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Characterization of newly isolated *Pseudonocardia* sp. N23 with high 1,4-dioxane-degrading ability

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This study was conducted to elucidate the 1,4-dioxane degradation characteristics of a newly isolated 1,4-dioxane-degrading bacterial strain and evaluate the applicability of the strain to biological 1,4-dioxane removal from wastewater. A bacterial strain (designated strain N23) capable of degrading 1,4-dioxane as the sole carbon and energy source was isolated from an enrichment culture prepared from 1,4-dioxane-contaminated groundwater. Strain N23 was phylogenetically identified as belonging to the genus *Pseudonocardia*, based on 16S rRNA gene sequencing. 1,4-Dioxane degradation experiments revealed that strain N23 is capable of constitutive 1,4-dioxane degradation. Further, this strain exhibited the highest specific 1,4-dioxane degradation rate of 0.230 mg-1,4-dioxane (mg-protein)$^{-1}$ h^{-1} among 1,4-dioxane-degrading bacteria with constitutively expressed degrading enzymes reported to date. In addition, strain N23 was shown to degrade up to 1100 mg L^{-1} of 1,4-dioxane without significant inhibition, and to maintain a high level of 1,4-dioxane degradation activity under a wide pH (pH 3.8–8.2) and temperature (20–35 °C) range. In particular, the specific 1,4-dioxane degradation rate, even at pH 3.8, was 83% of the highest rate at pH 7.0. In addition, strain N23 was capable of utilizing ethylene glycol and diethylene glycol, which are both considered to be present in 1,4-dioxane-containing industrial wastewater, as the sole carbon source. The present results indicate that strain N23 exhibits the potential for 1,4-dioxane removal from industrial wastewater.

[**Key words:** Biodegradation; 1,4-Dioxane-degrading bacterium; Isolation; *Pseudonocardia*; Wastewater treatment]

1,4-Dioxane, a polar cyclic diether, has been used mainly as a solvent for extraction and detergent in the manufacture of chemicals. It is also known that 1,4-dioxane is formed as a by-product during the manufacture of ethylene oxide and ethylene glycol. Owing to its suspected carcinogenic effects in humans (group 2B) (1), 1,4-dioxane was listed as a hazardous compound in the World Health Organization guidelines for drinking water in 2003. Owing to its physical and chemical properties of this compound, such as its high solubility in water, low volatility, low absorbability to solids, low hydrolyzability, and low biodegradability (2–5), 1,4-dioxane is stable and capable of persistence for a prolonged period once released into the environment. The development of appropriate treatment technologies for 1,4-dioxane-containing industrial wastewater, which may contain as high as several hundred mg L^{-1} of 1,4-dioxane (6–8), is critical for the prevention of 1,4-dioxane contamination in the environment.

The high stability of 1,4-dioxane renders most conventional biological and physicochemical water/wastewater treatment technologies ineffective for its removal from industrial wastewater (9–11). Advanced oxidation processes (AOPs), which combine multiple oxidation treatments such as ozonation, hydrogen peroxide treatment, and ultraviolet irradiation, have been shown to be effective only for 1,4-dioxane degradation (10,12–14). However, several technical limitations are associated with the use of AOPs; these include (i) high energy consumption and operational costs, and (ii) inhibition of 1,4-dioxane degradation by co-occurring compounds (10,15). Therefore, the development of cost- and energy-saving technologies with stable performance that are unaffected by the presence of other organic compounds is required.

A possible alternative to the use of AOPs is biological treatment using 1,4-dioxane-degrading bacterial strains. Although 1,4-dioxane was previously considered recalcitrant to biodegradation (16), bacterial strains capable of degrading 1,4-dioxane as the sole carbon and energy source have been recently isolated (4,17–24). These strains are classified into two groups based on whether their expression of 1,4-dioxane-degrading enzymes is inducible or constitutive (4). Because there exists certain threshold concentration at which the degrading enzymes are expressed in inducible 1,4-dioxane-degrading bacteria, they may not be effective for the treatment of wastewater containing low concentrations of 1,4-dioxane. For example, in our previous report on continuous biological wastewater treatment using pure culture of a 1,4-dioxane degrading bacterium, *Afipia* sp. D1 with inducible enzyme expression remained approximately 3 mg L^{-1} of 1,4-dioxane in the effluent (25). By contrast, *Pseudonocardia* sp. D17 with a constitutive catabolic activity could remove 1,4-dioxane from 5 mg L^{-1} to <0.5 mg L^{-1} (26). Therefore, the constitutive 1,4-dioxane degrading bacteria, which can maintain

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their 1,4-dioxane degrading ability in spite of the 1,4-dioxane concentration, show great promise for the treatment of industrial wastewater of which 1,4-dioxane concentrations are low or fluctuated. However, only 3 constitutive 1,4-dioxane-degrading strains, *Mycobacterium* sp. D6, *Pseudonocardia* sp. D17 and *Xanthobacter flavus* DT8, have been isolated to date (4,24). Moreover, the maximum specific 1,4-dioxane degradation rates of these strains were less than 40% of that of *Afipia* sp. D1, which degrades 1,4-dioxane inductively with the highest maximum specific degradation rate reported to date (4). Further, the 1,4-dioxane-degrading abilities of the latter strains are greatly reduced at temperatures and pH outside the optimum range (4). As a result, the applicability of these previously isolated constitutive 1,4-dioxane degrading strains is quite limited, necessitating the identification of more powerful constitutive 1,4-dioxane degrading strains capable of maintaining high degradation activity under a wide pH and temperature range.

In this study, a novel constitutive 1,4-dioxane-degrading bacterium was isolated from an enrichment culture of groundwater contaminated with 1,4-dioxane. In addition to taxonomic characterization, the isolated strain was characterized for its 1,4-dioxane-degrading ability. In particular, 1,4-dioxane degradation kinetics, the effects of pH and incubation temperature on 1,4-dioxane degradation, and the availability of various substrates for growth were evaluated to clarify the applicability of the isolated strain to biological 1,4-dioxane treatment systems under various conditions.

MATERIALS AND METHODS

Culture media and cultivation conditions For enrichment of 1,4-dioxane-degrading bacteria as well as for experiments performed to investigate 1,4-dioxane degradation and utilization of various substrates by the isolated strain, a basal mineral medium (BMM; 1000 mg L^{-1} K_2HPO_4, 1000 mg L^{-1} $(NH_4)_2SO_4$, 200 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 10 mg L^{-1} $FeCl_3$, 50 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 50 mg L^{-1} NaCl) supplemented with known concentrations of sole carbon source was used. Routine cultivation of 1,4-dioxane-degrading bacteria was conducted in CGY medium (4) or MGY medium (23), to which 1,4-dioxane was added if necessary. Unless otherwise stated, the initial pH of the medium was adjusted at 7.0–7.3, and the culture was incubated at 28 °C with shaking at 120 rpm. In order to prepare solid medium, agar was added to liquid medium at 1.5%.

Isolation of 1,4-dioxane-degrading bacteria A groundwater sample contaminated with 1.3 mg L^{-1} of 1,4-dioxane collected in Japan was centrifuged (10,000 ×*g*, 4 °C, 10 min), and the suspended solids in the sample were harvested and inoculated into a 300-mL flask containing 100 mL of BMM supplemented with 500 mg L^{-1} of 1,4-dioxane as the sole carbon source. The flask was sealed with a silicon cap and incubated. After cultivation for 2 weeks, the suspended solids in the medium were harvested by centrifugation and transferred to a fresh medium, after which further enrichment was performed under the same conditions as above. Then, 1,4-dioxane concentration was measured during the enrichment process to confirm degradation. After enrichment for about 3 months with transfer of the suspended solids to fresh medium at 2-week intervals, 1,4-dioxane-degrading bacteria in the enrichment culture were isolated by spreading on BMM agar plates supplemented with 500 mg L^{-1} of 1,4-dioxane. A 1,4-dioxane-degrading strain was isolated.

Physiological and phylogenic characterization Cell morphology and motility, Gram staining, and catalase tests were performed to characterize the isolated strain. Cells of the isolated strain were also observed by scanning electron microscopy. The culture of the isolated strain grown in CGY medium for 7 d was dropped on a nano-percolator filter (pore size 0.6 μm; JEOL, Tokyo, Japan). The cells on the filter were collected using a sterilized syringe, washed twice with 0.1% phosphate buffer (pH 7.2) containing 0.85% NaCl, and fixed with 2% glutaraldehyde buffered with 0.1% phosphate buffer (pH 7.2) containing 0.85% NaCl for 2 h at 25 °C. The fixed samples were dehydrated in graded ethanol series from 50% to 99.5% and dried using a JCPD-5 critical point dryer (JEOL). The dried cells were sputter-coated with gold using a JFC-1100 sputter coater (JEOL) and scanned with a JSM-5610LV scanning electron microscope (JEOL) at an accelerating voltage of 10 kV.

In addition, phylogenetic characterization was performed by 16S rRNA gene sequencing. Genomic DNA was extracted from the isolated strain by the proteinase K method (27). Partial sequence of 16S rRNA gene was amplified from the extracted DNA using the 8F and 1429R primer set (28) under the following thermal profile: initial denaturation at 94 °C for 10 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min; and final extension at 72 °C for 10 min. The 16S rRNA gene sequence was determined using an Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA). The obtained sequence was compared with sequences in the NCBI database, using the BLAST search program (http://www.ncbi.nih.gov/blast/), and closely related sequences were obtained from GenBank. The sequences were then aligned using CLUSTAL W (29), and a phylogenetic tree was produced by TreeView X (30).

1,4-Dioxane degradation experiments The isolated strain was pre-grown in CGY medium supplemented with and without 500 mg L^{-1} of 1,4-dioxane for 7 d. Cells of this strain were harvested by centrifugation (10,000 ×*g*, 4 °C, 10 min) and washed twice with BMM without carbon source. The washed cells were suspended in BMM without carbon source, and the cell suspension was used for 1,4-dioxane degradation experiments. The concentration of cell suspension was set at 200–5820 mg-protein L^{-1}, and 1 mL of cell suspension was inoculated into a 100-mL flask containing 19 mL of BMM supplemented with 10–1000 mg L^{-1} of 1,4-dioxane. The pH of BMM was adjusted to 3–8, and incubation temperature was set at 20–45 °C to elucidate the effects of pH and temperature on 1,4-dioxane degradation by the isolated strain. Control systems without bacterial inoculation were also prepared for evaluation of 1,4-dioxane volatilization. Then, 1,4-dioxane degradation experiments were conducted up to 108 h, and temporal variations in 1,4-dioxane concentrations were investigated. If necessary, cell growth concomitant with 1,4-dioxane degradation was monitored by measuring dry cell weight. All experiments were conducted in duplicate or triplicate.

The 1,4-dioxane degradation kinetics of the isolated strain was analyzed with various initial 1,4-dioxane concentrations. The specific 1,4-dioxane degradation rate (k) was determined by dividing the volumetric 1,4-dioxane degradation rate (V) by the cell protein concentration (p_0) at the beginning of experiments (Eq. 1). The volumetric 1,4-dioxane degradation rate (V) was obtained by subtracting the slope of the decline of 1,4-dioxane concentration in the control system (the 1,4-dioxane volatilization rate (v_1)) from that in the test system (v_2) during the linear decreasing period ($r^2 \geq 0.95$) (Eq. 2). The cell yield on 1,4-dioxane was calculated based on the consumption of 1,4-dioxane and the increase in cell protein for 6 h of incubation time.

$$k = V/p_0 \quad (1)$$

$$V = v_2 - v_1 \quad (2)$$

In the experiments, *Pseudonocardia dioxanivorans* CB1190, which is capable of inductively degrading 1,4-dioxane as a sole carbon and energy source (18), was used as the comparative strain. *P. dioxanivorans* CB1190 registered as *P. dioxanivorans* JCM13855^T was provided by the RIKEN BioResource Center through the National Bio-Resources Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The pre-cultivations and preparation of cell suspensions of strain CB1190 were conducted according to the procedures described above for the isolated strain.

Utilization experiments for various substrates Cell suspensions were prepared according to the procedure described above, using cells of the isolated strain pre-cultivated in MGY medium. The concentration of the cell suspension was set at approximately 200 mg-dry cells L^{-1}. Five milliliters of cell suspension were inoculated into 50-mL vials containing 15 mL of BMM supplemented with 100 mg-C L^{-1} of one of the following carbon sources: acetic acid, 1,4-butanediol, 1-butanol, 1,4-dioxane, diethylene glycol, ethylene glycol, glucose, glycolic acid, glyoxal, glyoxylic acid, phenol, tetrahydrofuran, and triethylene glycol. The vials were incubated for 7 d, and the dry cell weights were measured on 0 d and 7 d. The experiments were conducted in triplicate.

Analytical procedures Samples for measurement of 1,4-dioxane concentrations were filtered through a cellulose acetate filter (pore size 0.45 μm; Advantec, Tokyo, Japan). The 1,4-dioxane concentration was determined by headspace-gas chromatography/mass spectrometry (HS-GC/MS). A GC/MS QP-2010 plus (Shimadzu, Kyoto, Japan) combined with a TurboMatrix HS40 headspace sampler (PerkinElmer, Waltham, MA, USA) and equipped with an Rtx-624 capillary column (60 m × 0.32 mm i.d.; film thickness 1.8 μm; Restek, Bellefonte, PA, USA) was used. Helium gas was used as the carrier gas with a flow rate of 2.42 mL min^{-1}. The column temperatures were held at 40 °C for 5 min, ramped at 20 °C min^{-1} to 230 °C, and held at 230 °C for 5 min. The detector temperature was set at 200 °C. The mass spectrometer was operated in the electron ionization (EI) mode at 0.7 kV. The selected ion monitoring (SIM) mode was used to acquire the signals at m/z 58 and 88 for 1,4-dioxane and m/z 64 and 96 for 1,4-dioxane-d_8 as the internal standard. The detection limit was 0.001 mg L^{-1}. The amounts of cell protein were determined as previously described (31), and the detection limit was 6 mg-protein L^{-1}. The dry cell weight was measured as follows: 10 mL culture sample was filtered through a glass microfiber filter (grade GF/B, pore size 1.0 μm; Whatman, Maidstone, UK) pretreated with ultrapure water, and the filter was washed with ultrapure water and dried for 2 h at 105 °C; the dry cell weight was calculated as the difference between the filter weights with and without dried cells.

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