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XYL, a Nonconjugative Xylene-Degradative Plasmid in *Pseudomonas* Pxy

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Pseudomonas Pxy metabolizes *p*- or *m*-xylene through intermediate formation of the corresponding methylbenzyl alcohol and toluic acid via the *meta* pathway. The strain *Pseudomonas* Pxy spontaneously loses its ability to grow with xylene or toluate, and the rate of loss of this ability is greatly enhanced by treatment of the cells with mitomycin C. The assay of a number of enzymes involved in xylene degradation in xylene-negative Pxy cells indicates the loss of the entire enzyme complement of the pathway. The genes specifying all the xylene-degradative enzymes, including those of the *meta* pathway, appear to be borne on a nonconjugative plasmid and can be transferred to xylene-negative Pxy or *P. putida* strain PpG1 cells only in the presence of a transfer plasmid termed factor K. When transferred to strain PpG1, the xylene-degradative plasmid, termed XYL, coexists stably with factor K, but transduction of XYL is not accompanied by a cotransfer of factor K. XYL appears to be compatible with all the other known degradative plasmids in *P. putida*. The xylene pathway is inducible in wild-type Pxy as well as in Pxy and PpG1 exconjugants, suggesting the cotransfer of regulatory genes along with the plasmid. The enzymes converting xylene to toluate are induced by xylene, methylbenzyl alcohol, or the aldehyde derivatives but not significantly by toluate, whereas catechol dioxygenase and other enzymes are induced by toluates and presumably by xylene as well.

A *Pseudomonas* species, designated strain Pxy, oxidizes *p*- and *m*-xylene through the intermediate formation of the corresponding methylbenzyl alcohols and toluic acids (Fig. 1). These pathways, which are utilized by strain Pxy and *Pseudomonas aeruginosa*, were established by the identification of several metabolic intermediates and also by demonstration of the requisite enzyme activities (5, 6, 10, 12). Until recently, the genetic basis of xylene degradation by these organisms was obscure. However, several catabolic pathways in *Pseudomonas* species are known to be specified by gene clusters which are not on the chromosome but are borne on conjugative plasmids (2, 3, 7, 14, 16–18). In this report, we present data which suggest that the genes for the entire xylene-degradative pathway in *Pseudomonas* Pxy are borne on a nonconjugative plasmid. This nonconjugative plasmid is transmissible in the presence of a transfer plasmid, factor K (4). The presence of a conjugative plasmid in *Pseudomonas putida* (*arvilla*) mt-2 which specifies the xylene catabolic pathways has been recently reported by Worsey and Williams (18).

MATERIALS AND METHODS

Growth of organisms. All *Pseudomonas* Pxy and *P. putida* PpG1 strains were maintained on L agar (9) slants. The xylene-positive Pxy and PpG1 strains were maintained on mineral salts (15) agar slants which contained *m*-toluic acid (0.1%) and L-methionine (50 μg/ml). Some of the genotypic and phenotypic properties of the strains are shown in Table 1.

Each organism was grown in mineral salts liquid medium (15) containing succinate (0.1%) and the appropriate aromatic substrates. *p*-Xylene and *p*-tolualdehyde were supplied in the vapor phase as described for toluene by Gibson et al. (8). *p*-Methylbenzyl alcohol and *p*-toluic acid were added to the mineral salts-succinate medium to give a final concentration of 0.1%. For organisms PpG1 AC 143, AC 148, and AC 10 the medium was also supplemented with L-methionine (50 μg/ml). Cells were harvested in the logarithmic phase of growth, washed twice with 0.02 M KH_2PO_4 buffer, pH 7.2, and either used immediately for whole cell oxidation studies or stored at −15°C until required for preparation of cell extracts.

Preparation of cell extracts. Cells were suspended in two volumes of 0.05 M KH_2PO_4 buffer, pH 7.5, which contained acetone (10%), and disrupted by sonic oscillation in a Biosonik II ultrasonic disin-

tegrator. After addition of deoxyribonuclease (1 mg/g [wet weight] of cells) the cell debris was removed by centrifugation at 13,000 × *g* for 20 min. The supernatant solution was centrifuged at 105,000 × *g* for 60 min. The clear supernatant solution obtained was used as the source of crude cell extract. Heat-treated extracts were prepared by heating crude cell extract at 55°C for 10 min. Denatured protein was removed by centrifugation at 13,000 × *g* for 30 min.

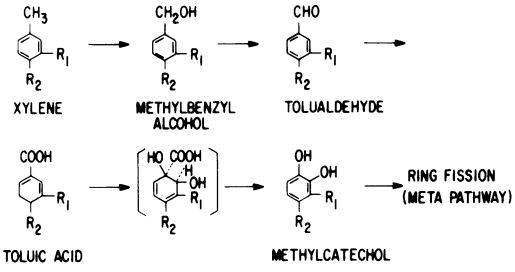

FIG. 1. *Pathway for the oxidation of m- and p-xylene by Pseudomonas Pxy. For m-xylene R_1 is CH_3 and R_2 is H, and for p-xylene R_1 is H and R_2 is CH_3.*

Protein content of whole cells and cell extracts was determined by the method of Oyama and Eagle (13).

Curing, transduction, and transmissibility of the plasmids. Methods describing the conditions for optimal curing and the transmissibility of plasmids were as previously described (2). All transductions were performed with phage pf16 (14).

Enzyme assays. Catechol 2,3-dioxygenase (EC 1.13.11.2) was assayed according to Nozaki et al. (11). The same assay procedure was used for 3-methylcatechol 2,3-dioxygenase (ϵ_{388} = 13,800) and 4-methylcatechol 2,3-dioxygenase (ϵ_{382} = 28,100), except that initial reaction rates were measured at the wavelengths given. Extinction coefficients for the ring-fission products formed from 3- and 4-methylcatechols are those reported by Bayly et al. (1). *p*-Methylbenzyl alcohol dehydrogenase activity was measured spectrophotometrically by following the reduction of nicotinamide adenine dinucleotide at 340 nm. Reaction mixtures (3.0 ml) contained in micromoles: KH_2PO_4 buffer (pH 7.5), 140; nicotinamide adenine dinucleotide, 3.0; *p*- or *m*-methylben-

TABLE 1. *List of Pseudomonas strains*[a]

Strain designation	Phenotype	Genotype		Derivation[a]	Source
		Chromosomal	Plasmid[b]		
Pseudomonas Pxy[c]					
AC142	Xyl$^+$	*wt*	XYL		(5)
AC 139	Xyl$^-$	*wt*		S, curing	AC 142 (this manuscript)
AC 138	Leu$^-$ Xyl$^+$	*leu*	XYL	NG	AC 142 (this manuscript)
AC 141	Ilv$^-$ Xyl$^-$ Tol$^+$	*ilv*	XYL-*a*	NG	AC 142 (this manuscript)
P. putida PpG1					
AC 2	Trp$^-$	*trpB*		NG	AC 30 (4)
AC 4	Trp$^-$	*trpB*	K	Conj	AC 2 (4)
AC 10	Met$^-$	*met*		NG	AC 30 (4)
AC 12	Ilv$^-$	*ilv*		NG	AC 30 (4)
AC 13	His$^-$ Sm^r	*his*		NG	AC 30 (4)
AC 27	WT		CAM	Conj	AC 30 (14)
AC 29	WT		SAL	Conj	AC 30 (2)
AC 30	WT				
AC 97	Trp$^-$	*trpB*	RP1	Conj	AC 2 (4)
AC 143	Met$^-$ Xyl$^+$	*met*	XYL	Conj	AC 10 (this manuscript)
AC 144	WT XYL$^+$		XYL	Conj	AC 30 (this manuscript)
AC 148	Met$^-$ Xyl$^+$	*met*	XYL	Trd	AC 10 (this manuscript)

[a] Abbreviations used: WT, Wild type; S, spontaneous; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Conj, conjugation; Trd, transduction; Xyl, xylene; Leu, leucine; Ilv, isoleucine-valine; Tol, *m*- or *p*-toluate; Trp, tryptophan; Met, methionine; His, histidine; Sm, streptomycin; XYL-*a*, represents the xylene gene cluster with a mutation in the first step (i.e., phenotypically Xyl$^-$ Tol$^+$).

[b] The plasmids represent: CAM, the plasmid specifying the camphor-degradative pathway (14); SAL, the plasmid specifying the salicylate-degradative pathway (2); RP1, the drug resistance plasmid of P1 incompatibility group (4); XYL, the plasmid specifying the *p*- or *m*-xylene-degradative pathway.

[c] *Pseudomonas* Pxy is the wild-type bacterium (AC 142) previously characterized (5), from which all other derivatives have been isolated. *P. putida* PpG1 wild type (AC 30) is normally xylene negative, from which all other mutants and exconjugants have been derived.

zyl alcohol, 1.0; and enzyme (1.0 to 2.0 mg of protein). The reaction was initiated by the addition of nicotinamide adenine dinucleotide.

In all enzyme assays substrates were used at saturating levels, and the rate of the reaction was proportional to protein concentration. Initial velocity measurements were obtained by drawing tangents to the recorded curves obtained during the first 15 s of the reaction.

One unit of enzyme activity is defined as the amount of enzyme required to produce 1.0 μmol of product (ring fission or reduced nicotinamide adenine dinucleotide) per min. Specific activities are expressed as units per milligram of protein.

Analytical methods. Oxygen consumption was measured polarographically with a Clark oxygen electrode. Details of individual experiments are given in the legends of the appropriate tables. All enzyme assays were recorded on a Cary model 14 recording spectrophotometer.

Chemicals. *p*-Xylene (99.95%) and *m*-xylene were obtained from Phillips Petroleum Co. The following compounds were obtained from Aldrich Chemical Co.: *p*-methylbenzyl alcohol, *p*-toluic acid, *m*-toluic acid, *p*-tolualdehyde, *m*-tolualdehyde, pyrocatechol, 3-methylcatechol, and 4-methylcatechol. *m*-Methylbenzyl alcohol was from K and K Laboratories. Deoxyribonuclease and nicotinamide adenine dinucleotide were from Sigma Chemical Co.

RESULTS

Curing of the xylene pathway. Table 2 shows that Pxy (AC 142) spontaneously loses the ability to grow with *p*- and *m*-xylene. When cells of this organism were treated with mitomycin C the loss of the ability to grow with *p*- and *m*-xylene was increased. Xylene-negative cells, obtained spontaneously or by treatment with mitomycin C, did not revert to the Pxy (AC 142) phenotype.

Transfer of the xylene pathway to other strains. Under normal mating conditions a Leu$^-$ Xyl$^+$ strain of Pxy (AC 138) was incapable of transferring the Xyl$^+$ phenotype to the wild-type strain of *P. putida* PpG1 AC 30, which is incapable of growing with xylene, or to a Xyl$^-$ strain of Pxy (Table 3, experiments 1 and 5). Under the same conditions, Pxy cells harboring a mutation at the first step of the xylene pathway (see AC 141, Table 1) could not transfer the Tol$^+$ phenotype to PpG1 (Table 3, experiment 7). However, when PpG1 AC 4, a Trp$^-$ mutant of PpG1 which harbors a conjugative plasmid, factor K, was included in the mating experiments, the Xyl$^+$ character was transferred to both Xyl$^-$ Pxy and PpG1 (Table 3, experiments 2, 3, 6, and 8). Also, the presence of PpG1 AC 4 in the mating experiments facilitated the transfer of Tol$^+$ properties from Pxy AC 141 to the wild-type strain of PpG1 (Table 3, experiment 8). The donor strain in this experiment harbors a mutation that prevents the oxidation of *p*- and *m*-xylene to the corresponding benzyl alcohols. This property was retained in the exconjugant and suggests that the genes for the xylene degradation are transferred as a unit. The failure to recover Trp$^+$ recombinants, when an auxotrophic mutant of PpG1 (AC 2) was mated with Pxy AC 138 in the presence of PpG1 AC 4 (Table 3, experiment 4), and similar experiments using a variety of other auxotrophic mutants suggest that the genes for xylene degradation are not part of the chromosome.

TABLE 2. *Curing of xylene-degradative pathway from Pseudomonas Pxy (AC 142) by mitomycin C*

Mitomycin C (μg/ml)	Colonies examined		Xyl$^-$ (%)
	Total	Xyl$^-$[a]	
0	370	1	0.27
2.5	215	3	1.4
5	85	2	2.3
10	165	5	3.0
25	100	6	6

[a] For scoring Xyl$^-$ phenotype, single colonies obtained after mitomycin C treatment were applied to mineral salts agar plates with glucose or *m*-toluate as growth substrate. Colonies that grew with glucose but not with *m*-toluate were always tested for growth with *p*- and *m*-xylene to confirm the Xyl$^-$ nature of such colonies.

Support for the extrachromosomal nature of the xylene catabolic pathway is given in Table 4. The Xyl$^+$ PpG1 exconjugants AC 144 and AC 143 showed a high frequency of spontaneous loss of the ability to grow with both *p*- and *m*-xylene, and this frequency was increased in the presence of mitomycin C. In addition, the Xyl$^+$ phenotype was transmissible from the PpG1 AC 143 exconjugant to Xyl$^-$ PpG1 strains and to a Xyl$^-$ segregant of Pxy (Table 5). Table 5 also shows that there was chromosomal gene transfer from PpG1 AC 143 to the auxotrophic mutant PpG1 AC 13. In contrast, chromosomal gene transfer from PpG1 AC 143 to auxotrophic mutants of strain Pxy was not observed.

Transduction experiments. The transfer of xylene catabolic genes from Pxy to PpG1 and the availability of a transducing phage for PpG1 makes these genes amenable to transductional genetic analysis. Table 6 shows that Xyl$^+$ characteristics can be transduced from Xyl$^+$ PpG1 exconjugants (AC 144 and AC 143) at a rather low frequency. These Xyl$^+$ transductants could spontaneously lose the ability to grow with *p*- and *m*-xylene, and this loss was enhanced by treatment with mitomycin C.

A notable difference between the PpG1 Xyl$^+$ transductants and the Xyl$^+$ exconjugants is the inability of the transductants to conjugally

TABLE 3. *Transfer of XYL plasmid in presence of factor K*[a]

Expt	Donor	Recipient	Select	Transfer frequency	Exconjugant phenotype[b]
1	AC 138	AC 30	Xyl$^+$	$<10^{-8}$	
2	AC 138 + AC 4	AC 30	Xyl$^+$	2×10^{-7}	Xyl$^+$ PpG1
3	AC 138 + AC 4	*AC 2*[c]	Xyl$^+$	1×10^{-7}	Xyl$^+$ Trp$^-$ PpG1
4	AC 138 + AC 4	AC 2	Trp$^+$	$<10^{-8}$	
5	AC 138	AC 139	Xyl$^+$	$<10^{-8}$	
6	Ac 138 + AC 4	AC 139	Xyl$^+$	5×10^{-8}	Xyl$^+$ Pxy
7	AC 141	AC 30	Tol$^+$	$<10^{-8}$	
8	AC 141 + AC 4	AC 30	Tol$^+$	1×10^{-7}	Xyl$^-$ Tol$^+$ PpG1

[a] The cells were grown overnight in L broth with shaking at 32°C. Portions (0.2 ml) of Xyl$^+$ donors or AC 4 were then inoculated in 2.0 ml of fresh L broth, either singly or in combination, and grown for 8 h at 32°C with mild shaking. The donors or donor-AC 4 mixtures were then mixed with recipients, diluted 10-fold in fresh L broth, and grown overnight before plating.

[b] Since *Pseudomonas* Pxy and strain PpG1 have different phenotypic properties, such as sensitivity toward phage pf16 or growth with *p*-hydroxybenzoate, the designation of PpG1 or Pxy has been made to identify the exconjugants.

[c] AC 2 has the same *trp* mutational site as AC 4.

TABLE 4. *Curing of XYL from Xyl$^+$ PpG1 exconjugants*[a]

Strain	Mitomycin C (μg/ml)	Frequency of curing (%)
AC 144	0	17
	2.5	84
	5	98
	10	No growth
AC 143	0	21
	2.5	92
	5	96

[a] The method of scoring XYL$^-$ colonies is essentially as described in the footnote to Table 2.

TABLE 5. *Transmissibility of XYL from AC 143*[a]

Recipient	Select	Frequency of transfer	Phenotype[b]
AC 30	XYL	10^{-2}	Xyl$^+$
AC 13	XYL	10^{-2}	Xyl$^+$ His$^+$ Str^r (99%)
			Xyl$^+$ His$^-$ Str^r (1%)
AC 13	His$^+$	10^{-1}–10^{-2}	His$^+$ Str^r Xyl$^+$ (11%)
			His$^+$ Str^r Xyl$^-$ (89%)
AC 139	XYL	10^{-4}	Xyl$^+$ Pxy

[a] The donor and recipient cells were grown overnight in L broth with shaking at 32°C. Equal portions were then mixed and plated on minimal *m*-toluate plates for scoring Xyl$^+$ character and on minimal glucose plates for scoring chromosomal recombinants.

[b] The direction of chromosomal gene transfer has always been shown to be effective from K$^+$ AC 143 to the K$^-$ recipients, as determined by genotypic (Str^r, His$^-$, etc.) and phenotypic (phage sensitivity, growth on selective substrates, etc.) properties of the recombinants.

transfer either Xyl$^+$ properties or chromosomal genes. In contrast to Xyl$^+$ PpG1 exconjugants, which can transfer chromosomal genes and Xyl$^+$ properties at a high frequency (Table 5), all Xyl$^+$ transductants tested so far do not transfer Xyl$^+$ properties by conjugation. However, it is possible to transfer Xyl$^+$ properties from transductants to other cells if transductants are grown in the presence of Xyl$^-$ PpG1 cells which harbor factor K.

Compatibility with other plasmids. Several catabolic and drug resistance plasmids are available in PpG1. Table 7 shows that although the presence of SAL, CAM, or RP1 plasmids reduces the frequency of transfer of Xyl$^+$ properties, all of them appear to be compatible with the genes for the degradation of *p*- and *m*-xylene.

Induction of the xylene catabolic enzymes in strain Pxy and Xyl$^+$ strains. The loss or the transfer of Xyl$^+$ properties could be due to one or more enzymes specified by a plasmid-borne gene(s). Alternatively, the enzymes for the entire catabolic pathway could be specified by such a plasmid. Table 8 shows the oxidation of intermediates in the *p*-xylene pathway by strains of Pxy and PpG1 after enzyme induction with *p*-methylbenzyl alcohol. Essentially identical results have been obtained after induction with *p*-xylene, although the values were comparatively lower. Similar results (not shown) were also obtained for *meta*-substituted metabolic intermediates. All of the intermediates were oxidized by Xyl$^+$ Pxy AC 142, the transductant PpG1 AC 148 and the exconjugant PpG1 AC 143. Although the specific activities of the Xyl$^+$ PpG1 strains induced with *p*-xylene were lower than those obtained for the wild-type strain of Pxy, they are significantly higher than the activities given by cells of the Xyl$^-$ Pxy segregant (AC 139) and the Xyl$^-$ PpG1 auxotroph (AC 10). Both Xyl$^-$ strains gave spe-

cific activities similar to the values obtained when all of the strains were grown on succinate in the absence of an inducing substrate.

The activities of methylbenzyl alcohol dehydrogenases and methylcatechol oxygenases in cell extracts are given in Table 9. The Pxy segregant (AC 139) and the PpG1 auxotroph (AC 10) do not contain detectable levels of these enzymes. Low specific activities were observed in extracts prepared from the PpG1 exconjugant (AC 143) and transductant (AC 148). This could be due to the high rate of spontaneous loss of the plasmid from such cells.

Studies on the specificity of induction of the *p*-xylene-degradative pathway are shown in Table 10. The wild-type strain of Pxy (AC 142) was grown on succinate in the presence of intermediates in the *p*-xylene-degradative pathway. Cells grown in the presence of *p*-xylene, *p*-methylbenzyl alcohol, and tolualdehyde oxidized all substrates tested. In contrast, cells grown in the presence of *p*-toluic acid oxidized *p*-tolualdehyde, *p*-toluic acid, and 4-methylcatechol. No activity was observed with *p*-xylene and *p*-methylbenzyl alcohol.

DISCUSSION

The ability of certain strains of *P. putida* to grow with octane, camphor, salicylate, naphthalene, toluene, and methylbenzoates can be lost spontaneously, and the frequency of loss can be increased by treatment with mitomycin C (2, 3, 7, 14, 17, and 18). In the case of aromatic substrates, the organisms also lost the ability to degrade catechol intermediates by the *meta* pathway (2, 7, 16, 17, and 18). These results have been correlated with the extrachromosomal (plasmid) nature of the genes responsible for the expression of these pathways. In this report, the plasmid nature of the xylene-degradative pathway in *Pseudomonas* Pxy is indicated by its spontaneous loss and the enhancement of the rate of loss on treatment of cells with mitomycin C. Cells of *Pseudomonas* Pxy

TABLE 6. *Transduction of XYL plasmid from XYL⁺-PpG1 exconjugants to other PpG1 recipients by phage pf16*[a]

Donor	Recipient	Select	Frequency of transduction	Transductant phenotype
AC 144 (XYL⁺ PpG1 WT)	AC 10 (Met⁻ PpG1)	XYL⁺	5×10^{-8}	Xyl⁺ Met⁻
	AC 10 (Met⁻ PpG1)	Met⁺	1×10^{-6}	Met⁺ Xyl⁻
AC 143 (XYL⁺ Met⁻ PpG1)	AC 10 (Met⁻ PpG1)	XYL⁺	1×10^{-7}	Xyl⁺ Met⁻
	AC 30 (PpG1 WT)	XYL⁺	1×10^{-7}	Xyl⁺

[a] The recipient cells were grown overnight in L broth at 32°C with shaking and infected at a multiplicity of infection of about 0.1 with phage pf16 grown previously with the donors. The cells were finally harvested by centrifugation, resuspended in saline containing pf16 antiserum, and plated on minimal *m*-toluate plates supplemented with necessary amino acids.

TABLE 7. *Compatibility of XYL with other plasmids*

Donor	Recipient	Resident plasmid	Transfer frequency	Exconjugant phenotype[a]
AC 143 (XYL⁺ Met⁻ PpG1)	AC 30 (PpG1 WT)	None	10^{-2}	Xyl⁺
	AC 29 (SAL⁺ PpG1 WT)	SAL	10^{-4}	Sal⁺ Xyl⁺
	AC 27 (CAM⁺ PpG1 WT)	CAM	10^{-5}	Cam⁺ Xyl⁺
	AC 97 (RP1⁺ Trp⁻ PpG1)	RP1	10^{-4}	Trp⁺ Xyl⁺ Cb^r Nm^r Tc^r

[a] Abbreviations used: Xyl, *p*- or *m*-Xylene; Sal, salicylate; Cam, camphor; Trp, tryptophan; Cb, carbenicillin; Nm, neomycin; Tc, tetracycline. The mating experiments have been done as described in the footnotes to Table 5.

TABLE 8. *Oxidation of p-xylene metabolites by washed-cell suspension*[a]

Assay substrate	Organisms and growth conditions[b]							
	Xyl+ Pxy (AC 142)		Xyl- Pxy (AC 139)		Xyl+ PpG1[c] transductant (AC 148)		Xyl+ PpG1 exconjugant (AC 143)	
	succ + *p*-MBA	succ	succ + *p*-MBA	succ	succ + *p*-MBA	succ	succ + *p*-MBA	succ
p-Xylene	510	6	0	0	60	13	520	10
p-Methylbenzyl alcohol	560	3	7	0	99	11	980	4
p-Tolualdehyde	420	13	2	20	52	6	930	18
p-Toluic acid	360	3	0	2	42	11	450	12
4-Methylcatechol	3,800	33	0	0	770	14	6,500	21

[a] Values are reported as nanomoles of oxygen consumed per minute per milligram of protein. Substrates were added in *N,N*-dimethylformamide. Results are corrected for endogenous oxygen consumption in the absence of substrate.

[b] Abbreviations used: Xyl, *p*-Xylene; *p*-MBA, *p*-methylbenzyl alcohol; succ, succinate.

[c] Identical values for AC 10.

TABLE 9. *Oxidation of aromatic compounds by cell extracts*[a]

Organism	Substrate			
	p-MBA	*m*-MBA	3-MC	4-MC
Xyl+ Pxy AC 142	0.23 (0.69)[b]	0.16 (0.43)	2.6 (12.76)	3.69 (23.17)
Xyl- Pxy AC 139	<0.003	<0.003	<0.001	<0.001
Xyl+ PpG1 AC 148	0.07 (0.2)	0.05 (0.14)	0.45 (1.43)	0.64 (1.43)
Xyl+ PpG1 AC 143	0.016 (0.107)	0.017 (0.07)	0.80 (6.43)	1.42 (9.09)
Xyl- PpG1 AC 10	<0.003	<0.003	<0.001	<0.001

[a] Assays performed as described in Materials and Methods. Abbreviations used: *p*-MBA, *p*-Methylbenzyl alcohol; *m*-MBA, *m*-methylbenzyl alcohol; 3-MC, 3-methylcatechol; 4-MC, 4-methylcatechol.

[b] Values in parentheses are for heat-treated extracts.

TABLE 10. *Oxidation of p-xylene metabolites by washed-cell suspension of Pseudomonas Pxy (wild type)*[a]

Assay substrate	Growth conditions[b]				
	succ + Xyl	succ + *p*-MBA	succ + *p*-TALD	succ + *p*-TA	succ
p-Xylene	130	510	52	9	6
p-Methylbenzyl alcohol	200	560	99	6	3
p-Tolualdehyde	170	420	120	64	13
p-Toluic acid	130	360	110	31	3
4-Methylcatechol	1,000	3,800	890	90	33

[a] Values are reported as nanomoles of oxygen consumed per minute per milligram of protein. Substrates were added in *N,N*-dimethylformamide. Results are corrected for endogenous oxygen consumption in the absence of substrate.

[b] Abbreviations used: Xyl, *p*-Xylene; *p*-MBA, *p*-methylbenzyl alcohol; *p*-TALD, *p*-tolualdehyde; *p*-TA, *p*-toluic acid; succ, succinate.

that could not utilize *p*- or *m*-xylene as growth substrates also lost the ability to catalyze "meta" cleavage of the aromatic nucleus. The observations that Xyl+ transductants and exconjugants also spontaneously lose the ability to grow with *p*- and *m*-xylene, a property which is increased in the presence of mitomycin C, also suggests that the genes for xylene degradation are carried on a plasmid. Further, covalently closed circular duplex molecules have

been isolated from XYL$^+$ K$^+$, XYL$^+$ K$^-$, and XYL$^-$ K$^+$ cells, but not from XYL$^-$ K$^-$ segregants, confirming the plasmid nature of this pathway (data not shown). Thus, we propose to designate the gene complement for xylene degradation in *Pseudomonas* Pxy as the XYL plasmid.

In contrast to a number of catabolic plasmids that can be transferred by conjugation, XYL appears to be nonconjugative and is rendered transmissible only in the presence of the factor K transfer plasmid. Consistent with this conclusion is the observation that transductional transfer of the XYL plasmid from XYL$^+$ K$^+$ PpG1 exconjugants to other PpG1 mutants produces transductants which inherit only the XYL plasmid. The transductants are incapable of transferring the XYL plasmid unless they are grown in the presence of K$^+$ cells. Thus, XYL replicates as a nonconjugative plasmid inside the transductants. TOL, a naturally occurring conjugative plasmid (18), in contrast, is highly transmissible from the TOL$^+$ PpG1 transductants.

The loss, either spontaneous or on treatment with mitomycin C, of the ability of Pxy to utilize xylene as a growth substrate does not give any indication as to the range of genetic entities that may be involved. However, the Xyl$^+$ strains PpG1 AC 148 and 143 can oxidize *p*-xylene, *p*-methylbenzyl alcohol, *p*-tolualdehyde, *p*-toluic acid, and 4-methylcatechol when they are grown in the presence of *p*-xylene or *p*-methylbenzyl alcohol. These oxidative capacities are lost in the Pxy segregant AC 139. Also, cell extracts of the Xyl$^+$ PpG1 strains, but not the Pxy segregant, show significant dehydrogenase and oxygenase activity for the respective methylbenzyl alcohols and methylcatechols. These observations provide further evidence that the xylene catabolic pathway is carried on the XYL plasmid.

The substrate specificity studies also provide preliminary evidence for the existence of two separate regulatory units. It appears that *p*-xylene oxygenase and *p*-methylbenzyl alcohol dehydrogenase are induced by *p*-xylene and *p*-methylbenzyl alcohol. In contrast, *p*-toluic acid induces the enzymes necessary for its own catabolism. Strongly polar Xyl$^-$ Tol$^+$ PpG1 exconjugant mutants have been isolated. Cells of these mutants, when grown in the presence of *p*-xylene, *p*-methylbenzyl alcohol, or *p*-tolualdehyde, do not oxidize any of the intermediates in xylene degradation. Induction with *p*-toluic acid elicits the formation of the necessary enzyme complement responsible for its catabolism. A detailed genetic analysis of the linkage relationship in the XYL plasmid might confirm the presence of separate genetic regulatory units in the xylene catabolic pathways in *Pseudomonas* Pxy.

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