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Ubiquity of Plasmids in Coding for Toluene and Xylene Metabolism in Soil Bacteria: Evidence for the Existence of New TOL Plasmids

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Thirteen bacteria have been isolated from nine different soil samples by selective enrichment culture on *m*-toluate (*m*-methylbenzoate) minimal medium. Eight of these were classified as *Pseudomonas putida*, one as a fluorescent *Pseudomonas* sp., and four as nonfluorescent *Pseudomonas* sp. All 13 strains appeared to carry TOL plasmids superficially similar to that previously described in *P. putida* mt-2 in that: (i) all the wild-type strains could utilize toluene, *m*-xylene, and *p*-xylene as sole carbon and energy sources, (ii) these growth substrates were metabolized through the corresponding alcohols and aldehydes to benzoate, *m*-toluate, and *p*-toluate, respectively, and thence by the divergent *meta* (or α-ketoacid) pathway, and (iii) the isolates could simultaneously and spontaneously lose their ability to utilize the hydrocarbons, alcohols, aldehydes, and acids, particularly during growth on benzoate, giving rise to cured strains which could grow only on benzaldehyde and benzoate of the aromatic substrates by the alternative *ortho* (or β-ketoadipate) pathway. Eight of the isolates were able to transfer their TOL plasmids into their own cured strains, but only five were able to transfer them in interstrain conjugation into the cured derivative of *P. putida* mt-2. However, *P. putida* mt-2 was able to transfer its TOL plasmid into 11 of the cured isolates, and eight of these were able to retransmit this foreign plasmid in intrastrain conjugation with their own cured derivatives. Three of the isolates, MT 14, MT 15, and MT 20, differed significantly from the others in that the wild-type strains dissimilated the *p*-methyl-substituted substrates poorly, and also, during growth on benzoate, in addition to the cured derivatives, they gave rise to derivatives with a phenotype intermediate between the cured and wild-type strains, the biochemical and genetic nature of which has not been elucidated.

The genus *Pseudomonas* is notable for the large number and variety of compounds that serve as carbon and energy sources for its members. Recent work in a number of laboratories has indicated that, in certain strains, the genes coding for the enzymes responsible for catabolism of some of the less common substrates are carried on transmissible plasmids. Camphor (8), salicylate (1), octane (3), naphthalene (4), benzoate and toluates (11, 12), benzene sulfonic acid (J. B. Johnston and R. B. Cain, Fed. Eur. Biochem. Soc., Dublin, abstr. 137, 1973), and toluene and xylenes (13) are all compounds the breakdown of which appears to be plasmid coded in certain *Pseudomonas* strains.

As yet there has been little attempt to discover how general a phenomenon plasmid coding is for any particular biochemical pathway either within *Pseudomonas* or within the microbial population as a whole. In only one case, that of naphthalene catabolism, has it been argued that the same pathway can apparently be determined by plasmid or chromosomal genes in different strains (4).

Using toluate metabolism as an experimental system, this paper attempts to discover the generality of plasmid coding for this pathway and to assess the extent to which transfer of plasmids can occur. All of 13 random soil isolates, selected from nine separate soil samples for their ability to degrade *m*-toluate, were found to carry plasmids, which, although superficially similar to the TOL plasmid we have described in *Pseudomonas putida (arvilla)* mt-2 (11, 13), did show some variation in their properties.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study are listed in Table 1. PaW 1, the wild type of *P.*

TABLE 1. *Bacterial strains*

Classification	Strain designation	Phenotype[a]	Genotype[b]	Parent strain	Method of production or reference[c]
P. putida (arvilla) mt-2	PaW 1	Tol$^+$	wt/TOL		
	PaW 8	Tol$^-$	/TOL^d	PaW 1	(11)
	PaW 15	Tol$^+$Leu$^-$	*leu-1*/TOL	PaW 1	(11)
	PaW 17	Tol$^-$Str^r	*str-1*/TOL^d	PaW 8	(11)
P. putida	MT 1	Tol$^+$	wt/TOL1		
	MT 1-B1	Tol$^-$	/TOL1^d	MT 1	B
	MT 1-B2	Tol$^-$Str^r	*str-11*/TOL1^d	MT 1-B1	S(100)
	MT 1-2	Tol$^+$Arg$^-$	*arg-11*/TOL1	MT 1	NG
	MT 1-B1TOL	Tol$^+$	/TOL	MT 1-B1	C
Pseudomonas sp.	MT 3	Tol$^+$	wt/TOL3		
	MT 3-B1	Tol$^-$	/TOL3^d	MT 3	B
	MT 3-B2	Tol$^-$Str^r	*str-31*/TOL3^d	MT 3-B1	S(10)
P. putida	MT 5	Tol$^+$	wt/TOL5		
	MT 5-B1	Tol$^-$	/TOL5^d	MT 5	NA
	MT 5-B2	Tol$^-$Str^r	*str-51*/TOL5^d	MT 5-B1	S(100)
	MT 5-B1TOL	Tol$^+$	/TOL	MT 5-B1	C
Pseudomonas sp.	MT 12	Tol$^+$	wt/TOL12		
	MT 12-B1	Tol$^-$	/TOL12^d	MT 12	B
	MT 12-1	Tol$^+$His$^-$	*his-121*/TOL12	MT 12	NG
Pseudomonas sp.	MT 13	Tol$^+$	wt/TOL13		
	MT 13-B1	Tol$^-$	/TOL13^d	MT 13	B
	MT 13-11	Tol$^+$His$^-$	*his-131*/TOL13	MT 13	NG
	MT 13-B1TOL	Tol$^+$	/TOL	MT 13-B1	C
	MT 13-B1TOL 7	Tol$^+$Arg$^-$	*arg-131*/TOL	MT 13-B1TOL	NG
Pseudomonas sp.	MT 14	Tol$^+$	wt/TOL14		
	MT 14-B1	Tol$^-$	/TOL14^d	MT 14	B
	MT 14-B2	Tol$^-$Str^r	*str-141*/TOL14^d	MT 14-B1	S(2.5)
	MT 14-B3	Mxy$^+$Pxy$^-$Tln$^+$	NK	MT 14	B
	MT 14-B4	Mxy$^+$Pxy$^-$Tln$^+$Str^r	*str-142*/NK	MT 14-B3	S(2.5)
	MT 14-4	Tol$^+$His$^-$	*his-141*/TOL14	MT 14	NG
	MT 14-B1TOL	Tol$^+$	/TOL	MT 14-B1	C
	MT 14-B3TOL	Tol$^+$	/TOL	MT 14-B3	C
P. putida	MT 15	Tol$^+$	wt/TOL15		
	MT 15-B1	Tol$^-$	/TOL15^d	MT 15	B
	MT 15-B2	Tol$^-$Str^r	*str-151*/TOL15^d	MT 15-B1	S(10)
	MT 15-B3	Mxy$^+$Pxy$^-$Tln$^+$	NK	MT 15	B
	MT 15-B4	Mxy$^+$Pxy$^-$Tln$^+$Str^r	*str-152*/NK	MT 15-B3	S(10)
	MT 15-8	Tol$^+$His$^-$	*his-151*/TOL15	MT 15	NG
	MT 15-B1TOL	Tol$^+$	/TOL	MT 15-B1	C
	MT 15-B3TOL	Tol$^+$	/TOL	MT 15-B3	C
P. putida	MT 16	Tol$^+$	wt/TOL16		
	MT 16-B1	Tol$^-$	/TOL16^d	MT 16	B
	MT 16-B2	Tol$^-$Str^r	*str-161*/TOL16^d	MT 16-B1	S(50)
	MT 16-B1TOL	Tol$^+$	/TOL	MT 16-B1	C
P. putida	MT 17	Tol$^+$	wt/TOL17		
	MT 17-B1	Tol$^-$	/TOL17^d	MT 17	B
	MT 17-B2	Tol$^-$Str^r	*str-171*/TOL17^d	MT 17-B1	S(100)
	MT 17-B1TOL	Tol$^+$	/TOL	MT 17-B1	C
P. putida	MT 18	Tol$^+$	wt/TOL18		
	MT 18-B1	Tol$^-$	/TOL18^d	MT 18	B
	MT 18-B2	Tol$^-$Str^r	*str-181*/TOL18^d	MT 18-B1	S(100)
	MT 18-B1TOL	Tol$^+$	/TOL	MT 18-B1	C
P. putida	MT 19	Tol$^+$	wt/TOL19		
	MT 19-B1	Tol$^-$	/TOL19^d	MT 19	B
	MT 19-B2	Tol$^-$Str^r	*str-191*/TOL19^d	MT 19-B1	S(10)
	MT 19-11	Tol$^+$Leu$^-$	*leu-191*/TOL19	MT 19	NG
	MT 19-B1TOL	Tol$^+$	/TOL	MT 19-B1	C

TABLE 1. *Continued*

Classification	Strain designation	Phenotype[a]	Genotype[b]	Parent strain	Method of production or reference[c]
P. putida	MT 20	Tol$^+$	wt/TOL20		
	MT 20-B1	Tol$^-$	/TOL20^d	MT 20	B
	MT 20-B2	Tol$^-$Str^r	*str-201*/TOL20^d	MT 20-B1	S(5)
	MT 20-B3	Mxy$^+$Pxy$^-$Tln$^+$	NK	MT 20	B
	MT 20-B4	Mxy$^+$Pxy$^-$Tln$^+$Str^r	*str-202*/NK	MT 20-B3	S(5)
	MT 20-3	Tol$^+$His$^-$	*his-201*/TOL20	MT 20	NG
	MT 20-B1TOL	Tol$^+$	/TOL	MT 20-B1	C
	MT 20-B3TOL	Tol$^+$	/TOL	MT 20-B3	C
P. putida	MT 21	Tol$^+$	wt/TOL21		
	MT 21-B1	Tol$^-$	/TOL21^d	MT 21	B
	MT 21-B2	Tol$^-$Str^r	*str-211*/TOL21^d	MT 21-B1	S(100)
	MT 21-B1TOL	Tol$^+$	/TOL	MT 21-B1	C

[a] Phenotype abbreviations: Leu, leucine; His, histidine; Arg, arginine; Str, streptomycin; Mxy, *m*-xylene; Pxy, *p*-xylene; Tln, toluene; Tol, the linked ability to metabolize *m*-xylene, *p*-xylene, toluene, *m*-toluate, and *p*-toluate.

[b] wt, Wild type; NK, not known. The plasmid of PaW 1 is referred to as TOL; the plasmids found in the other strains are designated TOL, followed by the MT strain number.

[c] NG, Nitrosoguanidine mutagenesis: B, spontaneous selection after growth on benzoate; S, spontaneous selection on nutrient agar plates containing streptomycin sulfate (concentration in micrograms per milliliter in parentheses); NA, spontaneous occurrence after growth on nutrient broth; C, after conjugation with PaW 15.

putida (arvilla) mt-2 (ATCC 23973), is from a clone maintained in our laboratory and used in previous work (6, 11, 13). The other wild-type bacteria were isolated from soil samples by selective enrichment culture in 5 mM *m*-toluate minimal salts medium. Partial classification was carried out by the National Collection of Industrial Bacteria, Aberdeen, Scotland. MT 1, MT 5, MT 15, MT 16, MT 17, MT 18, MT 19, MT 20, and MT 21 were motile, gram-negative rods, which produced green fluorescent pigments and were unable to hydrolyze gelatin, give a positive egg yolk reaction, or grow at 42 C and best fit *P. putida*. MT 1, MT 5, MT 15, MT 19, and MT 20 conform to biotype B by the criterion of the ability to grow on L-tryptophan and D-galactose, and MT 16, MT 17, MT 18, and MT 21 conform to biotype A by their inability to utilize these substrates. MT 12 and MT 13 had similar nutritional characteristics but appeared unable to produce fluorescent pigments and can form long chains of cells; they could be atypical *P. putida* biotype A. MT 3 was fluorescent and differed only slightly from other strains. MT 14 was nonfluorescent and differed further, in particular by the ability to slowly liquefy gelatin. MT 3, MT 12, MT 13, and MT 14 are tentatively named *Pseudomonas* sp.

Maintenance of strains, culture conditions, and growth determination. PaW 1, the wild-type isolates, and plasmid-containing strains were maintained on 5 mM *m*-toluate minimal agar slopes and plates, and derivative and mutant strains were maintained on nutrient agar slopes and 10 mM succinate minimal agar plates. The composition of the minimal medium is as described (6). Ability to grow on substrates was determined on minimal agar plates either with the substrate incorporated at 5 mM or, for toluene, *m*-xylene, *p*-xylene, benzaldehyde, *m*-tolualdehyde, and *p*-tolualdehyde, using the vapor phase as described (13).

Preparation of cell extracts and enzyme assays. Cells were grown, harvested, and stored and extracts were prepared as described (6); catechol 1,2-oxygenase (EC 1.13.1.1) (6) and catechol 2,3-oxygenase (EC 1.13.1.2) (9) were assayed in extracts by published procedures.

Curing experiments. Single colonies from stock plates were inoculated into culture tubes containing 5 ml of nutrient broth (NB). After overnight growth at 30 C, 0.05 ml of a 10^{-3} dilution of the culture was added to 5 ml of either NB or 5 mM benzoate minimal medium, which was incubated at 30 C until the cultures were fully grown (overnight in NB, 1 to 2 days in benzoate medium). Appropriate dilutions were made and spread onto nonselective agar plates (nutrient agar or 10 mM succinate minimal). Single colonies were picked and replicated onto plates to test their phenotype.

Matings. Donor and recipient cultures in NB were incubated overnight at 30 C without shaking. Equal volumes were mixed and stood for 90 min at 30 C before dilution and plating. In earlier matings the donor was diluted 1:15 in NB before mating, which increases the efficiency of transfer but reduces the possibility of detecting conjugation at low frequency. After mating, 0.1-ml aliquots of the mixture and of dilutions of it were spread on selection plates, which were incubated at 30 C for 3 to 4 days. The matings involved selecting against the donor either by using auxotrophic mutants of it and omitting its auxotrophic requirement from the selection

plates or by streptomycin sensitivity. In the latter case, spontaneous streptomycin-resistant mutants of the recipient were used, and selection plates incorporated the lowest concentration of streptomycin effective against the wild-type donor. In both cases, selection against the recipient was achieved by use of *m*- or *p*-toluate as the sole carbon source in the selection plates.

Isolation of mutant strains. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was carried out by standard procedures (7). Auxotrophic mutants were selected after mutagenesis by their ability to grow on 10 mM succinate agar plates containing 200 μg of NB per ml but inability to grow on 10 mM succinate minimal agar. Streptomycin-resistant strains were isolated as spontaneous mutants by plating 10^8 to 10^9 cells on nutrient agar plates containing a concentration of streptomycin sulfate sufficient to reduce the number of colonies appearing to <50/plate (see Table 1 for the appropriate concentrations).

RESULTS

Isolation and growth characteristics. The 13 organisms isolated in this investigation were selected and purified after incubating nine different soil samples in medium containing 5 mM *m*-toluate as sole carbon source for 2 to 4 days. With three of the samples more than one organism from each was isolated and retained since they showed differences in colony morphology or fluorescent pigment production. These were (i) MT 1 and MT 3, (ii) MT 12, MT 13, and MT 14, and (iii) MT 19 and MT 20, and subsequent investigation indicated biochemical or genetic differences between strains from the same source. The soil samples were taken from different sites in an area of several miles.

The growth characteristics of the isolates are shown in Table 2. All grow on *p*-toluate and benzoate and also on *m*-xylene, *p*-xylene, and toluene. In this respect their metabolism appears identical to that of *P. putida* mt-2, which metabolizes the three hydrocarbons to benzoate and the toluates via the corresponding alcohols and aldehydes (13; Fig. 1). This similarity is further borne out by the ability of all the isolates to grow on the alcohols and aldehydes, as does *P. putida* mt-2. There are some differences, however, since MT 14, MT 15, and MT 20 grow poorly on all the *p*-methyl compounds, in particular *p*-tolualdehyde, compared to their growth on the unsubstituted and the *m*-methyl derivatives; it is also noticeable with these strains that, when growing in liquid or on plates on the *p*-methyl derivatives, there is accumulation of a yellow color, probably the *meta* ring fission product of 4-methylcatechol (6). Preliminary evidence suggests that this growth pattern is an indication of a difference in the

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

Growth substrate	Strains				
	MT 1, MT 3, MT 5, MT 12, MT 13, MT 16, MT 17, MT 18, MT 19, MT 21	MT 14, MT 15, MT 20	MT 1-B1, MT 3-B1, MT 5-B1, MT 12-B1, MT 13-B1, MT 14-B1, MT 15-B1, MT 16-B1, MT 17-B1, MT 18-B1, MT 19-B1, MT 20-B1, MT 21-B1	MT 14-B3, MT 15-B3, MT 20-B3	MT 1-B1TOL, MT 5-B1TOL, MT 13-B1TOL, MT 14-B1TOL, MT 15-B1TOL, MT 16-B1TOL, MT 17-B1TOL, MT 18-B1TOL, MT 19-B1TOL, MT 20-B1TOL, MT 21-B1TOL, MT 14-B3TOL, MT 15-B3TOL, MT 20-B3TOL
o-Xylene	−	−	−	−	−
m-Xylene	+	+	−	+	+
m-Methylbenzyl alcohol	+	+	−	−	+
m-Tolualdehyde	+	+	−	−	+
m-Toluate	+	+	−	−	+
p-Xylene	+	±	−	−	+
p-Methylbenzyl alcohol	+	±	−	−	+
p-Tolualdehyde	+	±	−	−	+
p-Toluate	+	±	−	−	+
Toluene	+	+	−	+	+
Benzyl alcohol	+	+	−	+	+
Benzaldehyde	+	+	+	+	+
Benzoate	+	+	+	+	+

[a] +, Good growth; ±, slow growth; −, no growth.

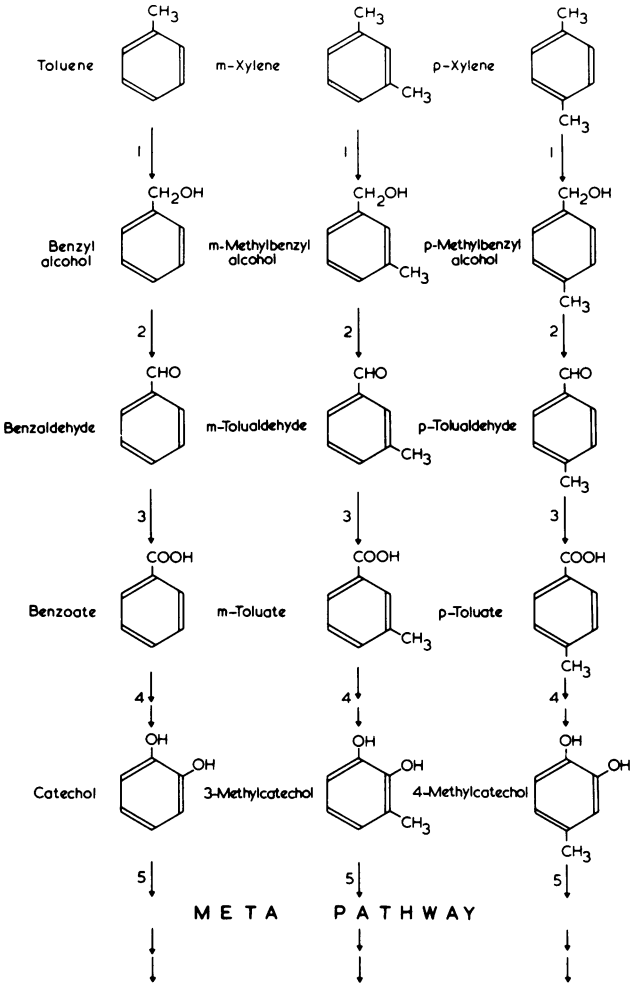

FIG. 1. *Metabolism of toluene and the xylenes by P. putida mt-2. (1) Toluene oxidase; (2) benzyl alcohol dehydrogenase; (3) benzaldehyde dehydrogenase; (4) benzoate oxidase; (5) catechol 2,3-oxygenase.*

biochemistry and possibly also the regulation of the dissimilatory pathway in these bacteria (unpublished data).

The superficial similarity between the isolates and *P. putida* mt-2 in their metabolism is augmented by the observation that all of them appear to use the *meta* (or α-ketoacid) pathway for further metabolism of *m*-toluate (6); cells grown on *m*-toluate show the characteristic yellow color of 2-hydroxymuconic semialdehyde when incubated with catechol in the cleavage test (10), and cell extracts prepared from them contain induced levels of catechol 2,3-oxygenase (Table 3).

Curing experiments. One of the main criteria for determining whether a catabolic pathway is coded by plasmid-carried genes is to produce strains that have lost the ability to utilize the particular growth substrate(s). The main agent used to cure catabolic plasmids in *Pseudomonas* has been mitomycin C. However, we have shown that the proportion of cells of *P. putida* mt-2 cured of their TOL plasmid is greater after growth from a small inoculum on benzoate minimal medium (11). It appears that spontaneously cured cells have a selective advantage since they use the chromosomally coded *ortho* (or β-ketoadipate) pathway for the dissimilation of benzoate, by which they grow faster than wild-type cells (11); because of differences in the regulation of the *meta* and *ortho* pathways, the *ortho* pathway is not normally expressed in cells containing the plasmid-coded *meta* pathway. Since the *ortho* pathway is specific for benzoate, these cured cells lose the ability to metabolize *m*- and *p*-toluates.

TABLE 3. *Catechol oxygenase activities in wild-type and cured strains*

Strain	Catechol 2,3-oxygenase activity[a]			Catechol 1,2-oxygenase activity[a]	
	10 mM succinate[b]	5 mM *m*-toluate	20 mM acetate + 5 mM *m*-toluate	10 mM succinate	5 mM benzoate
MT 1	0.14	4.0	ND	ND	ND
MT 1-B1	ND	ND	<0.001	<0.002	0.10
MT 3	0.07	2.1	ND	ND	ND
MT 3-B1	ND	ND	<0.001	<0.002	0.27
MT 5	0.13	2.7	ND	ND	ND
MT 5-B1	ND	ND	<0.001	<0.002	0.19
MT 12	0.09	3.8	ND	ND	ND
MT 12-B1	ND	ND	<0.001	<0.002	0.28
MT 13	0.04	4.0	ND	ND	ND
MT 13-B1	ND	ND	<0.001	<0.002	0.60
MT 14	0.09	1.4	ND	ND	ND
MT 14-B1	ND	ND	<0.001	<0.002	0.16
MT 15	0.11	1.6	ND	ND	ND
MT 15-B1	ND	ND	<0.001	<0.002	0.12
MT 16	0.10	6.7	ND	ND	ND
MT 16-B1	ND	ND	<0.001	<0.002	0.26
MT 17	0.11	7.2	ND	ND	ND
MT 17-B1	ND	ND	<0.001	<0.002	0.30
MT 18	0.09	11.4	ND	ND	ND
MT 18-B1	ND	ND	<0.001	<0.002	0.26
MT 19	0.13	4.6	ND	ND	ND
MT 19-B1	ND	ND	<0.001	<0.002	0.13
MT 20	0.08	3.2	ND	ND	ND
MT 20-B1	ND	ND	<0.001	<0.002	0.13
MT 21	0.16	10.3	ND	ND	ND
MT 21-B1	ND	ND	<0.001	<0.002	0.45

[a] Expressed as enzyme units per milligram of protein, where 1 unit is the amount of enzyme required to convert 1 μmol of substrate to product in 1 min. ND, Not determined.

[b] Growth substrate.

We therefore determined the proportion of cured cells for all the isolates after growth on benzoate from a small inoculum. The results, together with those of cells similarly grown on NB, are shown in Table 4. In most cases, cured strains that had lost the ability to grow on *m*-toluate (Mtol$^-$) were found in greater numbers after growth on benzoate than on NB. The frequency of curing produced after benzoate growth varies from 1 to 100% with different isolates. Cured strains were patched onto King, Ward, and Raney A & B plates (5) together with wild-type colonies and incubated for several days; even in the case of nonfluorescent strains, we found this to be an excellent diagnostic means of distinguishing between the various isolates and for determining that cured strains were derived from the wild type since the colony morphology in particular and the fluorescence, if any, were very characteristic of each individual isolate and its derivatives.

We also tried curing with mitomycin C using the method of Chakrabarty (1) and in many cases obtained derivatives identical in phenotype with those obtained from growth on benzoate, but at a lower frequency. However, our experience with this compound leads us to conclude that it is not a good general agent for plasmid curing. Some of the bacteria were very sensitive to the antibiotic and produced virtually no viable cells at concentrations of only 0.25 μg/ml. More importantly a number of the organisms produced cells with a much reduced growth rate or other phenotypic alteration, suggesting that mitomycin C can produce mutants at a comparable frequency to cured cells.

Apart from MT 14, MT 15, and MT 20, all the cured strains obtained after growth on benzoate or, where detected, after growth on NB showed identical growth characteristics (Table 3) to PaW 8, the cured strain from PaW 1, having lost the ability to grow on all the aromatic substrates of the pathway with the exception of benzaldehyde and benzoate: these strains were designated the B1 derivatives. The difference in growth rate on benzoate is very apparent for most of the bacteria, since a colony or patch of cured cells after 2 days of incubation produced noticeably more growth on benzoate minimal agar than an adjacent colony or patch of its wild type.

After growth of the B1 strains on 20 mM

TABLE 4. *Curing frequencies of isolates*

Strain	No. of colonies tested after growth on:		No. of colonies Mtol⁻	Curing frequency (%)
	NB	5 mM benzoate		
MT 1	140		8	6
		88	87	99
MT 3	61		0	<1
		176	169	96
MT 5	88		2	2 [a]
		176	3	2 [a]
MT 12	88		0	<1
		155	43	27
MT 13	176		0	<1
		83	14	16
MT 14	176		0	<1
		63	63	100
MT 15	188		1	1
		176	176	100
MT 16	176		2	1
		176	11	6
MT 17	176		1	1 [a]
		176	2	1 [a]
MT 18	176		0	<1
		176	13	8
MT 19	176		3	2
		87	2	2
MT 20	176		0	<1
		176	176	100
MT 21	176		1	1 [a]
		176	4	2 [a]

[a] Figures are correct to the nearest whole numbers.

acetate in the presence of 5 mM *m*-toluate as inducer, the activities of catechol 2,3-oxygenase were undetectable, being less than 1% of the activities in uninduced wild-type cells (Table 3). None of the B1 derivatives reverts to wild type at a detectable frequency ($<10^{-8}$) on either *m*-toluate or *p*-toluate minimal agar plates.

By these criteria and by comparison with *P. putida* mt-2 it appears that all the isolates carry plasmids coding for the enzymes responsible for the catabolism of toluene and the xylenes *via* benzoate and the toluates and then by the *meta* pathway, and that during growth on benzoate there is positive selection for strains that have lost these plasmids. This indicates that all of the strains have a pathway, probably chromosomally determined, which, in most, permits faster growth on benzoate. This alternative pathway appears to be the *ortho* pathway in all isolates since we have demonstrated the *ortho* cleavage of catechol by the Rothera test (10) in benzoate-grown cells of all the B1 derivatives and have shown induced levels of catechol 1,2-oxygenase in their cell extracts (Table 3).

After growth of MT 14, MT 15, and MT 20 on benzoate, cells of the B1 phenotype were found, but in addition cells of a different phenotype were also obtained. These cells (MT 14-B3, MT 15-B3, and MT 20-B3) had lost the ability to grow on *m*-toluate, *p*-toluate, and *p*-xylene but retained the ability to grow on *m*-xylene and toluene (Table 2), although, particularly in the case of MT 15-B3, the growth rate on *m*-xylene is considerably slower than that of wild type. These B3 derivatives are found at a rather variable frequency but one that is comparable to that of the B1 derivatives (Table 5). No explanation for the frequent and spontaneous occurrence of this phenotype either at a genetic or a biochemical level can be offered at present.

Conjugation experiments. In addition to the production of cured, nonrevertible strains, the ability of the wild type to transfer the genes back into the cured strains by conjugation is a major criterion, which has been applied to characterize a set of genes as being plasmid carried.

Difficulties were experienced in quantitating transfer frequencies owing to the considerably reduced numbers of exconjugants observed on plates at higher dilutions compared with the numbers expected from plates at the lower dilutions. This was previously observed with *P. putida* mt-2 (11) and is presumably due to mating occurring on the plate. The transfer frequencies recorded are calculated from the plate with the smallest number of colonies, but the experimental variation so introduced is obviously increased.

The majority of matings were carried out using streptomycin selection against the donor, but in addition auxotrophic mutants of several of the isolates were made; this was essential for MT 12 and MT 13, which were very resistant to streptomycin.

The results of the intrastrain matings are given in Table 6. MT 16, MT 17, MT 18, and MT 21 were able to transfer their putative TOL plasmids back into their cured strains at a frequency comparable to *P. putida* mt-2; these

TABLE 5. *Curing of MT 14, MT 15, and MT 20 after growth on benzoate*

Strain	No. of colonies tested	No. of colonies with phenotype:	
		Mtol⁻ Mxy⁺	Mtol⁻ Mxy⁻
MT 14	176	129	47
	177	169	5
MT 15	176	39	137
	174	144	30
MT 20	176	51	125
	174	7	167

TABLE 6. *Intrastrain transfer of TOL plasmids from wild types into the corresponding cured strains*

Donor	Recipient	Frequency of transfer[a]	
		Streptomycin selection (μg/ml)[b]	Auxotroph selection[c]
PaW 1	PaW 17	3×10^{-4} (100)	
MT 1	MT 1-B2	$<10^{-8}$ (100)	
MT 1-2	MT 1-B1		$<2 \times 10^{-7}$
MT 3	MT 3-B2	$<10^{-7}$ (10)	
MT 5	MT 5-B2	$<10^{-7}$ (100)	
MT 12-1	MT 12-B1		2×10^{-6}
MT 13-11	MT 13-B1		10^{-6}
MT 14	MT 14-B2	$<5 \times 10^{-8}$ (2.5)	
MT 14-4	MT 14-B1		$<10^{-7}$
MT 14	MT 14-B4	$<5 \times 10^{-8}$ (2.5)	
MT 14-4	MT 14-B3		$<10^{-7}$
MT 15	MT 15-B2	$<10^{-7}$ (10)	
MT 15-8	MT 15-B1		$<10^{-7}$
MT 15	MT 15-B4	$<10^{-7}$ (10)	
MT 15-8	MT 15-B3		$<10^{-7}$
MT 16	MT 16-B2	10^{-3} (100)	
MT 17	MT 17-B2	10^{-3} (100)	
MT 18	MT 18-B2	10^{-4} (100)	
MT 19	MT 19-B2	3×10^{-5} (5)	
MT 19-11	MT 19-B1		4×10^{-5}
MT 20	MT 20-B2	10^{-5} (5)	
MT 20-3	MT 20-B1		10^{-5}
MT 20	MT 20-B4	$<10^{-7}$ (5)	
MT 20-3	MT 20-B3		$<10^{-7}$
MT 21	MT 21-B2	2×10^{-4} (100)	

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

[b] Selection plates contained 5 mM *m*-toluate as carbon and energy source, with streptomycin sulfate added to the concentration given in parentheses.

[c] Selection plates contained 5 mM *m*-toluate except for MT 12-1 and MT 13-11, where transfer was slightly higher on 5 mM *p*-toluate plates.

organisms were also indistinguishable from *P. putida* mt-2 in their colony morphology and fluorescence. MT 12, MT 13, MT 19, and MT 20 were also able to transfer their plasmids at a lower, but detectable, frequency into their cured derivatives, but no transfer of TOL20 from MT 20 into the abnormal "cured" strain MT 20-B3 could be detected. No measurable transfer from MT 1, MT 3, MT 5, MT 14, and MT 15 into their cured strains was observed.

A second series of conjugation experiments was performed to assess the ability of the isolates to transfer their plasmids by interstrain conjugation into cured strains of PaW 1 using both streptomycin or auxotroph selection (Table 7). Again, MT 16, MT 17, MT 18, and MT 21 were able to transfer their plasmids at high frequency and MT 19, at a lower frequency. None of the other bacteria appeared able to effect this transfer at detectable frequencies. On the other hand, the transfer of the TOL plasmid from *P. putida* mt-2 into the cured derivatives of the other bacteria could be detected in all cases except into MT 3-B1 and MT 12-B1 (Table 8). It is of note that in this conjugation system, TOL is transferred into both B1

TABLE 7. *Interstrain transfer of TOL plasmids from wild types into cured strains of P. putida (arvilla) mt-2*

Donor	Recipient	Frequency of transfer[a]	
		Streptomycin selection (μg/ml)[b]	Auxotroph selection
PaW 1	PaW 17	3×10^{-4} (100)	
MT 1	PaW 17	$<10^{-7}$ (100)	
MT 1-2	PaW 8		$<10^{-7}$
MT 3	PaW 17	$<5 \times 10^{-8}$ (10)	
MT 5	PaW 17	$<10^{-7}$ (100)	
MT 12-1	PaW 8		$<10^{-7}$
MT 13-11	PaW 8		$<10^{-7}$
MT 14	PaW 17	$<10^{-7}$ (2.5)	
MT 14-4	PaW 8		$<10^{-7}$
MT 15	PaW 17	$<10^{-7}$ (10)	
MT 15-8	PaW 8		$<10^{-7}$
MT 16	PaW 17	2×10^{-3} (100)	
MT 17	PaW 17	10^{-3} (100)	
MT 18	PaW 17	10^{-3} (100)	
MT 19	PaW 17	10^{-6} (5)	
MT 19-11	PaW 8		2×10^{-6}
MT 20	PaW 17	$<10^{-7}$ (5)	
MT 20-3	PaW 8		$<8 \times 10^{-8}$
MT 21	PaW 17	10^{-4} (100)	

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

[b] Selected on 5 mM *m*-toluate minimal agar plates containing streptomycin sulfate added to the concentration given in parentheses.

TABLE 8. *Interstrain transfer of TOL plasmid from P. putida (arvilla) mt-2 into cured strains with PaW 15 as donor*

Recipient	Frequency of transfer[a]
PaW 8	10^{-3}
MT 1-B1	2×10^{-5}
MT 3-B1	$<6 \times 10^{-8}$
MT 5-B1	10^{-3}
MT 12-B1	$<6 \times 10^{-8}$
MT 13-B1	2×10^{-5}
MT 14-B1	7×10^{-4}
MT 14-B3	7×10^{-6}
MT 15-B1	6×10^{-3}
MT 15-B3	6×10^{-4}
MT 16-B1	2×10^{-3}
MT 17-B1	10^{-3}
MT 18-B1	10^{-3}
MT 19-B1	10^{-3}
MT 20-B1	10^{-3}
MT 20-B3	2×10^{-4}
MT 21-B1	2×10^{-3}

[a] Defined as the number of exconjugants per donor cell; selection on 5 mM *p*-toluate minimal agar plates.

and B3 derivatives of MT 14, MT 15, and MT 20, but with all three bacteria the frequency is greater by a factor between 5 to 100 into the B1 than into the B3 derivatives. The exconjugants from all these matings, containing the TOL plasmid from *P. putida* mt-2, all showed an identical growth pattern to PaW 1 itself (Table 2).

All the exconjugants derived from the matings of PaW 15 with the cured strains were tested for their ability to transfer this foreign plasmid into their own cured derivatives (Table 9). Again the derivatives of MT 16, MT 17, MT 18, and MT 21 were able to transfer the TOL plasmid received from *P. arvilla* mt-2 at a high frequency, and in addition MT 5, MT 13, MT 19, and both derivatives of MT 20, MT 20-B1TOL and MT 20-B3TOL, were able to transfer. The greater frequency with which it appears possible to transfer into the B1 strains than the B3 strains (Table 8) is further confirmed in that both MT 20-B1TOL and MT 20-B3TOL transfer TOL at a higher frequency into the streptomycin-resistant mutant of MT 20-B1 (MT 20-B2) than into that of MT 20-B3 (MT 20-B4). MT 5 appears unique in that it is the only isolate unable to transfer its native plasmid TOL5 (Table 6) yet able to transfer the plasmid received from *P. putida* mt-2: in the other strains the ability to transfer both plasmids appears to be the same.

TABLE 9. *Intrastrain transfer of P. putida (arvilla) mt-2 TOL plasmid from exconjugants of cured strains into the cured strains*

Donor	Recipient	Frequency of transfer (μg/ml)[a]
MT 1-B1TOL	MT 1-B2	$<10^{-7}$ (100)
MT 5-B1TOL	MT 5-B2	5×10^{-5} (100)
MT 13-B1TOL 7	MT 13-B1	10^{-6} (0)
MT 14-B1TOL	MT 14-B2	$<10^{-7}$ (2.5)
MT 14-B1TOL	MT 14-B4	$<10^{-7}$ (2.5)
MT 14-B3TOL	MT 14-B2	$<10^{-7}$ (2.5)
MT 14-B3TOL	MT 14-B4	$<10^{-7}$ (2.5)
MT 15-B1TOL	MT 15-B2	$<10^{-7}$ (10)
MT 15-B1TOL	MT 15-B4	$<10^{-7}$ (10)
MT 15-B3TOL	MT 15-B2	$<10^{-7}$ (10)
MT 15-B3TOL	MT 15-B4	$<10^{-7}$ (10)
MT 16-B1TOL	MT 16-B2	5×10^{-3} (100)
MT 17-B1TOL	MT 17-B2	3×10^{-4} (100)
MT 18-B1TOL	MT 18-B2	2×10^{-4} (100)
MT 19-B1TOL	MT 19-B2	4×10^{-5} (5)
MT 20-B1TOL	MT 20-B2	2×10^{-4} (5)
MT 20-B1TOL	MT 20-B4	3×10^{-6} (5)
MT 20-B3TOL	MT 20-B2	2×10^{-4} (5)
MT 20-B3TOL	MT 20-B4	5×10^{-6} (5)
MT 21-B1TOL	MT 21-B2	4×10^{-4} (100)

[a] Defined as the number of exconjugants per donor cell. Selected on 5 mM *m*-toluate minimal agar plates containing streptomycin sulfate added to the concentration given in parentheses (micrograms per milliliter). In the case of MT 13-B1TOL 7, selection plates contained 5 mM *p*-toluate.

DISCUSSION

The presence of TOL plasmids in all the isolates we have studied is strongly suggested by the evidence, in particular the facility with which there occurs spontaneous and simultaneous loss of their ability to grow on toluene, *m*-xylene, *p*-xylene, *m*-toluate, and *p*-toluate coupled with the loss of detectable catechol 2,3-oxygenase activity. In the majority of the strains (8 out of 13) transfer of the putative plasmid back into the corresponding cured derivative can also be detected.

A number of implications follow from the argument that the enzymes of toluene and xylene metabolism are plasmid coded in each of these organisms. Firstly, at least as far as this catabolic pathway is concerned, the plasmid-borne nature of the genes appears to be a much more general phenomenon than might have been expected. Secondly, in each case the plasmid appears not only to carry the genes for toluate metabolism, for which the organisms were selected, but also, as in *P. putida* mt-2, apparently carries them linked to the genes responsible for conversion of toluene and xylenes through to benzoate and toluates (13). It is therefore possible that the toluates, if not benzoate, for which there is an alternative pathway, are not important substrates in the environment but are merely intermediates of xylene metabolism; thus pathways for toluate metabolism have only evolved linked to that of the hydrocarbons.

The selection of cured strains during growth on benzoate is surprising, not because of its novelty but because it appears to be a general phenomenon: not only do all the strains which harbor the putative TOL plasmids appear to have the alternative chromosomally coded pathway for benzoate catabolism but, from the ease with which the curing takes place, the growth rate by the alternative pathway in many cases appears higher than that by the TOL-determined pathway. In those instances where the frequency of curing is 100%, either this differential rate of growth is very great or else the plasmid-coded enzymes cannot adequately dissimilate benzoate and the chromosomally determined pathway can only be expressed in the absence of the plasmid.

The frequency of curing in benzoate culture

cannot be taken as a measure of the stability of the plasmids within the strains but is a reflection of the biochemistry of the system; a better reflection of the stability would be the frequency of curing after growth under nonselective conditions in NB, and there is little difference between the strains under these conditions.

Consideration of the properties of the organisms isolated and of the plasmids they contain indicates that they are heterogenous. Strains MT 5, MT 12, MT 13, and MT 14 represent the first nonfluorescent pseudomonads, indeed the first representatives of *Pseudomonas* species other than *P. putida*, shown to harbor catabolic plasmids, although transfer of some of the plasmids into other species under laboratory conditions has been achieved (1, 2, 8).

Strains MT 16, MT 17, MT 18, and MT 21 are all very similar to *P. putida* mt-2, both in the frequencies of their curing and in their high frequency of transmission to their own cured strains and to the cured strains of *P. putida* mt-2; unlike the other isolates, they are indistinguishable in colony morphology and fluorescence to *P. putida* mt-2. The most similar organism to this group with regard to plasmid transfer is MT 19, which is the only other isolate to give detectable transfer in all of the conjugation experiments, except that it does so at somewhat lower frequency than MT 16, MT 17, MT 18, and MT 21; this organism is noticeably different from *P. putida* mt-2 in colonial morphology and fluorescence.

MT 14, MT 15, and MT 20 clearly differ from the other isolates since they grow slowly and accumulate a yellow color on the *p*-methyl substrates. This characteristic phenotype is not observed when the strains are cured and reinfected with TOL from *P. putida* mt-2, showing that it is a property of their plasmids and not an effect of the host cell. In addition, these three strains differ from the other 10 in their ability to give rise to the B3 phenotype, although it is not clear at present whether this is a property of the plasmids or of the host cells.

The plasmid in MT 5 must also differ from the plasmid in *P. putida* mt-2 in that transfer of TOL from MT5-B1TOL into MT 5-B2 can be detected, whereas transfer of TOL5 from MT 5 into MT 5-B2 cannot.

MT 12 and MT 13 are very similar in their properties: both are nonfluorescent, show a tendency to form long chains of cells, have a high streptomycin resistance, and transfer their plasmids to their cured derivatives at low frequency. However, they differ both in their colony morphology and in their ability to receive TOL from *P. putida* mt-2. It is interesting that they both originated from the same soil sample together with MT 14, also nonfluorescent but with properties obviously very different, and raises the possibility that the particular conditions prevalent in the environment from which that sample was taken favored the growth of nonfluorescent pseudomonads.

MT 3 is unique in that we were neither able to transfer TOL or TOL3 into its cured derivative.

Our failure to detect plasmid transfer in some matings need not mean that the plasmids are nontransmissible. Firstly, our in vitro conjugation system may not represent optimum conditions for some transfers, which might be met by some alternative in vitro system or in the natural environment of the bacteria. Secondly, the lowest transfer frequency detectable under our conditions was about 10^{-7}/donor cell. Two catabolic plasmids, NAH (4) and OCT (3), have been reported as having transfer frequencies lower than this. The third point, which emerges clearly from our results, is that the host cell can have a marked effect on the transmissibility of a plasmid. For example, although TOL may be readily transferred from *P. putida* mt-2 into the cured derivatives of MT 1, MT 14, and MT 15, these organisms cannot retransfer it at detectable frequencies to their own cured strains.

It is interesting to speculate whether the ability of bacteria such as *P. putida* mt-2, and presumably MT 16, MT 17, MT 18, and MT 21, to transmit their plasmids with considerable facility into organisms that cannot further retransmit them may play a role in the environment in maintaining in the soil bacterial population maximum biochemical flexibility coupled with minimum genetic burden. In this respect it is noteworthy that, whereas *P. putida* mt-2 can readily transfer TOL into all of the cured isolates except MT 3-B1 and MT 12-B1, the ability to transfer into PaW 8 and PaW 17 is limited to those strains which very closely resemble *P. putida* mt-2 and MT 19. Furthermore, because of the effect which the host bacteria has on the transmissibility of these plasmids, we have no evidence as to the properties of or interrelationships between the plasmids in MT 1, MT 3, MT 12, MT 13, and MT 19; indeed it is possible that they are all very similar to TOL and have been received from an organism such as *P. putida* mt-2 during the selection procedure.

Our results indicate that there are a range of organisms in the soil microbial population that harbor TOL plasmids and that these plasmids show differences from one another. To date no

organism has been detected with properties suggesting a chromosomal location for the enzymes involved in toluene, xylene, and toluate degradation.

Perhaps the most unexpected of our findings is the frequent occurrence of cells of the B3 phenotype after growth of certain strains on benzoate. Any explanation of these strains must accommodate the fact that they retain the ability to utilize *m*-xylene but not *m*-toluate, which is normally an intermediate in its degradation (6).

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