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Isolation of a *Pseudomonas stutzeri* Strain That Degrades *o*-Xylene

GRAZIA BAGGI,[1] PAOLA BARBIERI,[2] ENRICA GALLI,[2]* AND STEFANO TOLLARI[3]

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche,[1] Dipartimento di Genetica e di Biologia dei Microrganismi,[2] and Dipartimento di Chimica Organica e Industriale,[3] Università degli Studi di Milano, 20133 Milan, Italy

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A *Pseudomonas stutzeri* strain capable of growing on *o*-xylene was isolated from enrichment cultures. The organism grew on 2,3- and 3,4-dimethylphenol but not on 2-methylbenzyl alcohol, *o*-tolualdehyde, or *o*-toluate. *P. stutzeri* was not able to utilize *m*-xylene, *p*-xylene, or 1,2,4-trimethylbenzene, but growth was observed in the presence of the corresponding alcohols and acids. From the *Pseudomonas* cultures supplied with *o*-xylene, 2,3-dimethylphenol was isolated and identified. When resting *P. stutzeri* cells were incubated with 2,3-dimethylphenol, the reaction mixture turned greenish yellow and showed spectral properties identical to those of the 3,4-dimethylcatechol *meta* ring fission product. Catechol 2,3-oxygenase was induced by growth on *o*-xylene or on 2,3- or 3,4-dimethylphenol. The suggested hypothesis is that the first metabolic steps of growth on *o*-xylene involve the direct oxygenation of the aromatic nucleus, followed by *meta* pathway reactions.

Bacteria belonging to the genus *Pseudomonas* are known to be the most versatile in metabolizing aromatic substrates, including methyl-substituted compounds such as toluene and xylenes. *Pseudomonas* strains which utilize *p*- and *m*-xylene have been isolated from soil by the enrichment culture technique. Both compounds are oxidized to the corresponding methylbenzyl alcohols, tolualdehydes, and toluic acids and then to 4- and 3-methylcatechols, respectively, which undergo extradiol cleavage of the aromatic ring (4, 5). Toluene may be catabolized by the same route, resulting in the formation of benzoate as an intermediate (13). Degradation of toluene and of *m*- and *p*-xylene has been shown to be encoded mostly by TOL-type plasmids (12).

In an alternative to metabolic pathways which start with the oxidation of a methyl substituent, Gibson et al. (7), using a *Pseudomonas putida* strain, showed that *p*- and *m*-xylene are oxidized directly on the aromatic ring, resulting in the formation of *cis*-3,6-dimethyl-3,5-cyclohexadiene-1,2-diol (*p*-xylene *cis*-dihydrodiol) and *cis*-3,5-dimethyl-3,5-cyclohexadiene-1,2-diol (*m*-xylene *cis*-dihydrodiol), respectively; these compounds are subsequently dehydrogenated to 3,6- and 3,5-dimethylcatechol, respectively.

None of the microorganisms capable of utilizing *p*- and *m*-xylene were able to grow on *o*-xylene, which suggests the importance of the position on the aromatic ring of the methyl substituent in the selection of microorganisms able to grow on the xylenes (5). On the other hand, no reports have appeared on the isolation of microorganisms which utilize *o*-xylene as growth substrate. Only Raymond et al. (11) reported the cooxidation of *o*-xylene to *o*-toluic acid by a *Nocardia* strain isolated on *n*-paraffins, which indicates that the oxidation of *o*-xylene occurs at a methyl substituent.

In the present work, we report the isolation of a *Pseudomonas stutzeri* strain able to degrade *o*-xylene when it is supplied as the only C and energy source.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *o*-Xylene-degrading *P. stutzeri* was grown on minimal medium M9 (6) supplied with *o*-xylene as the only carbon source.

Growth surveys were conducted on minimal medium M9 in liquid form or solidified with Bacto-Agar (18 g/liter; Difco Laboratories, Detroit, Mich.). Water-soluble substrates were added at 0.05% (wt/vol). The cultures with volatile hydrocarbons were incubated under a saturated atmosphere of the tested compound. For resting-cell experiments, the cells were harvested in late log phase, washed twice by centrifugation with 0.1 M potassium phosphate buffer (pH 7), and resuspended in the same buffer (5 mg [dry weight]/ml).

Preparation of cell extracts. The cells, harvested and washed as described above, were disrupted by passage through a French pressure cell (American Instruments Co., Inc., Washington, D.C.) at 4°C. The broken-cell suspension, after treatment with 100 μg of DNase for 10 min, was clarified by centrifugation twice at 38,000 × *g* at 2°C for 30 min. The clear supernatant solution, the source of soluble enzymes, was diluted to give a total protein content of 20 mg/ml, as determined by the method of Layne (10), with bovine serum albumin as a standard (Laboratory Chemical Division, BDH, Poole, United Kingdom).

O_2 uptake measurements. Oxygen consumption by whole-cell suspensions and cell extracts was determined by using the conventional Warburg apparatus at 30°C. The reaction mixture contained the following constituents in a final volume of 3 ml: potassium phosphate buffer (pH 7; 32 μmol), substrate (3 μmol), and washed-cell suspension or cell extract (1 ml).

Enzyme assays. The catechol 2,3-dioxygenase (EC 1.13.11.2; catechol:oxygen 2,3-oxidoreductase) assay consisted of measuring the rate of formation of the ring fission product of catechol (375 nm; E = 33,000 M^{-1}), 3-methylcatechol (388 nm; E = 13,400 M^{-1}), 4-methylcatechol (382 nm; E = 28,100 M^{-1}), and 3,4-dimethylcatechol (322 nm; E = 13,200 M^{-1}) in a 3-ml reaction mixture which contained 0.3 mM substrate, 50 mM phosphate buffer (pH 7.5), and cell extract. Specific activity was defined as the amount of activity required to convert 1 nmol of substrate per min per mg of protein. All spectrophotometric assays were carried out at room temperature in a spectrophotometer (model 550S; The Perkin-Elmer Corp., Norwalk, Conn.).

Chemical determinations. Phenols were qualitatively detected by the method of Boyland and Wiltshire (3).

* Corresponding author.

TABLE 1. Rates of oxygen uptake with aromatic compounds by washed *o*-xylene-degrading *P. stutzeri* cells

Assay substrate	Rate of O_2 uptake after growth on the following substrate[a]			
	o-Xylene	2,3-Dimethyl-phenol	3,4-Dimethyl-phenol	Glutamate
o-Xylene	31.2	32	29.2	0
2,3-Dimethylphenol	35.5	50.8[b]	49.1	0
3,4-Dimethylphenol	40	55.2[b]	53.7	0
3,4-Dimethylcatechol	28.8[b]	16.5[b]	13.8[b]	0.6
2-Methylbenzyl alcohol	0.3	0.2	0.1	ND[c]
o-Tolualdehyde	0.4	1.8	2.1	ND
o-Toluate	0.1	0.8	0.7	ND

[a] Rates are expressed as microliters of O_2 consumed per hour per milligram (dry weight) of cell. All results have been corrected for endogenous respiration.

[b] During O_2 consumption, the reaction mixture turned greenish yellow.

[c] ND, Not determined.

Isolation of 2,3-dimethylphenol. *P. stutzeri* was inoculated into 750-ml Erlenmeyer flasks containing 150 ml of mineral salt medium under an atmosphere saturated with *o*-xylene. After 24 h of incubation, the cultures were collected, the cells were removed by centrifugation at 10,000 × *g*, and the supernatant was extracted three times with ethyl acetate (150 ml each time) at pH 7. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure at 30°C. The residue was dissolved in 1 ml of methanol and treated with an ethereal solution of diazomethane. The presence of 2,3-dimethylphenol (see no. II in Fig. 2) was previously demonstrated by silica gel thin-layer chromatography with chloroform as the eluent and then by gas chromatography in comparison with an authentic specimen. This analysis was confirmed by comparing, by the same methods, the corresponding methyl ether (see no. V in Fig. 2) with an authentic specimen. Mass spectra of the components were obtained by the gas chromatography-mass spectrometry technique by using a Varian MAT 112 instrument equipped with a glass column (2 m by 3 mm) containing 3% SE30 on Chromosorb W (carrier gas, helium; flow rate, 25 ml/min; column temperature, 70 to 250°C) operating with a 10°C/min temperature gradient. The retention time of both 2,3- and 3,4-dimethylphenol (see no. II and III in Fig. 2) was 3.4 min; that of the corresponding ethers (see V and VI in Fig. 2) was 3.8 min.

Chemicals. *o*-, *m*-, and *p*-xylene, 1,2,4-trimethylbenzene, *m*- and *p*-toluate, benzyl alcohol, benzoate, catechol, and 4-methylcatechol were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany). 2-Methylbenzyl alcohol, *o*-tolualdehyde, *o*-toluate, and 2,3- and 3,4-dimethylphenol were from Fluka AG (Buchs, Switzerland). 4-Methylbenzyl alcohol and 3,4-dimethylbenzoate were from Aldrich Chemical Co., Inc. (Gilligham, United Kingdom). 3-Methylcatechol was from Pfaltz and Bauer (Flushing, N.Y.). 3,4-Dimethylcatechol was prepared by the oxidation of 2-hydroxy-3,4-dimethylacetophenone, as described by Baker et al. (2). All other chemicals were of the highest purity available.

RESULTS

Microorganism. A short, gram-negative, motile rod was isolated from activated sludges of a wastewater treatment plant by the enrichment technique in the presence of *o*-xylene as the sole C and energy source. Pyoverdin and phenazine pigments were never produced. Gelatin, starch, and urea were not hydrolyzed. Tween 80 was hydrolyzed. The oxidase and catalase reactions were positive. Nitrate was reduced to nitrogen. Acid was produced from glucose, fructose, galactose, mannose, xylose, mannitol, and sorbitol but not from lactose, sucrose, maltose, or rhamnose.

β-Galactosidase, ornithine and lysine decarboxylase, and arginine dihydrolase were not produced. On the basis of these biochemical characteristics and the criteria of Gilardi (8), the organism was tentatively classified as *P. stutzeri*.

Growth characteristics. *P. stutzeri* grew on *o*-xylene and on 2,3- and 3,4-dimethylphenol but not on 2-methylbenzyl alcohol, *o*-tolualdehyde, or *o*-toluate. Growth on phenol and cresol was also observed. The organism was not able to grow on *m*- or *p*-xylene or on 1,2,4-trimethylbenzene, but the organism grew on the corresponding alcohols and acids. The only additional methylbenzene which supported growth of *P. stutzeri* was toluene.

Oxygen uptake experiments. Washed-cell suspensions of *P. stutzeri* grown on *o*-xylene rapidly oxidized *o*-xylene, 2,3- and 3,4-dimethylphenol, and 3,4-dimethylcatechol. 2-Methylbenzyl alcohol, *o*-tolualdehyde, and *o*-toluate were not oxidized. Similar results were obtained with resting cells grown on 2,3- and 3,4-dimethylphenol. No O_2 uptake was observed with glutamate-grown cells incubated in the presence of the same compounds (Table 1).

As indicated in Table 1, a yellow color was produced in some reaction mixtures during oxygen consumption, which suggested that a semialdehyde was excreted. Such reaction mixtures, from which cells had been removed by centrifugation, showed spectral properties (λ_{max} = 320 nm at pH 7, 390 nm at pH 11, and 310 nm at pH 2.5) (Fig. 1) corresponding to those of 2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate, the ring fission product of 3,4-dimethylcatechol (9).

Cell extract experiments. Cell extracts obtained from *P. stutzeri* grown either on *o*-xylene or on 2,3- or 3,4-dimethylphenol oxidized catechol, 3-methylcatechol, 4-methyl-

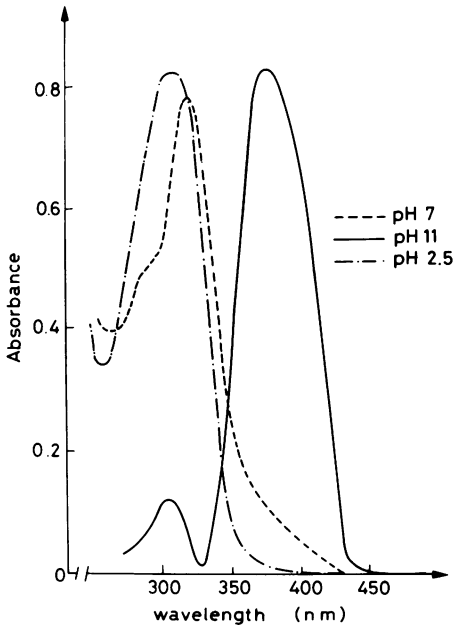

FIG. 1. Absorption spectra, at different pH values, of the ring fission product obtained from oxidation of either 2,3-dimethylphenol, 3,4-dimethylphenol, or 3,4-dimethylcatechol by resting *P. stutzeri* cells grown on 2,3-dimethylphenol.

TABLE 2. Levels of catechol 2,3-dioxygenase in cell extracts of *o*-xylene-degrading *P. stutzeri*

Inducer	Sp act (nmol/min per mg)			
	Catechol	3-Methyl-catechol	4-Methyl-catechol	3,4-Dimethyl-catechol
o-Xylene	1,605	647	1,126	362
2,3-Dimethylphenol	357	175	331	305
3,4-Dimethylphenol	341	182	368	100
Glutamate	100	58	89	22

catechol, and 3,4-dimethylcatechol, with the consumption of 1 mol of O_2 per mol of substrate. The levels of catechol 2,3-oxygenase in the same cell extracts are shown in Table 2. The enzyme was induced by all substrates, although the best inducer was *o*-xylene. The highest activity was observed with catechol and 4-methylcatechol. Cell extracts of glutamate-grown cells contained less than 10% of the activity measured in extracts of cells grown on *o*-xylene.

When extracts of *o*-xylene-grown cells were incubated with 3,4-dimethylcatechol, the reaction mixture turned greenish yellow and showed spectral properties identical to those found in experiments with resting cells (Fig. 1).

Isolation of 2,3-dimethylphenol. When *P. stutzeri* was grown in the presence of *o*-xylene, spectrophotometric analysis of culture fluid sampled at different incubation times showed the appearance of an absorption band (λ_{max} = 270 nm) whose intensity increased with time. In addition, after 24 h of incubation, the reaction of Boyland and Wiltshire (3) was positive, indicating the formation of phenols.

The supernatants of several cultures were pooled and processed as described in Materials and Methods. The extract was then methylated with ethereal diazomethane. This treatment was performed by dissolving the extract in methanol and adding an ethereal solution of diazomethane in three portions at 30-min intervals. This method had been proved to methylate phenols completely. Both the starting material and its methyl ether were subjected to gas chromatographic-mass spectrometric investigation in comparison with authentic specimens of the three possible metabolites: 2,3-dimethylphenol (no. II), 3,4-dimethylphenol (no. III), and *o*-methylbenzyl alcohol (no. IV) and their methyl ethers (no. V to VII) (Fig. 2). The mass spectra of both *o*-methylbenzyl alcohol (no. IV) and its methyl ether (no. VII) were different from that of the sample obtained from *o*-xylene metabolism, which suggested that *o*-xylene did not undergo an oxidation reaction of one of its methyl groups. The mass spectra of the two isomeric dimethylphenols (no. II and III) and the corresponding methyl ethers (no. V and VI) were very similar and resembled closely the mass spectrum of the *o*-xylene metabolite. The structure of the metabolite could be assigned by inspecting relative abundances of fragments in its mass spectrum in comparison with those of reference samples. The mass spectrum of the metabolite was basically that of 2,3-dimethylphenol (no. II) (Fig. 3). A contamination with a small amount of the isomeric 3,4-dimethylphenol could be present in the sample derived from the metabolism of *o*-xylene.

DISCUSSION

The isolation of a *P. stutzeri* strain able to grow on *o*-xylene but not on *m*- or *p*-xylene suggests a substrate specificity for this strain similar to that of *P. putida* strains which utilize only *m*- and *p*-xylene and not the *ortho*- isomer.

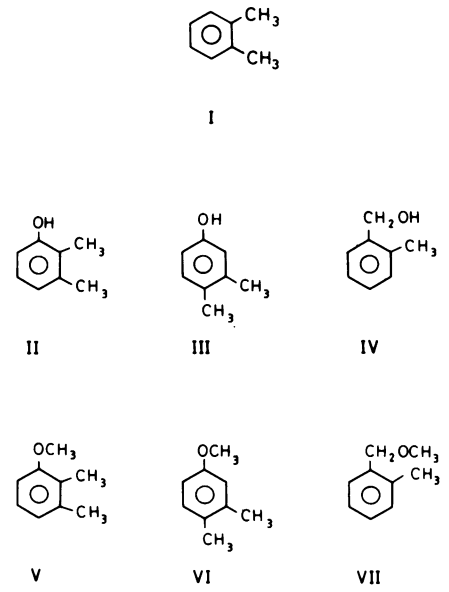

FIG. 2. Possible metabolites from *o*-xylene (I): 2,3-dimethylphenol (II), 3,4-dimethylphenol (III), 2-methylbenzyl alcohol (IV), and the corresponding methyl ethers (V, VI, and VII, respectively).

This substrate specificity means that the position of methyl groups on the aromatic ring plays a role in the selection of the microorganisms able to grow on xylenes. However, *P. stutzeri* has been shown to be able to utilize aromatic alcohols and acids derived from the oxidation of *m*- and *p*-xylene, which are not utilized for growth.

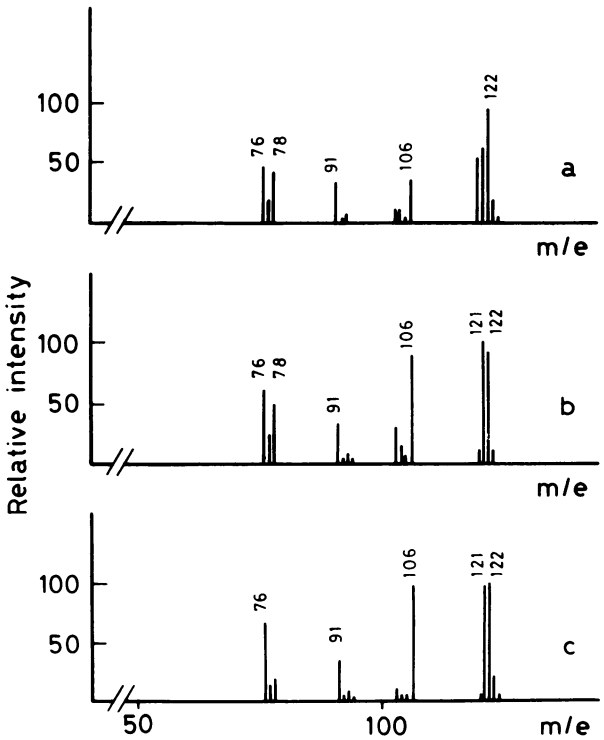

FIG. 3. Relative abundances of mass fragments in the spectra of (a) the metabolite from *o*-xylene, (b) 2,3-dimethylphenol, and (c) 3,4-dimethylphenol.

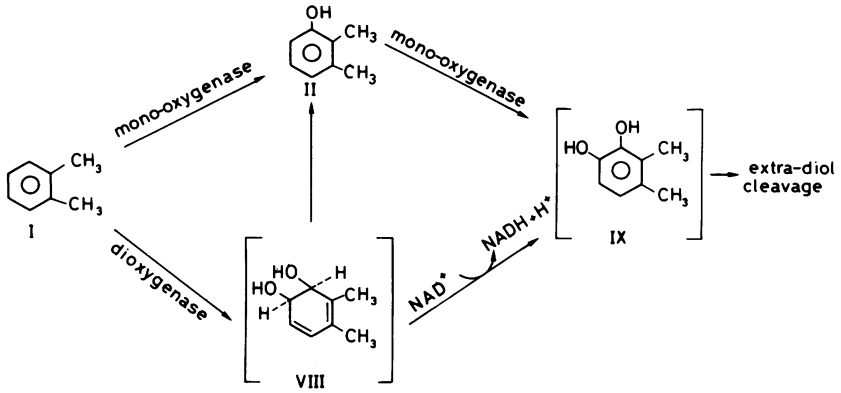

FIG. 4. Proposed initial steps for the metabolism of *o*-xylene by *P. stutzeri*. I, *o*-Xylene; II, 2,3-dimethylphenol; VIII, 3,4-dimethyl dihydrodiol; IX, 3,4-dimethylcatechol.

The failure of *P. stutzeri* to grow on 2-methylbenzyl alcohol, *o*-tolualdehyde, and *o*-toluate suggests that the first metabolic steps of *o*-xylene utilization could proceed through sequences which do not involve the oxidation of a methyl to a carboxyl group. From the *P. stutzeri* cultures supplied with *o*-xylene, 2,3-dimethylphenol was isolated and identified, but the presence of a small amount of the isomeric 3,4-dimethylphenol cannot be excluded. Growth on 2,3- and 3,4-dimethylphenol was demonstrated; both phenols were also oxidized by washed *o*-xylene-grown cells with an immediate oxygen uptake.

These results do not clearly elucidate the mode of initial attack upon *o*-xylene. In fact, the phenols could derive from the direct oxygenation of the aromatic ring catalyzed by a mono-oxygenase, or they could have been formed by the corresponding dihydrodiols by spontaneous conversion to phenols under mildly acidic conditions. This last hypothesis was supported by Davey and Gibson (4), Kunz and Chapman (9), and Baggi et al. (1), who reported the accumulation of 3-methylsalicylic acid, 3-ethylsalicylic acid, and 3,4-dimethylphenol, respectively, from the oxidation of *m*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene, respectively.

The further metabolism of *o*-xylene is consistent with sequences proceeding via the *meta* pathway. Growth on either *o*-xylene, 2,3-dimethylphenol, or 3,4-dimethylphenol induced *P. stutzeri* to synthesize a catechol 2,3-oxygenase with a wide substrate specificity, as evidenced by the comparable levels of specific activity measured in the presence of different catechols. When the 2,3-dimethylphenol-grown cells were incubated with either 2,3- or 3,4-dimethylphenol, the reaction mixture turned greenish yellow, suggesting the formation of a semialdehyde-related compound whose spectral properties corresponded to those of the 3,4-dimethylcatechol *meta* cleavage product. On the basis of these assumptions, the metabolic pathway depicted in Fig. 4 is proposed.

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