in 26 mL-serum bottles, followed by the addition of 0.1 mL of filter-sterilized vitamin solution (0.01 g of *p*-aminobenzoic acid and 0.01 mg of biotin per liter), 0.3 mL of ferric citrate (0.6 g/L), 0.5 mL of glucose (10 g/L), and 0.1 mL of enrichment culture. Prior to the addition of the vitamin, ferric citrate, glucose, and enrichment culture, the headspaces of the vial were aseptically flushed with pure nitrogen, and sealed with Teflon-lined rubber septa and aluminum crimp caps. The KYT-1 strain was identified as a gram-positive, endospore-forming, motile bacterium, 2.5 to 3.0 μm in length, with no flagella (Fig. 1). During the 7 days of incubation, at lower temperatures and pH, the growth and degradation activity of strain KYT-1 were lower than was observed under optimum conditions, but strain KYT-1 proved able to degrade *cis*-DCE in a range of temperature from 15 to 37°C, with an optimum occurring at 30°C, and at a pH range of 5.5 to 8.5, with an optimum occurring at 7.2 (Fig. 2).

The 16S rRNA gene of strain KYT-1 was amplified by PCR using 27F and 1525R primers and then sequenced. A DNA sequencer was employed in order to determine the 1,421 bp nucleotide sequence. A BLAST search was applied to the sequence results to determine homology, and revealed a 99% nucleotide sequence homology with *Clostridium beijerinkii*, *Clostridium acetobutylicum*, and *Clostridium diolis*. Also, CLUSTAL W analysis on the obtained nucleotide sequence revealed the closest relationship with *Clostridium butyricum*.

In order to characterize the degradation of *cis*-DCE by strain KYT-1, 10 mL of culture medium was placed into 26-mL vials, and cultured under anaerobic and Fe (III)-reducing conditions at 30°C. Halogenated aliphatic compounds and *cis*-DCE in the anaerobic cultures were quantified via the injection of 250 μL of headspace gas into a gas chromatograph (GC-17A; Shimadzu Co., Japan), equipped with a capillary column, VOCOL (0.25 mm × 30 m; Supelco Inc., USA) and a flame ionization detector (FID). The column temperature was maintained for 2 min at 35°C, and then raised to 180°C at a rate of 4°C/min. The injector and detector temperatures were maintained at 100 and 160°C, respectively. The chlorine ion concentration in the culture liquid after *cis*-DCE degradation was assessed via the colorimetric method of Bergman and Sanik [18]. Bacterial growth was monitored via optical density readings at a wavelength of 660 nm (OD_{660}), using a spectrophotometer (UV-1600; Shimadzu).

The growth of strain KYT-1 began after 12 h of incubation, and growth achieved a steady state after 36 h of incubation (Fig. 3). The growth of strain KYT without *cis*-DCE was similar to that observed in the sample with *cis*-DCE (data not shown). The growth and degradation of *cis*-DCE increased gradually after 12 h of incubation, and when the growth of strain KYT-1 stopped, degradation also ceased. From this result, it can be concluded that the degradation of *cis*-DCE is closely related with the growth of strain KYT-1. During the degradation of *cis*-DCE, carbon dioxide and small amounts of hydrogen were produced, but toxic VC and ethylene which is a general innocuous byproduct of the reductive dechlorination of PCE, were not detected. VC and ethylene never accumulated as intermediates of the anaerobic degrada-

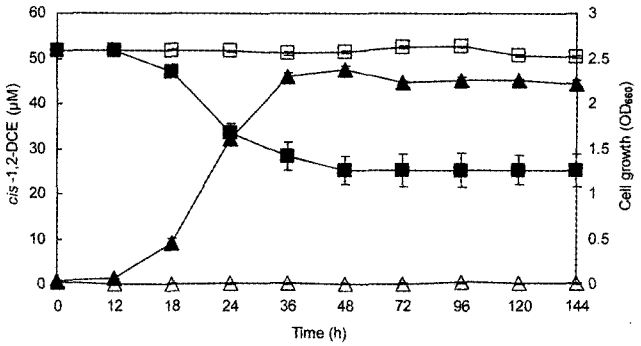

Fig. 3. Time course of *cis*-DCE degradation and growth profiles in the medium using strain KYT-1. Results are expressed as the means ± SD of triplicate experiments. Symbols: □, *cis*-DCE (without cells); ■, *cis*-DCE; △, OD_{660} (without cells); ▲, OD_{660}.

Table 1. Degradation of aliphatic compounds by *Clostridium* sp. KYT-1

Halogenated aliphatic compounds	Degradation (%)
Vinyl chloride	36.9 ± 2.2
1,1-Dichloroethylene	44.8 ± 3.1
trans-1,2-Dichloroethylene	59.9 ± 1.2
cis-1,2-Dichloroethylene	52.1 ± 3.1
Trichloroethylene	58.5 ± 1.9
Tetrachloroethylene	66.1 ± 1.2
1,2-Dichloroethane	11.6 ± 2.1
1,1,1-Trichloroethane	41.8 ± 6.5
1,1,2-Trichloroethane	21.2 ± 3.4

Percent degradation compared with controls without cells. Initial concentration of each compound was 5 mg/L. Data are means ± SD for triplicate experiments.

tion of DCE and VC, and they were readily oxidized to carbon dioxide [19-22]. In these experiments, substrates labeled with ^{14}C were employed under anaerobic conditions, including Fe (III)-reduction, sulfate-reduction, and methane generation conditions. ^{14}C-VC and a portion of the ^{14}C-DCE were converted to the inorganic compound, $^{14}CO_2$. It has been also reported that an organic compound, such as humic acid, might be utilized as an electron acceptor for the oxidation of VC and DCE. Thus, intermediate products such as VC and ethylene do not accumulate [19-22]. As is shown in Table 1, strain KYT-1 effects the degradation of PCE, and it was difficult to obtain ^{14}C-labeled DCE, ^{14}C-labeled PCE was utilized for radioisotope experiments. The origin of carbon in CO_2 gas, from PCE or from the materials used in the medium, was our primary concern. The generated $^{14}CO_2$ was then absorbed using CO_2 absorption liquid (Carbo Sorb E, Perkin Elmer, USA) after incubation with PCE [1,2-^{14}C] (American Radiolabeled Chemicals Inc.), and the collected $^{14}CO_2$ was analyzed via liquid scintillation counting. As a result, the RI activity was not observed in the CO_2 absorption liquid. Thus, it was determined that PCE [1,2-^{14}C] was not converted into $^{14}CO_2$. This indicates that strain KYT-1 is not capable of metabolizing PCE into CO_2, and the metabolic reaction is halted in the state of some organic compound forms, with the exceptions of TCE, DCEs, and VC.

In general, the microorganism-mediated dechlorination of chlorinated organic compounds can be verified via measurements of the chlorine ion. In this study, only a small amount of chloride ions was observed during the *cis*-DCE degradation effected by strain KYT-1 (dechlorination rate 1.3 ± 0.7%). Therefore, it appears that the unknown chlorinated organic compounds may represent a metabolic product. From the literature survey, chlorinated epoxy ethane, ethanol, acetaldehyde, and acetic acid have been implicated as aerobic metabolism products [23,24]. Strain KYT-1 was found to be capable of partially metabolizing *cis*-DCE, but approximately 50% of the *cis*-DCE was not degraded. It is possible that the degradation was halted via the accumulation of intermediate metabolites.

However, it has also been verified that strain KYT-1 evidences the ability to degrade a variety of halogenated aliphatic compounds, including VC, isomers of DCE (1,1-dichloroethylene, *trans*-1,2-dichloroethylene, *cis*-DCE), TCE, PCE, 1,2-dichloroethane, 1,1,1-trichloroe- thane, and 1,1,2-trichloroethane. The percentages of these compounds were determined to be 37, 45, 60, 52, 59, 66, 12, 42, and 21%, respectively (Table 1). *In situ* contamination frequently involves a mixture of complex chemicals, *i.e.*, halogenated aliphatic compounds harboring *cis*-DCE [25]. Therefore, it would clearly be beneficial to determine the conditions allowing for the simultaneous degradation of several halogenated aliphatic compounds.

Clostridium sp. KYT-1 degrades *cis*-DCE under anaerobic conditions. A similar result was observed using *Clostridium* sp. strain DC1, which was isolated from the same sludge at the waste landfill site [26]. However, the classification of the strain is different. In the case of strain DC-1, *cis*-DCE degradation occurs after full growth. Hata *et al.* reported that other *Clostridium* species, such as *C. butyricum* and *C. acetobutylicum*, also evidenced the ability to degrade *cis*-DCE without any accumulation of VC and ethylene [26]. Although the *cis*-DCE degradation pathway exploited by strain KYT-1 remains uncertain, the advantages of strain KYT-1 with regard to *cis*-DCE degradation are as follows: (1) the absence of accumulation of the harmful intermediate VC during anaerobic *cis*-DCE degradation, (2) the ability to degrade a variety of volatile organic compounds, and (3) the ability to degrade *cis*-DCE even at temperatures as low as 15°C (in nature, a ground water temperature condition of 15°C prevails).

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REFERENCES

[1] Vogel, T. M., C. S. Criddle, and P. L. McCarty (1987) Transformations of halogenated aliphatic compounds. *Environ. Sci. Technol.* 21: 722-736.

[2] Quinton, G. E., R. J. Buchanan, D. E. Ellis, Jr., and S. H. Shoemaker (1997) A method to compare groundwater cleanup technologies. *Remediation* 8: 7-16.

[3] Humayra, A. S., Y. Hasegawa, I. Nomura, Y. C. Chang, T. Sato, and K. Takamizawa (2005) Evaluation of different culture conditions of *Clostridium bifermentans* DPH-1 for cost effective PCE degradation. *Biotechnol. Bioprocess Eng.* 10: 40-46.

[4] Scholz-Muramatsu, H., A. Neumann, M. Messmer, E. Moore, and G. Diekert (1995) Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch. Microbiol.* 163: 48-56.

[5] Gerritse, J., V. Renard, T. M. Pedro Gomes, P. A. Lawson, M. D. Collins, and J. C. Gottschal (1996) *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. *Arch. Microbiol.* 165: 132-140.

[6] Holliger, C., D. Hahn, H. Harmsen, W. Ludwig, W. Schumacher, B. Tindall, F. Vazquez, N. Weiss, and A. J. B. Zehnder (1998) *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch. Microbiol.* 169: 313-321.

[7] Chang, Y. C., M. Hatsu, K. Jung, Y. S. Yoo, and K. Takamizawa (2000) Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, *Clostridium bifermentans* DPH-1. *J. Biosci. Bioeng.* 89: 489-491.

[8] Suyama, A., R. Iwakiri, K. Kai, T. Tokunaga, N. Sera, and K. Furukawa (2001) Isolation and characterization of *Desulfitobacterium* sp. strain Y51 capable of efficient dehalogenation of tetrachloroethene and polychloroethanes. *Biosci. Biotechnol. Biochem.* 65: 1474-1481.

[9] He, J., K. M. Ritalahti, K. L. Yang, S. S. Koenigsberg, and F. E. Löffler (2003) Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature* 424: 62-65.

[10] Maymo-Gatell, X., Y. Chien, J. M. Gossett, and S. H. Zinder (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethane. *Science* 276: 1568-1571.

[11] Müller, J. A., B. M. Rosner, G. Von Abendroth, G. Meshulam-Simon, P. L. McCarty, and A. M. Spormann (2004) Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. *Appl. Environ. Microbiol.* 70: 4880-4888.

[12] Sung, Y., K. M. Ritalahti, R. P. Apkarian, and F. E. Löffler (2006) Quantitative PCR confirms purity of Strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. *Appl. Environ. Microbiol.* 72: 1980-1987.

[13] Ottow, J. C. (1968) Evaluation of iron-reducing bacteria in soil and the physiological mechanism of iron-reduction in *Aerobacter aerogenes*. *Z. Allg. Mikrobiol.* 8: 441-443.

[14] Zeikus, J. G. (1977) The biology of methanogenic bacteria. *Bacteriol. Rev.* 41: 514-541.

[15] Pfenning, N., F. Widdel, and H. G. Trüper (1992) The dissimilatory sulfate-reducing bacteria. pp. 926-940. In: A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.). *The Prokaryotes.* 2nd ed., Vol. 1. Springer-Verlag, New York, NY, USA.

[16] Hobbie, J. E., R. J. Daley, and S. Jasper (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33: 1225-1228.

[17] Bromfield, S. M. (1954) The reduction of iron oxide by bacteria. *J. Soil Sci.* 5: 129-139.

[18] Bergmann, J. G. and J. Sanik (1957) Determination of trace amounts of chlorine in naphtha. *Anal. Chem.* 29: 241-243.

[19] Bradley, P. M. and F. H. Chapelle (1996) Anaerobic mineralization of vinyl chloride in Fe(III)-reducing, aquifer sediments. *Environ. Sci. Technol.* 30: 2084-2086.

[20] Bradley, P. M. and F. H. Chapelle (1998) Microbial mineralization of VC and DCE under different terminal electron accepting conditions. *Anaerobe* 4: 81-87.

[21] Bradley, P. M., F. H. Chapelle, and D. R. Lovley (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* 64: 3102-3105.

[22] Bradley, P. M. and F. H. Chapelle (1997) Kinetics of DCE and VC mineralization under methanogenic and Fe(III)-reducing conditions. *Environ. Sci. Technol.* 31: 2692-2696.

[23] Van Hylckama Vlieg, J. E. T., W. de Koning, and D. B. Janssen (1996) Transformation kinetics of chlorinated ethenes by *Methylosinus trichosporium* OB3b and detection of unstable epoxides by on-line gas chromatography. *Appl. Environ. Microbiol.* 62: 3304-3312.

[24] Hashimoto, A., K. Iwasaki, N. Nakasugi, M. Nakajima, and O. Yagi (2002) Degradation pathways of trichloroethylene and 1,1,1-trichloroethane by *Mycobacterium* sp. TA27. *Biosci. Biotechnol. Biochem.* 66: 385-390.

[25] Verschueren, K. (1983) *Handbook of Environmental Data on Organic Materials.* 2nd ed., Van Nostrand Reinhold Co., New York, NY, USA.

[26] Hata, J., N. Miyata, E. S. Kim, K. Takamizawa, and K. Iwahori (2004) Anaerobic degradation of *cis*-1,2-dichloroethylene and vinyl chloride by *Clostridium* sp. strain DC1 isolated from landfill leachate sediment. *J. Biosci. Bioeng.* 97: 196-201.

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