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Organization and Regulation of *meta* Cleavage Pathway Genes for Toluene and *o*-Xylene Derivative Degradation in *Pseudomonas stutzeri* OX1

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***Pseudomonas stutzeri* OX1 *meta* pathway genes for toluene and *o*-xylene catabolism were analyzed, and loci encoding phenol hydroxylase, catechol 2,3-dioxygenase, 2-hydroxymuconate semialdehyde dehydrogenase, and 2-hydroxymuconate semialdehyde hydrolase were mapped. Phenol hydroxylase converted a broad range of substrates, as it was also able to transform the nongrowth substrates 2,4-dimethylphenol and 2,5-dimethylphenol into 3,5-dimethylcatechol and 3,6-dimethylcatechol, respectively, which, however, were not cleaved by catechol 2,3-dioxygenase. The identified gene cluster displayed a gene order similar to that of the *Pseudomonas* sp. strain CF600 *dmp* operon for phenol catabolism and was found to be coregulated by the *tou* operon activator TouR. A hypothesis about the evolution of the toluene and *o*-xylene catabolic pathway in *P. stutzeri* OX1 is discussed.**

In bacteria, aerobic catabolic pathways for aromatic hydrocarbon degradation can schematically be divided into two major biochemical steps. First, early reactions, the so-called upper pathways or peripheral routes, channel the hydrocarbons towards the formation of partially oxidized aromatic intermediates. Then, dihydroxylated aromatic molecules that can undergo the cleavage of the ring are produced and further processed to give compounds that can enter the tricarboxylic acid cycle. Whereas a wide variety of very different peripheral routes for the oxidation of many different aromatic hydrocarbons exists, only a limited number of dihydroxylated compounds that can be cleaved and productively processed to enter the tricarboxylic acid cycle are known.

A good example of this is represented by the diversity of the known toluene catabolic pathways. Toluene is oxidized through different routes: via progressive oxidation of the methyl group (TOL pathway) (6), via dioxygenation (25), or via monooxygenations of the aromatic ring in different positions (18, 22, 31). Most of these pathways give rise to (methyl)catechols further processed through *meta* cleavage pathways. At least in one strain, *Pseudomonas mendocina* KR1, protocatechuate is produced and then cleaved in intradiol position (27). The genes coding for upper and lower pathways may be clustered in one (32), two (6), or more (18, 29) operons, independently but coordinately regulated.

The combination of different upper operons with one or more lower operons can thus increase not only the number of pathways through which a certain molecule can be degraded but also the range of substrates utilized for growth (10), and it is recognized as a mode for the evolution of new catabolic pathways (23, 28).

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Pseudomonas stutzeri OX1 is able to utilize toluene and *o*-xylene as the sole carbon and energy sources. For both compounds the degradation proceeds through two successive monooxygenations of the aromatic nucleus catalyzed by toluene-*o*-xylene monooxygenase (ToMO) followed by extradiol ring cleavage (3). Here we investigate the organization of genes involved in the further degradation of toluene and *o*-xylene derivatives produced by the action of ToMO.

Identification of a *P. stutzeri* OX1 phenol hydroxylase activity. The maps of pFB3401 and its derivatives (see Table 1 for a description of strains and plasmids) are reported in Fig. 1. Previously, a catechol 2,3-dioxygenase (C2,3O) gene was roughly mapped to the 7.2-kb *Eco*RI fragment at the right end of the pFB3401 insert (3). As the ToMO-encoding operon did not contain genes involved in the lower pathway (1, 4), it was supposed that the C2,3O gene was part of a distinct *meta* operon.

In an attempt to verify if the C2,3O expression was controlled by the ToMO operon transcriptional activator TouR, *E. coli* JM109(pFB1021) cells were transformed with the compatible plasmid pRZ7085 carrying the regulatory gene *touR* in order to assay a possible increase in C2,3O activity upon exposure to the phenolic compounds that were demonstrated to be TouR effectors (1). Surprisingly, a yellow color, indicative of the accumulation of a ring fission product, developed in cultures exposed for 1 h to *o*-cresol (Fig. 1). When the same experiment was performed with cells carrying pRZ7085 and pFB1036, a faint yellow color was detectable only after prolonged incubation of more than 24 h. These results suggested that in the pFB1021 insert, a gene or genes coding for a phenol hydroxylase activity, able to catalyze the conversion of the phenolic compound into a catechol, might be present.

Mapping of *meta* pathway genes. To localize genes involved in *meta* pathway reactions, several overlapping fragments from the insert of pFB1021 and of pFB3411 were subcloned in *Escherichia coli* IPTG (isopropyl-β-D-thiogalactopyranoside)-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strain		
P. putida PaW340	Cre$^-$ Dmp$^-$ C2,3O$^-$ Trp$^-$ Str^r	9
P. putida PaW340::*touR*	Teh^r *P. putida* PaW340 derivative; contains a copy of *touR* integrated in the chromosome	This work
E. coli JM109	*recA1 hsdR17 thi* Δ*(lac-proAB)* (F′ *traD36 proAB lacI*^{q}ZΔM15)	30
Plasmid		
pGEM-3Z	Ap^r; cloning vector	Promega Corp.
pGEM-7Z	Ap^r; cloning vector, compatible with pLAFR vectors	Promega Corp.
pJB3Km1	Km^r; cloning vector	5
pUC19	Ap^r; cloning vector	24
pFB3401	Tc^r; 28-kb *Eco*RI fragment from *P. stutzeri* OX1 chromosome cloned in pLAFR1	3
pFB3411	Tc^r; 22.4-kb *Eco*RI fragment from *P. stutzeri* OX1 chromosome cloned in pLAFR1	3
pFB1021	Tc^r; partial deletion of pFB3411	3
pFB1036	Tc^r; partial deletion of pFB3411	3
pRZ7085	Ap^r; pFB3401 8.5-kb *Apa*I fragment cloned in pGEM-7Z	This work
pJEX159	Km^r; pFB3401 5.9-kb *Eco*RI-*Xba*I fragment cloned in pJB3Km1	This work
pJSX148	Km^r; pJEX159 4.8-kb *Sac*I-*Xba*I fragment cloned in pJB3Km1	This work
pBZ3120	Ap^r; pFB3401 2-kb *Bam*HI-*Xho*I fragment cloned in pGEM-3Z	This work
pVS1934	Ap^r; pFB3411 3.4-kb *Xba*I-*Sac*I fragment cloned in pUC19	This work
pVS1921	Ap^r; pFB3411 2.1-kb *Mlu*I-*Sma*I fragment cloned in pUC19	This work

[a] Abbreviations: Cre, *o-*, *m-*, *p*-cresol; Dmp, 2,3-dimethylphenol and 3,4-dimethylphenol; Trp, tryptophan; Str, streptomycin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; Teh, tellurite.

inducible vectors. The insert of pFB3411 partially overlapped and extended beyond the right end of pFB3401, and it was unable to direct the conversion of both hydrocarbons and phenols into catechols (3). The plasmids obtained were examined for phenol hydroxylase (PH), C2,3O, 2-hydroxymuconate semialdehyde hydrolase (HMSH), and 2-hydroxymuconate semialdehyde dehydrogenase (HMSD) activities, and the encoding loci were mapped (Fig. 2). PH activity was determined in resting cells by monitoring the decrease in concentration of phenol in the medium, using a colorimetric assay described previously (3, 15). C2,3O, HMSH, and HMSD were assayed in crude extracts by measuring the formation rate (17) or the decrease (21) of the catechol ring fission product.

Quantitative assays performed on cells carrying the plasmids depicted in Fig. 2 or plasmids carrying the same inserts cloned in the opposite direction with respect to the *Plac* promoter of the vector (not shown) suggested that the genes mapped are transcribed in the same direction, from the left to the right with respect to the maps shown in Fig. 2.

Substrate range of the cloned phenol hydroxylase. Plasmids pJEX159 and pJSX148 were also examined and found not to confer upon cells the ability to convert toluene and *o*-xylene to phenolic compounds. The activity cloned in pJEX159 and pJSX148 is thus specific for the introduction of a hydroxyl group on phenolic compounds. The substrate range of the cloned PH was investigated in *E. coli* JM109(pJSX148) cells. Specific activities are reported in Table 2 and found comparable for all the phenols tested. *p*-Cresol, 2,4-dimethylphenol

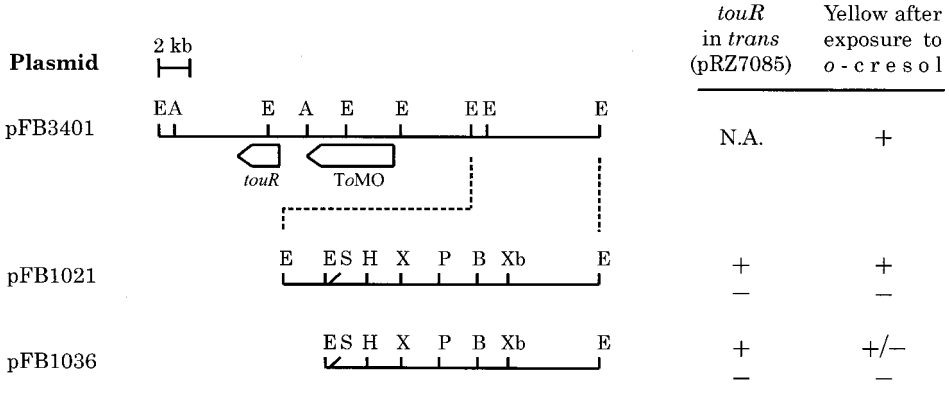

FIG. 1. Restriction endonuclease maps of the *P. stutzeri* OX1 chromosomal DNA fragment cloned in pFB3401 and its derivatives (3). pFB3401 allowed the host cells to convert toluene, *o*-xylene, and their phenolic derivatives into the corresponding ring fission products. The ToMO-encoding operon and the regulatory gene (*touR*) controlling its expression are indicated (1, 4). The point of the arrows indicates the direction of transcription. The ability (+) or inability (−) of the plasmids to direct the conversion of *o*-cresol into ring fission products in the absence (−) or in the presence (+) of *touR*, cloned in pRZ7085 and provided in *trans*, is indicated to the right of the restriction maps. +/−, mildly positive reaction after prolonged exposure. Abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N.A., not applicable; P, *Pst*I; S, *Sac*I; X, *Xho*I; Xb, *Xba*I. Only relevant restriction sites are indicated.

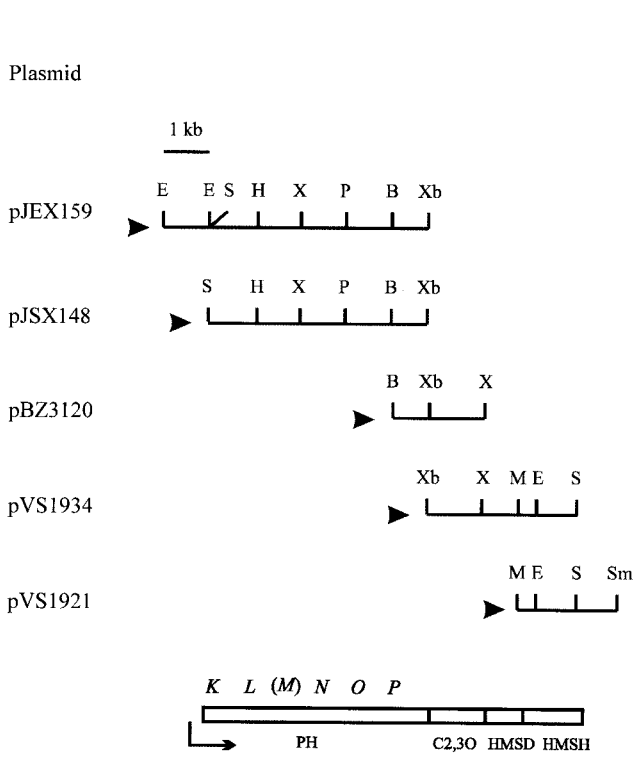

FIG. 2. Restriction endonuclease maps of DNA fragments subcloned from pFB1021 (pJ and pBZ series) and from pFB3411 (pVS series). Only the plasmids containing the minimal region coding for PH, C2,3O, HMSH, and HMSD are reported. The black arrowheads indicate the position of the *Plac* of the vector. To the right of the restriction maps, the enzymatic activities expressed from the indicated plasmids in uninduced (−) or IPTG-induced (+) *E. coli* JM109 cells are reported. At the bottom, the map obtained by subcloning, Southern, and sequence analyses (see text for details) displaying the gene arrangement and the putative promoter is shown. The presence of the gene indicated in parentheses can be hypothesized but has not been investigated. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; ND, not detectable; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I. Only relevant restriction sites are indicated.

(2,4-DMP), and 3,4-DMP, due to the presence of a substituent *para* to the hydroxyl group, were undetectable in this assay. High-pressure liquid chromatography (HPLC) analyses, however, demonstrated that these phenols were oxidized by the cloned enzyme.

To identify the reaction products, the supernatants of *E. coli* JM109(pJSX148) cell reactions, with different phenols supplied at a final concentration of 0.5 to 3 mM depending on the compound, were extracted with a 1/5 volume of acetonitrile; the organic phase was filtered, deoxygenated with N_2, and then analyzed by reverse-phase HPLC; and the retention times were compared with those of authentic standards (Table 2). The supernatants were also treated with partially purified C2,3O expressed from pBZ3120 and checked by HPLC analyses for the complete disappearance of PH reaction products. The spectral properties of the yellow compounds produced after C2,3O reaction were identical to those determined for the extradiol ring fission products obtained from authentic stan-

TABLE 2. Substrate range and reaction product identification for the *P. stutzeri* OX1 PH from IPTG-induced *E. coli* JM109(pJSX148) cells

Substrate	PH sp act (nmol min^{-1} mg of $protein^{-1}$)	HPLC retention time of product (min)	Product identified	HPLC retention time for standard of predicted product (min)	Cleaved by C2,3O
Phenol	8.2 ± 0.5	3.55	Catechol	3.51	+
o-Cresol	10.8 ± 1.0	3.44	3-Methylcatechol	3.40	+
m-Cresol	8.7 ± 0.7	3.35	3-Methylcatechol	3.40	+
p-Cresol	NA[a]	3.16	4-Methylcatechol	3.13	+
2,3-DMP	12.7 ± 0.5	4.25	3,4-Dimethylcatechol	4.05	+
3,4-DMP	NA	4.17	3,4-Dimethylcatechol	4.05	+
2,4-DMP	NA	4.10	3,5-Dimethylcatechol[b]	NAv[c]	−
3,5-DMP	11.4 ± 1.0	4.07	Not analyzed	NAv	−
2,5-DMP	14.4 ± 2.8	4.21	3,6-Dimethylcatechol[b]	NAv	−

[a] NA, Not applicable.
[b] Identified by NMR analysis.
[c] NAv, not available.

dards of catechol, 3-methyl-, 4-methyl-, and 3,4-dimethylcatechol (data not shown). The further data confirmed the identification of the reaction products and suggested that *m*-cresol and 3,4-DMP were mainly converted into 3-methyl- and 3,4-dimethylcatechol, respectively, although the presence of small amounts of other isomers could not be excluded. The reactions catalyzed by PH thus appear to be redundant with respect to those catalyzed by ToMO, at least as regards the phenols derived from toluene and *o*-xylene.

Identification of the products derived from 2,4-DMP and 2,5-DMP. The *P. stutzeri* OX1 PH acts on a very broad range of substrates, as it was able to produce more polar compounds, presumably dimethylcatechols, even from 2,4-DMP, 3,5-DMP, and 2,5-DMP, which are not growth substrates for *P. stutzeri* OX1. As standards of 3,5- and 3,6-dimethylcatechols for HPLC analyses were not available, we set out to identify these compounds by nuclear magnetic resonance (NMR). The reaction products obtained upon exposure of *E. coli* JM109(pJSX148) cells to 3 mM 2,4-DMP and 2,5-DMP were extracted from the supernatants with ethyl acetate, separated by flash column chromatography, and analyzed by ^{1}H-NMR. The protonic signals had the following features: δ 6.55 (s, 2H), δ 5.20 (br s, 2H, D_2O); and δ 2.25 (s, 6H) for the product obtained from 2,4-DMP and δ 6.60 (s, 2H), δ 5.60 (br s, 2H, D_2O), and δ 2.23 (s, 6H) for the product obtained from 2,5-DMP. Reactions of 5.20 and 5.60 protons, respectively, with D_2O confirmed the assignment of the signals to hydroxyl groups. The registered spectra were consistent with those reported in literature for 3,5-dimethylcatechol (26) and for 3,6-dimethylcatechol (14). Thus, the *P. stutzeri* OX1 PH displays precise regioselectivity, as it produces catechols from all the phenols tested. The product from 3,5-DMP was not analyzed.

The dimethylcatechols from 2,4-DMP, 2,5-DMP, and, presumably, 3,5-DMP did not disappear in the supernatants treated with C2,3O, nor did the typical yellow color of extradiol ring fission products develop (Table 2). To further assess if the *P. stutzeri* C2,3O was unable to catalyze the cleavage of 3,5- and 3,6-dimethylcatechol, specific activities were measured and found to be 0.01 and 0.05%, respectively, of the activity measured using catechol as the reaction substrate.

In *P. stutzeri* OX1, 2,4-DMP and 2,5-DMP were derived from the ToMO-catalyzed hydroxylation of *m*-xylene and *p*-xylene and are not used for growth (2, 3). Thus, in *P. stutzeri* OX1, catechol 2,3-dioxygenase represents the bottleneck for *m*-xylene and *p*-xylene degradation through the monooxygenation pathway.

Arrangement of the PH genes. The length of the minimal DNA region to detect PH activity in *E. coli* cells suggested that the *P. stutzeri* OX1 PH might be a multicomponent enzyme system. Southern hybridization analyses of pJEX159 were thus performed using the *dmp* genes coding for the subunits of the *Pseudomonas* sp. strain CF600 multicomponent PH (16) as probes. Hybridization signals were detected when pJEX159 was probed with *dmpL, dmpN, dmpO*, and *dmpP* but not when the probes were *dmpK* and *dmpM* (data not shown).

Sequencing of approximately 1,000 nucleotides (accession no. AJ309239) at the 5′ end of the PH locus revealed a sequence identical to the −12, −24 consensus of σ^{54}-dependent promoters followed by a putative open reading frame (ORF) (nucleotides 247 to 507) whose deduced amino acid sequence shared 62.6% identity (74% similarity) to the *Pseudomonas* sp. strain CF600 PH DmpK subunit. The map obtained is depicted in Fig. 2 and evidenced a conserved gene order with respect to that of the *Pseudomonas* sp. strain CF600 PH gene cluster (16). In the *Pseudomonas* sp. strain CF600 PH, DmpK is dispensable for the enzyme activity in vitro and it was suggested to play a role in assembly of the active form of the enzyme (20). In pJSX148, which expresses a high level of PH activity, the DmpK-like ORF is partially deleted, suggesting that its integrity is not essential for the enzyme to be assembled correctly enough to function.

TABLE 3. PH and C2,3O activity in *P. putida* PaW340(pFB3411) cells expressing or not expressing TouR

Strain	Induction with *o*-cresol	Sp act (nmol min^{-1} mg of $protein^{-1}$)	
		PH	C2,3O
PaW340::*touR*(pFB3411)	−	2	232
	+	61	1,870
PaW340(pFB3411)	−	1	420
	+	2	300

The *P. stutzeri meta* operon putative promoter is located in the 1.1-kb *Eco*RI fragment deleted in pFB1036 (Fig. 1), and its deletion may explain the scarce and delayed production of extradiol ring fission products observed in cells carrying pFB1036 and *touR* in *trans* and exposed to *o*-cresol.

PH and C2,3O are controlled by TouR. The ability of pFB1021 to direct the conversion of phenols into ring cleavage products when in *trans* with pRZ7085 (Fig. 1) together with the presence of a putative σ^{54}-dependent promoter upstream from the PH-encoding genes (Fig. 2) suggested that the expression of the identified *meta* pathway genes could also be controlled by TouR, the phenol-responsive regulator of the *tou* upper operon (1). The regulatory gene *touR* was thus integrated in the *Pseudomonas putida* PaW340 chromosome by means of minitransposon (8, 11), and the recombinant strain was transformed with pFB3411. Upon induction with *o*-cresol, the specific activities of PH and C2,3O increased 30- and 8-fold, respectively, in comparison to uninduced samples (Table 3). The inability of *P. putida* PaW340(pFB3411) to convert phenolic compounds into ring cleavage products (3) can thus be ascribed to the absence of a suitable transcriptional activator.

Conclusive remarks. The *P. stutzeri* OX1 genes coding for toluene and *o*-xylene catabolism are organized into at least two operons, one coding for the toluene-*o*-xylene-monooxygenase (4) and another for phenol catabolism, the latter organized as the *Pseudomonas* sp. strain CF600 *dmp* operon in which the multicomponent PH genes are followed by C2,3O, HMSH, and HMSD encoding genes (19). Both operons are inducible by phenols under the control of a σ^{54}-dependent promoter and TouR. The presence of a *dmp*-like operon together with a phenol-responsive regulator in the *P. stutzeri* OX1 genome suggests that, in this strain, the toluene and *o*-xylene catabolic pathway evolved by vertical expansion of a preexisting route for phenol catabolism through the incorporation of the ToMO gene cluster (Fig. 3). In this perspective, the redundancy between the hydroxylation of phenols catalyzed by ToMO and

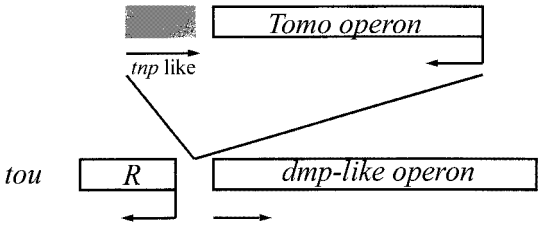

FIG. 3. A model for the evolution of the toluene and *o*-xylene catabolic pathway in *P. stutzeri* OX1. See text for details. The arrows indicate the direction of transcription. *tnp*, transposase-like ORF (4).

the PH activity can be fortuitous due to the acquisition of a monooxygenase endowed with a very broad range of substrates. The scarce specificity of σ^{54}-dependent promoters (13), which makes the P_{ToMO} promoter activatable by TouR, may be a factor that contributed to the successful acquisition of the ToMO gene cluster.

P. stutzeri OX1 is the first toluene-degrading strain in which the toluene monooxygenase-encoding operon has been found associated to a *dmp*-like operon. Toluene monooxygenases similar to the *P. stutzeri* OX1 ToMO have been found in other bacterial strains (7, 31), but they are associated with very different lower pathways (12, 29). These observations further confirm the modular organization of aromatic hydrocarbon catabolic pathways and suggest that the toluene monooxygenase operon represents an independent module.

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