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Degradation of 1,4-Dioxane by an Actinomycete in Pure Culture

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An actinomycete capable of sustained aerobic growth on 1,4-dioxane was isolated from a dioxane-contaminated sludge sample. The actinomycete, CB1190, grows on 1,4-dioxane as the sole carbon and energy source with a generation time of approximately 30 h. CB1190 degrades 1,4-dioxane at a rate of 0.33 mg of dioxane min^{-1} mg of $protein^{-1}$ and mineralizes 59.5% of the dioxane to CO_2. CB1190 also grows with other cyclic and linear ethers as the sole carbon and energy sources, including 1,3-dioxane, 2-methyl-1,3-dioxolane, tetrahydrofuran, tetrahydropyran, diethyl ether, and butyl methyl ether. CB1190 is capable of aerobic autotrophic growth on H_2 and CO_2.

1,4-Dioxane is a cyclic ether used as a solvent or formed as an undesired by-product in many industrial processes. It is highly water soluble and is a suspected carcinogen. Consequently, it has become a serious groundwater pollutant (6, 9, 13). In general, cyclic ethers such as 1,4-dioxane and tetrahydrofuran (THF) have not been amenable to biodegradation (1, 2, 12, 15, 21).

Bacterial cultures enriched on morpholine have been reported to degrade 1,4-dioxane; however, neither sustained growth with 1,4-dioxane as the sole carbon source nor mineralization of the compound was demonstrated (10). Recent reports have shown *Mycobacterium vaccae* to transform 1,4-dioxane to hydroxylated cyclic compounds, but the organism could not be grown on 1,4-dioxane nor did it mineralize the cyclic ether (5, 24). Pure cultures of a *Rhodococcus* sp. have been shown to degrade 1,4-dioxane (3), but evidence of sustained growth on 1,4-dioxane or mineralization was not presented. Mineralization of 1,4-dioxane has been demonstrated with mixed microbial populations (7, 23).

In this paper, we describe the isolation in pure culture of a nocardioform actinomycete capable of sustained growth on 1,4-dioxane. Evidence for 1,4-dioxane mineralization by the actinomycete is also presented.

MATERIALS AND METHODS

Media and growth conditions. All enrichments and growth of pure cultures were performed with basal salts medium (BSM) or ammonium mineral salts medium (AMS). One liter of BSM contained 100 ml of BSM buffer stock and 100 ml of BSM trace metal stock. The BSM buffer stock contained (per liter) 32.4 g of K_2HPO_4, 10.0 g of $NaH_2PO_4 \cdot H_2O$, and 20.0 g of NH_4Cl. The BSM trace metal solution contained (per liter) 1.23 g of nitrilotriacetic acid (disodium salt), 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.12 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot H_2O$, 0.03 g of $ZnSO_4 \cdot 7H_2O$, and 0.01 g of $CoCl_2 \cdot 6H_2O$. One liter of AMS contained 0.66 g of $(NH_4)_2SO_4$, 1.0 g of $MgSO_4 \cdot 7H_2O$, and 0.015 g of $CaCl_2 \cdot 2H_2O$ (salts solution), 1.0 ml of AMS trace elements, 1.0 ml of stock A, and 20 ml of 1.0 M phosphate buffer (added after sterilization). The AMS trace elements contained (per liter) 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.4 g of $ZnSO_4 \cdot 7H_2O$, 0.02 g of $MnSO_4 \cdot H_2O$, 0.015 g of H_3BO_3, 0.01 g of $NiCl_2 \cdot 6H_2O$, 0.25 g of EDTA, 0.05 g of $CoCl_2 \cdot 6H_2O$, and 0.005 g of $CuCl_2 \cdot 2H_2O$. The AMS stock A contained (per liter) 5.0 g of Fe-Na EDTA and 2.0 g of $NaMoO_4 \cdot 2H_2O$. The 1 M phosphate contained 113.0 g of K_2HPO_4 and 47.0 g of KH_2PO_4. 1,4-Dioxane was supplied to the enrichment cultures at concentrations ranging from 1 to 110 mM. Unless stated otherwise, all incubation of cultures and enrichments took place at 30°C. Liquid cultures were aerated at 250 rpm in reciprocal shaking incubators. For solid minimal media, 1.5% (wt/vol) Noble agar (Difco) was added to AMS or BSM. Luria broth was prepared by the method of Davis et al. (8) and solidified with 1.5% (wt/vol) Difco Bacto agar.

All growth was monitored as optical density at 660 nm (OD). Growth was confirmed by increases in the protein concentration in the cultures. Generation times were determined from the slope of OD plotted against time (semilog) during exponential phase.

A Tris-maleate buffer, useful over the pH range from 5.0 to 8.5, was used to determine the pH optimum for CB1190. The buffer was prepared by mixing 10 ml of 1 M maleic acid, 10 ml of 1 M Tris-HCl (pH 7.6), and 2 ml of 1 M phosphate (pH 7.0). The pH was adjusted to the desired value with NaOH, the total volume was brought to 180 ml, and the buffer was filter sterilized. pH-modified AMS media were prepared by combining 180 ml of buffer with 20 ml of a sterile 10-fold concentrate of AMS salts solution, trace elements, and stock A.

Enrichment and isolation. Enrichment cultures were started with industrial sludge from a 1,4-dioxane-contaminated site in Darlington, South Carolina. Sludge was incubated aerobically in BSM plus 0.1% (wt/vol) yeast extract (Difco) and 1.5 to 4.0 mM THF. Degradation of THF was observed within 3 weeks. Enrichments were transferred and maintained on THF, and transfers into media containing THF plus 1.0 to 5.5 mM 1,4-dioxane were made. All enrichment cultures capable of degrading both 1,4-dioxane and THF were combined at room temperature in an aerated 10-liter fermentor containing BSM, 0.1% yeast extract, THF, and 1,4-dioxane. After the THF was degraded, only 1,4-dioxane was supplied and, eventually, the

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culture was capable of degrading 10 mM 1,4-dioxane daily. This mixed population was designated CB1184, and consisted of irregularly branched filamentous organisms, typical bacterial rods, cocci, and various eucaryotes (protozoans and ciliates).

Attempts to isolate 1,4-dioxane-degrading organisms by plating on minimal medium with 1.0 to 55.0 mM 1,4-dioxane incorporated or with 1,4-dioxane supplied by vapor were unsuccessful. Treatment of CB1184 cultures with the procaryotic antibiotics tetracycline, penicillin, and streptomycin (100 mg/liter each) inhibited 1,4-dioxane degradation. In contrast, treatment with cycloheximide, an inhibitor of eucaryotic protein synthesis, did not affect 1,4-dioxane degradation and appeared to eliminate the eucaryotic organisms from the culture. These results indicated that procaryotic microorganisms were responsible for 1,4-dioxane degradation. CB1184 was then continually transferred in the presence of cycloheximide (100 mg/liter) and high levels of 1,4-dioxane (55 to 110 mM) and in the absence of yeast extract. The resulting culture, designated CB1185, was maintained on 1,4-dioxane and appeared to be a stable population consisting of at least 20 different types of bacteria that could be isolated on Luria agar or AMS containing 10 mM succinate. Only one isolate (a dry white colony that routinely grew slowly on minimal medium plates, regardless of whether any carbon source was present) was capable of degrading 1,4-dioxane when inoculated into liquid culture.

Analysis of 1,4-dioxane and THF. Cultures were filtered through 0.45-μm-pore-size nylon filters (Gelman Sciences, Ann Arbor, Mich.), and 1-μl samples were analyzed with a Hewlett-Packard 5890 series II gas chromatograph (GC) equipped with a DB624 megabore column (0.53-mm inner diameter, 3-μm film thickness, and 30-m length; J&W Scientific, Folsom, Calif.) and a flame ionization detector. The column was operated at an initial temperature of 70°C, and the temperature was increased to 220°C at a rate of 20°C per min for 7.5 min. The minimum detection limit for this method was 10 μM. Detection to 0.55 μM with 1-μl aqueous samples was achieved by monitoring for the 88-atomic-mass-unit ion, the molecular ion for 1,4-dioxane, with a mass spectrometry detector operated in the selected ion-monitoring mode. For this a Hewlett-Packard 5971A chromatograph with a mass spectrometry detector was used with a DB624 capillary column of 0.25-mm inner diameter and 1.4-μm film thickness.

Chemical oxygen demand (COD). COD was determined by the Hach COD closed reflux micro method (Hach Company, Loveland, Colo.) with both the Hach low-range (0- to 150-mg/liter) and high-range (0- to 1,500-mg/liter) ampoules.

Specific activity assay. CB1185 and CB1190 were grown in BSM or AMS with 1,4-dioxane. Cells were washed and resuspended in the same medium to an OD of 0.5 to 1.0. Dioxane was added at time zero and was then monitored by GC over a 6-h period. Protein from the cell cultures was also monitored during this time by the Lowry assay (20), with bovine serum albumin as the standard.

Analysis of CO_2. Headspace gas (100-μl samples) from mineralization experiments was analyzed with a Tracor 9000 series GC equipped with a Carbosieve S-II column (0.64-cm outer diameter, 2-mm inner diameter, 182.9-cm length, and 80/100 mesh; Supelco, Bellefonte, Pa.) and a thermal conductivity detector. The column was operated at an initial column temperature of 165°C, and the temperature was increased to 225°C at a rate of 15°C per min. CO_2 from the culture medium was measured by converting HCO_3^- and CO_3^{2-} to CO_2 by the addition of 100 μl of 10 N H_2SO_4 to 1 ml of culture medium in an 18-ml sealed tube. After shaking of the tube to transfer the CO_2 to the headspace, a sample was taken and analyzed as described above.

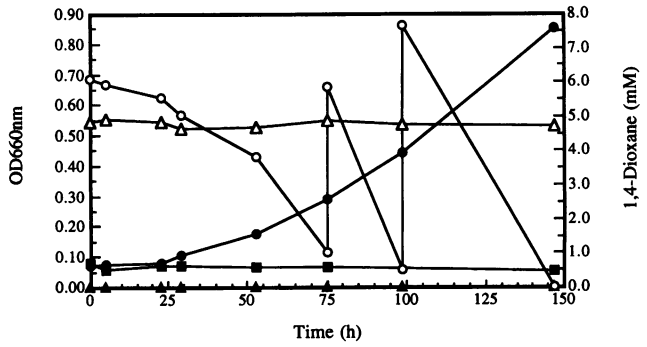

FIG. 1. Removal of 1,4-dioxane (open symbols) during growth of CB1190 (solid symbols). ● and ○, CB1190 with 1,4-dioxane; ■, CB1190 with no carbon source; ▲ and △, uninoculated controls. Additional 1,4-dioxane was provided to the growing culture at 75 and 100 h.

RESULTS

Isolation and identification of CB1190. The 1,4-dioxane-degrading organism (designated CB1190) was judged to be pure after microscopic observation of cell suspensions (magnification, ×1,000) and visual observation of colonies on AMS plates containing 20 mM 1,4-dioxane, AMS containing 10 mM succinate, AMS with no carbon source, Luria agar, and Actinomycete Isolation Agar (Difco). No diffusible pigments or sporangia were observed after growth on any solid medium. The nonmotile cells were 1 μm in diameter and highly variable in length and degree of branching. The substrate mycelium typically fragmented into various rod and coccoid elements. CB1190 was catalase positive and oxidase negative.

Fatty acids characteristic of actinomycetes (16, 17), including saturated and unsaturated, iso- and anteisobranched fatty acids, as well as 10-methyl-branched fatty acids were identified in analyses performed on CB1190 by Panlabs, Inc. (Bothell, Wash.) and Microbial ID (Newark, Del.). These characteristics are consistent with those of a nocardioform actinomycete (18).

Further attempts to identify CB1190 by fatty acid analyses (Panlabs, Inc.; Microbial ID) or by menaquinone and cell wall sugar analyses (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) did not lead to a definitive genus assignment. All investigators agreed (on the basis of morphological and chemotaxonomic characterizations) that CB1190 is a pure culture of a nocardioform actinomycete and is probably a member of the family *Pseudonocardiaceae* (11). The pure CB1190 culture was deposited with the American Type Culture Collection as strain 55486.

Degradation of 1,4-dioxane by the pure culture. To demonstrate growth of CB1190 on 1,4-dioxane as the sole carbon and energy source, CB1190 was inoculated into sealed flasks containing AMS plus 1,4-dioxane and AMS with no carbon source (Fig. 1). 1,4-Dioxane removal and an increase in OD were seen only in flasks containing 1,4-dioxane and CB1190. No abiotic loss of 1,4-dioxane was observed in uninoculated controls, and no change in OD was seen in carbon-free controls. CB1190 had a doubling time of approximately 30 h when grown at 30°C in AMS minimal medium (pH 7.0) containing approximately 5.5 mM 1,4-dioxane.

Assays of cell suspensions of CB1190 with 5.5 mM 1,4-

TABLE 1. Growth rates for CB1190 on various substrates

Substrate	Generation time (h)
Ethers	
1,4-Dioxane	30
1,3-Dioxane	42
THF	11
Tetrahydropyran	23
2-Methyl-1,3-dioxolane	23
Butyl methyl ether	23
Diethyl ether	25
Nonethers	
Ethanol	17
Isopropanol	17
1-Butanol	11
2-Butanol	17
β-Hydroxybutyrate	12
Glucose	32
$H_2 + CO_2$	67

dioxane at 30°C in sealed bottles demonstrated CB1190 to have a specific activity for dioxane degradation of 0.33 mg of dioxane min^{-1} mg of $protein^{-1}$. Under the same conditions, the mixed culture (CB1185) had a specific activity of 0.24 mg min^{-1} mg of $protein^{-1}$. In these assays and with growing cultures of CB1190, 1,4-dioxane was degraded to <0.55 μM as determined by GC-mass spectrometry in the single-ion monitoring mode.

Mineralization of 1,4-dioxane by CB1190. Conversion of 1,4-dioxane carbon to CO_2 by CB1190 was tested in sealed 160-ml serum bottles. The cultures were grown in BSM containing 1,4-dioxane, washed, resuspended in BSM, and incubated with or without 4.0 mM 1,4-dioxane. After 18 h, dioxane was degraded to <10 μM, and CO_2 in the liquid and headspace of the cultures was measured by GC-thermal conductivity detector analysis. The production level of endogenous CO_2 was similarly determined in the cultures that did not receive dioxane. The average of three replicate samples showed that 59.5% ± 1.8% of the dioxane carbon consumed (345.8 ± 0.0 μmol) was converted to CO_2 (205.8 ± 5.8 μmol [322.5 ± 5.8 μmol of C from total CO_2 production minus 116.7 ± 0.0 μmol of C from endogenous CO_2]). No other products were observed by GC-flame ionization detection or GC-thermal conductivity detector analysis. COD analysis of the spent medium showed no detectable soluble COD. These results indicate that 1,4-dioxane is mineralized by CB1190 and strongly suggest that no other organic product (other than biomass) accumulated in the medium.

Substrate range of CB1190. In addition to 1,4-dioxane, CB1190 was capable of growth with a variety of cyclic and linear ethers as sole carbon sources (Table 1). The fastest growth rate on ethers tested was observed with THF. CB1190 did not grow on the following ethers: 1,3-dioxolane, 1,3,5-trioxane, isopropyl ether, *tert*-butyl methyl ether, *tert*-butyl ethyl ether, diethylene glycol, and ethylene glycol dimethyl ether. CB1190 was able to grow on several nonether compounds (Table 1). Autotrophic growth on hydrogen and carbon dioxide was sustained with a 60:25:10:5 ratio of H_2-N_2-CO_2-O_2. Nonether substrates that did not support growth of CB1190 included morpholine, cyclohexane, cyclopentane, toluene, citrate, formate, fumarate, maleate, succinate, propionate, glutamate, starch, lactose, fructose, maltose, asparagine, and Casamino Acids. CB1190 also grew very poorly or not at all on most types of rich media tested, including Luria agar, brain heart infusion (Difco), and tryptic soy agar (Difco).

The pH optimum for CB1190 growing at 30°C with 1,4-dioxane was 6.0, although the organism was capable of growth in the pH range of 5.0 to 8.0. The optimum temperature for CB1190 growing in AMS with 1,4-dioxane was about 30°C, and no growth on or degradation of 1,4-dioxane was observed at temperatures higher than 42°C. The addition of up to 2.0% (wt/vol) NaCl to CB1190 cultures had no effect on growth or dioxane degradation. Some growth and some degradation were observed with up to 4.0% NaCl, but no activity was seen with 8.0% NaCl.

DISCUSSION

Strain CB1190 is the first reported pure culture demonstrating sustained growth on 1,4-dioxane as the sole carbon and energy source. This organism was isolated from THF enrichments that were gradually transferred onto 1,4-dioxane. As has been observed by other workers (3), direct enrichments on 1,4-dioxane were unsuccessful, and our early enrichments appeared to be cometabolizing 1,4-dioxane in the presence of THF. While long-term enrichments eventually yielded a culture capable of growth on 1,4-dioxane alone, THF still appears to be the preferred growth substrate for CB1190. It is possible that we have isolated a mutant THF-degrading organism that can now be maintained on 1,4-dioxane as a result of regulatory, transport, and/or enzyme substrate range alterations.

Although a complete mass balance has not been determined, we have demonstrated that more than 50% of the carbon from 1,4-dioxane is converted to CO_2 by CB1190 and no organic products other than biomass accumulated in the growth medium, indicating that 1,4-dioxane is mineralized by CB1190. Since no intermediates of 1,4-dioxane degradation could be detected in spent medium, we have no information from which to construct a pathway for 1,4-dioxane degradation. In a previous report, a hypothetical pathway for THF degradation by a *Rhodococcus* sp. based on substrate utilization (3) was presented, and several products of 1,4-dioxane biotransformation by *M. vaccae* have been identified (24). Both reports suggested that the dioxane ring is hydroxylated prior to cleavage.

The taxonomy of actinomycetes has been rapidly evolving. New genera have recently been introduced, and many reclassifications have occurred (4, 11, 14, 19, 22, 25). Actinomycete classification, which had been based primarily on morphology, has been supplemented by chemotaxonomic characterization and, more recently, rRNA sequence comparisons. On the basis of morphology, growth properties, and fatty acid analyses, CB1190 may be closely related to members of the genera *Amycolata* and *Amycolatopsis*. Two strains of each genus obtained from the American Type Culture Collection (*Amycolata hydrocarbonoxydans* ATCC 15104, *Amycolata autotrophica* ATCC 19727, *Amycolatopsis mediterranei* ATCC 27643, and *Amycolatopsis orientalis* ATCC 19795) were not capable of growth on 1,4-dioxane; however, growth with THF was not tested. It is likely that rRNA sequencing will be necessary to determine whether CB1190 is a member of a known genus or should receive a new designation.

CB1190 has been shown to have the ability to grow on and mineralize 1,4-dioxane under a wide variety of conditions (1,4-dioxane concentration, pH, and salt concentration). The organism is also capable of degrading other ethers such as THF and 2-methyl-1,3-dioxolane that are likely to be found in sites contaminated with 1,4-dioxane. The identification of CB1190 has the potential to lead to a practical and economical process for the bioremediation of 1,4-dioxane-contaminated groundwaters and waste streams.

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