Isolation and Metabolic Characterization of a *Pseudomonas stutzeri* Mutant Able To Grow on the Three Isomers of Xylene

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From an *o*-xylene-degrading *Pseudomonas stutzeri* strain (OX1), we previously isolated mutant M1, which had acquired the ability to grow on *m*-xylene and *p*-xylene but lost the ability to utilize the *ortho* isomer. From M1 cultures we have now isolated a revertant strain (R1) which grows on *o*-xylene and retains the ability to grow with the *meta* and *para* isomers regardless of the selective pressure applied. In *P. stutzeri* R1, *o*-xylene is degraded through two successive monooxygenations of the aromatic ring, while *m*-xylene and *p*-xylene catabolism proceeds through the progressive oxidation of a methyl substituent, although unquantifiable amounts of these two substrates are transformed into the corresponding dimethylphenols, which are not utilized for further growth. The two catabolic pathways are inducible by all three xylene isomers.

Several bacteria are reported to degrade *m*-xylene and *p*-xylene through the progressive oxidation of a methyl group (10, 11). *o*-Xylene is not degraded through this catabolic route, as xylene monooxygenase, responsible for the conversion of *m*-xylene and *p*-xylene into the corresponding alcohols, is ineffective towards the *ortho* isomer (12). The studies on the very few microorganisms which have been isolated for their ability to degrade *o*-xylene show that *o*-xylene catabolism proceeds through the direct oxygenation of the aromatic ring (1, 3, 5, 9). In the few cases in which it was tested, supplying *m*-xylene or *p*-xylene to microorganisms which attacked the aromatic ring led to the accumulation of partially oxidized intermediates which were not used for growth (2, 5, 6). Moreover, the only report on microorganisms naturally endowed with the two catabolic routes, namely, the progressive oxidation of a methyl group and the direct oxygenation of the aromatic ring, suggested that the two catabolic pathways could not be expressed in the same cells (2). In fact, from *Pseudomonas stutzeri* OX1, which is able to grow on *o*-xylene (*o*-Xyl⁺) via the formation of 2,3-dimethylphenol (2,3-DMP) and 3,4-DMP (1, 3), we isolated spontaneous mutants (e.g., *P. stutzeri* M1) able to grow on *m*-xylene and *p*-xylene which soon lost the ability to grow on *o*-xylene (*o*-Xyl[−]) (2). Evidence suggested that in such mutants, *m*-xylene and *p*-xylene were degraded through the progressive oxidation of a methyl group (2, 4).

Selection and growth characteristics of *P. stutzeri* R1. From cultures of the *o*-Xyl[−] *m*-Xyl⁺ *p*-Xyl⁺ *P. stutzeri* M1 mutant, we isolated *o*-Xyl⁺ revertant clones by plating the cultures in the presence of *o*-xylene as the only carbon and energy source. After 7 days of incubation we found 10 to 1,000 *o*-Xyl⁺ revertants for 10⁷ plated cells. Five independent *o*-Xyl⁺ revertants were tested for the ability to grow on *m*-xylene and *p*-xylene, and all of them retained the ability to utilize both the isomers. One of these revertants, named R1, was chosen for further analyses.

R1 cells were pregrown in minimal medium M9 (7) supplied with 10 mM malate or with *o*-xylene, *m*-xylene, or *p*-xylene and then transferred into fresh media where one of the three iso-

mers was supplied as the sole carbon and energy source. Malate-grown R1 cells showed a long lag phase (approximately 24 to 30 h) when *m*-xylene and *p*-xylene were the growth substrates, while the lag phase in the presence of *o*-xylene was remarkably shorter (Fig. 1A). The long lag phase was significantly reduced when the cells were pregrown not only on *m*-xylene or *p*-xylene (Fig. 1C and D) but also on *o*-xylene (Fig. 1B), suggesting that the *m*-xylene and *p*-xylene catabolic enzymes might be induced by each of the xylene isomers, while R1 cells pregrown on *m*-xylene or *p*-xylene showed a slight inhibition of growth on *o*-xylene. For each culture, at different growth phases, we checked the plating efficiency on each of the three isomers separately and found it to be approximately 100% regardless of the different substrates and the growth phase, suggesting that the ability to utilize the three isomers was retained despite the selective pressure applied during growth.

Identification of the metabolites produced from xylenes. To detect metabolites produced from the xylene isomers by R1 cells, cultures grown on *o*-xylene, *m*-xylene, or *p*-xylene were harvested, washed in 0.1 M phosphate buffer, pH 7, resuspended in the same buffer, and exposed to each isomer separately (2 mM final concentration, dissolved in *N,N*-dimethylformamide). At different incubation times, samples were collected and analyzed by reverse-phase high-performance liquid chromatography (HPLC) with a Waters μ Bondapak C₁₈ column eluted with acetonitrile-water, 50:50, at a flow rate of 1 ml/min.

Exposure of resting R1 cells to *o*-xylene caused the formation of 2,3-DMP and 3,4-DMP (retention times, 6.71 and 6.13 min, respectively), which accumulated for at least 4 h, while in R1 cells growing on *o*-xylene the DMPs appeared transiently and were then rapidly metabolized.

During growth on *m*-xylene and *p*-xylene, R1 cells turned brown, suggesting the accumulation of partially oxidized intermediates; HPLC analyses of the supernatants of R1 cells exposed to *m*-xylene and *p*-xylene revealed 2,4-DMP (retention time, 6.66 min) and 2,5-DMP (retention time, 6.05 min), respectively. 2,4-DMP and 2,5-DMP did not disappear with further incubation, were not used as growth substrates by the R1 cells, and caused high cell lethality when supplied (2 to 4% survivors after 24 h). 2,4-DMP and 2,5-DMP were previously observed also in the supernatants of the wild-type strain OX1

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FIG. 1. Growth curves of *P. stutzeri* R1 when *o*-xylene, *m*-xylene, or *p*-xylene was supplied as the only carbon and energy source. The inocula were grown on malate (A), *o*-xylene (B), *m*-xylene (C), or *p*-xylene (D). OD, optical density.

exposed to *m*-xylene and *p*-xylene, respectively (2); their production was demonstrated to be due to the broad substrate range of the enzyme involved in the first step of the *o*-xylene catabolic route, toluene/*o*-xylene monooxygenase (3), and their accumulation led to cell lethality (2). However, exposure of the resting R1 cells to *m*-xylene and *p*-xylene also revealed, besides DMPs, small amounts, undetectable at longer incubation times, of other compounds which were not found in the supernatants of OX1 cells exposed to *m*-xylene and *p*-xylene and which showed the same retention times as and coeluted with standards of 3-methylbenzyl alcohol (3-MBOH) and 4-MBOH, respectively (retention times, 4.55 min for the product derived from *m*-xylene and 4.23 min for that from *p*-xylene). HPLC analyses demonstrated that exposure of resting R1 cells to *m*-xylene, *p*-xylene, or *o*-xylene always led to the detection of the cited alcohols and/or phenols regardless of the xylene used as a growth substrate. The presence of DMPs in the cultural broths of R1 cells which were never exposed to *o*-xylene could be due either to a basal activity of toluene/*o*-xylene monooxygenase or to the induction of this enzyme by the *meta* and *para* isomers.

3-MBOH and 4-MBOH, along with 2,3-DMP and 3,4-DMP, supported R1 growth and rapidly disappeared from the medium when supplied to the R1 cell suspensions; no intermediate metabolites were detected.

These data suggest that in R1 cells, *o*-xylene is metabolized through the formation of the corresponding dimethylphenols, as previously demonstrated for OX1 cells (3), while *m*-xylene and *p*-xylene degradation may proceed through the oxidation of a methyl group, as also suggested for the parent strain M1 (2, 4), although an unquantifiable amount of these substrates is transformed into 2,4-DMP and 2,5-DMP, respectively.

***P. stutzeri* R1 induction pattern.** The growth curve and HPLC analysis survey prompted us to investigate the R1 induction pattern. The activities of enzymes involved in the first steps of each pathway were assayed, and the R1 induction pattern was compared with that of the parent strain M1 and the wild-type strain OX1.

Toluene/*o*-xylene monooxygenase activity was assayed by monitoring changes in phenolic compound concentration in the medium with a colorimetric assay (3, 8). R1 cells grown on M9 medium with each separate isomer were harvested, washed in 0.1 M potassium phosphate buffer, pH 7, and resuspended in the same buffer. *o*-Xylene or *p*-xylene dissolved in *N,N*-dimethylformamide was added at a final concentration of 1 mM as the assay substrate. *m*-Xylene was not used as an assay substrate, as 2,4-DMP was undetectable by this assay. At 2-min intervals, for a total of 14 min, 1-ml samples were collected and mixed with 100 μ l of 1 M NH_4OH and 25 μ l of 4-aminoantipyrine (2%). After the addition of 25 μ l of $\text{K}_3\text{Fe}(\text{CN})_6$ (8%), the samples were centrifuged briefly and the optical density at 540 nm of the supernatant was measured. DMP concentration was calculated with a calibration curve.

Benzylalcohol dehydrogenase (BADH) (11), chosen as the representative enzyme of the early stage of the *m*-xylene and *p*-xylene degradative pathway, was assayed in crude extracts, obtained by passage through an Aminco French pressure cell, by monitoring the NAD^+ reduction at 340 nm in a reaction mixture containing 100 μ l of NAD^+ (5 mM), 100 μ l of methylbenzyl alcohol (40 mM), 1.2 ml of Tris-HCl (50 mM) (pH 8.7), and 100 μ l of crude extract. Total protein concentration was determined by the bicinchoninic acid method.

As shown in Table 1, BADH activity was inducible in R1 cells by each xylene isomer. The ability of *o*-xylene to induce the *m*-xylene and *p*-xylene catabolic pathway was also confirmed by the detection of high levels of BADH activity in M1 cells grown on malate in the presence of *o*-xylene. The modification in the BADH induction pattern from constitutive to regulated, which occurred in the conversion of strain OX1 to M1 (2), was retained in strain R1.

In OX1 cells, toluene/*o*-xylene monooxygenase activity was found to be inducible by each xylene isomer. Malate-grown R1 cells showed measurable basal levels of toluene/*o*-xylene monooxygenase activity which, however, increased upon induction with xylenes. Nevertheless, R1 cells pregrown on the *meta* or *para* isomer showed a lag phase when transferred on *o*-xylene (Fig. 1C and D). Although different hypotheses could be proposed regarding this topic, it was not further investigated.

Thus, it seems that the *o*-xylene, *m*-xylene, and *p*-xylene catabolic pathways can be expressed in the same strain, although cometabolism of *m*-xylene and *p*-xylene through the activity of toluene/*o*-xylene monooxygenase leads to the formation of toxic intermediates whose formation is favored when *m*-xylene and *p*-xylene act as inducers of toluene/*o*-xylene monooxygenase. In this context, it seems reasonable to hypothesize that the ability of strain R1 to grow on *m*-xylene and

TABLE 1. Specific activities of toluene/*o*-xylene monooxygenase and BADH in *P. stutzeri*

Strain	Inducer	Sp act (nmol min ⁻¹ mg of protein ⁻¹) of:			
		Toluene/ <i>o</i> -xylene monooxygenase ^a		BADH ^b	
		<i>o</i> -Xylene ^c	<i>p</i> -Xylene ^c	3-MBOH ^c	4-MBOH ^c
R1	<i>o</i> -Xylene	44 ± 9.90	46 ± 11.34	218	154
	<i>m</i> -Xylene	33 ± 6.48	30 ± 3.56	237	344
	<i>p</i> -Xylene	29 ± 7.04	41 ± 17.12	232	245
	None	12 ± 1.25	12 ± 2.16	8	4
M1	<i>o</i> -Xylene ^d	0	0	112	72
	<i>m</i> -Xylene	0	0	210	193
	<i>p</i> -Xylene	0	0	332	388
	None	0	0	11	10
OX1	<i>o</i> -Xylene	37 ± 4.92	36 ± 6.38	ND	113
	<i>m</i> -Xylene ^e	15 ± 4.49	15 ± 3.30	ND	ND
	<i>p</i> -Xylene ^e	20 ± 2.87	21 ± 5.31	110	89
	None	0	0	148	139

^a Data are means ± standard deviations of three independent experiments.^b Data are means of two independent experiments. ND, not determined.^c Assay substrate.^d Cells grown on 10 mM malate in the presence of *o*-xylene.^e Cells grown on 10 mM malate and then exposed to the inducer for 2 h.

p-xylene, even when retaining a functional *o*-xylene catabolic pathway, is due to a balance between the amount of *m*-xylene or *p*-xylene which is successfully metabolized through the oxidation of a methyl group and the amount of *m*-xylene or *p*-xylene which is misrouted through the *o*-xylene catabolic pathway.

As regards the hypothesis that such an induction pattern and cometabolic features are common to other strains which attack aromatic hydrocarbons through the oxygenation of the aromatic ring, these findings may explain the difficulties in isolating *o*-xylene-degrading microorganisms, which may be disadvantaged in an environment where a mixture of the three isomers is present.

This work was supported by the Consiglio Nazionale delle Ricerche, grant 93.01031 of the Target Project on Biotechnology and Bioinstrumentation, and by Piano Nazionale Biotechnologie Vegetali, MIRAAF (Rome).

We thank P. Di Gennaro for technical assistance in HPLC analyses.

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