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Alternative pathways for *o*-xylene or *m*-xylene and *p*-xylene degradation in a *Pseudomonas stutzeri* strain

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Abstract

Pseudomonas stutzeri OX1 is able to grow on *o*-xylene but is unable to grow on *m*-xylene and *p*-xylene, which are partially metabolized through the *o*-xylene degradative pathway leading to the formation of dimethylphenols toxic to OX1. *P. stutzeri* spontaneous mutants able to grow on *m*-xylene and *p*-xylene have been isolated. These mutants soon lose the ability to grow on *o*-xylene. Data from HPLC analyses and from induction studies suggest that in these mutants *m*-xylene and *p*-xylene could be metabolized through the oxidation of a methyl substituent. *P. stutzeri* chromosomal DNA is shown to share homology with pWW0 catabolic genes. In the mutant strains the region homologous to pWW0 upper pathway genes has undergone a genomic rearrangement.

Abbreviations: BADH – benzylalcohol dehydrogenase; cat – catechol; C23O – catechol 2,3-dioxygenase; 2,3-, 3,4-, 2,4-, 2,6-, 3,5-, 2,5-DMP – 2,3-, 3,4-, 2,4-, 2,6-, 3,5-, 2,5-dimethylphenol; 2-MBOH – 2-methylbenzyl alcohol; 3-MBOH – 3-methylbenzyl alcohol; 4-MBOH – 4-methylbenzyl alcohol; *m*-, *p*-tol – *m*-, *p*-toluate; *o*-, *m*-, *p*-xyl – *o*-, *m*-, *p*-xylene

Introduction

Methylbenzenes are used in several industrial processes and are thus widespread environmental contaminants; they are often present in polluted waters and soils as a mixture of isomeric compounds, i.e. xylenes. Several bacteria are reported to degrade *m*-xylene and *p*-xylene, whose catabolism proceeds via the oxidation of a methyl group to the corresponding alcohol, aldehyde and carboxylic acid. The pathway has been widely studied at both biochemical and genetic level: a model of this catabolic route and its regulation is represented by *P. putida* mt-2 (PaW1), in which the enzymes for the catabolism of *m*- and *p*-xylene, as well as toluene, are encoded by plasmid pWW0 (Worsey & Williams 1975; Worsey et al. 1978). In spite of the large number of strains able to grow on *m*-xylene and *p*-xylene, only a few microorganisms have been reported to degrade *o*-xylene: a *Corynebacterium* (Schraa et al. 1987) and a *Nocardia* (Gibson & Subramanian 1984) among Gram-positive, and a *P. stutzeri* (OX) (Baggi et al. 1987) among Gram-negative bacteria. It seems that the relative position of the methyl groups on the aromatic ring plays an important role in the selection of the microorganisms and also of the catabolic pathway; in fact the *Pseudomonas* strains, known to utilize *m*-xylene and *p*-xylene through the oxidation of a methyl substituent, are unable to grow on *o*-xylene. As far as *o*-xylene is

concerned, the pathway proposed is through the direct oxygenation of the aromatic ring. In particular, the first step of *o*-xylene degradation proposed for the two Gram-positive bacteria is a dioxygenation leading to the formation of *o*-xylene dihydrodiol which is then dehydrogenated to 3,4-dimethylcatechol. This pathway closely resembles the one described by Gibson et al. (1970) for toluene degradation in *P. putida* F1. The pathway suggested for *o*-xylene degradation in *P. stutzeri* OX proceeds via two successive hydroxylations leading to the formation of 2,3-dimethylphenol and 3,4-dimethylcatechol respectively, followed by meta pathway reactions, although the formation of *o*-xylene dihydrodiol cannot be excluded (Baggi et al. 1987). A similar pathway has been described for toluene degradation in *P. cepacia* G4 (Shields et al. 1989; Shields et al. 1991) and in *P. pickettii* PKO1 (Kaphamer et al. 1990; Kukor & Olsen 1990).

To the best of our knowledge no reports have appeared on microorganisms able to degrade the three isomers of xylene through the same catabolic pathway or on microorganisms in which both pathways, the one proceeding through the oxidation of a methyl group and the one through the direct oxygenation of the aromatic ring, are present.

In this work we describe the potential ability of *P. stutzeri* to degrade the three isomers of xylene through two different pathways and the possible causes of its inability to degrade these compounds simultaneously.

Materials and methods

Bacterial strains and plasmids

The *Pseudomonas* strains and the plasmids used in the present work are listed in Table 1.

Media and growth conditions

M9 minimal medium used for *Pseudomonas* growth was described by Kunz and Chapman (1981); volatile compounds (*o*-xylene, *m*-xylene, *p*-xylene) were supplied in the vapor phase; water soluble substrates were added to the minimal medium at a final concentration of 10 mM. When required, glutamate was added at a final concentration of 10 mM. Cultures were grown at 30° C. Growth was monitored by measuring absorbance at 540 nm.

Metabolite analyses

Cells of the different strains were grown in 2000 ml flasks with 500 ml of minimal medium (M9) under

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	References
Strain		
P. stutzeri		
OX1	*o*-Xyl$^+$, *m*-Xyl$^-$, *p*-Xyl$^-$ plasmid free (Hg^s)	(Barbieri et al. 1989)
M1	OX1 spontaneous mutant *o*-Xyl$^-$, *m*-Xyl$^+$, *p*-Xyl$^+$	This work
M2	OX1 spontaneous mutant *o*-Xyl$^-$, *m*-Xyl$^+$, *p*-Xyl$^+$	This work
P2	OX1 spontaneous mutant *o*-Xyl$^-$, *m*-Xyl$^+$, *p*-Xyl$^+$	This work
Plasmid		
pED3306	Amp^r, pBR322 derivative containing the 9.8Kb *Hin*dIII fragment of pWW0 which carries the upper pathway promoter, and *xylC,A,M* genes	(Mermod et al. 1986)
pGSH2836	Amp^r, pLV85 derivative containing a *Sal*I-*Hin*dIII fragment of pWW0 carrying *xylM,A* genes	(Harayama et al. 1989)
pKT570	Str^r, pKT231 derivative containing the 6.8Kb *Xho*I D fragment of pWW0 which carries *xylR,S* genes	(Mermod et al. 1986)
pGSH2960	Amp^r, pUC18 derivative containing the 2.25Kb *Xho*I I fragment of pWW0 which carries *xylE* gene	Obtained from S. Harayama

vapor of a hydrocarbon. After 48 h of incubation the cultures were collected under sterile conditions and washed twice by centrifugation with 0.1 M potassium phosphate buffer (pH 7). The cells were resuspended in 0.2 volume of the same medium and exposed to each xylene separately (2 mM) or to 2,3-dimethylphenol, 2,4-dimethylphenol, and 2,5-dimethylphenol separately (at both 2 mM and 1 mM); at 1 h intervals samples were collected and the supernatants analyzed by HPLC. The instrument was equipped with a reverse phase C18 column and the eluent was acetonitrile: H_2O (50:50); the detector was set at 254 nm. For gas-chromatography-mass-spectrometry analyses the cells were prepared as described above, exposed for 3 h to the different compounds, and then removed by centrifugation. The supernatants were extracted four times with dichloromethane (100 ml each time) at pH 7. The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure at 30° C; the residue was dissolved in 1 ml of methanol. Analyses were performed with a Varian Mat 112 equipped with a capillary CP-Sil-5 CB column (25 m; i.d. 0.32 mm; helium as gas carrier; flow rate 25 ml/min; column temperature 50° C to 250° C).

O_2 uptake measurements

Oxygen consumption by whole cell suspensions was determined using a Clark electrode. An exponentially growing culture was washed and resuspended in a phosphate buffer 50 mM, pH 7, with an OD of 0.5 at 540 nm. 1 µmol of substrate was added to 2 ml of cell suspension; hydrocarbons were dissolved in N,N-dimethylformamide:water (9:1). Assays were carried out at 30° C. All results were corrected for endogenous respiration.

Cell extracts and enzyme assays

Cells grown to the late exponential phase, harvested and washed in phosphate buffer were disrupted by passing through a French Pressure Cell. The crude extract was treated with 100 µg/ml of DNase and centrifuged twice at 38000 g at 4° C. Protein concentration was determined by the method of Layne (1957) with Bovine Serum Albumin as standard. Benzylalcohol dehydrogenase (BADH) activity was assayed by measuring the rate of NAD^+ reduction at 340 nm (Worsey & Williams 1975). Catechol 2,3-dioxygenase (C23O) activity was assayed by measuring the rate of formation of the ring fission product of catechol at 375 nm (Sala-Trepat & Evans 1971). Assays were carried out at 30° C.

DNA preparation, restriction and Southern analysis

Bacterial DNA was prepared by the method of Ljungquist and Bukhari (1977). Plasmid preparation from *E. coli* was performed by standard procedures (Maniatis et al. 1982) and from the *Pseudomonas* by the Hansen and Olsen method (1978). Purification by ethidium bromide-CsCl density gradient was as described (Duggleby et al. 1977). Restriction analyses, transfer to Hybond N filter and Southern hybridizations were by standard procedures (Maniatis et al. 1982). The probes were biotin-labeled using a Nick Translation Kit (BRL); detection of homologous sequences was performed as specified in the Blugene DNA Detection System (BRL).

Chemicals

o-, *m*-, *p*-xylene and 2-methylbenzyl alcohol were from Fluka AG. 2,3-, 3,4-, 2,4-, 2,6-, 3,5-, 2,5-DMP, 3-methylbenzyl alcohol, 4-methylbenzyl alcohol from Aldrich. *m*-Toluate and *p*-toluate from Merck.

Results

Growth and induction experiments with P. stutzeri *OX1*

P. stutzeri OX1 is able to grow on *o*-xylene, but not on *m*-xylene and *p*-xylene; glutamate-grown cells exposed to these compounds undergo high cell lethality (1–3% of survivors after 24 h of exposure) accompanied by the appearance in the cultural

broth of a brown color, becoming blackish with time. It may be suggested that *m*-xylene and *p*-xylene are partially metabolized by this strain, leading to the formation of toxic intermediates. In the same cells exposed for only 3 h (ca. 80–90% survivors) the two isomers did not induce a significant O_2 consumption with any substrate except catechol (Table 2).

In the presence of catechol, and regardless of the inducer, O_2 consumption was always measured and accompanied by the formation of a yellow color, suggesting that induction of C23O is not affected by the position of the methyl substituents on the aromatic ring.

Metabolite analyses

The supernatants of *P. stutzeri* OX1 cultures, grown in the presence of *o*-xylene and then exposed to *ortho, meta,* or *para* isomer, were examined by HPLC at 1 h intervals. Compounds that had the same retention time as specimens of 2,3-DMP, 2,4-DMP and 2,5-DMP respectively were detected; the peaks of these compounds had comparable areas in the three supernatants. The supernatants of cultures exposed to the different isomers for 3 h were extracted and subjected to GC-MS investigation; the metabolites were compared with specimens of: 2,3-DMP; 3,4-DMP; 2,4-DMP; 2,6-DMP; 3,5-DMP; 2,5-DMP; 2-methylbenzyl alcohol, 3-methylbenzyl alcohol, 4-methylbenzyl alcohol. The mass spectra of the dimethylphenols were very similar and closely resembled those of the metabolites from the three xylenes. The metabolite from *o*-xylene, *m*-xylene, and *p*-xylene had the same retention time in GC as 2,3-DMP, 2,4-DMP and 2,5-DMP, respectively (Fig. 1), although the production of 2,6-DMP and 3,5-DMP from *m*-xylene cannot be excluded. *P. stutzeri* cannot grow on 2,4-DMP and 2,5-DMP, and exposure to these compounds caused a cell lethality comparable to that observed after exposure to *m*-xylene and *p*-xylene. Furthermore, on supplying 2,3-DMP to *o*-xylene-grown cells, the broths turned greenish-yellow within 3 h and, in the same time, 2,3-DMP, monitored by HPLC, completely disappeared from the supernatant. On the contrary, 2,4-DMP did not disappear when supplied to *o*-xylene-grown cells and the supernatants showed the same brown color as was observed after exposure to *m*-xylene and *p*-xylene. By supplying 2,5-DMP, which has a retention time of 5.5 min, it did not disappear

Table 2. O_2 uptake (nmol min^{-1} ml^{-1} of cell suspension) in OX1 and OX1 mutants.

Strain	Inducer	Assay substrates						
		o-xyl	*m*-xyl	*p*-xyl	*m*-tol	*p*-tol	2,3DMP	Cat
OX1	*o*-Xylene	39	5	5	3	4	42	70
	m-Xylene[a]	9	7	7	7	7	18	88
	p-Xylene[a]	6	nd	5	nd	3	nd	61
	Glutamate	0	5	4	0	0	0	12
M1	*m*-Xylene	3	39	29	44	56	0	499
	o-Xylene[a]	0	10	9	5	5	0	82
	Glutamate	0	2	0	2	2	0	12
M2	*m*-Xylene	0	53	34	34	19	0	212
	o-Xylene[a]	0	25	15	14	11	0	69
	Glutamate	0	0	0	0	0	0	13
P2	*p*-Xylene	0	18	18	13	15	0	69
	o-Xylene[a]	0	0	0	0	0	0	263
	Glutamate	0	0	0	0	0	0	36

[a]Glutamate was used as growth substrate. Cells were exposed to the hydrocarbon for 3 h.
nd: not detectable.

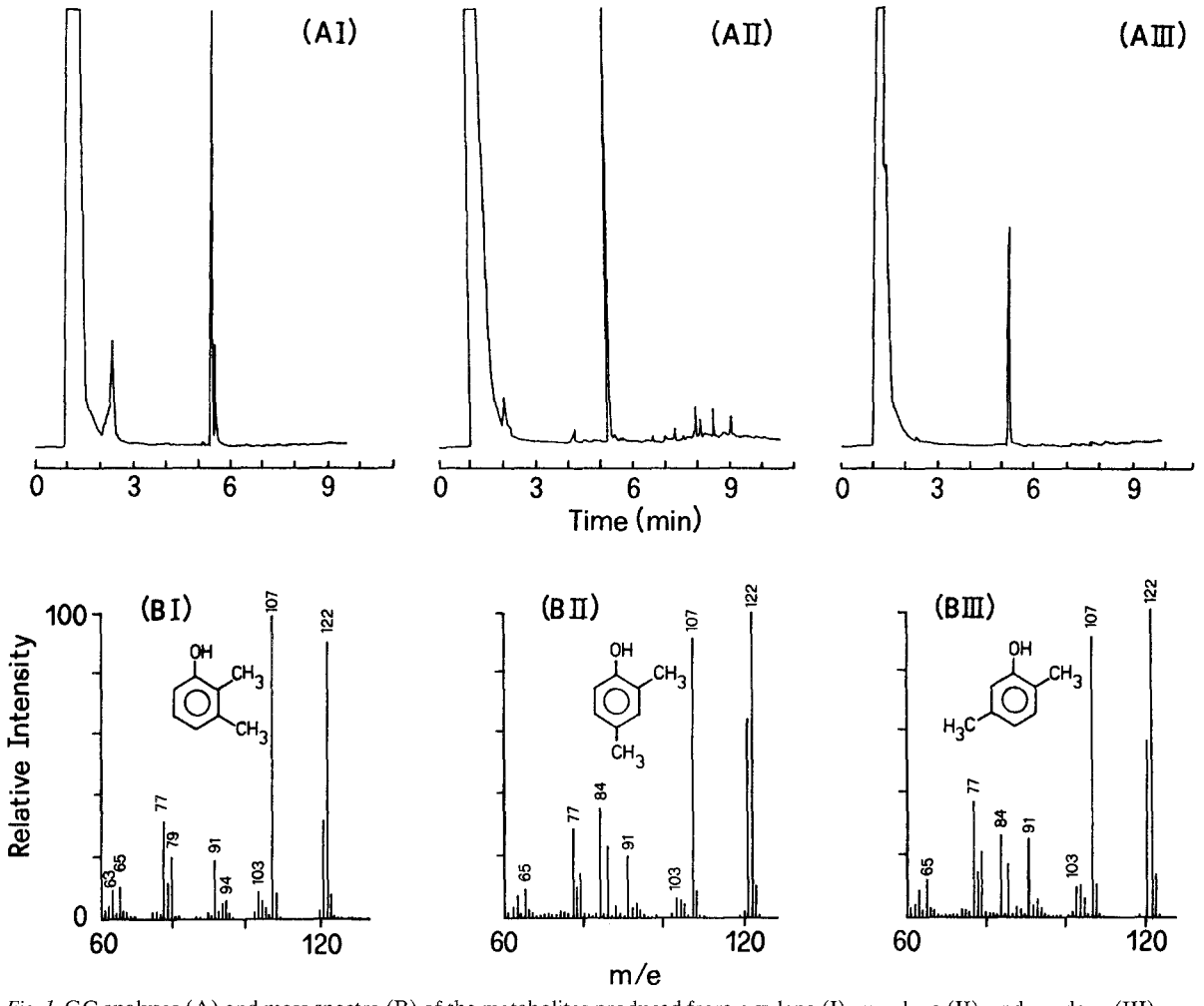

Fig. 1. GC analyses (A) and mass spectra (B) of the metabolites produced from *o*-xylene (I), *m*-xylene (II), and *p*-xylene (III).

but a secondary product, which has a retention time of 4,9 min in the HPLC profile, was formed and identified by GC/MS analysis as 2,5-dimethylhydroquinone or 2,5-dimethylresorcinol (Fig. 2). It has been found that the cells undergo high cell lethality after exposure to both compounds (data not shown).

Selection of spontaneous mutants able to grow on m-*xylene and* p-*xylene*

Although *P. stutzeri* OX1 is unable to utilize *m*-xylene and *p*-xylene, it can grow on the corresponding alcohols and acids. Moreover *P. stutzeri* OX1 DNA (Fig. 5, lane 4) shares homology with the pWW0 *xylA,M* genes which code for xylene monooxygenase. We thus started to select spontaneous mutants able to grow on *m*-xylene and *p*-xylene. Samples of *P. stutzeri* OX1 cultures were plated on minimal medium with *m*-xylene or *p*-xylene as the sole carbon and energy source. The frequency of mutants able to grow on *m*-xylene or *p*-xylene was 10^{-5}–10^{-6}. Some clones from each culture were streaked on glutamate and on the isolation substrate and single colonies were checked for growth on each of the three xylenes separately; all the colonies checked were able to grow on the three isomers, but this seemed to be only a transient phenotype: in fact, when subcultured on glutamate or on *m*-xylene or *p*-xylene and checked again, we found that ca. 50% of the colonies tested had retained the ability to

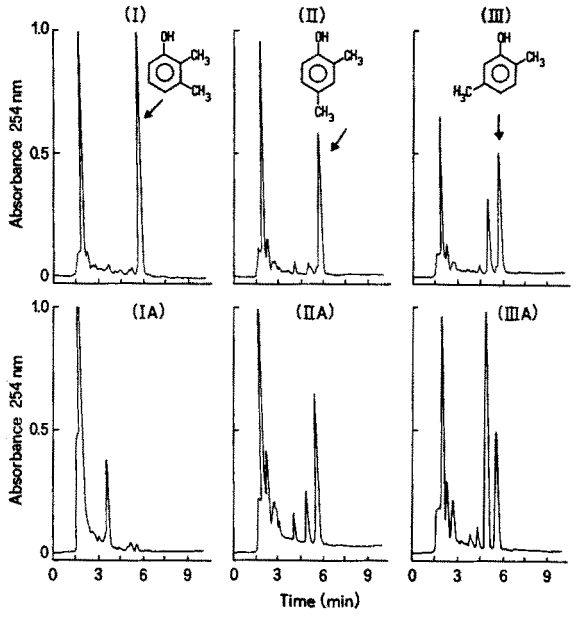

Fig. 2. HPLC of *P. stutzeri* OX1 cultural broths in the presence of: 2,3-DMP (I), 2,4-DMP (II), 2,5-DMP (III) all at T_0 and after 1 h (IA) or 5 h (IIA, IIIA), respectively, of incubation.

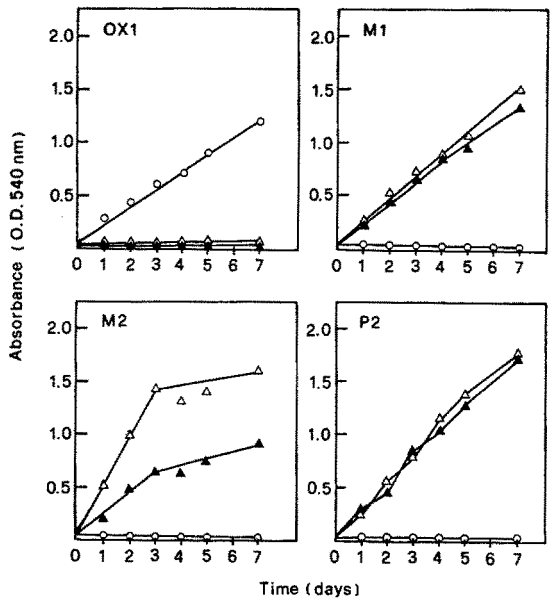

Fig. 3. Growth curves of *P. stutzeri* OX1 and of *m*-Xyl$^+$, *p*-Xyl$^+$ mutants on: ○, *o*-xylene; △, *m*-xylene; ▲, *p*-xylene. Each strain was pregrown on its isolation substrate.

grow on the *meta* and *para* isomer but had lost the ability to grow on the *ortho* isomer and, vice versa, those able to grow on the *ortho* isomer no longer grew on *m*-xylene and *p*-xylene. Three mutants which had retained the ability to grow on *m*-xylene and *p*-xylene, two from the cultures originally plated on *m*-xylene (M1 and M2) and one from *p*-xylene (P2), were chosen for further analyses. Typical growth curves of these strains in the presence of the three xylenes were compared with those of the parent strain *P. stutzeri* OX1 (Fig. 3). As expected, the three mutants were able to grow on both *m*-xylene and *p*-xylene, but not on *o*-xylene. Exposure of these mutants to *o*-xylene (60–100% of survival rate after 24 h exposure) did not cause the high cell lethality found when strain OX1 was exposed to *m*-xylene or *p*-xylene. Moreover, none of them accumulated 2,3-DMP and 3,4-DMP, nor was 2-methylbenzyl alcohol found in the cultural broths when they were exposed to *o*-xylene (Fig. 4). Dimethylphenols were neither found when the mutants were exposed to *m*-xylene and *p*-xylene (data not shown).

Induction pattern of m-*Xyl*$^+$, p-*Xyl*$^+$ *mutants*

The induction pattern of *m*-Xyl$^+$ and *p*-Xyl$^+$ mutants was analyzed by O_2 uptake experiments with resting cells. Cells were grown on *m*-xylene or *p*-xylene or on glutamate and then exposed to *o*-xylene for 3 h. The O_2 uptake in the presence of various potential intermediates was compared with that of the parent strain *P. stutzeri* OX1 (Table 2). In all mutants the growth in the presence of *m*-xylene or *p*-xylene induced O_2 uptake in the presence of *m*-xylene, *p*-xylene and toluates. In M1 and P2, *o*-xylene did not induce a significant O_2 consumption in the presence of any substrate except catechol. In strain M2, *o*-xylene seems to act as a gratuitous inducer of *m*-xylene and *p*-xylene catabolism. None of the xylenes induced O_2 uptake in the presence of *o*-xylene or 2,3-DMP.

On the hypothesis that M1, M2, and P2 metabolized *m*-xylene and *p*-xylene through the oxidation of a methyl substituent, benzylalcohol dehydrogenase (BADH) activity was evaluated in cell-free extracts after growth in the presence of different substrates and compared with those of the parent strain *P. stutzeri* OX1 (Table 3). In *P. stutzeri* OX1 BADH

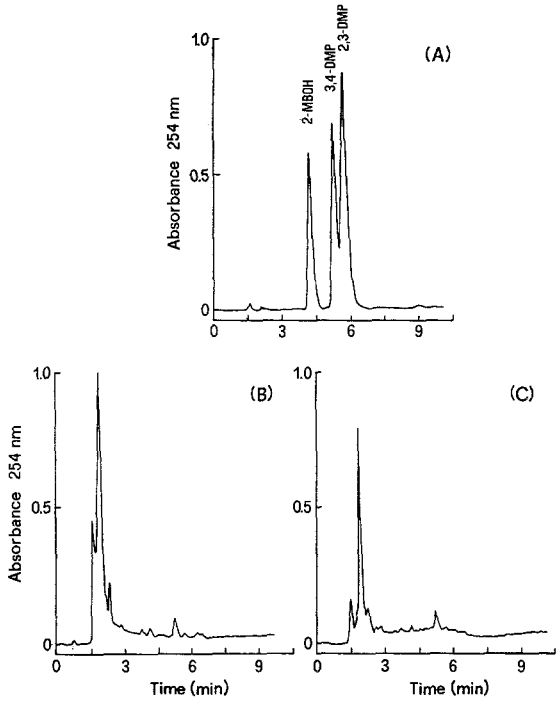

Fig. 4. HPLC elution profile of standards (A) and analysis of supernatants of M2 exposed (B) and not exposed (C) to *o*-xylene.

showed similar levels of specific activity regardless of the aromatic compound used as inducer, including *o*-xylene, where the metabolism does not proceed through the oxidation of a methyl group, or *p*-xylene, which is not utilized for growth. Comparable activity levels were also detected after growth in the presence of glutamate, suggesting that this enzyme is constitutively expressed in *P. stutzeri* OX1. In the mutant strains BADH revealed levels of activity quite different from those of the parent strain *P. stutzeri* OX1; in fact, in all of them, the BADH activity levels, after growth on *m*-xylene or *p*-xylene, were significantly higher than those of *P. stutzeri* OX1. After growth on toluates or on glutamate, differences in BADH specific activities were again found between *P. stutzeri* OX1 and the mutant strains; in particular, no BADH activity was detected in M2 after growth on *m*-toluate and glutamate, and in P_2 after growth on glutamate.

The same extracts were also evaluated for C23O activity. C23O was induced, although to a different specific activity, by all the xylenes tested.

Southern analyses

Southern analyses were performed on DNA of mutant strains using as probes catabolic genes of pWW0. Hybridization patterns were compared with those of *P. stutzeri* OX1. The probe containing the upper pathway genes *xylC,A,M* shares homology with two *P. stutzeri* OX1 DNA *Hin*dIII fragments of 6 Kbp and 4.5 Kbp, respectively (Fig. 5, panel A). The 6 Kbp fragment also hybridized with pGSH2836, carrying *xylA,M* which code for xylene monooxygenase (panel B), while the 4.5 Kbp fragment gave a weak signal with pKT570 carrying the regulatory genes *xylR,S* (panel C). In all the mutants analyzed these two signals had disappeared, while a new fragment of 8 Kbp shares homology with all three probes. Hybridization with pGSH2960 (*xylE* gene coding for C23O) gave two bands in the autoradiography; no evidence of rearrangement was detected in mutant strains (panel D).

Table 3. BADH and C23O specific activity (mU mg^{-1} of protein) in cell extracts of OX1 and OX1 mutants.

Strain	Inducer	BADH Assay substrates		C23O Assay substrate
		3-MBOH	4-MBOH	CATECHOL
OX1	*p*-Xylene[a]	110	89	643
	4-MBOH	156	204	1122
	p-Toluate	131	148	1293
	o-Xylene	nd	113	2032
	Glutamate	148	139	289
M1	*m*-Xylene	547	554	6290
	m-Toluate	25	37	4340
	Glutamate	77	87	958
M2	*m*-Xylene	334	223	4295
	m-Toluate	0	0	1279
	Glutamate	2	4	25
P2	*p*-Xylene	294	398	7471
	p-Toluate	108	108	424
	Glutamate	3	6	145

[a] Glutamate was used as growth substrate. Cells were exposed to *p*-xylene for 3 h.

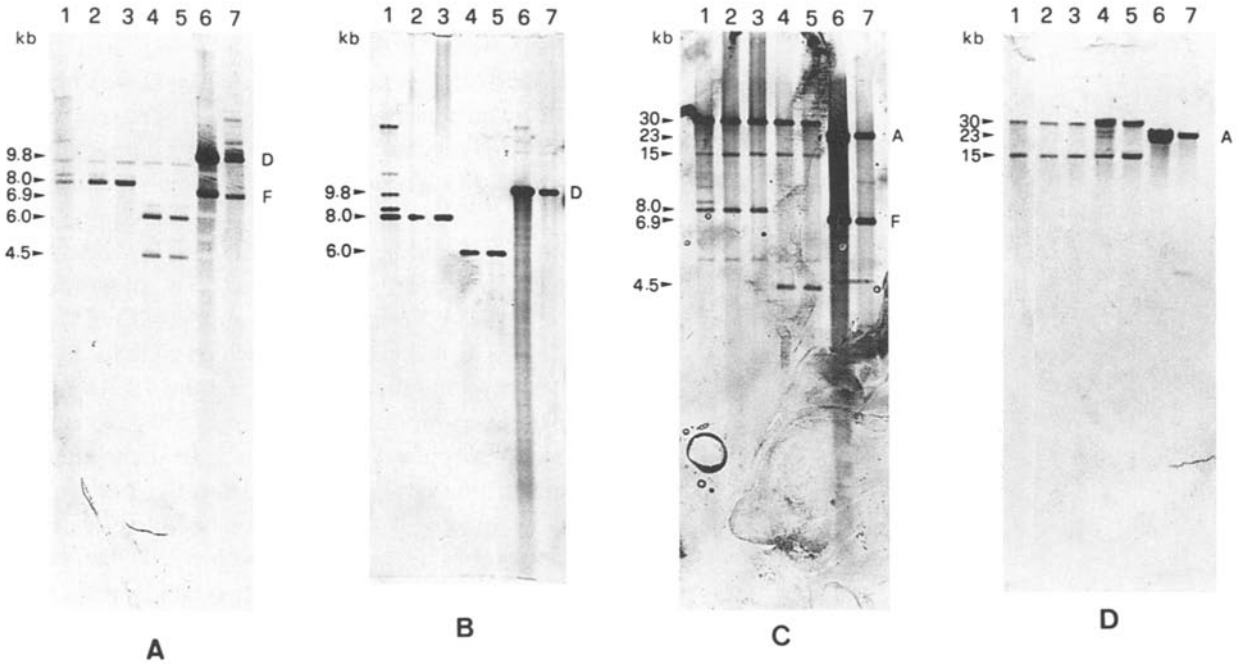

Fig. 5. Southern blot hybridization of OX1 and derivative mutants with pWW0 *xyl* probes. Samples were *Hin*dIII digested total DNA from: 1, P2; 2, M2; 3, M1; 4, OX1; 5, OX; 7, PaW1; lane 6, *Hin*dIII digested pWW0. The probes were: pED3306 (upper pathway, panel A); pGSH2836 (*xylA,M;* panel B); pKT570 (regulatory genes *xylR,S,* panel C); pGSH2960 (*xylE;* panel D). Molecular sizes were calculated by using as standards the pWW0 fragments, and the lambda *Hin*dIII digested fragments visualized by ethidium bromide staining before transfer to the filters. Letters are referred to pWW0 *Hin*dIII fragments.

Discussion

Studies on the degradation of aromatic hydrocarbons have received a lot of attention in recent years due to the potential toxicity of these molecules. The bacterial metabolism of *m*-xylene and *p*-xylene has been widely studied but very little information is available on the degradation of *o*-xylene, the most recalcitrant of these hydrocarbons; so far no strain is known to degrade all three isomers of xylene.

P. stutzeri OX1 is able to grow on *o*-xylene but not on *m*-xylene and *p*-xylene. Although this strain cannot use these two isomers for its growth, it can utilize 3-methylbenzyl alcohol, 4-methylbenzyl alcohol, *m*-toluate and *p*-toluate. It is worth noting that a prolonged exposure of *P. stutzeri* OX1 to *m*-xylene or *p*-xylene causes high cell lethality accompanied by the appearance of a brown color in the cultural broth, this brown becoming blackish with time. The enzymes involved in the dioxygenation or hydroxylation of a aromatic ring often show a low substrate specificity (Gibson et al. 1990; Kukor & Olsen 1990); we found that *m*-xylene and *p*-xylene can be partially attacked by *P. stutzeri* OX1, leading to the formation of 2,4- and 2,5-dimethylphenols, which have been characterized by HPLC and GC/MS analysis; these compounds not only cannot support growth but are also toxic to this strain. A partial oxidation of *p*-xylene through the direct oxygenation of the aromatic ring, leading to intermediates which are not used for growth, has also been reported for a *P. putida* and a *Nocardia corallina* strain (Gibson & Subramanian 1984). In some cases the accumulation of partially oxidized intermediates has toxic effects on the cells, as sometimes occurs with chlorocatechols (Bartels et al. 1984; Klecka & Gibson 1981; Reinecke & Knackmuss 1984). Thus it may be assumed that the inability of *P. stutzeri* OX1 to utilize *m*-xylene and *p*-xylene is not due to a lack of genetic information, as it is also demonstrated by the selection of spontaneous *m*-Xyl$^+$, *p*-Xyl$^+$ mutants, but rather by the fact that in *P. stutzeri* OX1 they are metabolized via the *o*-xylene degradative pathway

which is unproductive and leads to the accumulation of toxic intermediates.

From *P. stutzeri* OX1 cultures, spontaneous *m*-Xyl$^+$, *p*-Xyl$^+$ mutants have been isolated at a frequency of 10^{-5}–10^{-6}. These mutants retained for a short time the ability to grow also on *o*-xylene, but when subcultured they retained only the *m*-Xyl$^+$ and *p*-Xyl$^+$ phenotype and lost the *o*-Xyl$^+$ phenotype. As *m*-xylene and *p*-xylene are toxic to strain OX1 when they are metabolized through the *o*-xylene degradative pathway, it can be hypothesized that, when this pathway does not operate in *m*-Xyl$^+$, *p*-Xyl$^+$ mutants, the degradation of *m*-xylene and *p*-xylene could be more efficient, thus resulting in a greater stability of their phenotype. In fact cells of *m*-Xyl$^+$, *p*-Xyl$^+$ mutants did not consume O_2 in the presence of *o*-xylene or 2,3-DMP, nor were dimethylphenols detected in the cultural broths when they were exposed to the three xylenes. Moreover, in *m*-Xyl$^+$ and *p*-Xyl$^+$ mutants the BADH induction pattern, while retaining some features of the parent strain, seems to be more similar to that of *P. putida* PaW1, in which BADH is induced by hydrocarbons and alcohols but not by acids (Worsey & Williams 1975; Worsey et al. 1978). These findings, together with the absence of dimethylphenols in the cultural broths, suggest that *m*-Xyl$^+$, *p*-Xyl$^+$ mutants catabolize *m*-xylene or *p*-xylene through the subsequent oxidation of a methyl group according to the pathway reported for *P. putida* mt-2(PaW1). *o*-Xylene is not metabolized since xylene monooxygenase, which is responsible for the oxidation of *m*-xylene and *p*-xylene to the corresponding alcohol, seems to be unable to hydroxylate substituent groups in the *ortho* position (M.G. Wubbolts, pers. comm.).

As far as genetic information is concerned, in *P. stutzeri* the genes encoding enzymes involved in aromatic compound metabolism are chromosomally encoded (Barbieri et al. 1989). Hybridization experiments have shown that *P. stutzeri* chromosomal DNA shares homology with pWW0 catabolic genes. A chromosomal location of TOL genes has also been observed in other *Pseudomonas* strains (Sinclair et al. 1986; Polissi et al. 1990). The *m*-Xyl$^+$, *p*-Xyl$^+$ mutants showed a hybridization pattern different from that of the parent strain; in particular, they seem to have undergone the same genomic rearrangement: in fact it is possible in all cases to deduce the loss of one or more *Hin*dIII sites, which in *P. stutzeri* OX1 divide sequences homologous to *xylA,M* from other sequences homologous to the other upper pathway genes. Genomic rearrangements leading to drastic effects on phenotype and/or regulation pattern have been described in other *Pseudomonas* strains (Polissi et al. 1990), thus it seems possible that the rearrangements observed in the mutants are related to their phenotype.

We may conclude that *P. stutzeri* OX1 is potentially able to metabolize the three isomers of xylene, probably through two different upper pathways, which converge in a meta-cleavage pathway, but this metabolic potential is not simultaneously expressed.

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