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Characterization of a Spontaneously Occurring Mutant of the TOL20 Plasmid in *Pseudomonas putida* MT20: Possible Regulatory Implications

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Pseudomonas putida MT20 carries a plasmid (TOL20) that codes for the enzymes responsible for the catabolism of toluene, *m*- and *p*-xylene to benzoate, and *m*- and *p*-toluate, respectively, followed by *meta* cleavage of the aromatic ring. Growth on 5 mM benzoate selects very strongly for (i) strains that have been cured of the plasmid and (ii) strains with an intermediate growth pattern (the B3 phenotype) that retain the ability to grow on toluene, *m*-xylene, and benzoate but are unable to grow on *m*-toluate. Both types of strains were selected because they are no longer able to oxidize benzoate by the plasmid pathway but instead use an alternative route, the *ortho* or β-ketoadipate pathway, which is chromosomally coded and supports faster growth. Evidence that one strain with the B3 phenotype, MT20-B3, has a regulatory mutation that prevents induction of the *meta*-pathway enzymes by benzoate and *m*-toluate, but which enables them to be induced by toluene and *m*-xylene, is presented. The plasmid in this strain, as in most of the others with the same phenotype, is nonconjugative. Analysis of MT20-B3, together with revertants of it and other noninducible mutants, has led to a model for the regulation of the plasmid-coded enzymes in MT20, in which it is proposed that the early enzymes for degradation of *m*-toluate and benzoate are positively controlled by two regulator molecules, one of which interacts with toluene and *m*-xylene as inducers and the other of which interacts with benzoate and *m*-toluate. It is argued that MT20-B3 and strains with a similar phenotype arose as a result of a deletion of the gene coding for the second regulator molecule.

We have recently shown that pseudomonads capable of degrading toluene, *m*-xylene, and *p*-xylene through benzoate, *m*-toluate, and *p*-toluate, respectively, and thence by the *meta* pathway (Fig. 1), can readily be isolated from soil and that in each strain examined the genes coding for the catabolic enzymes appear to be present on a curable plasmid (8).

These plasmids, called the TOL plasmids, showed some differences from each other. In particular, three of them, of which we have selected TOL20 (8) for further study, were characterized by: (i) the relatively poor growth of the host cell on the *p*-methyl-substituted substrates and (ii) the strong counterselection against wild-type cells during growth on benzoate, resulting both in cells cured of the plasmid, with a growth pattern referred to as the B1 phenotype, and cells with a growth pattern intermediate between the wild type and the cured strains (the B3 phenotype; 8).

The reason why benzoate selects for cured cells of *Pseudomonas putida* MT20, the host of TOL20, appears to be the same as previously described for *P. putida* (*arvilla*) mt-2 (7). Like *P. putida* mt-2, *P. putida* MT20 possesses a second pathway for the dissimilation of benzoate, the *ortho* or β-ketoadipate pathway, which is presumed to be chromosomally coded; this pathway is common among saprophytic pseudomonads (5). Like the plasmid-coded *meta* pathway, the first step is the oxidation of benzoate to catechol, but thereafter the pathways diverge. Further utilization by the *ortho* pathway depends upon the conversion of catechol by basal levels of catechol 1,2-oxygenase (C12O) to *cis, cis*-muconate, its product inducer (5). The catechol 2,3-oxygenase (C23O) initiating the *meta* pathway is probably induced by benzoate itself (2, 3; this paper); thus, during growth on benzoate it is fully induced and will rapidly metabolize any catechol formed, preventing induction of the *ortho* pathway. In the wild type, where both pathways are present, benzoate is therefore degraded by the plasmid-coded *meta* pathway.

We have found empirically that growth on benzoate is a good method of selecting cells that have lost a resident TOL plasmid, and the cured cells, which use the chromosomal *ortho* pathway, appear to grow more rapidly than the wild type, as has been demonstrated for *P. putida* mt-2 (7, 8).

Although this argument clearly explains the selection of cured cells resulting from growth on benzoate, it does not explain or predict the simultaneous occurrence of cells having the B3 phenotype, also found at high frequencies after growth of *P. putida* MT20 on benzoate.

This paper reports findings on the biochemistry and genetics of a strain having this phenotype, which explain why it was readily selected during growth on benzoate. The findings, together with the results of further experiments suggested by them, have led to the formulation of a possible model for the regulation of some of the plasmid-coded enzymes present in *P. putida* MT20.

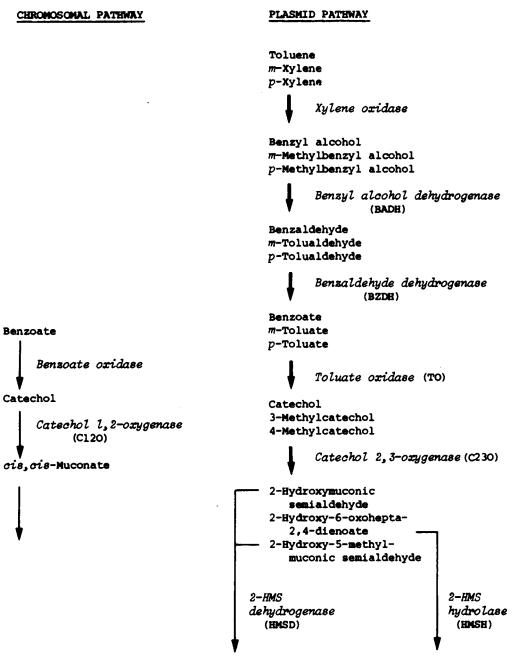

FIG. 1. *Early enzymes of aromatic metabolism in P. putida MT20.*

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this paper are listed in Table 1. The isolation and background of most of these strains, together with methods for their growth and maintenance, have been described (8).

Preparation of cell-free extracts and enzyme assays. In most experiments, cultures were grown at 30°C under forced aeration in 2-liter Pyrex bottles. Exponentially growing cells were harvested after centrifugation at 800 × *g* for 30 min at 4°C in an MSE Major centrifuge. The cells grown for the experiments listed in Table 5 were grown on a 10 mM succinate-minimal salts medium, harvested, and resuspended at 0.3 to 0.4 unit of absorbancy at 610 nm in a minimal salts medium modified as described (4). The appropriate carbon source was then added either directly to a final concentration of 0.5 mM or in four equal portions over a period of 6 h to a final concentration of 5 mM. In the former case, cells were stirred at room temperature; in the latter case, aeration was gentle at first but was gradually increased over the course of the experiment. Cells were harvested after 7 to 8 h. Concentrations of 2-hydroxymuconic semialdehyde were estimated by a method described previously (3). The following enzymes, referred to in the text by abbreviations shown in parentheses, were assayed by published procedures: catechol 1,2-oxygenase (C12O, EC 1.13.11.1; 3); catechol 2,3-oxygenase (C23O, EC 1.13.11.2; 6); benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH, EC

TABLE 1. *Strains of P putida*

Strain designation	Relevant genotype[a]	Parent strain	Method of production or reference[b]
PaW1	Wild type (TOL)		References 3 and 7
PaW15	*leu-1* (TOL)	PaW1	Reference 7
MT20	Wild type (TOL20)		Reference 8
MT20-B1	(TOL20[d])	MT20	Reference 8
MT20-B2	*str-201* (TOL20[d])	MT20-B1	Reference 8
MT20-B3	(TOL20-1)	MT20	Reference 8; this paper
MT20-B4	*str-201* (TOL20-1)	MT20-B3	Reference 8
MT20-B1TOL	(TOL)	MT20-B1	Reference 8
MT20-13	(TOL20-13)	MT20	Growth of MT20 on benzoate
MT20-5	(TOL20-5)	MT20-B3	Spontaneous reversion to Mtol$^+$
MT20-210	(TOL20-210)	MT20	NG mutagenesis
MT20-211	(TOL20-211)	MT20-210	Spontaneous reversion to Mxy$^+$
MT20-212	(TOL20-212)	MT20-210	Spontaneous reversion to Mxy$^+$

[a] Abbreviations: *leu*, Leucine; *str*, streptomycin. The native plasmids resident in PaW1 and MT20 are designated TOL and TOL20, respectively.

[b] NG, Nitrosoguanidine; Mtol$^+$, ability to grow on *m*-toluate; Mxy$^+$, ability to grow on *m*-xylene.

1.2.1.28; 9); 2-hydroxymuconic semialdehyde hydrolase (HMSH; 7); and 2-hydroxymuconic semialdehyde dehydrogenase (HMSD), which was assayed as outlined (5), except that only 0.05 μmol of 2-hydroxymuconic semialdehyde was added as substrate.

Oxygen uptake by whole cells. Measurements were carried out by published methods (3), except that cell suspensions were adjusted to a constant absorbance of 0.5 at 610 nm, and the uptake was calculated as micromoles of O_2 consumed per minute per milliliter.

Conjugation experiments. Procedures were as reported (8).

Mutagenesis. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis was carried out by the method of Ornston (5).

RESULTS

Growth on benzoate and production of the B3 phenotype. After about 20 generations of growth on benzoate, cultures of *P. putida* MT20 contained <1% of cells of the wild type (8). When individual colonies were tested, two phenotypic groups were found, the growth properties of which are shown in Table 2. One group, exemplified by MT20-B1, had lost the ability to grow on all of the aromatic substrates tested except benzaldehyde and benzoate, which were utilized by the chromosomal *ortho* pathway. This isolate does not regain the ability to grow on *m*-toluate or *m*-xylene at detectable frequencies ($<10^{-9}$), completely lacks the plasmid-coded C23O (8), and acts as a recipient for the TOL20 plasmid when mated with wild-type MT20 (Table 3). By these criteria, the MT20-B1 strain appears to have been cured of the TOL20 plasmid.

By contrast, cells having the B3 growth phenotype, exemplified by MT20-B3, retain a number of functions that are plasmid coded, particularly the ability to grow on toluene, *m*-xylene, benzyl alcohol, and *m*-methylbenzyl alcohol (Table 2).

By analogy with the selection of cured strains of *P. putida* mt-2 after growth on benzoate (7), the selection of MT20-B1 and MT20-B3 suggests that these should grow faster than MT20 on benzoate. This was confirmed by following their growth in 5 mM benzoate-minimal medium by determining viable cell counts (Fig. 2). After a brief lag, both MT20-B1 and MT20-B3 grew with a doubling time of about 80 min. By contrast, MT20 grew very slowly (doubling time, about 270 min) for a variable period (in this experiment, about 23 h), after which the growth rate increased. This increase corresponded with an increase in the proportion of cells having growth phenotypes similar to MT20-B1 and MT20-B3. The ratio of B1 to B3 strains fluctuated widely between experiments and presumably reflects a statistical variation in the small numbers of these cells present in different inocula. In general, cells of the B3 type occurred as frequently as B1 cells after growth on benzoate.

From the experiment shown in Fig. 2, it was estimated that between 10^{-4} and 10^{-5} of the cells of the inoculum were of the B^3 phenotype. It must be remembered that MT20 was main-

TABLE 3. *Effect of the B3 phenotype on plasmid transfer*

Donor	Recipient	Selection medium[a]	Frequency of transfer[b]
MT20	MT20-B2	mtol str	10^{-5}
MT20	MT20-B4	mtol str	$<10^{-7}$
MT20-B1TOL	MT20-B2	mtol str	2×10^{-4}
MT20-B1TOL	MT20-B4	mtol str	3×10^{-6}
PaW15	MT20-B1	mtol	10^{-3}
PaW15	MT20-B3	mtol	2×10^{-4}
MT20-B3	MT20-B2	mxy str	$<10^{-7}$
MT20-13	MT20-B2	mxy str	2×10^{-5}

[a] Selection plates contained 5 μg of streptomycin sulfate per ml where indicated plus 5 mM *m*-toluate (mtol)-minimal agar or minimal agar with small tubes of *m*-xylene (mxy) in the inverted lids.

[b] Number of transconjugants per donor cell.

TABLE 2. *Growth characteristics[a] of bacterial strains*

Growth substrate	Bacterial strains used:						
	MT20	MT20-B1	MT20-B3	MT20-5	MT20-210	MT20-211	MT20-212
Toluene	+	−	+	+	−	+	+
m-Xylene	+	−	+	+	−	+	+
p-Xylene	±	−	−	±	−	±	±
Benzyl alcohol	+	−	+	+	−	+	+
m-Methylbenzyl alcohol	+	−	±	+	−	+	+
p-Methylbenzyl alcohol	±	−	−	±	−	±	±
Benzoate	+	+	+	+	+	+	+
m-Toluate	+	−	−	+	+	+	+
p-Toluate	±	−	−	−	±	±	±

[a] Symbols: +, Good growth; ±, poor growth; −, no growth.

tained on *m*-toluate stock plates, which selects against growth of strains with this phenotype, and that not until a colony was put into nutrient broth for the inoculum for this experiment could any growth of B3 cells have occurred. This frequency is therefore remarkably high.

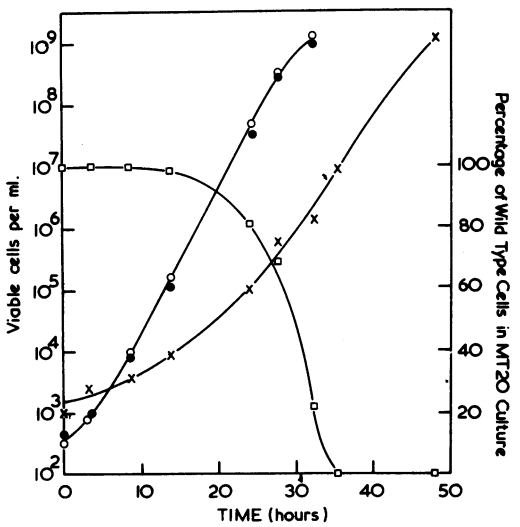

FIG. 2. *Growth of P. putida MT20 and its derivatives on benzoate. A single colony of strain MT20 maintained on a m-toluate agar plate was picked into nutrient broth and grown overnight at 30°C. A 0.1-ml amount of a 10^{-3} dilution was diluted into a 50-ml portion of benzoate-minimal salts medium which was shaken at 30°C in an Erlenmeyer flask. Samples were removed at intervals, diluted, and plated onto nutrient agar plates to determine the viable count. In the case of strain MT20, 176 colonies were then patched onto appropriate test plates to determine their growth phenotype. The growth phenotypes of strains MT20-B1 and MT20-B3 were tested and found to be the same at the beginning and end of the experiment. Symbols: MT20 (×); MT20-B1 (○); MT20-B3 (●); percentage of cells in MT20 culture with wild-type phenotype (□).*

Enzyme analysis of MT20-B3. The results of enzyme assays (Table 4) show that when MT20-B3 was grown on benzoate, C12O, the key enzyme of the *ortho* pathway, but not the *meta*-pathway enzymes C23O, HMSH, and HMSD (Fig. 1), was induced. Normal basal levels of these *meta*-pathway enzymes were, however, present (Table 4). The enzyme activities in *P. putida* MT20 cannot, of course, be assayed after growth on benzoate because of the strong selection against wild-type cells (Fig. 2). However, when succinate-grown cells of *P. putida* MT20 are resuspended in a minimal salts medium containing 5 mM benzoate, there is an appreciable induction of *meta*-pathway enzymes but not of C12O (Table 5). In a similar experiment with 5 mM *m*-toluate as an inducer, *meta*-pathway enzyme levels were about threefold higher, suggesting that benzoate is a relatively poor inducer.

These results show that because MT20 grows slowly on benzoate, utilizing the *meta* pathway,

TABLE 5. *Enzyme specific activities in extracts of P. putida MT20 incubated in the presence of benzoate, catechol, or m-toluate*

Incubation medium[a]	Sp act (mU of activity/mg of protein) of:			
	C23O	HMSH	HMSD	C12O
0.5 mM benzoate[b]	250	37	9.3	10
0.5 mM catechol[b]	28	11	1.9	4
5 mM benzoate[c]	440	66	18	14
5 mM *m*-toluate[c]	1,190	190	49	ND[d]

[a] Cells were grown in 10 mM succinate-minimal salts medium to 0.5 unit of absorbancy at 610 nm, harvested, and resuspended in minimal salts to which the appropriate carbon source was added.
[b] Stirred at room temperature.
[c] Gentle aeration at 30°C.
[d] ND, Not determined.

TABLE 4. *Specific activities of enzymes of aromatic metabolism in cell extracts*

Enzymatic activity of:	Sp act (mU of activity/mg of protein) of:								
	Strain MT20		Strain MT20-B3						
	m-Xylene	Acetate (10 mM)	Toluene	*m*-Xylene	Benzoate (5 mM)	Benzyl alcohol (5 mM)	Acetate (10 mM) + *m*-toluate (5 mM)	Acetate (10 mM) + *m*-methylbenzyl alcohol (5 mM)	Acetate (10 mM)
BADH	2,430	33	2,020	1,940	ND[a]	450	57	360	30
BZDH	580	11	190	370	ND	90	17	130	13
C23O	1,630	31	1,720	1,010	11	220	12	150	25
HMSH	95	6	115	92	3	25	3	78	3
HMSD	20	1.5	18	15	0.3	3.8	0.6	13	0.5
C12O	ND	ND	ND	ND	102	111	ND	10	2

[a] ND, Not determined

both MT20-B3 and MT20-B1 were selected because they could utilize the chromosomal *ortho* pathway and were able to grow faster. The simplest explanation of the failure of benzoate to induce the *meta*-pathway enzymes in MT20-B3 is that this strain has a regulatory mutation that renders them noninducible. The *meta*-pathway enzymes are also not induced when MT20-B3 is grown on acetate in the presence of *m*-toluate (Table 4).

Paradoxically, however, C23O, HMSH, and HMSD were fully induced when MT20-B3 was grown on *m*-xylene or toluene (Table 4).

Benzyl alcohol and *m*-methylbenzyl alcohol as inducers. MT20-B3 grows on benzyl alcohol and very poorly on *m*-methylbenzyl alcohol on plates (Table 2). Enzyme assays after growth on benzyl alcohol and on acetate in the presence of *m*-methylbenzyl alcohol demonstrate some induction of both BADH and BZDH; by this criterion, at least, neither alcohol appears to be as effective an inducer as the hydrocarbons (Table 4). Some induction of the *meta*-pathway enzymes was also observed.

In the benzyl alcohol-grown cells, it is clear that the C23O activity was insufficient to rapidly metabolize the catechol, since the chromosomal C12O was also induced, and as this is induced by *cis, cis*-muconate (5) it requires some accumulation of its substrate for induction. The benzyl alcohol may well be assimilated by both pathways simultaneously.

In the case of MT20-B3 grown in the presence of *m*-methylbenzyl alcohol, there was also a low level of induction of C23O but little significant induction of C12O, which is not surprising, since the *ortho* pathway cannot utilize methylcatechols. The low level of induction of C23O may explain why MT20-B3 grows so poorly on *m*-methylbenzyl alcohol. It should be pointed out, however, that the activities of HMSH and HMSD are relatively high (Table 4) and may indicate that these enzymes are regulated noncoordinately to the activity of C23O.

Finally, it should be stated that the relatively low enzyme levels in cells grown on the alcohols suggest that toluene and *m*-xylene themselves are the more effective inducers of the early enzymes of the *meta* pathway in MT20-B3 than the intermediates in their breakdown.

Oxygen uptake experiments. When cells of MT20-B3 grown on benzoate were tested for their ability to oxidize benzoate, *m*-toluate, and *p*-toluate, the relative rates of oxidation of these assay substrates were found to be very similar to those observed with benzoate-grown MT20-B1 but differed significantly from those of *m*-toluate-grown MT20 or of MT20-B3 grown on *m*-xylene or toluene (Table 6). This indicates that when MT20-B3 is grown on benzoate, it uses the chromosomal and more substrate-specific benzoate oxidase rather than the plasmid-coded toluate oxidase (TO), which does, however, appear to be used when MT20-B3 is grown on *m*-xylene or toluene (Table 6). It should also be noted that there was no evidence for significant oxidation of any of the substrates by MT20-B3 grown on acetate in the presence of *m*-toluate (Table 6), demonstrating that *m*-toluate does not induce the plasmid-coded TO in this strain.

MT20-B3 grown on *m*-xylene or toluene can oxidize *m*-toluate at an induced rate, as can be seen by comparing this rate with that observed with cells of *m*-toluate-grown MT20 and cells of MT20-B3 grown on acetate in the presence of *m*-toluate. The results obtained in these experiments were not sufficiently reproducible for a two-fold difference in the rate of oxidation between *m*-toluate-grown MT20 and toluene- or *m*-xylene-grown MT20-B3 to be considered significant.

Enzyme inducers. Since BADH and BZDH are not induced when *P. putida* MT20 is grown on *m*-toluate, they must be regulated independently from TO, C23O, HMSH, and HMSD (Table 7; Fig. 1).

TABLE 6. *Rates of oxygen uptake by washed cell suspensions*

Assay substrate	Rate of oxygen uptake (μmol of O_2/min per ml)[a] in:					
	Strain MT20	Strain MT20-B1	Strain MT20-B3			
	(*m*-toluate, 5 mM)	(benzoate, 5 mM)	*m*-Xylene	Toluene	Benzoate (5 mM)	Acetate (10 mM) + *m*-toluate (5 mM)
Benzoate	0.056 (52)[b]	0.086 (370)	0.030 (75)	0.062 (111)	0.063 (420)	0.001
m-Toluate	0.107 (100)	0.023 (100)	0.040 (100)	0.056 (100)	0.015 (100)	0.003
p-Toluate	0.050 (47)	0.011 (48)	0.028 (70)	0.030 (54)	0.010 (67)	0.002

[a] The endogeneous uptake was subtracted.
[b] Numbers in parentheses indicate relative uptake, with rate on *m*-toluate = 100.

TABLE 7. *Enzyme specific activities in extracts of strains of P. putida MT20*

Enzymatic activity of:	Sp act (mU of activity/mg of protein)[a] of:												
	Strain MT20			Strain MT20-5			Strain MT20-210			Strain MT20-211		Strain MT20-212	
	Acetate (10 mM)	*m*-Toluate (5 mM)	*m*-Xylene	Acetate (10 mM)	*m*-Toluate (5 mM)	*m*-Xylene	Acetate (10 mM)	Acetate (10 mM) + *m*-xylene	*m*-Toluate (5 mM)	Acetate (10 mM)	*m*-Xylene	Acetate (10 mM)	*m*-Xylene
BADH	33	59	2,430	25	36	470	13	13	13	32	710	81	230
BZDH	11	23	580	6	9	275	4	4	10	15	94	17	24
C23O	31	1,260	1,630	660	650	3,330	20	16	1,040	32	440	180	390
HMSH	6	144	95	72	160	630	5	5	85	8	120	34	113
HMSD	1.5	49	20	8.6	20	79	1.2	1.4	19	1.6	16.5	6.9	16

[a] One unit is the amount of enzyme required to convert 1 μmol of substrate to product per min.

It seems probable that when *P. putida* MT20 is grown on benzoate or *m*-toluate, these substrates are the inducers of at least C23O and presumably also TO. This inference is drawn from the observation that when *P. putida* MT20 is grown on succinate and resuspended with 0.5 mM benzoate, there is appreciable induction of C23O in contrast to a parallel experiment in which the cells were resuspended in 0.5 mM catechol (Table 4). There can be no doubt that catechol was entering the cells during the incubation period, since the concentration of 2-hydroxymuconic semialdehyde in the medium reached 0.07 mM within 1 h. The possibility that failure to detect C23O in this experiment was due to plasmid loss or mutation can be excluded, since a sample of cells were tested at the end of it and all were found to have the full wild-type phenotype.

Reversion of MT20-B3. MT20-B3 does not regain the ability to form colonies on 5 mM *p*-toluate plates at a detectable frequency ($<10^{-9}$). Revertants can, however, be isolated at a frequency of about 10^{-7} on 5 mM *m*-toluate plates. On the basis of their growth properties, revertants on *m*-toluate fell into two phenotypic groups. Nineteen class I revertants were phenotypically similar to MT20-5 and had regained the ability to grow on all substrates except *p*-toluate (Table 1). Nine class II revertants, on regaining the ability to grow on *m*-toluate, lost the ability to grow on the hydrocarbons; the enzymatic analysis of these mutants has proved complex and will be reported elsewhere.

Six independently isolated class I revertants of MT20-B3 were assayed after growth on acetate, and all were found to have constitutive levels of C23O, HMSH, and HMSD as shown for MT20-5 (Table 7).

Since MT20-5 produces *meta*-pathway enzymes constitutively, it seems surprising that it does not regain the ability to grow on *p*-toluate. However, MT20 itself grows very poorly on this substrate (8), which is probably toxic; MT20 produces visible colonies more rapidly on plates containing 2 mM *p*-toluate than on plates containing 5 mM *p*-toluate. Since the enzyme activities in MT20-5 are lower than those in fully induced cells, it may be that toxic intermediates in the metabolism of *p*-toluate accumulate and kill the cell. Because *para* compounds are such poor growth substrates and tend to be toxic, their inductive effects have not been investigated in MT20.

MT20-5, like all other revertants of MT20-B3, produced copious quantities of a black pigment when grown on *m*-toluate, and attempts to grow MT20-5 in liquid on 5 mM *m*-toluate failed, presumably due to the toxic effects of these oxidation products. To obtain sufficient cells for assay, it was necessary to grow the cells overnight on 1.5 mM acetate and over a 6-h period add four portions of *m*-toluate to reach a final concentration of 5 mM. Cells were harvested 1 to 2 h after the final addition of *m*-toluate. When MT20-5 was grown in this way, there was little, if any, increase in C23O activity compared with acetate-grown cells, but HMSH and HMSD activities were both increased (Table 7).

Growth of MT20-5 on *m*-xylene produced a surprising result. The activities of BADH and BZDH were lower than in *m*-xylene-grown MT20 or MT20-B3, whereas C23O and especially HMSH and HMSD were considerably higher (Table 7).

Characterization of mutants noninducible by toluene or *m*-xylene. After nitrosoguanidine mutagenesis of MT20, mutants that failed to grow on both the hydrocarbons and alcohols

but which grew normally on benzoate and *m*-toluate were readily isolated. Six independently isolated mutants were examined, and in each case neither BADH, BZDH, C23O, HMSH, nor HMSD was induced when grown on acetate in the presence of *m*-xylene. This is exemplified by one of them, MT20-210 (Table 7). This indicates that this strain is either a regulatory mutant noninducible by the hydrocarbons or alcohols or, alternatively, that it is impermeable to these substrates.

The comparative ease with which such mutants can be isolated, compared with our failure to detect other classes of mutants with the same growth phenotype, such as the BADH and BZDH structural gene mutants, is surprising. It has also proved difficult to isolate point mutants in structural genes coding for other enzymes in this strain. The reason for this is unknown.

MT20-210 has slightly reduced basal enzyme levels after growth on acetate compared with MT20, and these levels are not increased when the cells were grown in the presence of *m*-xylene (Table 7). Growth on *m*-toluate, however, induces the *meta*-pathway enzymes C23O, HMSH, and HMSD (Table 7).

If the mutation in MT20-210 is a regulatory mutation, then some revertants of it, selected for their ability to grow on *m*-xylene, should produce regulatory molecules altered in such a way that some or all of the enzymes controlled by them would be produced constitutively. Since the enzyme assay data obtained with MT20-B3 strongly suggest that the early enzymes of the *meta* pathway can be directly induced by *m*-xylene, we have screened revertants of MT20-210 for their ability to grow on plates containing 2.5 mM 3-methylcatechol as a carbon source. Mutants such as MT20-5, producing C23O constitutively, grew to some extent on such plates, whereas MT20 normally produced only a small amount of background growth (unpublished data). The results obtained with this toxic substrate have been rather variable, but it is at least a preliminary basis for screening for potential constitutive C23O producers. Out of a total of 256 spontaneous revertants of MT20-210 that were selected on *m*-xylene and tested for growth on 3-methylcatechol, eight were chosen for assay and six were found to have levels of C23O, HMSH, and HMSD after growth on acetate that were significantly higher than the levels in acetate-grown MT20. The results from the strain with the highest level, MT20-212, and one of the revertants which had normal uninduced enzyme levels, MT20-211, are shown in Table 7. In MT20-212, the uninduced levels of C23O, HMSH, and HMSD are consistently four- to fivefold higher than in uninduced cells of MT20, MT20-210, or MT20-211.

Growth on *m*-xylene of both revertants increases the activities of all the enzymes measured. However, in neither case do the activities of C23O appear to be fully induced.

Transfer and curing of TOL20. MT20-B3 and its streptomycin-resistant derivative, MT20-B4, are comparatively poor recipients both for TOL and TOL20 (Table 3), which suggests that they retain a plasmid that is capable of reducing conjugational transfer by means of either surface exclusion or a reduction of the efficiency of establishment of a superinfecting plasmid.

Further evidence that MT20-B3 retained a plasmid comes from the observation that it can spontaneously produce cells with the B1 phenotype. Out of 4,230 colonies tested after growth on nutrient broth and plating onto nutrient agar plates, 9 such strains equivalent to a frequency of 2×10^{-3} were found. It has proved difficult to enhance this frequency, since growth on benzoate has no selective effect (Fig. 2), and we have not found a satisfactory curing agent for MT20 and its derivatives. Acridine orange is generally ineffective against the TOL plasmid-containing strains, and MT20 is extremely sensitive to the toxic and mutagenic effects of mitomycin C.

Although the evidence indicates that MT20-B3 retains a plasmid, it is clear that it has lost the ability to transfer it (Table 3). Out of seven independently isolated B3 strains only one, MT20-13, retained the transfer ability of MT20 (Table 3).

The observation that strains having the same growth phenotype as MT20-B3 are usually pleiotropic mutants suggests that a deletion might have occurred in the plasmid.

From earlier results (Fig. 2) it was estimated that cells with the B3 phenotype were present at a frequency of 10^{-4} to 10^{-5} in MT20 cultures. To confirm this, MT20 was subcultured four times in nutrient broth and plated to single colonies on plates containing 5 mM *m*-toluate and 0.5 mM succinate as carbon sources; cells unable to grow on *m*-toluate form small colonies. Of a total of 17,429 colonies examined, 2 which had the same phenotype as MT20-B3 were found (Table 2), corresponding to a frequency of 10^{-4}.

DISCUSSION

The spontaneous mutant MT20-B3 was originally selected during growth on benzoate be-

cause it outgrew its parent strain, MT20 (8; Fig. 2). It could do this because the enzymes of the *meta* pathway had become noninducible by benzoate (Tables 4 and 6), and it was able to use the chromosomal *ortho* pathway and thus grow faster. The ability of cells to grow faster when utilizing benzoate by the chromosomal *ortho* pathway than by the plasmid-coded *meta* pathway appears to be a general phenomenon (8).

The *meta*-pathway enzymes in MT20-B3 are also noninducible by *m*-toluate (Table 4). The most probable explanation is that MT20-B3 has a regulatory mutation preventing induction of the *meta*-pathway enzymes by benzoate and *m*-toluate. An alternative explanation is that an alteration in permeability results in noninducibility by *m*-toluate, owing to lack of uptake, and that the growth of MT20-B3 on benzoate could result from uptake by a chromosomally coded permease that is specific for benzoate. The difficulties of such an hypothesis are that: (i) it does not explain the switch in utilizing benzoate from the *meta* to the *ortho* pathway with the concomitant increase in growth rate; (ii) it requires the assumption that *m*-xylene and toluene induce a permease that is able to take up *m*-toluate (Table 6); and (iii) it provides no explanation for the constitutive production of *meta*-pathway enzymes by some revertants of MT20-B3 that are able to grow on *m*-toluate (Table 7). MT20-B3 may be impermeable to *m*-toluate if a permease is under the control of a regulatory gene mutated in this strain, but this would be a secondary effect rather than the primary explanation.

Strains having the same unusual growth phenotype as MT20-B3 can be very readily isolated from cultures of MT20 grown on benzoate and occur spontaneously with frequencies as high as 10^{-4}. It seems probable that many, if not all, of them are also regulatory mutants. The high spontaneous frequency could be explained if the plasmid in MT20 had areas of genetic homology or regions of deoxyribonucleic acid analogous to insertion sequences and, thus, commonly produced deletions. Six out of seven independently isolated strains having the same growth phenotype as MT20-B3 are nonconjugative, which seems most readily explicable if a transfer gene(s) is linked to a regulatory gene(s) and deletions often extend from the latter into the former. Furthermore, MT20-B3 does not regain the full wild-type phenotype at a detectable frequency ($<10^{-9}$ on *p*-toluate), which also suggests that the mutation in this strain is a deletion.

The enzymes involved in toluene and xylene degradation fall into at least two regulatory units. BADH and BZDH are regulated independently of the enzymes involved in toluate degradation, as was shown by growth of MT20 on *m*-toluate (Table 7), and are induced by toluene, *m*-xylene, and to a lesser extent by the alcohols derived from them. TO and C23O are probably induced by benzoate and *m*-toluate (Table 5) and also by toluene and *m*-xylene, as was demonstrated by the properties of mutant MT20-B3 (Table 4).

The inducers of HMSH and HMSD are more difficult to ascertain. The only mutants lacking C23O which we have been able to isolate are pleiotropic and probably deletions. The properties of these strains are complex and will be discussed elsewhere, but although they have enabled us to demonstrate that HMSH and HMSD are induced by toluene and *m*-xylene (unpublished data), they have not allowed unambiguous demonstration of whether these enzymes are regulated independently of TO and C23O. Several instances of apparent lack of coordination have been noted during the course of the experiments described in this paper.

It has been argued above that the mutation in MT20-B3 is a deletion in a gene coding for a regulatory molecule, and the inference that such mutants occur spontaneously at a high frequency has been drawn. Thus, it is difficult to understand how TO and C23O remain inducible by toluene and *m*-xylene in MT20-B3 if these inducers interact with the same regulatory molecule as do benzoate and *m*-toluate. It seems more probable that these enzymes are under the control of two regulatory molecules, one interacting with benzoate and *m*-toluate as inducers and another which interacts with toluene and *m*-xylene (Fig. 3).

If this hypothesis is correct, then the properties of MT20-210 can also be readily explained. Since revertants of this strain that are able to grow on *m*-xylene and have low constitutive levels of C23O, HMSH, and HMSD can be readily produced, it seems most probable that the mutation in this strain is in a gene coding for a regulatory molecule that can interact with *m*-xylene and toluene to induce BADH and BZDH and also the early enzymes of the *meta* pathway (Fig. 3). It is not surprising that some of the revertants should have genetically altered regulatory molecules that result in enhanced expression of C23O, HMSH, and HMSD activities.

According to the scheme proposed in Fig. 3, MT20-B3 has a mutation, probably a deletion, in the R2 gene. Thus, *m*-toluate can no longer act as an inducer, but *m*-xylene can still be utilized for growth by interacting with the R1

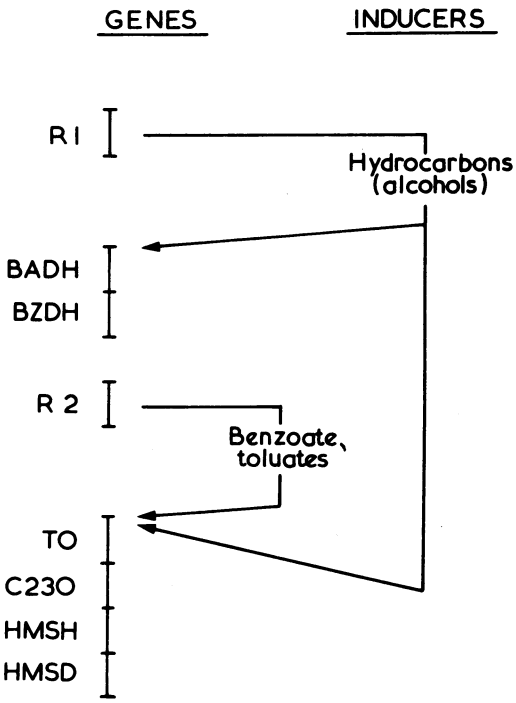

FIG. 3. *Proposed model for the regulation of the early enzymes of the TOL20 specified pathway. A regulator molecule specified by the R1 gene combines with the hydrocarbons and, to a lesser extent, the alcohols as inducers. A molecule specified by a second regulatory gene, R2, controls only the second block of enzymes. In the absence of strong contradictory evidence, enzymes sharing common inducers are represented as forming part of the same regulatory block.*

gene product to induce at least BADH, BZDH, TO, and C23O. Since wild-type MT20 has a functional R2 gene product, *m*-toluate can induce the *meta*-pathway enzymes and is therefore a growth substrate for MT20. In *m*-xylene-grown MT20, TO and C23O could be induced as a result of the interactions of both *m*-xylene with the R1 gene product and of any *m*-toluate transiently formed during the breakdown of *m*-xylene with the R2 gene product.

Revertants of MT20-B3 able to grow on *m*-toluate as a result of enzyme constitutivity could be two types, either operator constitutive mutants or mutants in the R1 gene coding for a modified regulatory molecule. The analysis of MT20-5 is perhaps more consistent with the latter hypothesis in that when this strain was grown on *m*-xylene, BADH and BZDH activities were somewhat lower than in *m*-xylene-grown MT20 or MT20-B3, whereas C23O, HMSH, and HMSD activities were considerably higher.

The hypothesis that genes coding for a set of enzymes may be controlled by two independent regulatory molecules is most consistent with the enzymes being under positive control. If both R1 and R2 genes code for a repressor, it is difficult to see how MT20 could grow on *m*-toluate, since this substrate would not prevent repression by R1 (Fig. 3). If the mutation in MT20-B3 is the result of a deletion in the R2 gene and if mutations in this gene produce a noninducible phenotype at a high frequency, then this also suggests a positive regulatory role for the R2 gene product (1).

The model presented in Fig. 3 provides a convenient rationalization of the data obtained to date on enzyme regulation in MT20 and a working hypothesis for further study. Progress depends upon the development of a transductional system to enable an ordering of the sites of mutation in the various strains and the isolation and characterization of new mutants.

The ability to degrade benzoate by the *ortho* pathway is widespread among soil bacteria, from which it may be deduced that benzoate is a common metabolite. The strong selective effect which growth of MT20 on benzoate has under laboratory conditions is, therefore, rather surprising. Since such strong selective effects are not properties of all TOL plasmid-carrying strains (8), it is interesting to speculate whether the behavior of MT20 in producing cured derivatives and regulatory mutants at such high frequencies after growth on benzoate has any adaptive significance.

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