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ORIGINAL PAPER

Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp. PH-06

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Abstract A new bacterial strain PH-06 was isolated using enrichment culture technique from river sediment contaminated with 1,4-dioxane, and identified as belonging to the genus *Mycobacterium* based on 16S rRNA sequencing (Accession No. EU239889). The isolated strain effectively utilized 1,4-dioxane as a sole carbon and energy source and was able to degrade 900 mg/l 1,4-dioxane in minimal salts medium within 15 days. The key degradation products identified were 1,4-dioxane-2-ol and ethylene glycol, produced by monooxygenation. Degradation of 1,4-dioxane and concomitant formation of metabolites were demonstrated by GC/MS analysis using deuterium labeled 1,4-dioxane (1,4-dioxane-*d8*). In addition to 1,4-dioxane, this bacterium could also transform structural analogues such as 1,3-dioxane, cyclohexane and tetrahydrofuran when pre-grown with 1,4-dioxane as the sole growth substrate. Our results suggest that PH-06 can maintain sustained growth on 1,4-dioxane without any other carbon sources.

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Introduction

The polar cyclic ether 1,4-dioxane is widely used as an industrial solvent and as a solvent stabilizer for some chlorinated compounds in various industrial processes, including the manufacture of pulp, textiles, electronics and other products (Zenker et al. 2003). In addition to its commercial uses, 1,4-dioxane can be formed as a by-product during the polymerization of ethylene glycol in polyester fiber synthesis and surfactant production (Lanigan 2000; Popoola 1991; Zenker et al. 2003). Due to its presence in waste effluents, it is frequently found in river water and groundwater (Abe 1999; Tanabe et al. 2006; Zenker et al. 2003). The U.S. Environmental Protection Agency (EPA) classifies 1,4-dioxane as a B2 pollutant, a probable human carcinogen, based on findings that it is a causative agent for cancer of the nasal cavity, liver carcinomas in rats and mice, and gall bladder carcinomas in guinea pigs. The International Agency for research on cancer (IARC) also classifies 1,4-dioxane as Group 2B, a possible human carcinogen (DeRosa et al. 1996).

A number of treatment methods have been developed for the removal of 1,4-dioxane, including photoremediation by UV light or ozonic destruction in the

presence of hydrogen peroxide. However, these processes require substantial infrastructure and consequently are associated with very high treatment costs, making them impractical for the in situ remediation of vast contaminated sites (Adams et al. 1994; Hill et al. 1997; Maurino et al. 1997; Son et al. 2006; Stefan and Bolton 1998). Bioremediation is a promising alternative technology because of its cost effectiveness, inherent eco-friendly characteristics, and potential for complete decomposition of harmful compounds. Despite these advantages only a few reports are available on the biological degradation or transformation of 1,4-dioxane using mixed microbial cultures or activated sludge (Burback and Perry 1993; Roy et al. 1994; Vainberg et al. 2006; Zenker et al. 2000). Furthermore, only two bacterial strains such as *Rhodococcus* sp. (Bernhardt and Diekmann 1991), *Pseudonocardia dioxanivorans* CB1190^{T} (Mahendra and Alvarez-Cohen 2005; Parales et al. 1994) and a fungus *Cordyceps sinensis* strain A (Nakamiya et al. 2005) have been found to be capable of sustained growth in the presence of 1,4-dioxane as the sole carbon source.

Recently, Mahendra et al. investigated the kinetics of biodegradation of 1,4-dioxane and its metabolic intermediates using various monooxygenase-expressing strains (Mahendra and Alvarez-Cohen 2006; Mahendra et al. 2007). These studies provide valuable information on the potential for biodegradation of 1,4-dioxane among various microorganisms. However, information is scarce on microorganisms known to grow with 1,4-dioxane in pure culture, and details of the biochemistry and genetics of 1,4-dioxane degradation are still lacking. Thus, further investigations in these areas are necessary in order to assess and develop efficient bioremediation processes. In this study, we describe the isolation of a new strain of *Mycobacterium* sp. which utilizes 1,4-dioxane as a sole carbon and energy source, and identify the metabolic intermediates of 1,4-dioxane and transformation products of its structural analogues. Previously, *Mycobacterium vaccae* JOB5 strain showed cometabolic degradation of 1,4-dioxane when pregrown with other substrates (Burback and Perry 1993; Mahendra et al. 2007), however, this strain could not grow with 1,4-dioxane as a sole carbon source. Therefore, this is the first report of the sustained growth with 1,4-dioxane by a *Mycobacterium* species.

Materials and methods

Chemicals

1,4-Dioxane, 1,4-dioxane-*d8*, 1,3-dioxane, ethylene glycol, cyclohexane, tetrahydrofuran, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and bovine serum albumin were purchased from Sigma–Aldrich (St. Louis, MO; Milwaukee, WI). Dimethylformamide (DMF), 85% *ortho*-phosphoric acid, ethyl acetate, cycloheximide, acetone, and nutrient agar were purchased from Merck (Darmstadt, Germany). Minimal salt medium (MSM) contained 3.5 g $Na_2HPO_4 \cdot 2H_2O$, 1 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2 \cdot 6H_2O$, 50 mg $Ca(NO_3)_2 \cdot 4H_2O$ and 1 ml of trace elements solution per liter of distilled water and adjusted to pH 7.2 (Fortnagel et al. 1990). All chemicals and solvents used were of the highest purity available and especially the purity of 1,4-dioxane in this study was 99%.

Enrichment, isolation and identification of bacterial strain PH-06

River sediment was obtained from the Nakdong River which was historically contaminated with 1,4-dioxane for more than decade in South Korea (Park et al. 2005). Prior to enrichment, 1 kg of sediment (wet weight) was divided into four equal parts and each was washed three times with sterile 20 mM phosphate buffer (pH 7.2) to remove dissolved natural organic carbon sources. The washed samples were suspended in 200 ml MSM in 1l Erlenmeyer flasks supplemented with 1 g/l 1,4-dioxane as the sole carbon source, and incubated for 2 weeks on a rotary shaker (160 rpm at 28°C). To suppress the growth of protozoa in the enrichment culture, the medium contains 1,4-dioxane was amended with 10 mg/l cycloheximide)(Kota et al. 1999). Bi-weekly for a 2 month period, 5% (v/v) of the enrichment culture was transferred into 200 ml of fresh MSM, at which time 50 µl samples of each of the resulting culture suspensions were plated onto solidified nutrient agar media, and morphologically distinct colonies were obtained. Individual colonies were transferred to 10 ml of MSM amended with 1 g/l 1,4-dioxane in 100 ml Erlenmeyer flasks, and a bacterial strain showing maximum growth in the presence of 1,4-dioxane was selected for further study. The selected isolate was identified by physiological and

biochemical tests, and by 16S rRNA gene sequencing of amplified PCR product obtained using the 27F and 1492R primers (Lane 1991). The nucleotide sequence was determined at the Macrogen DNA Sequencing Center (Seoul, Korea) and deposited in the National Center for Biotechnology Information (NCBI) Genbank (accession number EU239889). The strain was designated as PH-06.

Growth of strain PH-06 on 1,4-dioxane

The ability of strain PH-06 to utilize 1,4-dioxane as a sole growth substrate was evaluated by inoculation of pre-grown stationary phase cells (20 mg dry weight/l) into 100 ml Erlenmeyer flasks containing 10 ml sterilized MSM with 1 g/l 1,4-dioxane. Control samples were prepared by an inoculation of heat-killed cells. The flasks were incubated at 28°C and 160 rpm in a shaking incubator. A set of three flasks was removed for growth measurement every 3 days from 0 to 15 days, and stored at −20°C. The residual 1,4-dioxane concentration and its degradation products in all flasks were measured after 15 days. Growth of strain PH-06 on 1,4-dioxane was determined from the change in total dry weight of cell biomass, which was measured by filtration of 10 ml culture suspension onto membrane filters (0.2 μm pore size, Advantec MFS, CA) followed by oven drying (80°C for 30 min). Additionally, growth study with 1 g/l of ethylene glycol was also performed to evaluate the ability of strain PH-06 to use ethylene glycol as the carbon source using the same procedure as in 1,4-dioxane. Spectrophotometry could not be used to accurately measure bacterial growth in culture suspensions due to the aggregation and settling of cells. All experiments were performed in triplicate.

Identification of degradation products

A culture suspension (800 ml) of PH-06 grown on 1,4-dioxane as a sole carbon source was harvested from four Erlenmeyer flasks (1l) during the exponential growth phase and washed three times with sterile phosphate buffer (pH 7.2). After removal of media components, the cells (1 g dried cell biomass/l) were resuspended in 250 ml of fresh MSM. Turnover experiments were conducted by addition of 25 ml of cell suspension to sterile 250 ml Erlenmeyer flasks containing 10 mg of 1,4-dioxane, 1,4-dioxane-*d8*, 1,3-dioxane, tetrahydrofuran or cyclohexane. As controls, heat inactivated (70°C for 40 min) and NaN_3-killed cells (10 mM) were prepared and incubated with the respective substrates as well as zero time samples. After 2 days of incubation, all cultures were subjected to chemical analysis for identification of degradation intermediates and transformation products.

Sample preparation and analytical procedures

Residual 1,4-dioxane in culture suspensions was quantified using reverse phase high performance liquid chromatography (RP-HPLC) coupled with a diode array detector system (Agilent 1100 series, Waldbronn, Germany). At the end of the growth measurement experiment, all flasks were thawed and 1.5 ml of culture suspension was removed and centrifuged at 13,000*g* for 10 min. The supernatant was filtered through a 0.45 μm pore size membrane syringe filter (Acrodisc, Pall Cooperation, MI). The filtrates were stored in auto-sampler vials with Teflon-coated caps and analyzed using a ZORBAX SB-C18 column (Agilent, Palo Alto, CA) with 20% aqueous acetonitrile as the mobile phase and a constant flow rate of 1.0 ml/min. All chromatograms were obtained at a wavelength of 200 nm and the lowest detection limit was 20 mg/l (Park et al. 2005; Scalia 1990).

Metabolic intermediates were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). The culture suspensions from the turnover experiments were extracted eight times with ice-cold ethyl acetate. After the sixth extraction, the remaining water phase was adjusted to pH 3.5 with 85% *ortho*-phosphoric acid, and then the final two extractions were conducted. Extracts were dried over anhydrous sodium sulfate and the ethyl acetate was evaporated under reduced pressure. Polar metabolites were derivatized by addition of 20 μl of BSTFA to final extracts in 1 ml acetone (Yu et al. 1998). The metabolic intermediates were identified by comparison of retention times, and mass spectra were obtained by a Trace GC 2000 equipped with a 60 m DB-5 capillary column (Agilent, Palo Alto, CA) and a Polaris Q Ion Trap MS (Thermoquest, San Jose, CA). The initial oven temperature (50°C) was maintained for 5 min, then increased (10°C/min) to 250°C and held for 5 min.

Results

Identification of strain PH-06

Seven strains were isolated from enrichments of river sediment contaminated with 1,4-dioxane. Only one isolate (PH-06) was able to effectively utilize 1,4-dioxane as the sole growth substrate from each growth test. PH-06 was strictly aerobic, Gram-positive, oxidase-positive and catalase-positive strain. The 16S rRNA gene sequencing revealed greater than 98% similarity with *Mycobacterium brisbanense* (AY012577), *Mycobacterium* sp. TMAH-W0418 (EF062506), *Mycobacterium cosmeticum* strains (AY449728, AY44979), and *Mycobacterium* sp. O228YA (DQ372728). Consequently, PH-06 is considered to be a species of the genus *Mycobacterium*.

Growth of strain PH-06 on 1,4-dioxane

Under aerobic conditions, *Mycobacterium* sp. PH-06 utilized 1,4-dioxane as a sole carbon and energy source. The growth curve (Fig. 1) showed a correlation between increase in dry cell mass and depletion of 1,4-dioxane. When grown with 1,4-dioxane in MSM, PH-06 cells tended to aggregate, which prevented accurate spectrophotometric measurements of growth. Therefore, change in dry biomass weight was used to quantify growth. Within 15 days the initial concentration of 1,4-dioxane (1 g/l) had decreased to 0.1 g/l, and the maximum dry cell biomass was 447 mg/l (Fig. 1). Ethylene glycol, the prospective intermediate, was also utilized by this strain, however maximum cell biomass was only 312 mg/l. PH-06 was also efficient in utilizing labeled 1,4-dioxane-*d8* as a substrate for growth, but no growth was observed in the presence of 1,3-dioxane, tetrahydrofuran or cyclohexane.

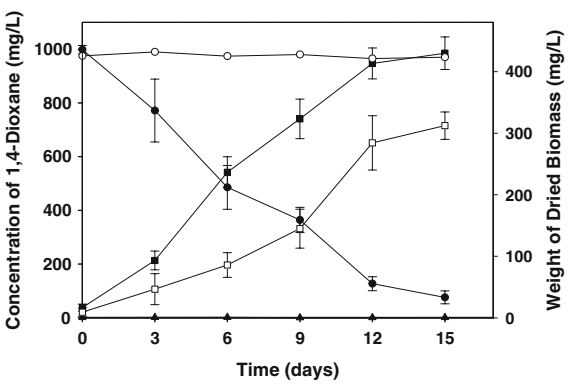

Fig. 1 Growth of *Mycobacterium* sp. PH-06 with 1,4-dioxane as a sole carbon and energy source. All values represent the mean of three independent measurements. (●, concentration of 1,4-dioxane of live samples; ○, concentration of 1,4-dioxane in control samples; ■, total weight of dried biomass of live samples grown with 1,4-dioxane; □, total weight of dried biomass of live samples grown with ethylene glycol; ▲, total weight of dried biomass of control samples)

Identification of degradation products

The turnover experiments for 1,4-dioxane and its different analogues were performed using exponential growth phase cells of strain PH-06 pre-grown on 1,4-dioxane. The first observed 1,4-dioxane degradation product (1,4-dioxane-2-ol) was confirmed by analysis of degradation products of isotopic 1,4-dioxane-*d8* (Fig. 2a, b). In the two MS spectra a mass/charge difference of seven was detected between 1,4-dioxane-2-ol and 1,4-dioxane-2-ol-*d7* at the same retention time. This difference is indicative of enzymatic substitution of one deuterated hydrogen atom by a hydroxyl group. Ethylene glycol was also detected in the trimethylsilated (TMS) form in the GC/MS analysis, suggesting that it was formed from the successive degradation of 1,4-dioxane-2-ol (Fig. 2a, c). Furthermore the mass/charge difference of four was also detected between ethylene glycol and ethylene glycol-*d4* at the same retention time.

Further, strain PH-06 could produce monohydroxylated compounds during the biotransformation of 1,4-dioxane-related substrates including 1,3-dioxane, tetrahydrofuran and cyclohexane. Cyclohexanol was identified as a TMS form from the turnover experiment with cyclohexane (Fig. 3a). Monohydroxytetrahydrofuran was also detected in the tetrahydrofuran-amended sample, but confirmation of the hydroxylated position in the tetrahydrofuran structure was not possible due to the unavailability of authentic standards for comparison (Fig. 3b). Therefore, calculation of parent molecular weight and interpretation of MS fragment patterns were performed to elucidate the structure of detected compounds, assisted by several MS library databases including NIST and Wiley. Hydroxylated metabolites were also detected in the biotransformation of 1,3-dioxane by strain PH-06.

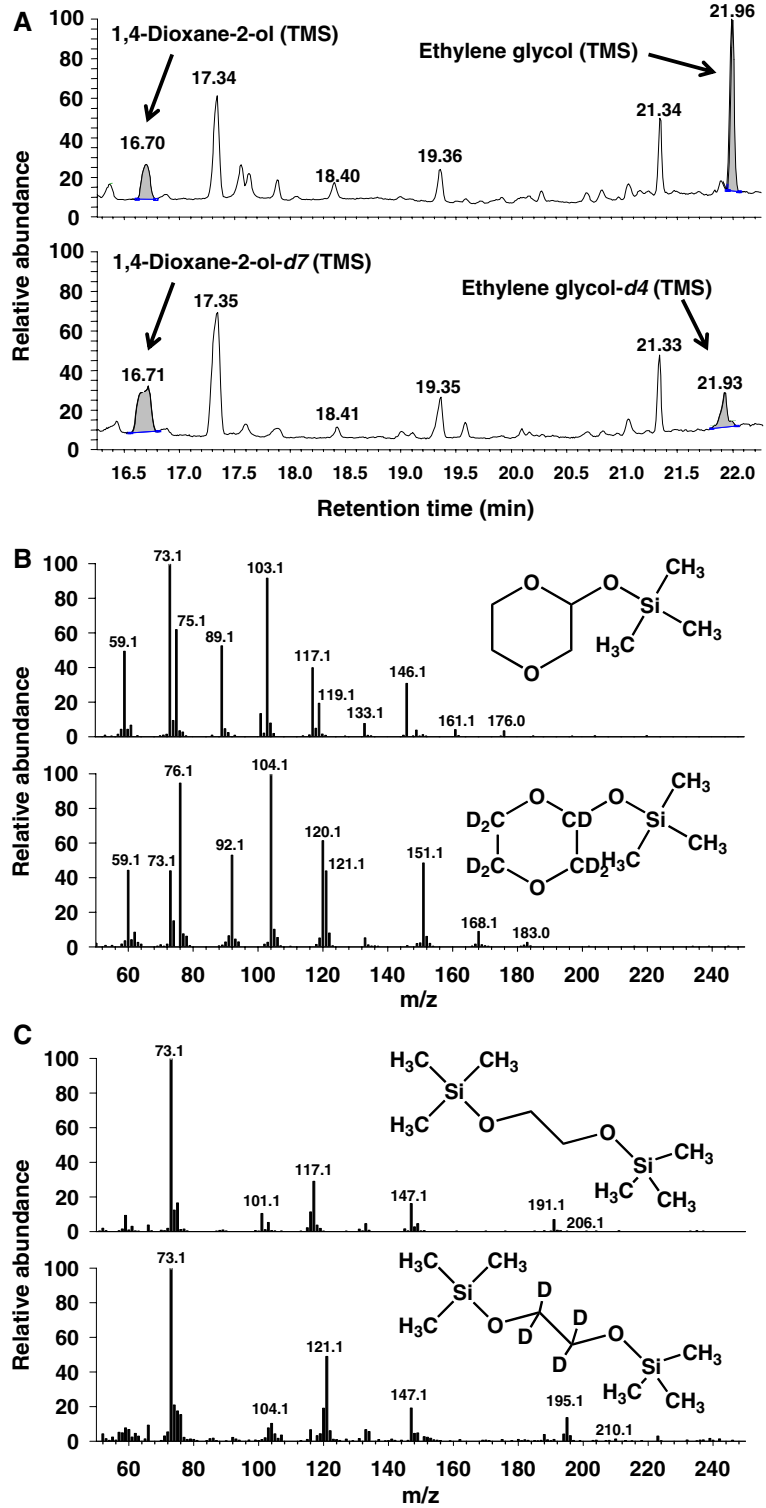

Fig. 2 Identification of metabolites in the biodegradation of 1,4-dioxane by strain PH-06. **a** chromatograms of GC/MS analysis of 1,4-dioxane (*top*) and 1,4-dioxane-*d8* (*bottom*), **b** mass spectra of TMS derivatized 1,4-dioxane-2-ol (*top*) and 1,4-dioxane-2-ol-*d7* (*bottom*), **c** mass spectra of TMS derivatized ethylene glycol (*top*) and ethylene glycol-*d4* (*bottom*)

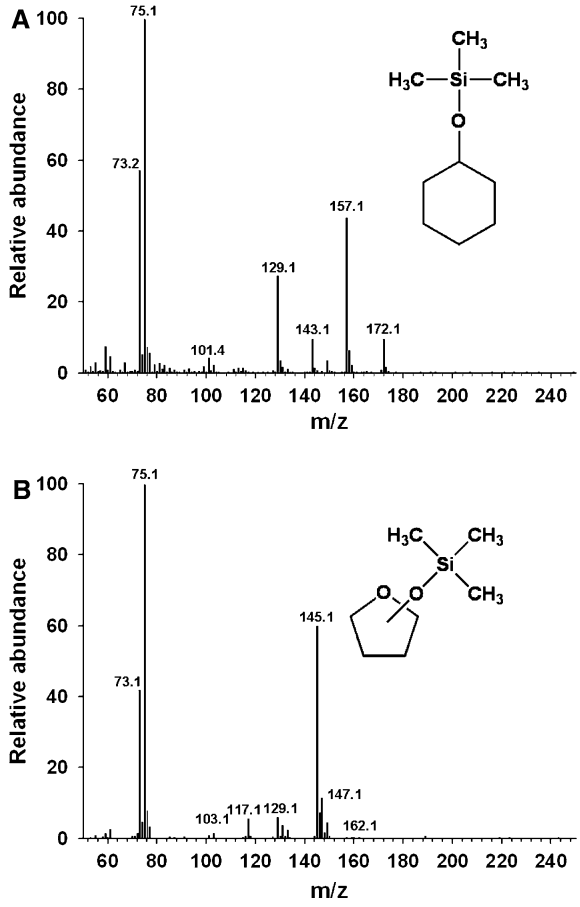

Fig. 3 Identification of metabolite in the biotransformation of cyclohexane and tetrahydrofuran. **a** Mass spectra of TMS derivatized cyclohexanol from cyclohexane, **b** mass spectra of TMS derivatized monohydroxytetrahydrofuran from tetrahydrofuran (exact hydroxylated position was not confirmed at the present time)

Discussion

Bacteria belonging to the genus *Mycobacterium* are known to degrade or transform a wide range of organic substances of environmental concern (Boldrin et al. 1993; Khan et al. 2002). In the present study, we isolated a new 1,4-dioxane-mineralizing bacterial strain PH-06 from contaminated river sediment using an enrichment culture technique. This strain had a 16S rRNA gene sequence matching that of *Mycobacterium* species in the NCBI GenBank database (>98% similarity). We investigated the cultural and biochemical characteristics of PH-06, and the metabolic intermediates formed during the biodegradation and transformation of related cyclic ether compounds.

Mineralization of 1,4-dioxane has been shown in mixed culture microbial cultures (Burback and Perry 1993; Roy et al. 1994; Vainberg et al. 2006; Zenker et al. 2000). The first report of degradation of 1,4-dioxane by a pure bacterial strain (*Rhodococcus* sp.) utilizing this chemical as a sole carbon source was made by Bernhardt and Diekmann (1991). However their study lacked sufficient experimental evidence and only postulated a hypothetical catabolic pathway. There are few reports of the biotransformation of 1,4-dioxane in pure cultures by co-metabolism. For example, *Mycobacterium vaccae* JOB-5, which utilizes propane, acetone and toluene as growth substrates, is the only *Mycobacterium* species known to be involved in the transformation of 1,4-dioxane (Burback and Perry 1993). However, this strain lacked the ability to grow in the presence of 1,4-dioxane as a sole carbon source. In contrast, the *Mycobacterium* sp. PH-06, isolated in our study, utilizes 1,4-dioxane as the sole carbon source. Hence, strain PH-06 is the first known *Mycobacterium* strain and forth known microorganism capable of using 1,4-dioxane as the sole growth and energy source. Previously reported organism in this respect are *Rhodococcus* sp. and *Pseudonocardia* strains CB1190, and fungus *Cordyceps sinensis.*

From enrichments of industrial sludge, Parales et al. (1994) isolated a pure *Actinomycetes* strain which could degrade up to 0.88 g/l 1,4-dioxane per day. This strain was recently reclassified as *Pseudonocardia dioxanivorans* CB1190^{T} (Mahendra and Alvarez-Cohen 2005, 2006), and 2-hydroxyethoxyacetic acid (HEAA) was identified as the major degradation intermediate during the biodegradation of 1,4-dioxane by this bacterium (Mahendra et al. 2007). Vainberg et al. (2006) also observed HEAA as the main intermediate during cometabolic transformation of 1,4-dioxane by *Pseudonocardia* sp. strain ENV478 and proposed a partial degradation pathway. In contrast, HEAA was not detected as an intermediate in our study, but 1,4-dioxane-2-ol and ethylene glycol were identified, and confirmed by isotope labeling experiments. In addition, ethylene glycol was utilized by strain PH-06 as a sole carbon and energy source. Recently, Mahendra et al. (2007) proposed the complete degradation pathway of 1,4-dioxane using strain CB1190^{T} and other

monooxygenase expressing strains. Although 1,4-dioxane-2-ol has not been detected during 1,4-dioxane degradation by other strains, it has been proposed as the first product in the degradation by Vainberg et al. (2006) and confirmed by Mahendra et al. (2007). Our findings also confirm this and contribute to a better understanding of the degradation pathway in monooxygenase-producing bacteria (Fig. 4). In addition, degradation of 1,4-dioxane by the fungus *Cordyceps sinensis* strain A, isolated from garden soil, has also been reported (Nakamiya et al. 2005), and a degradation pathway was proposed based on detection in the culture medium of several intermediates including ethylene glycol, glycolic acid and oxalic acid but, similar to our study, no HEAA was detected in their study. Although they suggested the possible involvement of etherases or oxidases, the sequential enzymatic steps in the conversion of 1,4-dioxane to ethylene glycol were not elaborated.

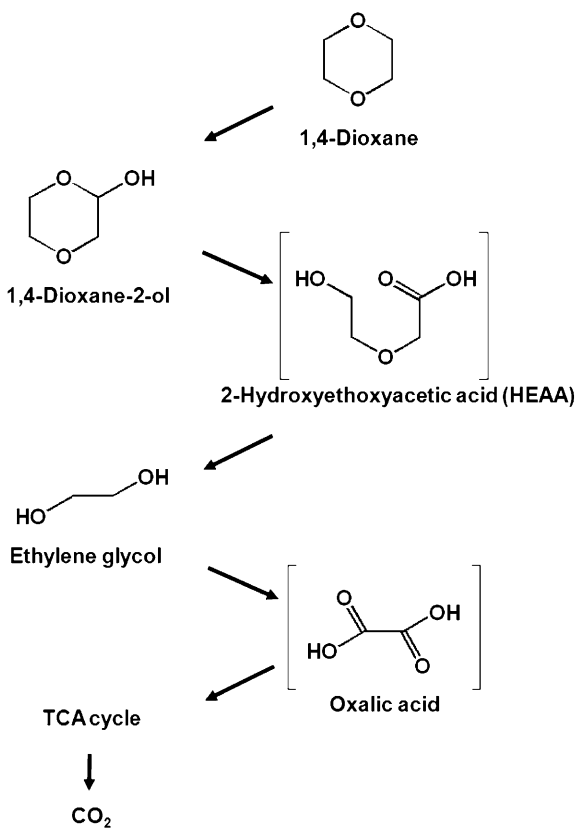

Fig. 4 Proposed pathway of the biodegradation of 1,4-dioxane by *Mycobacterium* sp. PH-06

Thiemer et al. (2003) proposed initial monooxygenation, similar to 1,4-dioxane, for tetrahydrofuran, and identified a responsible monooxygenase from *Pseudonocardia* sp. strain K1. Although monooxygenation product was observed as the initial metabolites from 1,4-dioxane degrading pure cultures, the exact enzyme system involved is unclear. Mahendra and Alvarez-Cohen (2006) proved that various monooxygenase expressing microorganisms that were induced with methane, propane, tetrahydrofuran, or toluene including *Methylosinus trichosporium* OB3b, *Mycobacterium vaccae* JOB5, *Pseudonocardia* K1, *Pseudomonas mendocina* KR1, *Ralstonia pickettii* PKO1, *Burkholderia cepacia* G4, and *Rhodococcus* RR1 cometabolically transformed 1,4-dioxane. The degradation of isotopic and non-isotopic 1,4-dioxane in our study clearly indicated that the initial conversion step of 1,4-dioxane by strain PH-06 involved monooxygenation. Similar activity was also observed with other analogous substrates, as confirmed by the formation of the monohydroxylated products cyclohexanol and monohydroxytetrahydrofuran (hydroxylated position could not be elucidated) from cyclohexane and tetrahydrofuran, respectively. From the above studies, it appears that all known pure cultures of 1,4-dioxane degrading strains have the similar initial monooxygenation reaction for 1,4-dioxane and its analogues however it is still unclear for fungal case.

We sought to measure the rates of oxygen consumption by strain PH-06, whose degradative enzymes had been induced by addition of 1,4-dioxane as the growth substrate (Kim et al. 2007), but the cell suspensions were not homogenous after washing due to cell aggregation and settling. Although the results were highly significant, they are not reported here as the culture characteristics noted above could have affected the accuracy of the measurements of oxygen consumption rates.

Conclusion

In this research, biological removal of 1,4-dioxane from aqueous medium was studied using *Mycobacterium* sp. PH-06. The conclusions are summarized as follows:

1. 1,4-Dioxane degrading bacterium, *Mycobacterium* sp. PH-06 was isolated and characterized

from 1,4-dioxane contaminated river sediment using enrichment culture technique.
2. The strain PH-06 was able to degrade 90% of 1,4-dioxane when the concentration of 1,4-dioxane was 1 g/l in MSM and also utilized ethylene glycol as its sole carbon and energy source.
3. The degradation metabolites, 1,4-dioxane-2-ol and ethylene glycol were identified through turnover experiments, and further confirmed by isotope experiments using 1,4-dioxane-*d8*.
4. The structural analogues such as 1,3-dioxane, tetrahydrofuran, and cyclohexane were also transformed to hydroxylated intermediates by monooxygenation activity of the strain PH-06.

To our knowledge, this is the first report of the biodegradation of 1,4-dioxane by a *Mycobacterium* species using this compound as a sole carbon and energy source. Although strain PH-06 effectively degrades 1,4-dioxane by an initial monohydroxylation, the enzyme systems involved are still unclear. Investigations aimed at elucidating these systems are currently underway.

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