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Microbial Hydrocarbon Co-oxidation

III. Isolation and Characterization of an α,α'-Dimethyl-*cis*,*cis*-Muconic Acid-producing Strain of *Nocardia corallina*

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A soil isolate identified as a strain of *Nocardia corallina* accumulated α,α'-dimethyl-*cis*,*cis*-muconic acid under co-oxidation conditions employing *n*-hexadecane for growth and *p*-xylene as the co-oxidizable substrate. *N. corallina* V-49 was postulated to have two pathways for the oxidation of *p*-xylene. One pathway proceeds through *p*-benzyl alcohol, *p*-tolualdehyde, and *p*-toluic acid to 2,3-dihydroxy-*p*-toluic acid, and the other pathway results in ortho ring cleavage of 3,6-dimethylpyrocatechol and hence accumulation of α,α'-dimethyl-*cis*,*cis*-muconic acid.

Many investigators have recently reported on the oxidation of simple aromatic ring structures by the genera *Pseudomonas* and *Mycobacterium*. In 1967, Atkinson and Newth (1) reported the oxidation of *p*-xylene to *p*-toluic acid (PTA) by *M. rhodochrous*. The yields were low and no evidence of ring cleavage was found. The work of Davis et al. (2) with *Pseudomonas* cultures demonstrated that the pathway of *p*-xylene oxidation proceeds through PTA, which is decarboxylated to 4-methylcatechol followed by meta ring cleavage to the 2-hydroxy-5-methylmuconic semialdehyde. Leavitt (5), with whole cells and soluble extracts of a *Pseudomonas* species, proposed a pathway for the oxidation of *p*-isopropyltoluene to *p*-isopropylbenzoic acid. He showed that the attack was only on the methyl group and that the ring was left intact. Very recently Gibson et al. (4) elucidated the initial attack on benzene which results in *cis*-benzene glycol as an intermediate to catechol formation.

In their co-oxidation studies, Raymond et al. (6) found that several *Nocardia* strains could carry out *o*-dihydroxylation of the ring. No evidence for ring cleavage was found. In the studies reported here, we found that a strain of *N. corallina* probably has two pathways for the oxidation of *p*-xylene. One of these pathways is identical to that descibed in the study mentioned above (6), whereas the other results in ortho ring cleavage of 3,6-dimethylpyrocatechol (DMPC).

MATERIALS AND METHODS

Microorganisms. All of the work described was carried out with *N. corallina* V-49. This culture was isolated from soil obtained in Montgomery, Ala. To the best of our knowledge the soil had never come in contact with hydrocarbons other than those normally present in plants. An 0.1-g sample of the air-dried soil was sprinkled over the surface of a phenol-red agar plate (6), and was incubated at 30 C in the presence of *n*-hexadecane for growth, with toluene as the co-oxidizable substrate. The culture was isolated because the intense yellow spot indicated that it was a strong acid-producing organism. It was purified by the usual procedures. Identification was carried out by the methods described in the *Manual of Microbiological Methods* (8), and classification was done according to *Bergey's Manual*.

Media and culture conditions. In all cases, the isolation, washed-cell, growth studies and the co-oxidation studies employed the techniques described previously (6, 7).

A 7.5-liter fermentor (model F-07) was used in a fermentor drive assembly (model FS-600; New Brunswick Scientific Co., New Brunswick, N.J.) for the accumulation of intermediate products. Liquid medium consisted of 0.2% peptone (Difco), 0.1% Beef Extract (Difco), and basal mineral salts. Fermentation experiments were conducted with 3,000 ml of medium. Operating conditions were as follows. Aeration rate after initiation of *p*-xylene addition (36 hr) was <400 ml/min. A 480-ml amount of IRA-93 resin was used in one system, and the *p*H was maintained at 6.5 by the addition of 10% NaOH. The system with no resin was maintained at a *p*H of 7.2. The temperature was 30 C, the stirring speed was 750 rev/min, and total *p*-xylene addition was 24 ml.

The mutation experiments were carried out with phosphate buffer and wild-type cells of *N. corallina* which were exposed to ultraviolet (UV) irradiation to obtain a 99.99% kill. The irradiated organisms were plated out on Nutrient Agar (Difco) and were

compared to the wild-type for morphological differences.

Hydrocarbons. All hydrocarbons used were of a purity previously described (6).

Product identification. Thin-layer-chromatographic (TLC) plates of Silica Gel G (Analtech, Inc., Wilmington, Del.) were employed. The composition of the solvent system for the development of the plates was ethyl alcohol (100%)-water-ammonium hydroxide (28%), 100:12:16. All other methods used were as previously described (6). The identification of the *p*-tolualdehyde and *p*-methyl benzyl alcohol was carried out on a Varian aerograph gas chromatograph (model 600-B; Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) consisting of a flame ionization detector, with a 1/8 inch (0.32 cm), 10-ft (3.04 m), copper AW DMCS 5% SE54, on a Chromosorb G 60/80 mesh column. Operating conditions were: column temperature, 130 C; nitrogen flow rate, 20 ml/min. Nuclear magnetic resonance (model A-60; Varian Associate, Palo Alto, Calif.) was used in confirmation of the *cis-cis* configuration of α,α'-dimethyl-*cis*,*cis*-muconic acid (DMMA). Further confirmation of the configuration was achieved by preparing the *cis-trans* and *trans-trans* acids.

Synthesis of DMMA. DMMA was prepared by the method of Elvidge et al. (3).

Synthesis of DMPC. DMPC was synthesized by the following procedure. A mixture of 0.7 g of 2,3-dimethoxy-*p*-xylene was dissolved in 15 ml of acetic acid (glacial) and 10 ml of hydriodic acid (47%) and refluxed for 45 min. This was poured into 150 ml of water and extracted with ether. The ether layer was washed five times with water and once with 10% $KHCO_3$ solution. The washed solution was dried and stripped. The residue weighed 0.475 g (81% yield). The residue was crystallized from ligroin and had a melting point of 98 to 99 C. A second crystallization resulted in a product with a melting point of 99 to 100 C. The mass spectrometer gave a molecular weight of 138 (theoretical 138).

TABLE 1. *Growth of N. corallina V-49 on hydrocarbons*

Hydrocarbon[a]	Growth
Ethane	−
n-Hexadecane	+
Benzene	+
Toluene	+
p-Xylene	−
o-Xylene	−
m-Xylene	−
Durene	−
Naphthalene	−
1-Methylnaphthalene	−
2-Methylnaphthalene	−
1,3-Dimethylnaphthalene	−
1,5-Dimethylnaphthalene	−
1,6-Dimethylnaphthalene	−
1,8-Dimethylnaphthalene	−
2,3-Dimethylnaphthalene	−
2,6-Dimethylnaphthalene	−
2,7-Dimethylnaphthalene	−

[a] In each case, the hydrocarbon vapor was the sole source of carbon. Growth was on an agar medium (6, 7).

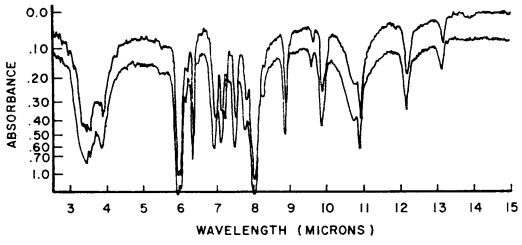

FIG. 1. *Infrared spectra of synthetic (upper scan) and biologically prepared (lower scan) DMMA (cis,cis isomers).*

RESULTS AND DISCUSSION

Description of culture. *N. corallina* V-49 was found to be a non-acid-fast, gram-positive rod. It formed long rods (1.5 to 3.5 μm) and showed

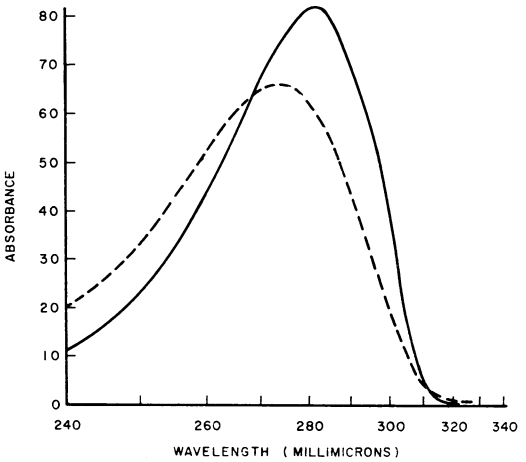

FIG. 2. *UV spectra of DMMA. Symbols: dashed line, cis,cis configuration; solid line, trans,trans configuration.*

TABLE 2. *p-Xylene oxidation by N. corallina V-49 in 7.5-liter New Brunswick fermentors in the presence and absence of IRA-93 resin*[a]

Products	IRA-93 resin	No resin
	g	g
DMMA	12.2	0.2
DHPT	0.7	
PTA	1.0	
DMPC	0.5	

[a] Yields are in grams per 7.5 liter.

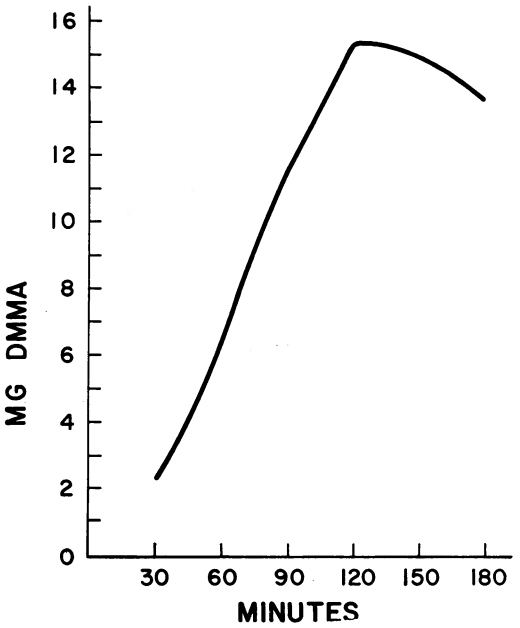

FIG. 3. *Oxidation of DMPC to DMMA by washed cells of N. corallina V-49, grown in nutrient broth, suspended in 0.03 M phosphate buffer (pH 6.8) at 30 C; 16.8 mg of substrate was used.*

branching in young cultures (24 to 36 hr); older cultures were mostly short, coccoidal rods. It grew on maltose, sorbitol, glucose, mannitol, lactose, L-arabinose, sucrose, fructose, and inositol with no evidence of acid formation. Tests for H_2S, indole, urease, and starch hydrolysis were all negative. Nitrites were formed from nitrates. There was no reaction in litmus milk. Colonial morphology was variable, but at no time was there any indication of aerial hyphae or motility. The culture grew readily on *n*-paraffins as well as on other hydrocarbons (Table 1).

Co-oxidation of p-xylene. With shaken flasks and resin-agar plates, two products were detected when *N. corallina* V-49 was grown on *n*-hexadecane with *p*-xylene as the co-oxidizable substrate. Examination of the first acidic spot by TLC gave an acid that had an R_F identical with PTA. This was also confirmed by UV and infrared (IR) measurements which gave spectra identical to those of a commercially available sample of PTA. The second acidic spot on the TLC plate was identical to that of the synthetically prepared

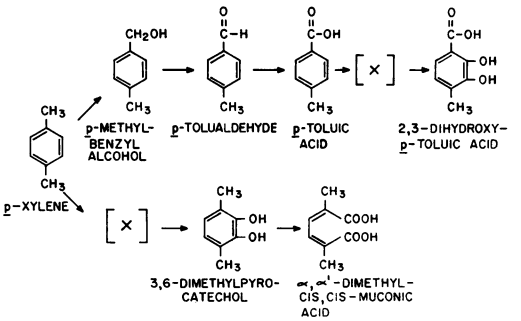

FIG. 4. *Proposed pathway for the oxidation of p-xylene by N. corallina V-49. The (X) intermediates have not yet been identified.*

TABLE 4. *Co-oxidation of cyclic aromatic hydrocarbons by N. corallina V-49*

Substrate	Principal product	Yield[a] (g/liter)
Benzene	Muconic acid	0.1
Toluene	Methylmuconic acid	0.2
p-Chlorotoluene	*p*-Chlorotoluic acid	1.5
1,2,4-Trimethylbenzene	3,4-Dimethylbenzoic acid	0.7
	2,3-Dihydroxy-4,6-dimethyl-benzoic acid	
	Trimethylmuconic acid	
m-Xylene	*m*-Toluic acid	0.7
2-Methylnaphthalene	2-Naphthoic acid	0.1
2,6-Dimethylnaphthalene[b]	6-Methyl-2-naphthoic acid	2.7
2,7-Dimethylnapthalene[b]	7-Methyl-2-naphthoic acid	6.2

[a] Growth substrate was *n*-hexadecane (0.5 ml/flask). A total of 250 mg of aromatic hydrocarbon was added to each flask and incubated for 8 days. Basal mineral salts medium was used (6, 7).

[b] A 1-g amount of substrate was used.

TABLE 3. *Morphological mutants of N. corallina V-49*[a]

Determination	Albino	Pink	Orange, moist	Orange, dry	Yellow	Light tan
No treatment, 155,000 colonies examined			1	1	5	1
UV irradiation (99.99% kill), 144,000 colonies examined	4	3	3	4	3	7

[a] The wild-type colony was orange.

DMMA. Figure 1 shows the IR spectra of the biologically made DMMA as compared with the synthetic material, and Fig. 2 shows UV spectra. Elemental analysis of the two types of DMMA is as follows. Synthetic: C, 56.7; H, 6.2; O, 37.1; unknown: C, 56.5; H, 5.9; O, 37.6; required for $C_8H_{10}O_4$: C, 56.5; H, 5.9; O, 37.6. Nuclear magnetic resonance studies demonstrated that all of the DMMA produced was of the *cis,cis* configuration.

When 7.5-liter New Brunswick fermentors were used with basal salts medium that contained peptone and beef extract, four products accumulated (Table 2): PTA, DMMA, 2,3-dihydroxy-*p*-toluic acid (DHPT), and an unidentified conversion product. The unknown product was shown to be DMPC, when compared with the synthetically made DMPC by TLC, UV, and IR methods.

The washed cells, which had been grown in Difco Nutrient Broth (Difco), oxidized DMPC to DMMA (Fig. 3).

Large-scale, pilot-plant runs revealed (P. Hosler and R. W. Eltz, Pure Appl. Chem., *in press*) more intermediate products. The fermentation broth was injected into a gas chromatograph and showed two peaks. These peaks were identified as *p*-methylbenzyl alcohol and *p*-tolualdehyde by comparing retention time with authentic samples.

In an effort to block the *p*-xylene to PTA pathway, 8 spontaneous and 24 UV mutants were obtained (Table 3). In all cases, the morphological changes in the mutants were stable after four or five transfers on nutrient agar. The hydrocarbon co-oxidation properties of the organism were not related to the production of carotenoid pigments, since a comparison of the albino mutants with the wild-type culture gave essentially the same results in the oxidation of *p*-xylene to DMMA.

The intermediate compounds that we have isolated and identified allowed us to postulate that *N. corallina* V-49 has two separate pathways for the oxidation of *p*-xylene (Fig. 4).

Other aromatic oxidation. Nine other cyclic aromatic hydrocarbons were tested in co-oxidation ferementation systems with 500-ml shaken flasks containing resin (Table 4). All products were identified by comparison with authentic material by use of TLC, UV, and IR methods. The oxidation of these cyclic aromatic hydrocarbons followed the previous experiments described with other *Nocardia* (6, 7). In summary, even though the aromatic hydrocarbons tested in co-oxidation systems do not support growth of *N. corallina* V-49, they are oxidized. Of the many cyclic aromatic hydrocarbons tested, only nine gave sufficient quantities of product for isolation and identification.

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