Journal of Hazardous Materials 167 (2009) 124–127

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Biodegradation of thiodiglycol, a hydrolyzate of the chemical weapon Yperite, by benzothiophene-desulfurizing bacteria

Leila El Bassi [a,b], Naoya Shinzato [a], Tomoyuki Namihira [a], Hirosuke Oku [a], Toru Matsui [a,*]

[a] Center of Molecular Biosciences, University of the Ryukyus, 1 Sembaru, Nishihara-cho, Okinawa 903-0213, Japan
[b] United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima, Japan

ARTICLE INFO

Article history:
Received 2 October 2008
Received in revised form 9 December 2008
Accepted 18 December 2008
Available online 30 December 2008

Keywords:
Benzothiophene
Desulfurization
Thiodiglycol
Nocardioform
Yperite

ABSTRACT

Microbial degradation of thiodiglycol (bis(2-hydroxyethyl)sulfide, TDG) with petroleum-desulfurizing soil bacteria was examined. Among the bacteria tested, several strains belonging to the genera *Rhodococcus* and *Gordonia* grew on TDG as the sole sulfur source. The selected strain *Rhodococcus* sp. strain T09, which was re-identified as *R. jostii*, showed TDG degradation activity only when grown in the presence of TDG as the sole sulfur source. Repeat batch degradation of TDG by using strain T09 could be continued for over 50 h, with a slight loss of activity.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Yperite, a chemical weapon known as sulfur mustard, must be destroyed under the provisions of the Chemical Weapons Convention (CWC); hence, several treatment processes for the destruction of sulfur mustard have been investigated. In an aqueous solution, sulfur mustard spontaneously hydrolyzes and generates thiodiglycol (bis(2-hydroxyethyl)sulfide, TDG) [1,26]. Thus, TDG as a hydrolyzate of sulfur mustard will accumulate in soil and remain in nature for long periods. TDG is included in the Schedule List of the CWC, and it is commercially used in textile dyeing and in printing solvents. There are many extensive reports on microbial TDG degradation by the gram-negative bacterium *Alcaligenes* sp., which utilizes TDG as the sole carbon and energy source [4–6,13,14,21]. Bioconversion/biodegradation of TDG by yeasts and basidiomycetes was also reported [7,9]. However, there is very little information on the degradation of TDG by gram-positive bacteria.

TDG is one of the aliphatic organosulfur compounds with a thioether bond. We previously reported that benzothiophene (BT)-desulfurizing gram-positive actinomycetes utilize not only thiophenic compounds but also noncondensed organosulfur compounds such as dimethyl sulfide, dimethyl sulfone, and dimethyl sulfate [16,20]. These results prompted us to further screen the TDG-degrading bacteria from our collection of BT-desulfurizing bacteria.

In addition to the treatment of abandoned chemical weapons such as Yperite, it is essential to study the fate of such compounds that pollute the surrounding environment. Nocardioforms such as *Rhodococcus* and *Gordonia* are frequently isolated from soil and have been shown to exhibit a wide range of degradative and/or oxidative functions, including hydroxylation, sulfoxidation, or dehalogenation [12]. These pathways are, in many cases, initiated by oxygenase coupled with coenzyme oxidoreductase. Several studies have reported that the oxygenase activity could be increased by heterologously expressing oxidoreductase [3,17,18].

In the present study, microbial degradation of TDG by gram-positive nocardioforms was investigated and their application to repeated degradation was evaluated.

2. Materials and methods

2.1. Bacterial strains and plasmids

DBT- and/or BT-desulfurizing strains from our collection were used (Table 1).

2.2. Cultivation

As the basal medium, AG, a sulfur-free medium, was used and prepared as described previously [16] with the following ingredients: 5 g glucose, 0.5 g KH_2PO_4, 4 g K_2HPO_4, 1 g NH_4Cl, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.02 g $CaCl_2$, 0.01 g NaCl, 10 ml metal solution, 1 ml vitamin mixture, and 1000 ml distilled water (pH 7.5). Cultivation and resting cell reactions were carried out aerobically at 30 °C. For

* Corresponding author. Tel.: +81 98 895 8973/8944; fax: +81 98 895 8973/8944.
E-mail address: tmatsui@comb.u-ryukyu.ac.jp (T. Matsui).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2008.12.097

Table 1
TDG degradation by growing cells of benzothiophene-desulfurizing bacteria.

Strain	Cell concentration (OD_{6060})		Residual TDG (mM)	Degradation (%)	Ref.
	+TDG	−TDG			
Rhodococcus sp. T09	1.33	0.10	0	100	[16]
Gordonia rubropertincta T08	0.24	0.02	nd*	nd	[20]
Sinoryzobium sp.KT55	0.58	0.55	0.20	0	[23]
G. terrae T14	1.47	0.16	0.16	20	[19]
Rhodococcus sp.KT462	0.81	0.18	0.05	75	[24]
Gordonia sp.T31	nd	nd	0.09	55	[19]
G. terrae T61	0.06	0.04	nd	nd	[19]
No cells	–	–	0.20	nd	

* nd, not determined. Cell concentration of strain T31 could not be measured due to cell flocculation.

preparing resting cell suspension, cultured cells were washed twice with sterilized water and once with 67 mM phosphate buffer (pH 7.5) and resuspended in the same buffer to adjust the cell concentration to an optical density at 660 nm $(OD)_{660}$ of 10. The reaction was started by adding the substrate TDG aqueous solution to the cell suspension.

2.3. Analysis

Cellular growth was estimated by measuring the OD at 660 nm. TDG concentrations were determined by HPLC at 210 nm with a Pham-Pak column (Waters Co., MA). HPLC running conditions were modified as described previously: column temperature, 45 °C; mobile phase, methanol/3 mM phosphoric acid = 5/95; flow rate, 1 ml/min.

The phylogenetic tree based on the 16S rRNA gene sequence was constructed by the neighbor-joining method [22] with the Kimura two-parameter model as a distance corrector [10] after alignment of sequences with the CLUSTALW program version 1.4 [25].

2.4. Sequencing

Genomic DNA were prepared with ISOPLANT (Nippon Gene, Tokyo, Japan) and used for polymerase chain reaction (PCR). The 16S rRNA gene locus was amplified by PCR with Ex-Taq DNA polymerase (Takara Bio, Shiga, Japan) and a Thermal Cycler type-PC818 (Astec Co., Fukuoka, Japan). The PCR primers were EUB11f (5′-TGR GTT TGA TCM TGG CTY AG-3′) and EUB1511r (5′-TGG HTA CCT TGT TAC GAC TT-3′). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min; and elongation at 72 °C for 10 min. The PCR-amplified 16S rRNA gene fragments (approximately 1.5 kb) were purified by agarose gel electrophoresis, ligated into the pT7blue vector (Merck Ltd., Darmstadt, Germany), and used for the transformation of *Escherichia coli* JM109. Nucleotide sequences of the 16S rRNA genes were determined on both strands by M13-47 and RV-M primers (Merck Ltd.) with the ABI model 3100 automated DNA sequencer and Big Dye terminator cycle sequencing kit ver.1.1 (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD) was used for gene homology search with the standard program default.

Nucleotide sequence accession number: Sequence of the fragments of 16S rRNA genes from strain T09 have been assigned DDBJ/EMBL/GenBank accession no. AB458522.

2.5. Chemicals

TDG was supplied by Wako Pure Chemicals Co., Japan. All other materials were of the highest purity commercially available and were used without further purification.

3. Results and discussion

TDG-degrading bacteria were screened from our DBT- and/or BT-desulfurizing bacteria library in a mineral medium with TDG as the sole sulfur source. As shown in Table 1, *Rhodococcus* sp. T09 and *Gordonia terrae* T14 grew well on TDG as the sole sulfur source, while *G. rubropertincta* T08, *G. terrae* T61, and the gram-negative bacterium *Sinorhizobium* sp. KT55 did not. Among the strains tested, only strain KT462 reported DBT desulfurizing activity, also showed TDG degradation in this study. There have been many reports for DBT^+ (DBT assimilation) albeit BT^- (no-BT assimilation) nocardioform bacteria [8,11]. Their nucleotide sequences for desulfurization gene claster were shown to be highly conserved

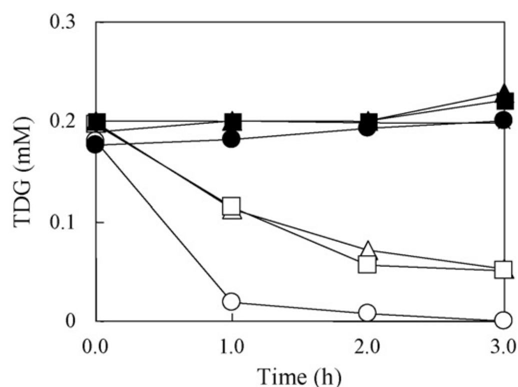

Fig. 1. TDG degradation by resting cells of benzothiophene-desulfurizing bacteria. Circles ((,(), T09; triangles ((, (), T08; squares (□,■), T14 cells; and crosses (+), no cells. Closed symbols ((, (,■) represent data obtained with heat-inactivated (121 °C, 5 min) cells. All the reactions were carried out with cells adjusted to OD_{660} of 10 at 30 °C.

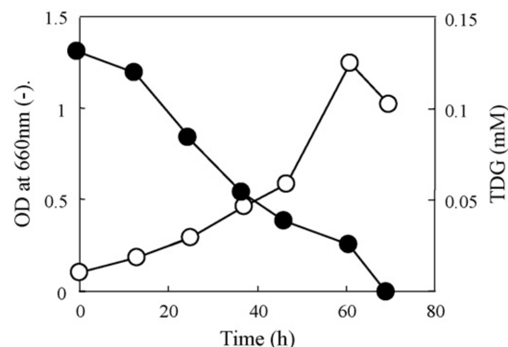

Fig. 2. Time course of strain T09 with TDG as the sole sulfur source under growing conditions. Closed circles ((), represent residual TDG concentrations, and open circles (() represent the cellular growth (expressed as OD_{660}). Cultivations were carried out aerobically at 30 °C.

Fig. 3. Time course of strain T09 with TDG in the presence of various sulfur sources under growing conditions. Circles ((,(), only with TDG; triangles ((, (), with TDG and 5 mM ammonium sulfate; squares (□,■), with TDG and 5 mM DMSO.

[2,11], and they did not utilize aliphatic sulfide, such as dimethyl sulfide [8], while the strain T09 (DBT$^-$, BT$^+$) did [16]. In addition, nocardioform bacteria of DBT+, BT$^-$ assimilated hydrophobic thiophenic derivatives (such as alkylated DBTs) but not hydrophilic derivatives (such as thiophene, and thiophene-calboxylate) [8]. This suggests that the TDG assimilation was not due to DBT desulfurization genes, *dsz*. The former bacterial species (*Rhodococcus* and *Gordonia* spp.) also degraded TDG in the resting cell state, as shown in Fig. 1. Because heat-treated cells did not degrade TDG, the TDG degradations were results of the microbial activity neither of the cell surface adhesion nor abiotic degradation. Among the tested strains shown in Fig. 1, strain T09 showed the highest degradation activity, which was calculated as 16.2 μM TDG/(h•OD), followed by strain T14 with a value of 8.6. On the basis of these results, strain T09 showed the highest degradation rate both in growing and resting cell conditions; therefore, it was selected for further study. Fig. 2 shows the time course of TDG degradation by strain T09 when grown in a mineral medium with 0.15 mM TDG as the sole sulfur source. The degradation proceeded with the cell growth, and most of TDG was depleted at 72 h. *Alcaligenes xylosoxydans* TD2, which could use TDG as the sole carbon source, was previously reported to produce thioglycolic acid, thiodiglycolicaldehyde, diglycolsulphoxide, dimethylether of thiodiglycolic acid and methyl ether of acetic acid as byproducts [4]. Under the conditions used in this study, no intermediates were detected in the broth in both growing and resting cell conditions after extraction with acidic ethyl acetate. This could be because the low TDG concentration in this study made undetectable level of byproduct. In addition, considering the BT desulfurization pathway of the strain T09, TDG may be converted *via* TDG-sulfone. Fig. 3 shows TDG degradation by strain T09 in the presence of various sulfur sources in the growing condition. The strain grew considerably better with ammonium sulfate or dimethyl sulfoxide than with TDG as the sole sulfur source; this suggested that the degradation activity was suppressed by these compounds, or that the strain predominantly utilized these compounds. A similar result was observed for ben-

Fig. 4. Phylogenetic analysis of *R. jostii* strain T09. The bar represents 5 nucleotide substitution per 100 nucleotides in the 16S rRNA gene sequences. The sequences of *Gordonia rubripertincta* (X80632) were used to root the tree. Bootstrap probabilities are indicated at the branch points. The accession numbers are shown in parentheses.

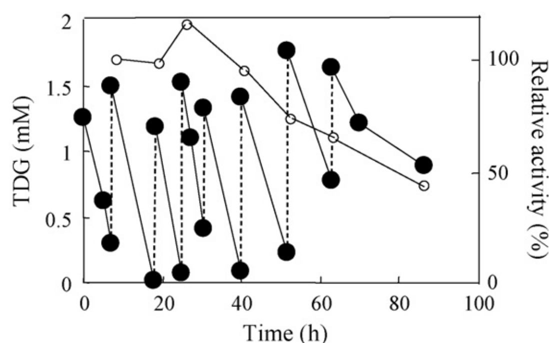

Fig. 5. Repeated degradation of TDG by resting cells of *R. opacus* T09. Closed circles ((), residual TDG concentration; open circles ((), relative specific activity of TDG degradation. The activity of the first batch is considered as 100.

zothiophene degradation by resting cells of strain T09 [16], while *Rhodococcus* sp. strain 1awq showed DBT desulfurization activity even when grown in a mixture of inorganic sulfate and dimethyl sulfoxide [15].

Because the BT-desulfurizing bacterium, named T09, was not identified to the genera level, 16SrRNA sequence of the strain was analyzed. Phylogenetic analysis (Fig. 4) showed the strain T09 belongs to a genera, *R. jostii*. The closest strain was *R. jostii* RHA-1 (accession no. AF435009, nucleotide identity of 100%).

3.1. Repeated degradation of TDG

To determine whether TDG degradation is possible on a practical scale, TDG was degraded by intermittent addition of TDG-grown resting cells. In the preliminary optimization experiment, the degradation rate was the highest at pH 7–7.5 and 30 °C (data not shown). As shown in Fig. 5, TDG could be degraded for 5 repeats (approximately 50 h) with a constant degradation rate, and the degradation rate then gradually decreased. At the end of the reaction, glucose feeding could not recover the activity. In this study, we found that one of the benzothiophene-desulfurizing bacteria, *R. jostii* strain T09, could degrade TDG, thus showing the possibility of repeated TDG degradation with resting cells of T09. To our knowledge, this is the first report of microbial degradation of TDG as the sole sulfur source and not carbon source. To determine whether degradation can occur for a longer period, we are currently examining a continuous operation with immobilized T09 cells after further optimizing the TDG degradation conditions.

References

[1] A.A. Brimfield, M.J. Novak, E. Hodgson, Thiodiglycol, the hydrolysis product of sulfur mustard: analysis of in vitro biotransformation by mammalian alcohol dehydrogenases using nuclear magnetic resonance, Toxicol. Appl. Pharmacol. 213 (2006) 207–215.

[2] G.F. Duarte, A.S. Rosado, L. Seldin, W. de Araujo, J.D. van Elsas, Analysis of bacterial community structure in sulfurous-oil-containing soils and detection of species carrying dibenzothiophene desulfurization (dsz) genes, Appl. Environ. Microbiol. 67 (2001) 1052–1062.

[3] T. Endoh, K. Kasuga, M. Horinouchi, T. Yoshida, H. Habe, H. Nojiri, T. Omori, Characterization and identification of genes essential for dimethyl sulfide utilization in *Pseudomonas putida* strain DS1, Appl. Microbiol. Biotechnol. 62 (2003) 83–91.

[4] I.T. Ermakova, I.I. Starovoitov, E.B. Tikhonova, A.V. Slepen'kin, K.I. Kashparov, A.M. Boronin, Bioutilization of thiodiglycol, the product of mustard detoxification: isolation of degrading strains, study of biodegradation process and metabolic pathways, Proc. Biochem. 38 (2002) 31–39.

[5] V. Garcia-Ruiz, L.E. Martin-Otero, A. Puyet, Transformation of thiodiglycol by resting cells of *Alcaligenes xylosoxydans* PGH10, Biotechnol. Prog. 18 (2002) 252–256.

[6] D.A. Irvine, J.P. Earley, D.P. Cassidy, S.P. Harvey, Biodegradation of sulfur mustard hydrolysate in the sequencing batch reactor, Water Sci. Technol. 35 (1997) 67–74.

[7] N. Itoh, M. Yoshida, T. Miyamoto, H. Ichinose, H. Wariishi, H. Tanaka, Fungal cleavage of thioether bond found in Yperite, FEBS Lett. 412 (1997) 281–284.

[8] Y. Izumi, T. Ohshiro, H. Ogino, Y. Hine, M. Shimao, Selective desulfurization of dibenzothiophene by *Rhodococcus* erythropolis D-1, Appl. Environ. Microbiol. 60 (1994) 223–226.

[9] H. Kawashima, Production of [(2-Hydroxyethyl)thio] acetic acid from thiodiglycol (2,2′-thiodiethanol) by resting cells of Candida rugosa IFO, 1364, Biosci. Biotechnol. Biochem. 59 (1995) 934–935.

[10] M. Kimura, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (1980) 111–120.

[11] M. Kobayashi, T. Onaka, Y. Ishii, J. Konishi, M. Takaki, H. Okada, Y. Ohta, K. Koizumi, M. Suzuki, Desulfurization of alkylated forms of both dibenzothiophene and benzothiophene by a single bacterial strain, FEMS Microbiol. Lett. 187 (2000) 123–126.

[12] M.J. Larkin, L.A. Kulakov, C.C. Allen, Biodegradation and *Rhodococcus*–masters of catabolic versatility, Curr. Opin. Biotechnol. 16 (2005) 282–290.

[13] T. Lee, M.Q. Pham, W.A. Weigand, S.P. Harvey, W.E. Bentley, Bioreactor strategies for the treatment of growth-inhibitory waste: an analysis of thiodiglycol degradation, the main hydrolysis product of sulfur mustard, Biotechnol. Prog. 12 (1996) 533–539.

[14] T. Lee, S.H. Chan, W.A. Weigand, W.E. Bentley, Biocatalytic transformation of [(2-Hydroxyethyl)thio]acetic acid and thiodiglycolic acid from thiodiglycol by *Alcaligenes xylosoxydans* ssp. *xylosoxydans* (SH91), Biotechnol. Prog. 16 (2000) 363–367.

[15] C.Q. Ma, J.H. Feng, Y.Y. Zeng, X.F. Cai, B.P. Sun, Z.B. Zhang, B.H.D. Blankespoor, P. Xu, Methods for the preparation of a biodesulfurization biocatalyst using *Rhodococcus* sp., Chemosphere 65 (2006) 165–169.

[16] T. Matsui, T. Onaka, Y. Tanaka, T. Tezuka, M. Suzuki, R. Kurane, Alkylated benzothiophene desulfurization by *Rhodococcus* sp. strain T09, Biosci. Biotechnol. Biochem. 64 (2000) 596–599.

[17] T. Matsui, K. Hirasawa, K. Koizumi, K. Maruhashi, R. Kurane, Dibenzothiophene desulfurizing genes copy number optimization to increase the desulfurization activity of recombinant *Rhodococcus* sp., Biotechnol. Lett. 23 (2001) 1715–1718.

[18] T. Matsui, K. Hirasawa, K. Koizumi, K. Maruhashi, R. Kurane, Effect of *dsz* D gene expression on benzothiophene degradation of *Rhodococcus* sp. strain T09, Proc. Biochem. 37 (2001) 31–34.

[19] T. Matsui, K. Hirasawa, J. Konishi, Y. Tanaka, K. Maruhashi, R. Kurane, Microbial desulfurization of alkylated dibenzothiophene and alkylated benzothiophene by recombinant *Rhodococcus* sp. strain T09, Appl. Microbiol. Biotechnol. 56 (2001) 196–200.

[20] T. Matsui, T. Onaka, K. Maruhashi, R. Kurane, Benzo[b]thiophene desulfurization by *Gordonia rubropertinctus* strain T08, Appl. Microbiol. Biotechnol. 57 (2001) 212–215.

[21] M.Q. Pham, S.P. Harvey, W.A. Weigand, W.E. Bentley, Reactor comparisons for the biodegradation of thiodiglycol, a product of mustard gas hydrolysis, Appl. Biochem. Biotechnol. 57–58 (1996) 779–789.

[22] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.

[23] Y. Tanaka, T. Onaka, T. Matsui, K. Maruhashi, R. Kurane, Desulfurization of benzothiophene by the gram-negative bacterium, *Sinorhizobium* sp. KT55, Curr. Microbiol. 43 (2001) 187–191.

[24] Y. Tanaka, T. Matsui, J. Konishi, K. Maruhashi, R. Kurane, Biodesulfurization of benzothiophene and dibenzothiophene by a newly isolated *Rhodococcus* strain, Appl. Microbiol. Biotechnol. 59 (2002) 325–328.

[25] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.

[26] H. Wariishi, N. Itoh, M. Yoshida, H. Tanaka, Complete degradation of Yperite, a chemical warfare agent, by basidiomycetes, Biotechnol. Lett. 24 (2002) 501–505.