Loss of the Toluene–Xylene Catabolic Genes of TOL Plasmid pWW0 during Growth of *Pseudomonas putida* on Benzoate Is Due to a Selective Growth Advantage of ‘Cured’ Segregants

By PETER A. WILLIAMS,* SUSAN D. TAYLOR AND LINDA E. GIBB

Department of Biochemistry & Soil Science, University College of North Wales, Bangor,
Gwynedd LL57 2UW, UK

(Received 29 October 1987; revised 22 February 1988)

During growth on benzoate-minimal medium *Pseudomonas putida* mt-2 (PaW1) segregates derivative (‘cured’) strains which have lost the ability to use the pathway encoded by its resident catabolic plasmid pWW0. Experiments with two plasmids identical to pWW0 but each with an insert of Tn401, which confers resistance to carbenicillin, suggested that the ‘benzoate curing’ occurs far more frequently by the specific deletion of the 39 kbp region carrying the catabolic genes than by total plasmid loss. This effect was not pH-dependent, and was not produced during growth on other weak organic acids, such as succinate or propionate, or when benzoate was present in the medium with an alternative, preferentially used carbon source such as succinate. Growth on benzoate did not cause loss from strain PaW174 of the plasmid pWW0-174, a derivative of pWW0 which has deleted the 39 kbp region but carries Tn401. Similarly the naphthalene-catabolic plasmid pWW60-1, of the same incompatibility group as pWW0, was not lost from PaW701 during growth on benzoate. Competition between wild-type PaW1 and PaW174, which has the ‘cured’ phenotype, showed that the latter has a distinct growth advantage on benzoate over the wild-type even when initially present as only 1% of the population: when PaW174 was seeded at lower cell ratios, spontaneously ‘cured’ derivatives of PaW1 took over the culture after 60–80 generations, indicating that they are present in PaW1 cultures at frequencies between 10^{-2} and 10^{-3} . We conclude that the progressive takeover of populations of PaW1 only occurs when benzoate is present as the sole growth source and that neither benzoate, nor other weak acids, affect plasmid segregation or deletion events: a sufficient explanation is that the ‘cured’ segregants grow faster than the wild-type using the chromosomally determined β -ketoadipate pathway.

INTRODUCTION

The observation that *Pseudomonas putida* mt-2 underwent an irreversible spontaneous phenotypic change which affected its growth on benzoate was first reported by Nakazawa & Yokota (1973), who noticed faster-growing colonies when the bacteria were spread on to agar plates containing benzoate-yeast extract medium. The phenomenon was explained when it was later shown that *P. putida* mt-2 harbours a plasmid which encodes the genes responsible for the catabolism of benzoate (and *m*- and *p*-toluates) by the *meta* cleavage pathway (see Fig. 1) (Williams & Murray, 1974). Segregants of strain mt-2 (also called PaW1) appeared after growth for > 20 generations on 5 mM-benzoate-minimal medium (BMM): they had irreversibly lost the entire set of catabolic genes encoded by the plasmid (pWW0), rendering them unable to grow on *m*-toluate (Mtol⁻) or *p*-toluate (Williams & Murray, 1974) or on the hydrocarbons toluene and *m*- and *p*-xylenes, which were subsequently shown to be the precursors of benzoate, *m*- and *p*-

Abbreviation: BMM, benzoate-minimal medium.

Fig. 1. Pathways for catabolism of aromatic compounds in *P. putida* mt-2 (PaW1) and its derivative strains. The pathway determined by TOL plasmids dissimilates toluene ($R_1 = R_2 = H$), *m*-xylene ($R_1 = CH_3$, $R_2 = H$) and *p*-xylene ($R_1 = H$, $R_2 = CH_3$), through benzoate, *m*-toluate and *p*-toluate respectively and then by the *meta* cleavage pathway to central metabolites. The chromosomally determined pathway will metabolize only benzoate via the *ortho* or β -ketoadipate pathway. The plasmid pathway is induced by both the hydrocarbons and the carboxylic acids substrates, and is thus utilized when they are presented as growth substrates. The primary inducer of *ortho* cleavage is *cis,cis*-muconate, the product of its first enzyme, catechol 1,2-oxygenase. This pathway is therefore only induced when the plasmid pathway is absent, either by loss of the plasmid or by deletion of the catabolic genes from the plasmid, such that some muconate can be formed by the action of the basal uninduced catechol 1,2-oxygenase upon catechol formed from benzoate.

toluates respectively on the plasmid-determined pathway (Worsey & Williams, 1975). The segregants retained the ability to grow well on benzoate (Ben^+) but they dissimilated it by the alternative, but chromosomally coded, *ortho* (or β -ketoadipate) pathway (Nakazawa & Yokota, 1973; Williams & Murray, 1974) which is ubiquitous in *P. putida* strains (Fig. 1). Williams & Murray (1974) proposed that the segregants had spontaneously lost the plasmid and, using the chromosomal route for benzoate catabolism, were able to grow faster than the wild-type, which was thus outcompeted during subsequent culture. Once physical isolation of the large *Pseudomonas* plasmids was possible, it was shown that the hypothesis was partly correct since some of the so-called 'benzoate-cured' segregants were in fact plasmid-free, but in others the same phenotype was found to be produced by a specific large deletion of 39 kbp from pWW0 (Bayley *et al.*, 1977); this 39 kbp region has been shown to carry all of the catabolic genes for the toluene-xylene pathway (Franklin *et al.*, 1981; Lehrbach & Timmis, 1983).

The procedure of growing strains on BMM has been used with nearly all *Pseudomonas* strains which have been isolated with plasmids carrying the same catabolic function as pWW0 (the TOL plasmids) and without exception it has been found that it exerts a strong selective pressure against the wild-type and results in the appearance either of plasmid-free segregants or of mutants, phenotypically distinguishable from the wild-type, with functional lesions in the pathway and deletions in the plasmids (Williams & Worsey, 1976; Worsey & Williams, 1977; Kunz & Chapman, 1981; Pickup & Williams, 1982; Pickup *et al.*, 1983; Keil & Williams, 1985). This phenomenon is particularly apparent with certain *Pseudomonas* strains which carry a subclass of large TOL plasmids (≥ 250 kbp), strains MT14, MT15 and MT20. These strains segregate mutants at very high frequency during growth on benzoate (Williams & Worsey, 1976), producing several classes of phenotypically distinguishable deletion mutants (Worsey & Williams, 1977; Pickup & Williams, 1982; Keil & Williams, 1985) and contain very related plasmids but ones structurally distinct from pWW0 (Keil & Williams, 1985).

It has always been assumed in this laboratory that the growth-rate hypothesis was sufficient to explain all these instances. However, the difficulties of accurately measuring small differences in growth rates in batch cultures have made the hypothesis largely untested, except in the case of *P. putida* MT20, where the cured and deletion mutants grow considerably more rapidly than the wild-type (Worsey & Williams, 1977).

Recently several papers from other laboratories have cast doubts on the hypothesis. Keshavarz *et al.* (1985) carried out long-term chemostat culture of a TOL-plasmid-containing isolate and suggested that the results could only be explained if benzoate had some effect on the partitioning of the plasmid. Stephens & Dalton (1987) have interpreted experiments with *P. putida* MT15 as demonstrating that the undissociated acid molecules of benzoate, and other weak organic acids, act to promote plasmid loss and/or deletion. Clarke & Laverack (1984) also showed that, at much higher concentrations than used in any of the growth experiments, benzoate could cause spontaneous loss, not only of TOL plasmids, but also of naphthalene catabolic plasmids.

In this paper we return to *P. putida* mt-2 (PaW1), host to the archetypal TOL plasmid pWW0, and demonstrate, with the use of genetically marked derivative strains, that 'benzoate curing', involving either plasmid loss or deletion, is entirely consistent with the occurrence of relatively frequent spontaneous events which are then amplified by a faster growth of the segregants on benzoate.

METHODS

Strains. A list of the strains of *P. putida* mt-2 used in this study is presented in Table 1.

Growth experiments. Cells were grown in liquid and solid minimal media, pH 7.0, containing (g l⁻¹): (NH₄)₂SO₄, 1.0; KH₂PO₄, 2.5; MgSO₄ · 7H₂O, 0.1; FeSO₄, 5 × 10⁻³; nitrilotriacetic acid, 0.25; NaOH, 0.55; and 1 ml l⁻¹ of the stock salts solution of Bauchop & Elsdon (1960). In some experiments the minimal medium was supplemented with KH₂PO₄ to a final concentration of 7.5 g l⁻¹; this was to increase its buffering capacity and thus minimize the effect of any increase of pH during growth. This medium is referred to as 'high phosphate'. Starting cultures were from single colonies inoculated into nutrient broth and grown overnight at 30 °C to cell densities of about 4–8 × 10⁸ ml⁻¹. Dilutions of 10⁻⁵ were made in sterile phosphate buffer, pH 7.5, and 0.1 ml was inoculated into 5 ml of the selected growth medium which was then incubated at 30 °C in a rotary shaker until turbid growth was seen: this was normally after 24 h. The cultures, which had cell densities of around 10⁸ ml⁻¹, were serially diluted and 0.1 ml volumes of 10⁻⁴ and 10⁻⁵ dilutions were spread on to nutrient agar plates. A 0.1 ml volume of the 10⁻⁵ dilution (usually containing about 50–200 cells) was also inoculated into 5 ml of fresh growth medium for the second cycle of growth, and so on. Each cycle was therefore about 22 generations, apart from the first of each series, with its inoculum from a nutrient broth culture, which was probably some 2–3 generations less. It follows from the fact that only of the order of 10² cells were transferred from one subculture to the next, that any strain in the population which drops to around 1% of the total cells stands a strong chance of being totally eliminated from the next subculture. After incubating the agar plates overnight, 88 isolated colonies were selected and transferred to appropriate selection plates in order to test their phenotype. Benzoate and *m*-toluate were incorporated into minimal salts agar at 5 mM and carbenicillin into Isosensitest agar at 2 mg ml⁻¹. For the early experiments using growth of PaW1 (data of Table 2 only), the phenotype of the colonies on the agar plates was examined by spraying

Table 1. *Pseudomonas putida* strains used

Strain	Plasmid	Relevant phenotype*	Notes and reference
PaW1	pWW0	Mtol ⁺ Ben ⁺	<i>P. putida</i> mt-2 wild-type (Williams & Murray, 1974)
PaW8	pWW0-8	Mtol ⁻ Ben ⁺	'Benzoate-cured' segregant of PaW1 (Williams & Murray, 1974)
PaW163	pWW0-163	Mtol ⁺ Ben ⁺ Cb ^r	Plasmid is pWW0::Tn401. Gift of J. Shapiro
PaW164	pWW0-164	Mtol ⁺ Ben ⁺ Cb ^r	Plasmid is pWW0::Tn401. Gift of J. Shapiro
PaW174	pWW0-174	Mtol ⁻ Ben ⁺ Cb ^r	'Benzoate-cured' segregant of PaW164 carrying a pWW0-8::Tn401 plasmid. This paper
PaW701	pWW60-1	Nah ⁺ Ben ⁺	Carries IncP9 naphthalene-catabolic plasmid (Cane & Williams, 1982)

* Phenotype designations: Mtol⁺, Ben⁺, Nah⁺, abilities to grow on *m*-toluate, benzoate, naphthalene as sole carbon sources; Cb^r, resistance to carbenicillin.

the plates with 100 mM-catechol; whereas wild-type cells (Mtol⁺ Ben⁺) turn yellow within 1–2 min due to the conversion of the catechol to 2-hydroxymuconic semialdehyde by the plasmid-coded catechol 2,3-oxygenase, the 'cured' strains (Mtol[–] Ben⁺) stay white.

Competition experiments. These were done as for the growth experiments above except that the two overnight starter cultures, assumed to be roughly of the same cell density, were diluted to different extents and mixed to give expected initial cell ratios (PaW1 : PaW174) of 1 : 1, 50 : 1 and 10³ : 1 which were then inoculated into 5 ml of BMM. The actual cell ratios were determined by doing plate counts on the two starter cultures. The mixed cultures were subsequently treated exactly as when growing from a single culture.

Plasmid isolation. Plasmids were isolated and their restriction digestion pattern examined according to Wheatcroft & Williams (1981).

RESULTS

Growth of single strains

In a preliminary experiment we confirmed that PaW1 segregated mutant strains which had lost the plasmid-coded pathway during growth on BMM at a range of initial pH values from 7.2 to 6.4 (Table 2). At all pH values the proportion of Mtol[–] strains increased through successive growth cycles and there was no apparent relationship with pH for either the first appearance of segregants or the rate of loss of wild-type. Rather, there was as great a variation between replicates at the same pH as at different pH values, suggesting that the onset of wild-type loss is dependent upon random or stochastic events. The use of high-phosphate mineral salts medium also had no significant effect. In these experiments we checked the pH at the end of each growth cycle and in all 12 determinations it was within ± 0.2 of the initial pH, showing that the effect could not be attributed to pH changes in the medium.

PaW174 carries a plasmid, pWW0-174, derived from pWW0. All the catabolic genes have been deleted between the 1.4 kbp direct repeats as in plasmid pWW0-8 (Fig. 2; Downing *et al.*, 1979); hence PaW174 will not grow on any of the usual substrates of the toluene-xylene pathway, such as *m*-xylene or *m*-toluate, but it will grow on benzoate via the chromosomal β -ketoadipate pathway. In addition it carries an insertion of Tn401 which confers resistance to carbenicillin. We grew three independent cultures of PaW174 for six cycles through BMM, pH 7.0, and were unable to detect a single colony, out of 528 examined, which had lost the plasmid and hence was Cb^s.

We repeated the experiments with PaW1 at initial pH 7.0 and 6.4 but growing the cells on minimal medium containing a preferred growth substrate (15 mM-succinate) in the presence of 5 mM-benzoate. Although we used high-phosphate medium, the pH in these experiments did change more than when benzoate alone was used (from 7.0 to about 7.3–7.45 and from 6.4 to 7.0–7.2) but still encompassed the same pH range as in the experiments with benzoate alone. After three cycles (Table 2) 98–100% of the colonies examined were wild-type (Mtol⁺), in stark contrast to the experiments with benzoate alone (Table 2). In these experiments we also measured the UV absorption spectra of the media after each growth cycle and generally less than 20% of the benzoate appeared to have been utilized. It follows that the presence of benzoate is insufficient to cause curing unless it is the major growth substrate. The final experiment in this series was to grow PaW1 on another weak organic acid (5 mM-propionate) through three subcultures: in four independent experiments, two at pH 7.0 and two at pH 6.4, we detected only two Mtol[–] colonies in the 88 examined from each experiment. It is clear from these growth experiments that pH, either directly or indirectly, by changing the degree of ionization of the organic acid growth substrate, has no effect, nor does growth on weak organic acids other than benzoate.

It is impossible to distinguish the phenotypes of the plasmid-free and the deletion mutants of PaW1 which occur during growth on BMM since they are both Mtol[–] Ben⁺ and they do not turn yellow when sprayed with catechol. They could be distinguished by the presence or absence of plasmid DNA, but demonstrating this is time-consuming and has only been done once on a very limited number of strains (Bayley *et al.*, 1977). However, deletion and plasmid loss can be distinguished if derivatives of pWW0 are used which carry a genetic marker such as a transposon inserted in the plasmid outside the 39 kbp region lost in the deletion event. PaW163

Fig. 2. Linear restriction map of pWW0. The sites for restriction enzymes *Hind*III and *Xho*I are shown together and the main fragments are lettered according to Downing & Broda (1978). The location of the *Tn401* insertions in pWW0-163 and pWW0-164 are indicated on the map of the gene organization together with the two 1.4 kbp direct repeats at the termini of the 39 kbp region deleted, the *xylCAB* operon (1), the *xylDLEGF(JK)IH* operon (2) and the *xylSR* regulatory gene region (3).

Table 2. Segregation of *Mtol*⁻ strains from *PaW1* grown on various media

Medium	Initial pH	Percentage of <i>Mtol</i> ⁺ cells after			
		1	2	3	4
5 mM-benzoate (low phosphate)	7.2	> 99	54	4	ND
	7.0	98	19	2	ND
	7.0	97	51	5	ND
	7.0	98	34	2	ND
	6.8	96	68	17	ND
	6.6	87	9	4	ND
	6.4	> 99	> 99	> 99	92
	6.4	81	12	2	ND
5 mM-benzoate (high phosphate)	7.0	> 99	73	9	1
	7.0	> 99	94	44	< 1
	6.4	92	10	2	ND
	6.4	> 99	74	5	ND
15 mM-succinate + 5 mM-benzoate (high phosphate)	7.0	ND	ND	98	ND
	7.0	ND	ND	> 99	ND
	6.4	ND	ND	99	ND
	6.4	ND	ND	> 99	ND

ND, Not determined.

and PaW164 are hosts to two such plasmids, which have inserts of *Tn401* carrying a β -lactamase gene conferring *Cb*^r. In pWW0-163 the insert is in the region common to fragments *Hind*III-E and *Xho*-H and in pWW0-164 it is in fragments *Hind*III-D and *Xho*-F (Fig. 2; Downing & Broda, 1978). Deletion of the 39 kbp region produces strains which retain the transposon and are *Mtol*⁻ *Ben*⁺ *Cb*^r, whereas plasmid loss results in *Mtol*⁻ *Ben*⁺ *Cb*^s strains. The presence of the *Tn401* on an otherwise cryptic plasmid in the putative deletion strains can be further confirmed by showing that the resistance can be transferred from them by conjugation.

PaW163 and PaW164 were each grown through three cycles in BMM, pH 7.0, in several independent experiments. Phenotypic analysis of the resulting cultures (Table 3) showed that in eight of the 11 experiments only deletion mutants were found and in two the deletion mutants were predominant: in only one case did plasmid-free segregants predominate, and in this

Fig. 3. Competition between PaW8 (Mtol⁻ Cb^s) and PaW174 (Mtol⁻ Cb^r) growing on benzoate at pH 7.0: the results are presented as the percentage of Cb^r cells in the population. The cell densities (cells ml⁻¹) at the start of the first subculture were: experiment A, PaW174 150, no PaW8 cells added; experiment B, PaW174 600, PaW8 130 (ratio 4.5:1); experiment C, PaW174 150, PaW8 130 (ratio 1.2:1); experiment D, PaW174 40, PaW8 130 (ratio 0.3:1).

Table 3. *Effect of growth on benzoate at pH 7.0 on strains carrying pWW0::Tn401 plasmids*

Strain	Experiment no.	No. of colonies with phenotypes		
		Mtol ⁺ Cb ^r	Mtol ⁻ Cb ^r	Mtol ⁻ Cb ^s
PaW163	1	1	87	0
	2	17	71	0
	3	1	87	0
	4	2	74	12
	5	2	86	0
PaW164	1	0	0	88
	2	3	53	32
	3	25	63	0
	4	1	87	0
	5	3	85	0
	6	0	88	0

experiment they were the only type found. As controls we demonstrated that PaW174, a deletion strain derived from PaW164, stably retained its Cb^r phenotype through five subcultures in BMM, pH 7.0, thus eliminating the possibility that the putative plasmid-free segregants could arise from excision of the transposon from the deleted plasmid (Fig. 3, line A) and also incidentally further confirming that pWW0-174 is totally stable under growth conditions where pWW0 segregates either deletions or plasmid-free derivatives (Table 2). We also showed that there was no selective advantage in the presence of Tn401 during growth on BMM and that in mixtures of PaW174 with PaW8 (Mtol⁻ Ben⁺ Cb^s) neither outcompeted the other (Fig. 3, lines B-D).

If it is assumed that the transposon does not influence the mode of segregation, and this assumption can be partly justified since plasmids with insertions in two different locations were used, this experiment (Table 3) indicates that deletion is a more frequent event than is plasmid loss. However, the differences between individual experiments again illustrate the random nature of the events which produce the strains which eventually predominate in the culture.

Fig. 4

Fig. 5

Fig. 4. Competition between PaW1 and PaW174 growing on benzoate at pH 7.0. (a) Percentage of wild-type cells (Mtol⁺ Cb⁺). (b) Percentage of 'cured' strains (---) with phenotypes Mtol⁻ Cb⁻ (PaW174) and (—) with phenotypes Mtol⁻ Cb⁺ (segregants of PaW1). The cell densities (cells ml⁻¹) at the start of the first subculture were: experiment A, PaW1 1.7×10^5 , PaW174 270 (ratio 630:1); experiment B, PaW1 8.5×10^3 , PaW174 270 (ratio 30:1); experiment C, PaW1 170, PaW174 270 (ratio 0.6:1).

Fig. 5. Competition between PaW1 and PaW174 growing on benzoate at pH 6.4. (a) Percentage of wild-type cells (Mtol⁺ Cb⁺). (b) Percentage of 'cured' strains (---) with phenotypes Mtol⁻ Cb⁻ (PaW174) and (—) with phenotypes Mtol⁻ Cb⁺ (segregants of PaW1). The cell densities (cells ml⁻¹) at the start of the first subculture were: experiment A, PaW1 1.3×10^5 , PaW174 80 (ratio 1600:1); experiment B, PaW1 6.8×10^3 , PaW174 80 (ratio 85:1); experiment C, PaW1 130, PaW174 80 (ratio 1.6:1).

Competition experiments

Because of the stable retention of pWW0-174 during growth on benzoate we set up competition experiments at pH 7.0 and pH 6.4 in which mixtures of PaW1 and PaW174 were inoculated into BMM in different initial cell ratios, and the composition of the culture was monitored during successive subcultures. This enabled determination of the individual growth advantages of the two strains and simultaneously it was possible to distinguish between the 'cured' strain seeded into the culture (PaW174) and any spontaneous 'cured' derivatives of PaW1 which arose during the course of the experiment. The results at pH 7.0 are shown in Fig. 4. As was expected from the results of the earlier experiments, the wild-type was lost from the culture even when present initially in a 630:1 preponderance over PaW174 (Fig. 4 a, line A). When PaW174 was present at about 60% of the initial population (Fig. 4 b, lines C) or at about 3% (Fig. 4 b, lines B), it ultimately took over as the dominant strain (>95%), but when it was initially present as only about 1% (Fig. 4 b, lines A), the final culture contained a higher proportion of Mtol⁻ Ben⁺ Cb⁺ strains, which must have arisen from PaW1 either by deletion

(probably) or by plasmid loss (possibly). The control experiment in which PaW174 was competed with PaW8, a 'cured' strain but with a deleted plasmid pWW0-8 (Bayley *et al.*, 1977; Downing *et al.*, 1979), is shown in Fig. 3; neither strain has a selective growth advantage on BMM, pH 7.0.

Qualitatively similar results were found when the experiment was repeated at pH 6.4 (Fig. 5), when Mtol⁻ Ben⁺ Cb^s strains ultimately predominated when the initial ratio of PaW1 : PaW174 was about 1600 (Fig. 5 *b*, lines A) but at lower ratios PaW174 became predominant.

The result showing that PaW174 takes over the population when initially present even as a minority component, resulting in loss of wild-type PaW1 from the population at a comparable rate to that seen when PaW1 is grown on BMM alone (compare Table 2 with Figs 4 and 5), must lead to the conclusion that the normal 'benzoate curing' of PaW1 (Table 2) can be explained by the simple selection on benzoate of faster-growing strains. Additionally the data of Figs 4 and 5 allow a rough estimate of the frequency of spontaneous occurrence within populations of PaW1 of the strains which give rise to the 'curing'. The implication of the data is that such strains are present at the surprisingly high frequency of 10^{-2} – 10^{-3} , so that when the culture is inoculated with another 'cured' strain (as PaW174) at cell ratios below this, the spontaneously formed strains ultimately predominate, whereas when the seeded strain is added at higher ratios than 10^{-2} , it will ultimately take over.

DISCUSSION

The results presented here show conclusively that the progressive takeover of populations of PaW1 by segregants which have lost the *meta* pathway genes either by deletion or by plasmid loss can be adequately explained by their faster rate of growth under the experimental conditions, batch culture on BMM, which have routinely been used by us and others in studies of TOL plasmids (Williams & Worsey, 1976; Worsey & Williams, 1977; Kunz & Chapman, 1981; Pickup & Williams, 1982; Pickup *et al.*, 1983; Keil & Williams, 1985).

There is no evidence with these studies that the segregation of the Mtol⁻ strains is due to a direct effect of benzoate or other anions as proposed by Stephens & Dalton (1987). (1) There is no effect of lowering the pH from 7.0 to 6.4 on the efficacy of 'curing'. (2) Growth of PaW1 on 5 mM-propionate at pH 7.0 and pH 6.4 does not cause any segregation of Mtol⁻ strains. (3) Growth of PaW174 on benzoate causes no loss of its plasmid pWW0-174: since this plasmid contains the complete replicon of pWW0 and lacks only the 39 kbp region, whose only known role is to encode the catabolic genes, any direct effect of benzoate on plasmid segregation should cause loss similar to that found with pWW0 itself.

We have shown that the loss of the Mtol⁺ phenotype only occurs when there is the selective advantage of growth on benzoate available for the segregants: when cells are grown on media where there is an alternative carbon source which requires only constitutive enzymes, such as 15 mM-succinate, even when 5 mM-benzoate is present, no segregation takes place.

Strain PaW701, a *P. putida* mt-2 host carrying the naphthalene catabolic plasmid pWW60-1, does not segregate any mutants of the plasmid-coded pathway after growth on benzoate. This confirms both of the arguments of the preceding paragraphs. Firstly pWW60-1 is of the same incompatibility group, IncP9, as pWW0 and therefore presumably shares similar, if not identical, replication and partition mechanisms: any effect of benzoate on plasmid replication and partition would be expected to act equally against this very similar plasmid as against pWW0. The lack of any such mutants after growth on benzoate therefore suggests that there is no such effect and at the same time reinforces our argument about selection. In the case of TOL plasmids, benzoate can be utilized either by the plasmid pathway or by the chromosomal pathway but, because of the regulatory differences between the two routes, only the plasmid pathway is expressed in the TOL⁺ wild-types: only by loss or mutational malfunction of the plasmid pathway can the chromosomal route be used and, as is proposed here, such strains will outcompete the wild-type because of their faster growth rate. However, no such selection can occur in the case of PaW701 since benzoate is not a substrate of the naphthalene-plasmid-determined pathway and therefore it is catabolized by the chromosomal pathway whether

plasmid is present, absent or mutated. Thus Nah⁻ mutants would not be expected on the growth-rate hypothesis but would be if benzoate caused plasmid loss or deletion. The fact that Clarke & Laverack (1984) reported a high frequency of loss of naphthalene and TOL plasmids (including pWW0) is not relevant to this argument since their protocol involved plating strains on to very high concentrations of benzoate (20–50 mM), just below growth-inhibitory levels where undoubtedly other factors operate.

The growth-rate hypothesis is confirmed by our competition experiments where cultures of PaW1 were inoculated at various dilutions with PaW174, carrying a transposon-marked plasmid. At initial cell concentrations of about 1% of the wild-type, PaW174 becomes the predominant strain after 60–80 generations and the curves follow the theoretical curves for growth-rate-determined competition (Stephens & Dalton, 1987). At lower initial cell ratio, although PaW174 is still present as a significant component of the population after 100 generations, 'cured' derivatives of PaW1 predominate and this suggests that their presence within unselected populations of PaW1 is between 10⁻² and 10⁻³: this is an order of magnitude higher than the value of about 4 × 10⁻⁴ estimated by Nakazawa & Yokota (1973) from fluctuation analysis with their clone of strain mt-2.

This paper also demonstrates for the first time that plasmid deletion appears to occur more frequently than does plasmid loss in these experiments. This is perhaps not surprising since pWW0 contains two direct repeats of 1.4 kbp (Meulien *et al.*, 1981) at either end of the 39 kbp region deleted (Fig. 2), which would serve as regions upon which the Rec system might act. A testable consequence of this might be that benzoate selection should be far less effective in a Rec⁻ host. We have evidence that some of the strains which are hosts to other different TOL plasmids which undergo loss of wild-type during growth on benzoate, including *P. putida* MT15 used by Stephens & Dalton (1987), have even larger regions of DNA homology within the plasmid due to extensive duplications of catabolic genes. These might also serve as templates for Rec-dependent deletion events (Chatfield & Williams, 1986; Keil *et al.*, 1987; K. J. O'Donnell & P. A. Williams, unpublished results) causing loss or malfunction of expression of the toluene-xylene catabolic genes and giving rise to host cells which use the chromosomal pathway and which are then positively selected during subsequent growth on benzoate because of their faster growth rate.

REFERENCES

- BAUCHOP, T. & ELSDEN, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *Journal of General Microbiology* **23**, 457–469.
- BAYLEY, S. A., DUGGLEBY, C. J., WORSEY, M. J., WILLIAMS, P. A., HARDY, K. G. & BRODA, P. (1977). Two modes of loss of the Tol function from *Pseudomonas putida* mt-2. *Molecular and General Genetics* **154**, 203–204.
- CANE, P. A. & WILLIAMS, P. A. (1982). The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1. *Journal of General Microbiology* **128**, 2281–2290.
- CHATFIELD, L. K. & WILLIAMS, P. A. (1986). Naturally occurring TOL plasmids in *Pseudomonas* strains carry either two homologous or two nonhomologous catechol 2,3-oxygenase genes. *Journal of Bacteriology* **168**, 878–885.
- CLARKE, P. H. & LAVERACK, P. D. (1984). Growth characteristics of *Pseudomonas* strains carrying catabolic plasmids and their cured derivatives. *FEMS Microbiology Letters* **24**, 109–112.
- DOWNING, R. G. & BRODA, P. (1978). A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. *Molecular and General Genetics* **168**, 189–191.
- DOWNING, R. G., DUGGLEBY, C. J., VILLEMS, R. & BRODA, P. (1979). An endonuclease map of the plasmid pWW0-8, a derivative of the TOL plasmid of *Pseudomonas putida* mt-2. *Molecular and General Genetics* **177**, 97–99.
- FRANKLIN, F. C. H., BAGDASARIAN, M., BAGDASARIAN M. M. & TIMMIS, K. N. (1981). Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of the entire regulated aromatic ring meta cleavage pathway. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 7458–7462.
- KEIL, H. & WILLIAMS, P. A. (1985). A new class of TOL plasmid deletion mutants in *Pseudomonas putida* MT15 and their reversion by tandem gene amplification. *Journal of General Microbiology* **131**, 1023–1033.
- KEIL, H., KEIL, S. & WILLIAMS, P. A. (1987). Molecular analysis of regulatory and structural *xyl* genes of the TOL plasmid pWW53-4. *Journal of General Microbiology* **133**, 1149–1158.
- KESHAVARZ, T., LILLY, M. D. & CLARKE, P. H. (1985). Stability of a catabolic plasmid in continuous culture. *Journal of General Microbiology* **131**, 1193–1203.
- KUNZ, D. A. & CHAPMAN, P. J. (1981). Isolation and characterization of spontaneously occurring TOL

- plasmid mutants of *Pseudomonas putida* HS1. *Journal of Bacteriology* **146**, 952–962.
- LEHRBACH, P. R. & TIMMIS, K. N. (1983). Genetic analysis and manipulation of catabolic pathways in *Pseudomonas*. *Biochemical Society Symposia* **48**, 191–291.
- MEULIEN, P., DOWNING, R. G. & BRODA, P. (1981). Excision of the 40 kb segment of the TOL plasmid from *Pseudomonas putida* mt-2 involves direct repeats. *Molecular and General Genetics* **184**, 97–101.
- NAKAZAWA, T. & YOKOTA, T. (1973). Benzoate metabolism in *Pseudomonas putida* (arvilla) mt-2: demonstration of two benzoate pathways. *Journal of Bacteriology* **115**, 262–275.
- PICKUP, R. W. & WILLIAMS, P. A. (1982). Spontaneous deletions in the TOL plasmid pWW20 which give rise to the B3 regulatory mutants of *Pseudomonas putida* MT20. *Journal of General Microbiology* **128**, 1385–1390.
- PICKUP, R. W., LEWIS, R. J. & WILLIAMS, P. A. (1983). *Pseudomonas* sp. MT14, a soil isolate which contains two large catabolic plasmids, one a TOL plasmid and one coding for phenylacetate catabolism and mercury resistance. *Journal of General Microbiology* **129**, 153–158.
- STEPHENS, G. M. & DALTON, H. (1987). The effect of lipophilic weak acids on the segregational stability of TOL plasmids in *Pseudomonas putida*. *Journal of General Microbiology* **133**, 1891–1899.
- WHEATCROFT, R. & WILLIAMS, P. A. (1981). Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. *Journal of General Microbiology* **124**, 433–437.
- WILLIAMS, P. A. & MURRAY, K. (1974). Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. *Journal of Bacteriology* **120**, 416–423.
- WILLIAMS, P. A. & WORSEY, M. J. (1976). Ubiquity of plasmids in coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. *Journal of Bacteriology* **125**, 818–828.
- WORSEY, M. J. & WILLIAMS, P. A. (1975). Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. *Journal of Bacteriology* **124**, 7–13.
- WORSEY, M. J. & WILLIAMS, P. A. (1977). Characterization of a spontaneously occurring mutant of the TOL20 plasmid in *Pseudomonas putida* MT20: possible regulatory implications. *Journal of Bacteriology* **130**, 1149–1158.