Evidence for the involvement of multiple pathways in the biodegradation of 1- and 2-methylnaphthalene by Pseudomonas putida CSV86

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Abstract. Pseudomonas putida CSV86, a soil bacterium, grows on 1- and 2-methylnaphthalene as the sole source of carbon and energy. In order to deduce the pathways for the biodegradation of 1- and 2-methylnaphthalene, metabolites were isolated from the spent medium and purified by thin layer chromatography. Emphasis has been placed on the structural characterisation of isolated intermediates by GC-MS, demonstration of enzyme activities in the cell free extracts and measurement of oxygen uptake by whole cells in the presence of various probable metabolic intermediates. The data obtained from such a study suggest the possibility of occurrence of multiple pathways in the degradation of 1- and 2-methylnaphthalene. We propose that, in one of the pathways, the aromatic ring adjacent to the one bearing the methyl moiety is oxidized leading to the formation of methylsalicylates and methylcatechols. In another pathway the methyl side chain is hydroxylated to -CH2_OH which is further converted to -CHO and -COOH resulting in the formation of naphthoic acid as the end product. In addition to this, 2-hydroxymethylnaphthalene formed by the hydroxylation of the methyl group of 2-methylnaphthalene undergoes aromatic ring hydroxylation. The resultant dihydrodiol is further oxidised by a series of enzyme catalysed reactions to form 4-hydroxymethyl catechol as the end product of the pathway.

Key words: Pseudomonas – Methylnaphthalenes – Metabolism – Hydroxylation – Oxygenases – Aromatic ring cleavage

Polycyclic aromatic hydrocarbons (PAHs) are compounds of environmental and human health concern, since some of the PAHs and their biotransformation products have been shown to be toxic, mutagenic and carcinogenic in nature (Griffin et al. 1983). Naphthalene and methyl substituted naphthalenes are among the most toxic components in the water soluble fraction of crude and fuel oils (Boylan and Tripp 1971; Lee et al. 1974; Winters et al. 1976). Naphthalene is widely used as a moth repellent and methylnaphthalenes along with naphthalene have been detected in commercial mosquito repellents (Wirtz et al. 1981) and aromatic solvents (Sparling et al. 1978). Exposure to naphthalene and methylnaphthalenes has been reported to cause a decrease in haemoglobin concentration, inhibition of oxygen consumption and pulmonary damage in various experimental organisms (Darville and Wilhm 1984; Struble and Hermon 1983; Honda et al. 1990).

Pseudomonads have been shown to degrade methylnaphthalenes by oxidation of the aromatic ring giving rise to methylcatechols as end products (Dean-Raymond and Bartha 1975; Williams et al. 1975; Cane and Williams 1982). In the present study, we demonstrate the occurrence of multiple pathways in the degradation of 1- and 2-methylnaphthalene by a newly isolated strain of Pseudomonas putida CSV86. One pathway serves as the sole source of carbon and energy by hydroxylating one of the aromatic rings and its subsequent oxidation to methylcatechols. In the second pathway, the methyl group is hydroxylated to form hydroxymethylnaphthalene which in turn is converted to naphthoic acid as the end product. In addition, the degradative pathway for 2-methylnaphthalene also proceeds with the simultaneous hydroxylation of the aromatic ring and the methyl moiety.

Materials and methods

Media

A mineral salt medium (pH 7.0) prepared in deionised water was used throughout these studies. Its composition per litre is as follows: K2HPO4, 6.3 g; KH2PO4, 1.8 g; NH4NO3, 1.0 g; FeSO4·7H2O, 5.0 mg; MgSO4·7H2O, 100 mg; MnSO4·H2O, 1.0 mg;
CuSO$_4$$\cdot$5H$_2$O, 1.0 mg; H$_2$BO$_3$, 1.0 mg; CaCl$_2$$\cdot$2H$_2$O, 5.0 mg; ZnSO$_4$$\cdot$7H$_2$O, 1.0 mg; Na$_2$MoO$_4$, 1.0 mg. Fine powder of 1- or 2-methylnaphthalene was used as a carbon source for the bacterial culture.

**Culture**

Enrichment culture technique was used to isolate the bacterium from the soil. It was identified as *Pseudomonas putida* CSV86. It could grow on naphthalene and methylnaphthalenes as the sole source of carbon and energy.

**Cell suspensions and cell free extracts**

For respiration studies, the cells were grown in 200-ml lots of mineral salt medium in 500/ml flasks with shaking at 30°C. Either 1- or 2-methylnaphthalene was used as a carbon source. The cells were collected by centrifugation at 12,000 × g for 10 min, washed twice with 50 mM phosphate buffer (K$_2$HPO$_4$-KH$_2$PO$_4$) pH 7.5 and suspended in the same buffer at a concentration of 100 mg of wet cells/ml.

For the preparation of cell free extracts, cells were grown at 30°C in a Biostat-E fermenter containing 5 l of mineral salt medium supplemented with 0.15% 1- or 2-methylnaphthalene. The cells were passed through a Miracloth to remove unutilized substrate and harvested on a continuous flow rotor at 3000 × g, washed twice with 50 mM phosphate buffer pH 7.5 and stored as a pellet at -20°C until further use. The frozen pellet was thawed on ice and suspended in 50 mM phosphate buffer containing 10% ethanol (v/v) and 10% glycerol (v/v). The cell suspension was sonicated at 4°C in six 10-s bursts using a Branson B-30 sonifier and subjected to successive centrifugation at 27,000 × g for 30 min and 100,000 × g for 1 h. The clear supernatant obtained was termed as cell free extract. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

**Respiration rate measurements**

Measurements were made polarographically on a Gilson Oxigraph fitted with Clark’s O$_2$ electrode. One ml reaction mixture contained 50 nmol phosphate buffer pH 7.5, 100 nmol of substrate in 10 μl of tetrahydrofuran and appropriate amount of cells. Oxygen uptake rates are expressed as nmol of O$_2$ consumed/min mg of cells. The values are corrected for endogenous respiration and small stimulation caused by the organic solvent.

**Enzyme assays**

The activity of naphthalene dioxygenase was monitored by measuring the rate of formation of [3H] naphthalene dihydrodiol from [3H] naphthalene as described previously by Ensley et al. (1982). One unit of enzyme activity is defined as the amount of protein required to form 1 nmol of product per min. cis-Naphthalene dihydrodiol dehydrogenase was measured spectrophotometrically by following the reduction of NAD$^+$ at 340 nm (Patel and Gibson 1974); one unit is defined as 1 μmol of NAD$^+$ reduced per min. Salicylate hydroxylase activity was measured polarographically at 30°C (Barnsley 1975) and one unit of enzyme activity is defined as 1 μmol of oxygen consumed per min. Catechol 2,3-dioxygenase was assayed spectrophotometrically by monitoring the appearance of different semialdehydes from catechol, 3- and 4-methyl catechols which have a ρ max at 375, 388 and 382 nm, respectively (Bayly et al. 1966). One unit of enzyme activity is defined as 1 μmol of product formed per min. Specific activities of all the enzymes are expressed as units per mg of protein.

**Isolation of metabolites**

The bacterium was grown in a Biostat-E fermenter (Braun, Melsungen, Germany) containing 4 l of mineral salt medium supplemented with 0.15% of either 1- or 2-methylnaphthalene for 28 h and 20 h, respectively, at 30°C with agitation at 250 rpm. Sterile air was pumped at the rate of 10 l/min. Cells were harvested by centrifugation on a continuous flow rotor at 7000 × g. The spent medium was acidified to pH 2.0 with 6 N HCl and the metabolites were extracted in equal volumes of ethyl acetate, dried over anhydrous sodium sulfate and evaporated in vacuo at 35°C. The concentrate was dissolved in a small amount of ethyl acetate and the metabolites were purified by thin layer chromatography on silica gel-G coated glass plates (20 × 20 cm size, 0.5 mm thick silica gel-G coating), using chloroform: acetone (4:1 v/v) and cyclohexane: chloroform:acetone (4:1:1 v/v) as solvent systems for 1- and 2-methylnaphthalene metabolites, respectively.

**Physical techniques employed**

Gas chromatography-mass spectrometry. A JOEL JMS DX-300 mass spectrometer attached to a gas chromatograph was used for the structural characterization of TLC purified metabolites.

**GC analysis of 1-methylnaphthalene metabolites.** The capillary column SE-30 of 30 m length was used. Temperature gradient during the run was 100°C to 200°C at a rate of 16°C/min (hold time at 200°C for 2 min) and 200°C to 250°C at a rate of 8°C/min (hold time at 250°C for 20 min). Helium was used as a carrier gas at the rate of 20 ml/min. Samples were dissolved in 50 μl of ethyl acetate and 5 μl was injected into the column.

**GC analysis of 2-methylnaphthalene metabolites.** The capillary column SE-30 of 0.25 mm × 50 m length was used. Temperature gradient during the run was 50°C to 230°C at a rate of 4°C/min. Helium gas was used as a carrier gas at the rate of 30 ml/min. Samples were dissolved in 50 μl of ethyl acetate and 5 μl was injected into the column.

**Mass spectrometry.** The conditions for the mass spectrometric analysis of the metabolites were: ionization at 70 eV, scan speed: 1 s, Resolution: 1000 (on low resolution mode), mass range: 50-500 m/z, chamber temperature: 150°C. Data acquisition was done on an SM-23 computer. Direct probe mass spectrometry was performed on samples which gave a single peak on GC.

**Results**

**Identification of 1-methylnaphthalene metabolites**

The metabolites extracted from the spent medium resolved into five distinct spots on TLC using chloroform-acetone (4:1 v/v).

| Table 1. Separation of 1-methylnaphthalene metabolites by thin layer chromatography using the solvent system chloroform:acetone (4:1 v/v) |
|-----------------|-----------------|-----------------|-----------------|
| TLC spots       | R$_f$ values    | UV fluorescence | Color reaction  |
|                 |                 |                 | with Gibbs’ reagenta |
| I               | 0.95            | Blue            | –               |
| II              | 0.83            | Green           | Brick red       |
| III             | 0.69            | Blue            | Yellow          |
| IV              | 0.55            | Blue            | Yellow          |
| V               | 0.33            | Pale blue/brown | Brown           |

a Gibbs’ reagent: 0.05% v/v 2,6-dichloroquinone-4-chloroimide in methanol followed by spraying with aqueous saturated NaHCO$_3$,