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Metabolism of Benzoate and the Methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for the Existence of a TOL Plasmid

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Mutant strains of *Pseudomonas putida* (*arvilla*) mt-2 which have lost the ability to grow at the expense of *m*- or *p*-toluate (methylbenzoate) but retain the ability to grow with benzoate arise spontaneously during growth on benzoate; this genetic loss occurs to a lesser extent during growth on nonaromatic carbon sources in the presence of mitomycin C. The mutants have totally lost the activity of the enzymes of the divergent *meta* pathway with the possible exception of 2-oxopent-4-enoate hydratase and 4-hydroxy-2-oxovalerate aldolase; unlike the wild type they utilize benzoate by the *ortho* pathway. Evidence is presented that these mutants have lost a plasmid coding for the enzymes of the *meta* pathway, which may be transmitted back to them or into other *P. putida* strains. Preliminary results from these mutants and from a mutant defective in the regulation of the plasmid-carried pathway suggest that the wild type contains two benzoate oxidase systems, one on the plasmid which is nonspecific in both its catalysis and its induction and one on the chromosome which is more specific to benzoate as substrate and is specifically induced by benzoate.

Pseudomonas putida (*arvilla*) mt-2 metabolizes benzoate, *m*-, and *p*-toluate (methylbenzoate) by the divergent *meta* (or α-ketoacid) pathway (7) (Fig. 1), a characteristic which separates it from other *P. putida* strains which metabolize benzoate by the *ortho* (or β-ketoadipate) pathway. However, *P. arvilla* has genes coding for the *ortho* pathway as was demonstrated by incubating uninduced cells with catechol (7) and subsequently by growth on catechol (unpublished results). Recently Nakazawa and Yokota (9) described spontaneous mutants of this organism which changed their mode of benzoate utilization from the *meta* pathway to the *ortho* pathway, apparently caused by a deletion of at least the early enzymes of the *meta* pathway.

Independently we have isolated mutants of apparently the same phenotype and probably the same genotype as those described previously (9) while screening for mutants for growth on benzoate and the toluates during attempts to produce mutants blocked in the *meta* pathway; the pleiotropic mutants have retained the ability to grow on benzoate but have lost the ability to grow on the toluates. Work presented here indicates that the majority, if not all, of the enzymes of the *meta* pathway are coded for on a transmissible plasmid carried by the wild-type strain, mt-2.

MATERIALS AND METHODS

Bacterial strains. *P. arvilla* mt-2 was originally given to us by G. D. Hegeman, Department of Microbiology, Indiana University. The wild-type and mutant strains derived from it have been given PaW (*P. arvilla* Wales) designations. *P. putida* NC1B 10015 was obtained from the National Collection of Industrial Bacteria, Torrey Institute, Aberdeen, Scotland. *P. putida* PRS 2000 (ATCC 12633) was given us by L. N. Ornston, Department of Biology, Yale University.

Media and culture conditions. *P. arvilla* wild type (PaW 1) was maintained on 5 mM *m*-toluate minimal salts agar slopes, and all other strains were maintained on 10 mM succinate or 1% yeast extract (YE) minimal salts agar slopes. The composition of the media and conditions of culture, harvesting, and preparation of cell-free extracts have all been described (7).

Enzyme assays. Enzyme assays were carried out as described (8). 2-Hydroxymuconic semialdehyde hydrolase was assayed by measuring the disappearance of 2-hydroxy-6-oxohepta-2,4-dienoate, the *meta* cleavage product of 3-methylcatechol which is the substrate most rapidly hydrolyzed by the enzyme and which is not attacked by the other ring-fission product metabolizing enzyme, 2-hydroxymuconic semialde-

hyde dehydrogenase. Therefore, we refer to it as 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (see Table 1); the specific activity thus measured is about 20 times that using 2-hydroxymuconic semialdehyde as substrate (7).

The low levels of catechol 2,3-oxygenase in cell-free extracts of PaW 3 were measured after heat treatment of the extracts, and the low levels of catechol 1,2-oxygenase in extracts of benzoate- and succinate-grown cells of PaW 1 were measured after H_2O_2 treatment (8).

Isolation of mutant strains. Mutagenesis was carried out by standard procedures involving exposure of cells to nitrosoguanidine (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) in citrate buffer (pH 6). In some cases this was followed by two cycles of penicillin G/D-cycloserine lysis to concentrate the mutants (11). The leucine auxotroph, PaW 15, was isolated as a spontaneous mutant by the penicillin-cycloserine method alone. The streptomycin-resistant strain PaW 17 was isolated as a spontaneous mutant by plating about 10^9 cells of PaW 8 onto nutrient broth plates containing 100 μg of streptomycin sulfate per ml.

Curing with mitomycin C. Experiments were carried out as described by Chakrabarty (2) except that nutrient broth, 10 mM succinate, or 10 mM benzoate minimal media was used in place of Luria broth. After incubation, tubes showing growth were diluted and spread onto succinate agar plates, and individual colonies were patched onto succinate, benzoate, and *m*-toluate plates.

Conjugation experiments. Donor and recipient cells were grown overnight at 30 C in 5 ml of 1% YE to a cell density of 10^8 to 7×10^8 cells/ml. About 30 min prior to the experiment, 0.2 to 0.4 ml of the donor culture was pipetted into 5 ml of fresh sterile 1% YE and shaken at 30 C. To initiate the conjugation, samples containing 0.5 ml of donor and recipient cultures were mixed and incubated at 30 C for 30 min without shaking, and concurrently separate tubes containing 0.5 ml of sterile minimal medium and either 0.5 ml of donor or recipient cultures were incubated as controls. The tubes with mixed donor and recipient cultures were centrifuged, washed, and suspended in 1 ml of minimal medium before plating 0.1 ml onto *p*-toluate agar plates (undiluted and diluted to 10^{-1} and 10^{-2}); control tubes were plated directly onto *p*-toluate agar plates and diluted appropriately for plating onto 1% YE agar plates to give cell counts. In no experiments did growth appear on any of the control *p*-toluate plates.

In making an assessment of the frequency of conjugation, we observed that dilution of a mixture after conjugation produced more than the expected reduction in the number of exconjugants, presumably because at higher cell densities there is still opportunity for conjugation to take place after plating. The frequency of conjugation was therefore calculated using the dilution which produced the lowest significant number of exconjugants, usually with less than 70 colonies per plate. With our technique, the vast majority of conjugations probably occur on the plate and not during the incubation in solution.

To allow for the possibility that cross-feeding might artificially raise the apparent number of exconjugants, single colonies were selected from the plates used for assessing the frequency of conjugation and were streaked onto fresh *m*- or *p*-toluate agar plates to establish that growth could take place in the absence of background cell mass.

Oxygen uptake experiments. Oxygen uptake was measured in a Clark oxygen electrode as was previously described (7).

RESULTS

During our earlier work on *P. arvilla* (7), we proposed from the specificities of the ring fission product-metabolizing enzymes that catechol and 4-methylcatechol, and their precursors benzoate and *p*-toluate, were dissimilated mainly by the oxidative or 4-oxalocrotonate branch of the *meta* pathway, and that 3-methylcatechol and its precursor *m*-toluate were dissimilated by the hydrolytic branch. In an attempt to prepare mutants blocked in one of the branches, we screened *P. arvilla* after mutagenesis for growth on benzoate and *m*-toluate. By far the most common strains obtained were able to grow on benzoate (*ben*$^+$) but were unable to grow on either *m*-toluate (*mtol*$^-$) or *p*-toluate (*ptol*$^-$). These strains were characterized by not producing a yellow color in liquid media during growth on benzoate, as does the wild type, and in producing a pink coloration but no growth when inoculated into *m*-toluate liquid media. On *m*-toluate agar plates, a brown diffuse area develops in the vicinity of mutant cells. No coloration is produced in liquid or on agar plates with *p*-toluate.

On assaying benzoate-grown cells of this strain type, a typical example of which is PaW 8, none of the *meta* pathway enzymes except for low uninduced levels of 2-oxopent-4-enoate hydratase and 4-hydroxy-2-oxovalerate aldolase were detected (Table 1). However, high induced levels of catechol 1,2-oxygenase and *cis,cis*-muconate lactonizing enzyme were found, indicating that benzoate was metabolized by this mutant via the *ortho* pathway. These strains appear to be extremely stable, and no revertants have been detected in our investigations; if revertants do occur, they do so at a frequency well below 10^{-9}.

The nature of the enzyme complement in these strains indicates that they have suffered a considerable deletion of the genes coding for at least the majority of the enzymes of the *meta* pathway. To test whether this was explicable in terms of loss of a plasmid as was recently demonstrated for several peripheral catabolic pathways in *Pseudomonas* (2, 3, 4, 12), we tried curing with mitomycin C by use of the method

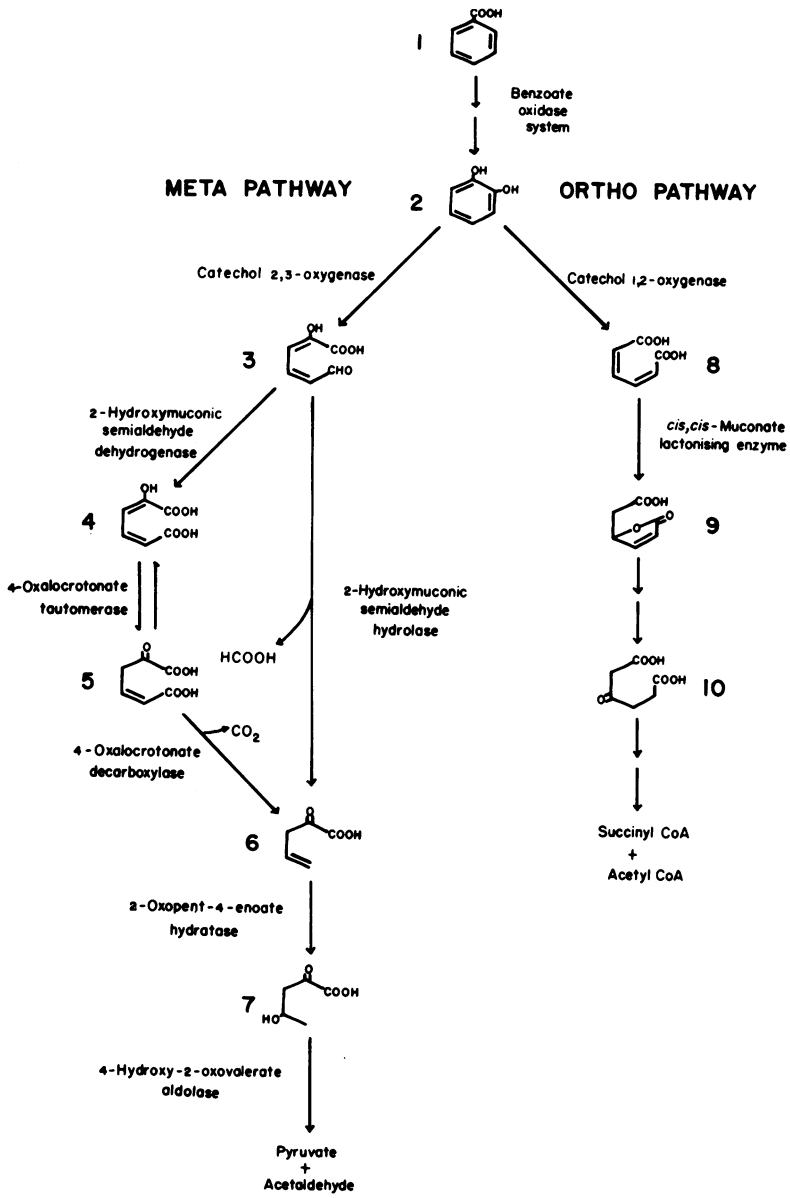

FIG. 1. *Metabolism of benzoate and catechol by Pseudomonas arvilla mt-2. Benzoate is metabolized by the meta pathway, catechol by the ortho pathway; m-toluate and p-toluate are metabolized by the meta pathway and the corresponding methyl derivatives (see reference 7). (1) Benzoate, (2) catechol, (3) 2-hydroxymuconic semialdehyde, (4) 4-oxalocrotonate (enol), (5) 4-oxalocrotonate (keto), (6) 2-oxopent-4-enoate, (7) 4-hydroxy-2-oxovalerate, (8) cis,cis-muconate, (9) muconolactone, (10) β-ketoadipate.*

described by Chakrabarty (2). In a number of screenings after growth in the presence of up to 20 μg of the antibiotic per ml, no colonies were detected which had lost the ability to grow on benzoate, but a small fraction (<1%) of the cells were found to be *ben*$^+$ *mtol*$^-$ *ptol*$^-$ and metabolized benzoate by the *ortho* pathway as determined by the Rothera test for β-ketoadipate (14); in all respects both phenotypically and genotypically, these cured strains appeared identical to those detected after nitrosoguanidine treatment. The strain chosen to represent this genotype, PaW 8, results from a mitomycin C curing experiment. We later found that growth upon benzoate after a small inoculum (~10^4 cells/ml) produced a greater degree of

curing (up to 7%), and the presence of mitomycin C in the benzoate medium did not increase the frequency of this spontaneous mutation. The spontaneous loss of *meta* enzymes on benzoate can be quite extensive: we found that on one of our older slopes, with benzoate agar, all the cells had reverted entirely to *ortho* cleavage of benzoate, and no wild-type cells could be found. We thereupon maintained the wild-type strain on *m*-toluate agar slopes. In the short term at least, the spontaneous loss on benzoate medium appears to be a function of cell growth and not an effect which occurs in stationary phase. In one experiment we inoculated a benzoate medium with wild-type cells, and monitored for loss of ability to grow on *m*-toluate. After 1 day, when growth was complete, the number of *mtol*$^-$ colonies was 3/200, and this proportion did not increase on letting the culture stand for 7 days. A likely reason for selection of these strains on benzoate medium is that their growth rate is somewhat faster than the wild type (~85 min for a PaW 8 as distinct from ~100 min for PaW 1).

The transmissible nature of the plasmid was demonstrated by conjugation between PaW 15, a *leu*$^-$ auxotroph of the wild-type strain which reverted to prototrophy at a frequency of 10^{-8} to 10^{-9}, and a number of other strains (Tables 2 and 3). The frequency of transfer was consistently high with PaW 8, the cured strain, and PaW 10 and PaW 11, both secondary mutants of PaW 8 which had been further mutated to loss of ability to grow on benzoate, and which are blocked mutants of the *ortho* pathway. The frequency of plasmid transfer into PaW 17, a streptomycin-resistant derivative of PaW 8, was about 10 times lower than into the parent

TABLE 1. *Relative specific activities of enzymes of aromatic metabolism in cell-free extracts of strains of P. arvilla mt-2*

Activity	Relative sp act[a]			
	PaW 1		PaW 8	PaW 3
	Benzoate 10 mM	Succinate 10 mM	Benzoate 10 mM	Benzoate 10 mM
meta pathway				
Catechol 2,3-oxygenase	100 (7.3)	0.3	<0.02	4.6
2-Hydroxy-6-oxohepta-2,4-dienoate hydrolase	100 (0.84)	0.1	<0.02	2.1
2-Hydroxymuconic semialdehyde dehydrogenase	100 (0.16)	1.9	<0.02	1.3
4-Oxalocrotonate tautomerase	100 (30)	0.8	0.3	2.5
4-Oxalocrotonate decarboxylase	100 (0.91)	1.8	<0.05	<0.05
2-Oxopent-4-enoate hydratase	100 (2.0)	0.5	3.5	8.0
4-Hydroxy-2-oxovalerate aldolase	100 (0.049)	5.0	16	6.0
ortho pathway				
Catechol 1,2-oxygenase	0.1	0.3	100 (1.04)	20
cis,cis-Muconate lactonizing enzyme	<0.02	0.7	100 (0.37)	105

[a] *meta* enzymes as percentage of activities in benzoate-grown cells of PaW 1 and *ortho* enzymes as percentage of activities in benzoate-grown cells of PaW 8. The numbers in parentheses are specific activities in units per milligram of protein.

TABLE 2. *Strains of P. arvilla mt-2*

Strain designation	Phenotype[a]	Genotype	Parent strain	Method of production[b]
PaW 1 (wild type)	Ben$^+$Mtol$^+$Ptol$^+$	wt/TOL		
PaW 3	Ben$^+$Mtol$^-$Ptol$^-$	/TOL-1	PaW 1	NG
PaW 5	Ben$^+$Mtol$^-$Ptol$^-$	/TOL-2	PaW 1	NG
PaW 8	Ben$^+$Mtol$^-$Ptol$^-$	/TOL[d]	PaW 1	MC
PaW 10	Ben$^-$Mtol$^-$Ptol$^-$	*ben-1*/TOL[d]	PaW 8	NG followed by PC
PaW 11	Ben$^-$Mtol$^-$Ptol$^-$	*pcaE1*/TOL[d]	PaW 8	NG followed by PC
PaW 15	Ben$^+$Mtol$^+$Ptol$^+$Leu$^-$	*leu-1*/TOL	PaW 1	PC
PaW 17	Ben$^+$Mtol$^-$Ptol$^-$Str^r	*str-1*/TOL[d]	PaW 8	S

[a] Phenotype abbreviations: Mtol, *m*-toluate; Ptol, *p*-toluate; Ben, benzoate; Leu, leucine; Str, streptomycin.
[b] NG, nitrosoguanidine mutagenesis; MC, mitomycin C curing; PC, penicillin G-D-cycloserine selection; S, spontaneous selection on nutrient agar plates containing 100 μg of streptomycin sulfate per ml.

TABLE 3. *Transfer of the meta pathway genes between strains of P. putida*

Donor	Recipient	Frequency of transfer[a]
PaW 15[b]	PaW 8[c]	2×10^{-3}–5×10^{-3}
	PaW 10[c]	10^{-3}
	PaW 11[c]	10^{-3}
	PaW 3[c]	10^{-3}
	PaW 5[c]	$<10^{-6}$
	PaW 17[c]	10^{-4}[d]
	PRS 2000[e]	10^{-5}
	NCIB 10015[e]	10^{-3}

[a] Defined as the number of exconjugants per donor cell.

[b] Reversion frequency to *leu*$^+$ about 10^{-8}.

[c] Reversion frequency to *ptol*$^+$ undetectable ($<10^{-9}$).

[d] Streptomycin sulfate added to the selection plates at 100 μg/ml.

[e] Did not grow on *p*-toluate or spontaneously mutate to *ptol*$^+$ at a frequency $>10^{-9}$.

strain when streptomycin was present in the selection plates, but was from two to five times higher when streptomycin was absent than when it was present. Since the exconjugants selected in the absence of streptomycin all retained streptomycin resistance (see below), it seems likely that the lower frequency in its presence must be due to at least some of the conjugation occurring on the plates and being deleteriously affected by the streptomycin. Two of the recipients, PaW 3 and PaW 5, have the same genotype as PaW 8 (*ben*$^+$ *mtol*$^-$ *ptol*$^-$) but contain measurable levels of *meta* pathway enzymes and so presumably still contain the plasmid. PaW 3 is a regulatory mutant (Table 1) and the nature of the lesion in PaW 5 is not certain since it contains induced levels of all the *meta* pathway enzymes after growth on benzoate or succinate in the presence of *m*-toluate, but cannot grow on *m*- or *p*-toluate; we think it may be defective at the level of benzoate oxidase (unpublished results). Transfer of the plasmid into PaW 5 occurs at a frequency undetectable in our experiments, which would agree with the difficulty of plasmid transfer into plasmid-containing strains demonstrated in other systems (4). The high frequency of transfer into PaW 3 is in direct opposition to this principle and warrants further investigation.

The plasmid can also be transferred to two other *P. putida* strains. *P. putida* NCIB 10015 already has a *meta* pathway when grown on phenol or the cresols (13), but cannot grow on *p*-toluate although it produces very poor patchy growth on *m*-toluate plates with much accumulation of background yellow color. Exconjugants of *P. putida* NCIB 10015 were produced at virtually the same rate as with PaW 8, and grew well on *p*-toluate and *m*-toluate with no background accumulation. Transfer of the plasmid into *P. putida* PRS 2000 which has no known *meta* pathway genes occurred at a lower frequency, 10^{-5}

Confirmation of the direction of the genetic transfer from donor to recipient is not possible with all the strains, and the results could always be produced by transfer of a *leu*$^+$ gene in the opposite direction. However, the direction of genetic exchange was definitely established with PaW 17 and with *P. putida* NCIB 10015 and *P. putida* PRS 2000. Exconjugants from PaW 17 were collected on *p*-toluate plates in the absence and presence of streptomycin; the numbers were roughly the same on both, differing by a factor of 2 to 5 in favor of the streptomycin-free plates, but all colonies on these plates retained streptomycin resistance when patched onto *p*-toluate-streptomycin plates, demonstrating that the genetic transfer was from PaW 15 to PaW 17. *P. putida* PRS 2000 is specifically lysed by the bacteriophage WOM (6), which does not lyse PaW 1, PaW 8, or exconjugants from PaW 8. Selected exconjugants from *P. putida* PRS 2000 were streaked onto nutrient agar plates and cross-streaked with the phage WOM; all were lysed. Exconjugants from *P. putida* NCIB 10015 can be distinguished from PaW 1 and PaW 8 by patching onto 0.1% nicotinate, malonate, or mannitol plates: whereas *P. arvilla* strains are *nic*$^+$ *mal*$^-$ *mann*$^-$, *P. putida* NCIB 10015 and exconjugants from it are *nic*$^-$ *mal*$^+$ *mann*$^+$.

PaW 8's ability to use benzoate despite plasmid loss suggests that the plasmid does not code for the enzymes converting benzoate to catechol, called here the benzoate oxidase system. This system cannot be assayed directly in cell-free extracts, but the rate of oxygen uptake by induced whole cells on the catechol and methylcatechols is considerably greater than on benzoate and the toluates (7). It is not unreasonable to conclude that the benzoate oxidase system is rate-limiting and, therefore, that at least the relative rates of respiration elicited by benzoate and the toluates are a measure of its specificity (7). We have found good experimental consistency in the relative rates, although the absolute value of the oxygen quotient is very sensitive to the state of the cells at the time of harvesting.

Comparison of the specificities of the benzoate oxidase systems in benzoate-grown cells of

wild type, PaW 1, and the plasmid⁻ strain PaW 8 (which we shall refer to as the TOL^d strain) shows distinct and reproducible differences (Table 4). In PaW 1 the activity is nonspecific and acts on benzoate and the two toluates at comparable rates, whereas in PaW 8 there is no activity on *p*-toluate and the activity on benzoate is three to four times that on *m*-toluate. Rates of uptake found in cells of PaW 8 grown on succinate in the presence of inducing levels of *m*-toluate are very low, insufficient for any significant induced levels of the benzoate oxidase system.

The results from PaW 8 are reinforced when the oxidase specificity in PaW 3 is compared (Table 4). From its enzyme levels (Table 1), PaW 3 appears to be a regulatory mutant strain which contains the TOL plasmid and, consequently, measurable levels of the *meta* pathway enzymes, but these are not induced by growth on benzoate. Consequently, benzoate is dissimilated by the *ortho* pathway and *m*- and *p*-toluates are not dissimilated at all. The benzoate oxidase specificity of this strain is identical to that of PaW 8, within experimental limits.

DISCUSSION

The deletion mutant strains we obtained, as exemplified by PaW 8, are identical in genotype to those described by Nakazawa and Yokota (9), by the criteria of their spontaneity of occurrence, their change in pathway of benzoate metabolism from *meta* to *ortho*, and their total absence of measurable levels of the early *meta* pathway enzymes. Our first detection after nitrosoguanidine treatment probably does not reflect any effect of the mutagen but is merely due to spontaneous loss of the plasmid during the procedure. In general we have found genuine blocked and regulatory mutant strains of the *meta* pathway in this organism very difficult to obtain, and the frequent occurrence of these plasmid deletants might well represent a higher frequency for plasmid loss than for a stable mutation.

It is interesting to note that the TOL plasmid appears to be somewhat insensitive to mitomycin C, particularly when compared to the high frequencies of curing found with the CAM (12) and the NAH (4) plasmids. The highest rate of curing of the TOL plasmid found with mitomycin C was 1%, and the rate of plasmid loss during growth on benzoate was not increased by the antibiotic.

We agree with Nakazawa and Yokota (9) that the spontaneous loss on benzoate is due probably not to a greater number of these events occurring, but to an amplification of the events due to a greater growth rate by the *ortho* pathway after plasmid loss than by the *meta* pathway before plasmid loss; however the difference on our media is not as pronounced. We also agree that the preferential expression of the *meta* pathway in the wild type is probably due to the differences in induction of the two pathways; whereas the *meta* pathway is induced from the top by the primary substrate, benzoate or the toluates, the *ortho* pathway is induced by the product of its first enzymes, *cis,cis*-muconate (10). This effect has been well described in several other systems (1, 5, 8). We are at present investigating the regulation of the *ortho* pathway in the cured strain, PaW 8, to confirm the hypothesis.

The results show conclusively that loss of the plasmid causes a total disappearance of all the *meta* enzymes from catechol 2,3-oxygenase to the two enzymes producing 2-oxopent-4-enoate. The level of 4-oxalocrotonate tautomerase is not considered significant because of the dubious nature of the assay at low specific activities.

TABLE 4. *Rates of oxygen uptake by washed cell suspensions of strains of P. arvilla mt-2*

Assay substrates	Rate of oxygen uptake				
	PaW 1		PaW 8		PaW 3
	Benzoate (5 mM)	*m*-Toluate (5 mM)	Benzoate (5 mM)	Succinate (5 mM) + *m*-Toluate (5 mM)	Benzoate (5 mM)
Benzoate	139 (146)	281 (117)	152 (330)	8	178 (390)
p-Toluate	40 (42)	192 (80)	0 (0)	0	0 (0)
m-Toluate	95 (100)	241 (100)	46 (100)	8	46 (100)
Endogenous	27	25	15	16	25

[a] In microliters of O_2 per minute per milligram (dry weight) (endogenous uptake subtracted). Numbers in parentheses indicate relative uptake, with rate on *m*-toluate-100%.

However, PaW 8 does contain low but significant levels of both 2-oxopent-4-enoate hydratase and 4-hydroxy-2-oxovalerate aldolase. It could be that these enzymes are not coded for on the plasmid but are determined by the chromosome, and are not induced in PaW 8 after growth on benzoate. If, however, both hydratase and aldolase are located on the plasmid, the low activities in PaW 8 would be due to the nonspecific action of other enzymes catalyzing other reactions in the cells' metabolism, a distinct possibility in light of the nature of the two reactions.

The results on the specificity of oxygen uptake strongly indicate that PaW 1 contains two benzoate oxidase systems, one coded for by genes on the plasmid which is nonspecific in action and nonspecifically induced by benzoate and *m*- and *p*-toluates, and one determined by the chromosome which is more benzoate-specific and which also appears to be specifically induced by benzoate or its metabolites. If the plasmid is lost, as in PaW 8, or if its regulatory mechanism is altered so as to make it uninducible, as in PaW 3, only the chromosomal oxidase system can be expressed.

The hypothesis of two oxidase systems is based only on oxygen uptake, but on this alone it would explain a discrepancy in our previous results (7) when we concluded, on the basis of the oxygen uptake results, that there was only one benzoate oxidase system whether *P. arvilla* was grown on benzoate or the toluates. Examination of the data for benzoate- and *m*-toluate-grown PaW 1 (Table 4) shows that the data do not fit too well with this idea, but if *m*-toluate induces only the nonspecific oxidase system and benzoate induces both, the discrepancy is explained—the specificity of uptake in benzoate-grown PaW 1 is midway between that of the nonspecific oxidase system in *m*-toluate-grown PaW 1 and the specific system in benzoate-grown PaW 8 or PaW 3.

The ability of *P. arvilla* mt-2 to transfer the genes coding for the enzymes of toluate metabolism represents another example of transmissible genetic information coding for peripheral metabolism in *Pseudomonas*, together with camphor (12), salicylate (2), naphthalene (4), and octane (3) metabolism. This method of storing genetic information has obvious advantages to saprophytic microorganisms as it involves least cost to the population as a whole in terms of deoxyribonucleic acid (DNA) synthesis when expression of the enzyme does not provide a positive selective force. It is of interest that three of these catabolic plasmids, coding for degradation of salicylate (2), naphthalene (4), and now the toluates, involve *meta* cleavage of catechol; no report has yet been made of an *ortho* pathway coded in this manner.

In the absence of alternative evidence it seems reasonable to assume that the explanation for the transmissibility and spontaneous loss of these catabolic pathways involves plasmids, in spite of the failure of attempts to demonstrate extrachromosomal elements of DNA in these organisms (G. D. Hegeman, personal communication), although the possibility of alternative explanations must not be discarded.

P. putida (*arvilla*) mt-2 represents an extreme case of nutritional versatility in that the loss of the TOL plasmid does not prevent it from utilizing the substrate on which it was first isolated in the laboratory, benzoic acid, but alters the pathway of its catabolism. Thus, the feature which distinguishes *P. arvilla* from other *P. putida* strains, its metabolism of benzoate by the *meta* pathway, is due not to its chromosomal genes but to a plasmid which can easily be lost and just as easily passed to other strains, and the regulation of which suppresses the expression of the chromosomal pathway. Findings such as this demonstrate the potential hazards of using nutritional screening as an aid to classification within genera of microorganisms able to carry the enzymes of peripheral metabolism on plasmids which are both easily lost and readily transmitted.

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