APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1993, p. 1504–1506
0099-2240/93/051504-03$02.00/0

Vol. 59, No. 5

Microbial Oxidation of Dimethylnaphthalene Isomers

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Received 9 November 1992/Accepted 1 March 1993

Three bacterial strains, identified as *Alcaligenes* sp. strain D-59 and *Pseudomonas* sp. strains D-87 and D-186, capable of growing on 2,6-dimethylnaphthalene (2,6-DMN) as the sole source of carbon and energy were isolated from soil samples. 2,6-Naphthalene dicarboxylic acid was formed in the culture broths of these three strains grown on 2,6-DMN. In addition, 2-hydroxymethyl-6-methylnaphthalene and 6-methylnaphthalene-2-carboxylic acid were detected in the culture broth of strain D-87. Strain D-87 grew well on 1,2-, 1,3-, 1,4-, 1,5-, 2,3-, and 2,7-DMN as the sole source of carbon and energy and accumulated 2-methylnaphthalene-3-carboxylic acid and 2,3-naphthalene dicarboxylic acid from 2,3-DMN, 4-methylnaphthalene-1-carboxylic acid from 1,4-DMN, and 7-methylnaphthalene-2-carboxylic acid from 2,7-DMN.

There have been many studies on the microbial oxidation and degradation of the methyl group on the benzene ring. The methyl group of toluene is oxidized to benzoic acid by the TOL pathway (10, 11). Xylene, *p*-cymene, and methylnaphthalene are converted to toluic acid, cumic acid, and naphthoic acid, respectively (1, 7, 9). However, there have been very few studies on the microbial oxidation and metabolism of dimethylnaphthalenes (DMNs), which are supplied from the light oil fraction of crude oil.

Co-oxidation of 1,3-, 1,6-, 2,3-, 2,6-, and 2,7-DMN to methylnaphthalene carboxylic acid, using *Nocardia coralina* (*Rhodococcus* sp.) A-6 and V-49 grown on aliphatic hydrocarbons, was reported earlier (4, 8). Degradation of 2,6-DMN with *Flavobacterium* sp. strain B233 grown on 2,6-DMN as the sole source of carbon and energy was reported (2). Barnsley (2) reported the oxidation of 2,6-DMN to 6-methylnaphthalene-2-carboxylic acid by strain B233 and the detection of 2-hydroxymethyl-6-methylnaphthalene, 6-methylnaphthalene-2-aldehyde, and 6-methylnaphthalene-2-carboxylic acid. He proposed a metabolic pathway for 2,6-DMN to 6-methylnaphthalene-2-carboxylic acid and further oxidation to 1-hydroxy-6-methylnaphthalene-2-carboxylic acid without the formation of 2,6-naphthalene dicarboxylic acid (NDCA). There have been no reports of the bacterial conversion of 2,6-DMN to 2,6-NDCA, which is used for polymers and liquid crystals. In this report, we describe the isolation of 2,6-DMN-utilizing bacteria, the identification of the isolates, the accumulation of 2,6-NDCA from 2,6-DMN, the identification of some metabolic intermediates of 2,6-DMN, and a proposal of a metabolic pathway of 2,6-DMN.

MATERIALS AND METHODS

Medium. For the screening and cultivation of 2,6-DMN-utilizing bacteria, a mineral salt medium was used. The mineral salt medium (MM) contained the following, per liter: $Na_2HPO_4 \cdot 12H_2O$, 1.2 g; KH_2PO_4, 0.4 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; Na_2CO_3, 0.1 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot nH_2O$, 20 mg; $CaCl_2 \cdot 2H_2O$, 10 mg. The pH of the medium was adjusted to 7.0. Sterilized 2,6-DMN (1.5 g) was added. Sterilized 2,6-DMN was prepared by the following method. An ethereal solution of 2,6-DMN was passed through a 0.2-μm-pore-size filter to a sterile mortar, and the ether was allowed to evaporate. 2,6-DMN was crushed into powder with a mortar and pestle.

Isolation of microorganisms. The isolation of a bacterial strain capable of growing on 2,6-DMN as the sole source of carbon and energy was carried out by the following method (6). Soil samples (0.1 to 0.5 g) were added to 21-mm test tubes containing 10 ml of MM and 15 mg of 2,6-DMN as the sole source of carbon and energy. Reciprocal shaking cultures were incubated for 1 week at 30°C. After incubation, three loopfuls of the resulting suspensions were transferred several times to fresh medium. The isolates which passed the purity test were maintained on a slant agar medium. For the purity test, a mineral salt medium solidified with 1.5% agar was used, and 2,6-DMN was provided in the lid.

Identification of isolated microorganisms. Identification was carried out by the methods described in the *Manual of Methods for General Bacteriology* (3), and organisms were classified in accordance with *Bergey's Manual of Systematic Bacteriology* (5).

Culture conditions. Cells were grown in 21-mm test tubes containing 10 ml of MM and 15 mg of DMN or in 2-liter shaking flasks containing 1 liter of MM and 1.5 g of DMN. The purity of DMN isomers was checked by gas chromatography (GC). The test tubes or flasks were incubated with shaking at 30°C for 1 week.

To examine the intermediates of 2,6-DMN degradation, cells grown in 1 liter of MM were harvested by centrifugation and washed with 50 mM potassium phosphate buffer (pH 7.0). The washed cells were then poured into a 2-liter shaking flask with 1 g of 2,6-DMN. The flask was incubated with shaking at 30°C for 2 h.

Preparation of samples for analyses. In the preparation of samples for high-pressure liquid chromatography (HPLC), the cultures were centrifuged and passed through a 0.22-μm-pore-size filter. For GC-mass spectrometry (GC/MS) analysis of NDCA and the products of the DMN isomers, culture broths were combined and the cells were removed by centrifugation. The supernatant was acidified to pH 2.0 by HCl and extracted two times with equal volumes of ethyl acetate. This extract was dried over anhydrous Na_2SO_4 and concentrated with a vacuum evaporator. The residue was

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dissolved in chloroform and treated with an ethereal solution of diazomethane.

For GC/MS analysis of the metabolic intermediates of 2,6-DMN, the cells were removed after 2 h of incubation and the supernatant was extracted twice with equal volumes of ethyl acetate (neutral fraction). The aqueous layer was acidified to pH 2.0 by HCl and reextracted twice (acid fraction). The neutral and acid fractions were dried and analyzed without methylation.

Analytical procedures. The production of 2,6-NDCA was measured with a reversed-phase HPLC (LC-6AD; Shimadzu, Kyoto, Japan) equipped with a UV detector (wave length, 283 nm) column (6 by 150 mm; Shim-pack CLC-ODS; Shimadzu), using equal volumes of methanol-water adjusted to pH 2.25 by H_3PO_4 at a flow rate of 1.0 ml/min.

NDCA and related compounds were analyzed on a GC/MS (5971B; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a DB-WAX capillary column (length, 30 m; inner diameter, 0.25 mm). The operating conditions were as follows: column temperature, 60 to 230°C at 5°C/min; electron impact, 70 eV; and injector and detector temperatures, 250 and 280°C.

6-Methylnaphthalene-2-carboxylic acid and 2-hydroxymethyl-6-methylnaphthalene were analyzed with the same GC/MS system described above, except that a polyethylene glycol 20 M column (length, 25 m; inner diameter, 0.2 mm) was used, with a column temperature of 50 to 210°C.

The nuclear magnetic resonance (NMR) spectra were recorded at 90 MHz for ^1H in dimethyl sulfoxide-d_6 on a JEOL FX-90Q instrument with tetramethylsilane as the internal reference.

Oxygen uptake was measured with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) in a vessel (3 ml) held at a constant temperature (30°C). Oxygen uptake rates were corrected for endogenous respiration.

RESULTS AND DISCUSSION

Isolation and identification of bacteria. Two hundred sixty strains of microorganisms which grew on 2,6-DMN as the sole source of carbon were isolated from 530 soil samples. Three isolates, strains D-59, D-87, and D-186, accumulated 2,6-NDCA in the culture broth of 2,6-DMN. Strain D-59 was identified as the genus *Alcaligenes* and D-87 and D-186 were identified as the genus *Pseudomonas* on the basis of the following morphological and biochemical properties. Strains D-59, D-87, and D-186 were straight gram-negative rods (0.5 by 1.0 μm), oxidase and catalase positive, and urease negative, and formation of H_2S and indole was negative. Strain D-59 was motile by peritrichous flagella. Strains D-87 and D-186 were motile by polar flagella.

Substrate specificity of strain D-87 for DMN isomers. The substrate specificity of strain D-87 was examined with 1,2-, 1,3-, 1,4-, 1,5-, 1,8-, 2,3-, 2,6-, and 2,7-DMN. Strain D-87 grew on seven of the isomers (including 2,6-DMN) as the sole source of carbon and energy, but no growth was observed on 1,8-DMN.

Identification of 2,6-NDCA. After cultivation of strains D-59, D-87, and D-186 on 2,6-DMN, samples prepared for HPLC were analyzed. One of the peaks on HPLC had the same retention time of 10.1 min as authentic 2,6-NDCA.

After the incubation of strain D-87 on 2,6-DMN, the sample prepared for GC/MS was analyzed. The methylated metabolite had a molecular ion peak at m/z = 244, which corresponds to a molecular formula of $C_{14}H_{12}O_4$, and fragment ions as follows: m/z 213 (M^+ —OCH_3), 185 (M^+ —$COOCH_3$), 154 (M^+ —OCH_3, —$COOCH_3$), and 126 (M^+ —$COOCH_3$, —$COOCH_3$). These data are consistent with NDCA dimethyl ester (Fig. 1a). The positions of the two carboxylic acid groups of 2,6-NDCA were determined by the ^1H NMR spectrum of the ring protons. The spectrum showed two types of protons, δ ppm = 8.2 (4H, d) and δ ppm = 8.8 (2H, s), demonstrating that both of the methyl groups of 2,6-DMN were oxidized to carboxylic groups to form 2,6-NDCA. The formation of 2,6-NDCA from 2,6-DMN by strain D-87 indicates the presence of a metabolic pathway of 2,6-DMN different from that described by Barnsley. The concentration of 2,6-NDCA in the culture broth was 4.5 mg/liter, which was determined with HPLC. In addition, 2-hydroxymethyl-6-methylnaphthalene and 6-methylnaphthalene-2-carboxylic acid were detected as metabolic intermediates of 2,6-DMN. The sample of the neutral fraction had a molecular ion peak at m/z 172, which corresponds to a molecular formula of $C_{12}H_{12}O$, and fragment ions at m/z 143, 129, and 115. These are characteristic fragments of naphthyl alcohol. These data are consistent with 2-hydroxymethyl-6-methylnaphthalene (Fig. 1b). The sample of the acid fraction had a molecular ion peak at m/z 186, which corresponds to a molecular formula of $C_{12}H_{10}O_2$, and fragment ions at m/z 169 (M^+ —OH), 141 (M^+ —COOH), and 126 (M^+ —COOH, —CH_3). These data are consistent with 6-methylnaphthalene-2-carboxylic acid (Fig. 1c).

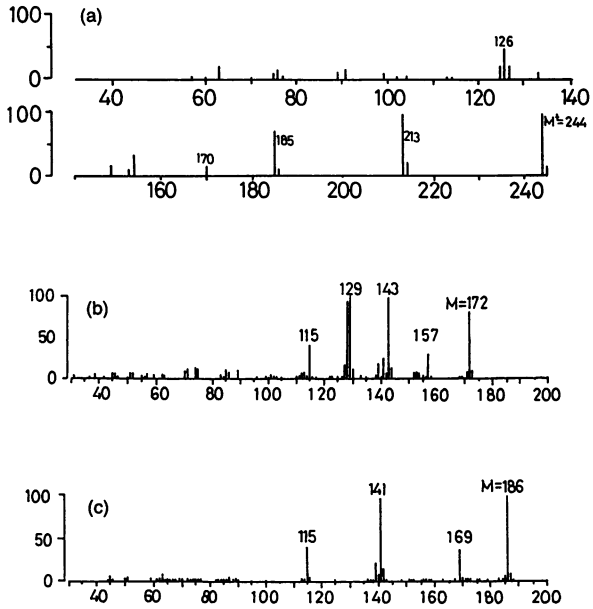

FIG. 1. Mass spectra of (a) the methylated product from 2,6-DMN by strain D-87 and of metabolic intermediates of 2,6-DMN, (b) product from neutral fraction, and (c) product from acid fraction.

Oxidation of DMN isomers. Strain D-87 grew on DMN isomers, and depending on the turbidity of growth of strain D-87 on the DMN isomers, the products from 2,3-, 1,4-, 2,7-, and 1,2-DMN were analyzed.

Two metabolites were obtained from 2,3-DMN. One of the methylated metabolites had a molecular ion peak at m/z 244 (relative intensity, 65%), which corresponds to a molecular formula of $C_{14}H_{12}O_4$, and fragment ions at m/z 213 (100%, M^+ —OCH_3), 185 (37%, M^+ —$COOCH_3$), 154 (19%, M^+ —$COOCH_3$ —OCH_3), and 126 (26%, M^+ —$COOCH_3$ ×2). Another had a molecular ion peak at m/z 200 (96%), which

corresponds to a molecular formula of $C_{13}H_{12}O_2$, and fragment ions at *m/z* 185 (6%, $M^+ - CH_3$), 169 (100%, $M^+ - OCH_3$), 141 (96%, $M^+ - COOCH_3$), and 126 (10%, $M^+ - COOCH_3 - CH_3$). These data are consistent with those of mass spectra of 2,3-NDCA dimethyl ester and 3-methylnaphthalene-2-carboxylic acid methyl ester, respectively.

The methylated metabolite from 1,4-DMN had a molecular ion peak at *m/z* 200 (63%), which corresponds to a molecular formula of $C_{13}H_{12}O_2$, and fragment ions at *m/z* 185 (2%, $M^+ - CH_3$), 169 (100%, $M^+ - OCH_3$), 141 (48%, $M^+ - COOCH_3$), and 126 (3%, $M^+ - COOCH_3 - CH_3$). The formation of 4-methylnaphthalene-1-carboxylic acid was determined with ^1H and ^{13}C NMR spectra: ^1H NMR δppm = 2.72 (s, 3H, CH_3Ph), 7.34 (d, 1H, Ph), 7.52 to 7.61 (m, 2H, Ph), 8.03 (d, 1H, Ph), 8.18 (d, 1H, Ph), 9.01 (br s, 1H, Ph COOH); ^{13}C NMR δppm = 20.09 (CH_3Ph), 124 to 140 (10 peaks, naphthalene), and 170.36 (Ph COOH).

A further attempt to identify the proton and carbon resonances by analysis of the ^1H-^1H COSY NMR spectrum, measured in chloroform-d_1 (0.4 ml) and dimethyl sulfoxide-d_6 (0.1 ml), was made. The spectrum shows correlations between δ = 7.3 and δ = 8.2, δ = 7.54 and δ = 7.59, δ = 7.54 and δ = 8.0, and δ = 7.59 and δ = 9.1. These data are consistent with identification as 4-methylnaphthalene-1-carboxylic acid.

The methylated metabolite from 2,7-DMN had a molecular ion peak at *m/z* 200 (98%), which corresponds to a molecular formula of $C_{13}H_{12}O_2$, and fragment ions at *m/z* 185 (1%, $M^+ - CH_3$), 169 (100%, $M^+ - OCH_3$), 141 (77%, $M^+ - COOCH_3$), and 126 (3%, $M^+ - COOCH_3 - CH_3$). These data are consistent with those of 7-methylnaphthalene-2-carboxylic acid methyl ester.

The methylated metabolite from 1,2-DMN had a molecular ion peak at *m/z* 200 (69%), which corresponds to a molecular formula of $C_{13}H_{12}O_2$, and fragment ions at *m/z* 185 (6%, $M^+ - CH_3$), 169 (100%, $M^+ - OCH_3$), 141 (77%, $M^+ - COOCH_3$), and 126 (6%, $M^+ - COOCH_3 - CH_3$). These data are consistent with those of methylnaphthalene carboxylic acid methyl ester.

Strain D-87 grew on and oxidized 1,2-, 2,3-, 1,4-, 2,6-, and 2,7-DMN to the corresponding methylnaphthalene carboxylic acid and 2,3- and 2,6-NDCA. Raymond et al. (8) reported the co-oxidation of 1,3-, 1,6-, 2,3-, 2,6-, and 2,7-DMN to the corresponding methylnaphthalene carboxylic acid by a *Rhodococcus* sp. In contrast with the *Rhodococcus* sp., which could not co-oxidize 1,4-DMN to 4-methylnaphthalene-1-carboxylic acid, strain D-87 formed 4-methylnaphthalene-1-carboxylic acid from 1,4-DMN. Although it was reported by Raymond et al. (8) that the oxidation of the methyl group of DMN occurs mainly at the methyl group of the β-position, strain D-87 oxidizes not only the methyl group of the β-position but also the α-position, which was demonstrated by the oxidation of 2,6-DMN and 1,4-DMN by strain D-87.

Proposed metabolic pathway of 2,6-DMN. Strain D-87 grew on 2,6-DMN as the sole source of carbon and energy and oxidized 2,6-DMN to form 2-hydroxymethyl-6-methylnaphthalene, 6-methylnaphthalene-2-carboxylic acid, and 2,6-NDCA in a culture broth of 2,6-DMN. The conversion of 2,6-DMN to 2,6-NDCA indicated the presence of a new metabolic pathway of 2,6-DMN degradation (Fig. 2). Strain D-87 showed no oxygen uptake on 2,6-NDCA, showing that 2,6-NDCA is a dead-end product. The problem with the pathway obtaining carbon and energy is the same as with Barnsley's pathway and remains to be solved.

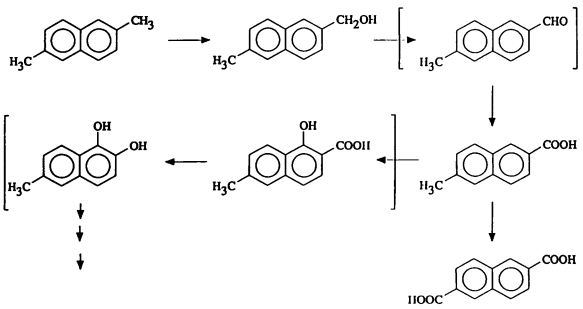

FIG. 2. Proposal for a metabolic pathway of 2,6-DMN in strain D-87. Structures are as follows (see arrows): 2,6-DMN, 2-hydroxymethyl-6-methylnaphthalene, 6-methylnaphthalene-2-aldehyde, 6-methylnaphthalene-2-carboxylic acid, 2,6-NDCA, 1-hydroxynaphthalene-2-carboxylic acid, and 1,2-dihydroxy-6-methylnaphthalene.

The strain D-87 enzymes involved in the degradation of DMNs have interesting characteristics. Further characterization of these properties is under way.

ACKNOWLEDGMENT

We thank the Petroleum Energy Center for financial support.

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