

## Spontaneous Deletions in the TOL Plasmid pWW20 which Give Rise to the B3 Regulatory Mutants of *Pseudomonas putida* MT20

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(Received 14 September 1981; revised 10 November 1981)

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The size of the TOL plasmid pWW20 from *Pseudomonas putida* MT20, as measured by analysis of agarose electrophoresis gels after restriction endonuclease hydrolysis, was 270–280 kilobase pairs (kb). During growth on benzoate, MT20 segregates strains carrying mutations in the plasmid regulatory gene *xylS*; these so-called B3 strains retain the ability to grow on *m*-xylene (Mxy<sup>+</sup>) but do not grow on its metabolite *m*-toluate (Mtol<sup>-</sup>) and have also lost the ability to transfer the plasmid (Tra<sup>-</sup>). Analysis of restriction digests of plasmid DNA from seven such segregants, independently isolated, showed that pWW20 had undergone extensive deletions of 90–100 kb. All the deleted plasmids had lost a common core of DNA, of about 72–80 kb, but in class A mutants the deletion extended at one end of this core and in class B mutants at the other end. Class A and B mutants also differed in their rate of growth on *m*-xylene as a result of differences in the level of expression of their plasmid-coded catabolic enzymes. This suggests that an additional gene, involved in regulating levels of gene expression, is located in the region uniquely deleted in the class B mutants.

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### INTRODUCTION

*Pseudomonas putida* MT20 carries a TOL plasmid, pWW20 (originally called TOL20), which encodes enzymes for the catabolism of toluene, *m*-xylene and *p*-xylene through benzoate, *m*-toluate and *p*-toluate, respectively, followed by the *meta* pathway for assimilation of the aromatic ring (Fig. 1) (Williams & Murray, 1974; Williams & Worsey, 1976; Worsey & Williams, 1975). As with strains carrying other isofunctional TOL plasmids (Williams & Murray, 1974; Williams & Worsey, 1976), growth in benzoate-minimal medium selects for spontaneous segregants which have lost the expression of the TOL pathway, but which catabolize benzoate by the chromosomal  $\beta$ -keto adipate (or *ortho*) pathway; this pathway is ubiquitous in *Pseudomonas* hosts, is not induced in the presence of a TOL plasmid (Williams & Murray, 1974; Williams & Worsey, 1976), and supports a faster growth rate than does the plasmid pathway which accounts for its selection (Williams & Worsey, 1976; Worsey & Williams, 1977). With most of the TOL plasmids studied, benzoate selection produces segregants which no longer grow on any of the TOL substrates, except benzoate, and are routinely tested as Ben<sup>+</sup>Mxy<sup>-</sup>Mtol<sup>-</sup> (unable to grow on *m*-xylene or *m*-toluate). With the archetype TOL plasmid, pWW0, this phenotype can result, with about equal frequency, either from total loss of the plasmid or from the specific excision of a 39 kilobase pairs (kb) section from the 117 kb plasmid (Bayley *et al.*, 1977). Strain MT20 is one of a small group of strains which on benzoate selection give rise not only to Ben<sup>+</sup>Mxy<sup>-</sup>Mtol<sup>-</sup> segregants but also to a novel class we have called the B3 strains (Williams & Worsey, 1976). These grow on the hydrocarbons by the complete TOL pathway, do not grow on *m*- or *p*-toluate, but grow on benzoate via the  $\beta$ -keto adipate pathway, and are routinely tested as Ben<sup>+</sup>Mxy<sup>+</sup>Mtol<sup>-</sup> (Williams & Worsey, 1976). In a detailed genetic and biochemical analysis of one such

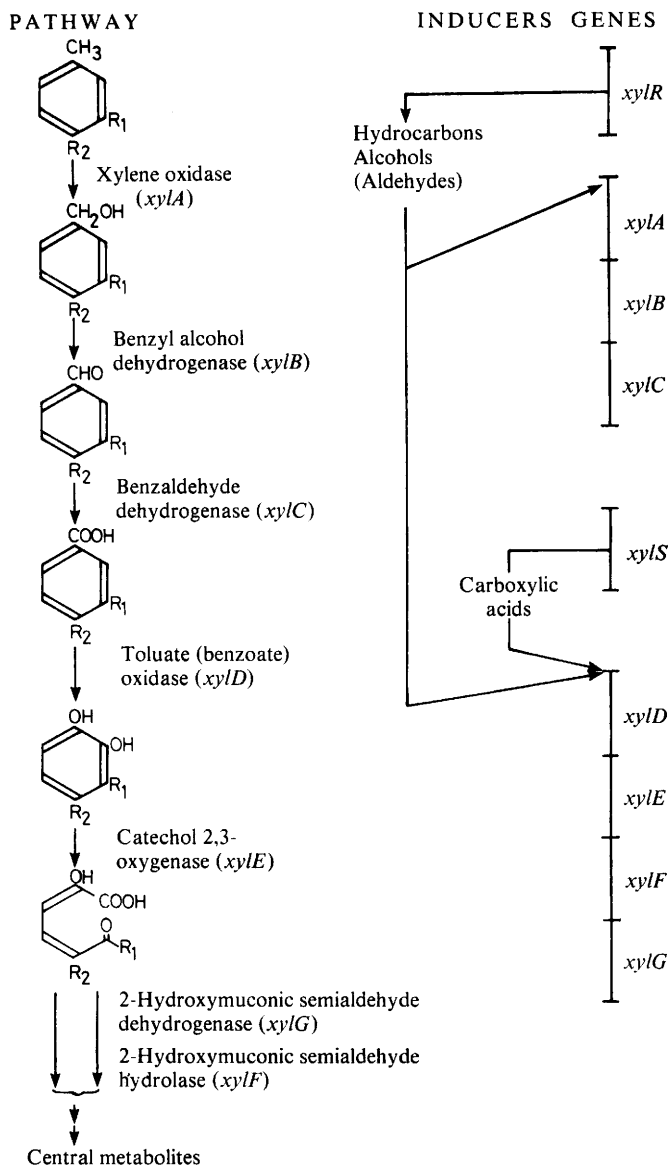


Fig. 1. The early enzymes of the TOL plasmid degradative pathway and their regulation. The primary metabolites of the pathway are toluene ( $R_1 = R_2 = H$ ), *m*-xylene ( $R_1 = CH_3$ ,  $R_2 = H$ ) and *p*-xylene ( $R_1 = H$ ,  $R_2 = CH_3$ ); *m*-toluate is the carboxylic acid metabolite of *m*-xylene. The regulatory model has been proposed from a study of pWW0 (Worsey *et al.*, 1978), but essentially the same model was proposed for pWW20 (Worsey & Williams, 1977) except that the regulation of *xylA* was not studied in this latter case. In the model, the hydrocarbon and alcohol substrates combine with the gene product of *xylR* to induce both *xylABC* and *xylDEFG*, whereas the carboxylic acids induce only the latter operon through the *xylS* gene product.

segregant (Worsey & Williams, 1977) we proposed that it was the result of the deletion of a regulatory gene corresponding to *xylS* (but originally referred to as R2; Fig. 1). The resultant plasmid had become non-conjugative in contrast to the wild-type plasmid, suggesting that essential *tra* gene(s) had also been deleted. Until recently, pWW20 plasmid DNA has proved refractory towards extraction by any of the methods previously used for degradative

plasmids. However, isolation of this DNA has now been achieved using a rapid method of preparation (Wheatcroft & Williams, 1981), and we have compared the structures of pWW20 and seven of its deletion derivatives.

#### METHODS

**Maintenance and growth of cultures.** Strains PaW1 (*Pseudomonas putida* mt-2, ATCC 33015) and *P. putida* MT20 were maintained on minimal salts agar containing 5 mM-*m*-toluate. The mutant B3 derivatives of strain MT20 were maintained on nutrient agar. Batch cultures in 2 litre minimal medium containing 10 mM-acetate were inoculated with 50 ml nutrient broth-grown starter cultures and incubated for 24 h at 30 °C under forced aeration. Inducing substrates were present from the start either in solution or, for *m*-xylene, as saturated vapour in the air supply.

**Preparation of extracts.** Cells were harvested by centrifugation for 1 h at 1000 g, washed in 0.1 M-phosphate buffer, pH 7.5, centrifuged a second time and stored in two aliquots at -20 °C. The frozen cells were resuspended in 5 ml of either 0.1 M-phosphate buffer, pH 7.5, supplemented with 10% acetone (for assay of catechol 2,3-oxygenase) or phosphate/NaCl/MgSO<sub>4</sub> (Worsey & Williams, 1975) (for assay of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase) and sonicated for six periods of 30 s at 0 °C in an MSE 100 W ultrasonic disintegrator. Cell debris was removed by centrifugation at 150000 g for 6 min at 4 °C.

**Enzyme assays.** The following enzymes were assayed as described by Worsey & Williams (1975): benzyl alcohol dehydrogenase, substrate benzyl alcohol; benzaldehyde dehydrogenase (EC 1.2.1.28), substrate benzaldehyde; catechol 2,3-oxygenase (EC 1.13.11.2), substrate catechol. Protein was determined by the biuret method.

**Plasmid isolation and digestion.** Plasmid DNA was isolated by the preparative method of Wheatcroft & Williams (1981) and was digested by restriction endonucleases (Uniscience, Cambridge, U.K.) according to the supplier's instructions. Gels containing 0.7 g agarose and 100 µg ethidium bromide in 100 ml of electrophoresis buffer were run submerged in electrophoresis buffer at 34–40 mA for 16–24 h and visualized as described previously (Wheatcroft & Williams, 1981).

**Sizing of plasmids.** Restriction enzyme digests of plasmids were always run beside *Xho*I digests of pWW0, the fragments of which have been accurately sized (Downing & Broda, 1979). Each plasmid was digested at least ten times and run for different lengths of time to resolve the larger or the smaller fragments. Interpretation of bands as double or triple bands was carried out by visual comparison of bands with adjacent bands in the same digests.

**Benzoate curing.** The B3 mutants of strain MT20 were prepared by growth on benzoate-minimal medium as described by Williams & Worsey (1976).

#### RESULTS AND DISCUSSION

We isolated seven independent B3 segregants of *P. putida* MT20 after growth in benzoate-minimal medium for 15–20 generations. All had the B3 phenotype (Ben<sup>+</sup>Mxy<sup>+</sup>Mtol<sup>-</sup>) but they differed in their rate of growth on *m*-xylene. Class A strains (MT20-22, MT20-23, MT20-27) grew as fast on *m*-xylene as did the wild-type MT20, but class B strains (MT20-21, MT20-24, MT20-25, MT20-26) grew more slowly and patches or colonies took several days to appear. Three of the TOL catabolic enzymes were assayed under different induction conditions in two representatives of each class (Table 1); benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were chosen as representative of the early enzymes under control of *xylR* only, and catechol 2,3-oxygenase as representative of the later enzymes controlled by both *xylR* and *xylS*. Both classes of mutants showed the same general induction pattern as described for the original B3 mutant (Worsey & Williams, 1977). Growth in the presence of *m*-toluate caused no induction of any of the enzymes, whereas growth on *m*-xylene induced all three enzymes assayed. This pattern differed from that for strain MT20 in which *m*-toluate induced catechol 2,3-oxygenase but not benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase. However, class A strains had fully induced levels of all three enzymes after growth on *m*-xylene, whereas class B strains had only about 10–30% of the fully induced levels of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase found in strain MT20, although catechol 2,3-oxygenase was fully induced. The uninduced levels of all three enzymes were also much reduced in class B mutants.

Table 1. *Enzyme specific activities in extracts of strains of P. putida MT20*

Enzyme	Inducer	Strain ...	Specific activity [mU (mg protein) <sup>-1</sup> ]*				
			MT20	MT20-22	MT20-27	MT20-25	MT20-26
Benzyl alcohol dehydrogenase	<i>m</i> -Xylene		2270	3970	1630	290	500
	<i>m</i> -Toluate		80	60	40	10	7
	None		50	90	100	5	7
Benzaldehyde dehydrogenase	<i>m</i> -Xylene		700	1000	1310	250	250
	<i>m</i> -Toluate		50	50	20	10	5
	None		30	40	50	5	6
Catechol 2,3-oxygenase	<i>m</i> -Xylene		2760	1980	1600	1930	2450
	<i>m</i> -Toluate		1080	20	30	10	2
	None		100	80	100	2	6

\* One unit (U) is the amount of enzyme required to convert 1  $\mu$ mol substrate  $\text{min}^{-1}$ . The values of specific activity quoted are the mean of at least three determinations.

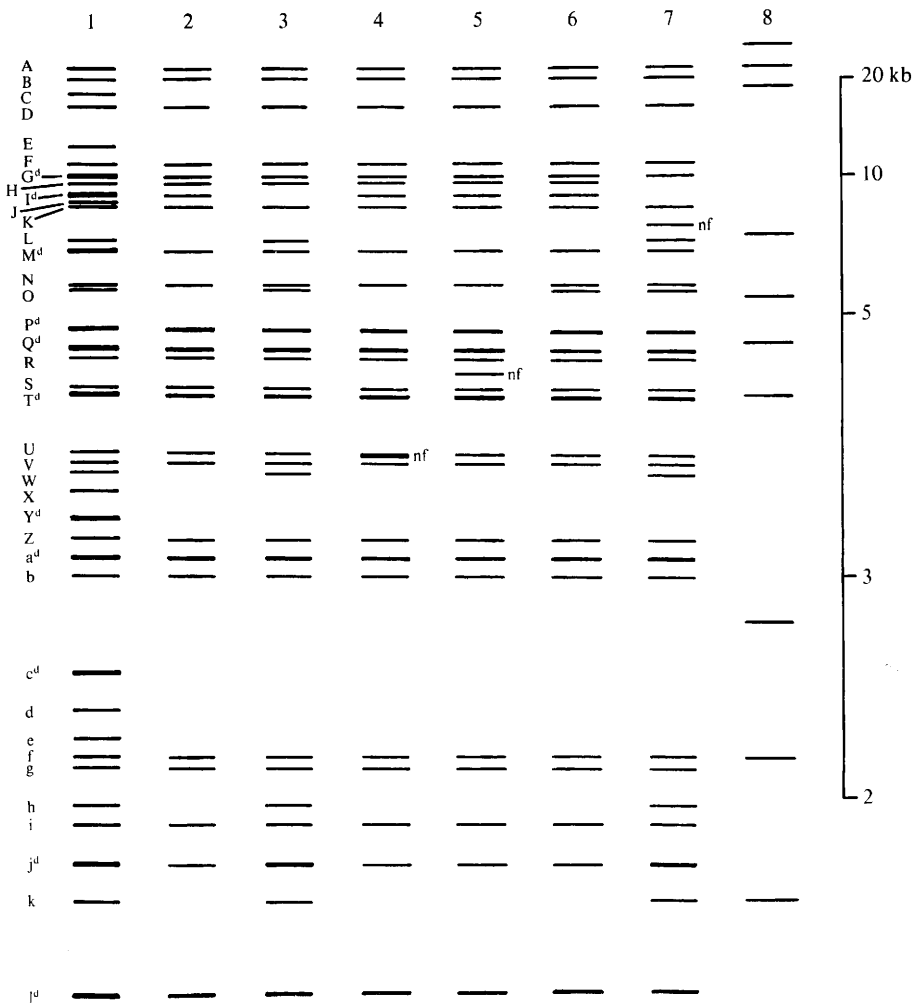


Fig. 2. Diagrammatic representation of bands visualized on agarose gels after electrophoresis of *Eco*RI digests of pWW20 and its derivatives (the plasmid numbers correspond to the MT numbers of the host strains). Double bands are shown as thick lines and are denoted with a superscript d after the fragment letter. Novel fragments are denoted nf. Track 1, pWW20; track 2, pWW20-21; track 3, pWW20-22 (identical to pWW20-23); track 4, pWW20-24; track 5, pWW20-25; track 6, pWW20-26; track 7, pWW20-27; track 8, *Xho*I digest of pWW0 from *P. putida* mt-2 as a size standard (Downing & Broda, 1979).

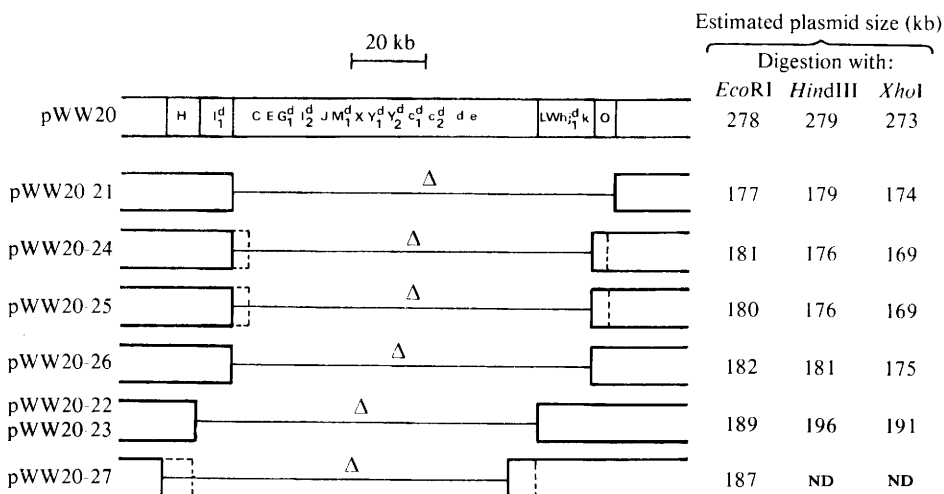


Fig. 3. Proposed structures for the deletions in the plasmids of B3 mutants of *P. putida* MT20 and estimates of the size of the plasmids. The structure of the deletions was derived from the *EcoRI* digests shown in Fig. 2: a substantially similar picture was obtained from analysis of the *HindIII* and *XhoI* digests (data not shown). Dashed lines at the end of a deletion represent the positional uncertainty inherent in the analysis; however, as the deletions could be sized quite accurately, they must run from either the right-hand ends of both 'boxes' (dashed lines) at one extreme or from the left-hand ends (continuous lines) at the other. The plasmid sizes were estimated by summation of the digest fragments after agarose gel electrophoresis, sized by comparison with pWW0 digests of known fragment size (Downing & Broda, 1979). Plasmids pWW20-21, pWW20-24, pWW20-25 and pWW20-26 confer a class B growth phenotype and pWW20-22, pWW20-23 and pWW20-27 a class A phenotype. (ND, Not determined because of poor resolution of digest fragments on electrophoresis.)

By these criteria, the original B3 strain would have been placed in class A (Worsey & Williams, 1977).

Digestion of the plasmid DNA from strain MT20 and its derivatives with endonucleases *HindIII*, *EcoRI* or *XhoI* gave a complex mixture of fragments. Estimates of the number of fragments from digestion of pWW20 itself were: *HindIII* 52 (including 12 doubles, 1 triple), *EcoRI* 49 (11 doubles) and *XhoI* 51 (12 doubles, 1 triple). By sizing the fragments from their electrophoretic mobility compared with pWW0 digest fragments of known size (Downing & Broda, 1979), the estimates of the total size of pWW20 were 279 kb (*HindIII*), 278 kb (*EcoRI*) and 273 kb (*XhoI*). Errors in this analysis are likely to arise from (a) errors in sizing of individual fragments, particularly the larger ones, (b) assigning the wrong multiplicity to a particular band and (c) difficulties in resolving contiguous bands in congested areas of the gel.

Comparison of the digests of the plasmid DNA from the B3 mutants with that of pWW20 showed that all had undergone extensive deletion (Fig. 2). Estimates of the sizes of the plasmids from digests with the same three enzymes (Fig. 3) indicated deletions of about 90 kb in plasmids from class A strains and 100 kb in those from class B strains.

Apart from pWW20-22 and pWW20-23 which were found to be identical, digests of all the other deleted plasmids differed in one or more fragments, showing that no specific excision event had occurred. All the plasmids had lost a common block of fragments amounting to 80 kb in *EcoRI* or *XhoI* digests and 72 kb in *HindIII* digests. The plasmids in class A strains had lost, in addition to the common block, a set of fragments different from the plasmids in class B strains: the pattern of loss was consistent with the deletions in class A continuing from the opposite end of the common block to those in class B. Using these data a partial ordering of some of the fragments was carried out, as shown in Fig. 3 for the *EcoRI* digest. A similar and remarkably consistent estimate of the relative positions of the deletions was obtained from the *XhoI* and *HindIII* digestions (data not shown). Undoubtedly there are errors in our interpretation of the plasmid structures, particularly where novel fragments occurred in the digests of deleted derivatives: this has partly been taken account of in the positional

uncertainty in the termini of the deletions marked in Fig. 3. Because of the large size of the plasmids, we can see little hope at present of achieving a more complete analysis.

The evidence presented proves conclusively the earlier contention that the B3 phenotype results from deletion of pWW20 DNA (Worsey & Williams, 1977), although the sizes of the deletions were unexpectedly large. If our earlier analysis of the regulation is correct, the *xyIS* gene should lie in the deleted region and probably in the core fragments lost from all the plasmids. Since all the derivative plasmids are non-conjugative, presumably one or more *tra* genes also lie in that region. We have also demonstrated that extension of the deletion to one side of the common region (arbitrarily assigned to the right in Fig. 3), as in the class B strains, reduces the level to which benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, but not catechol 2,3-oxygenase, can be induced. Presumably, therefore, some other gene which affects their expression but is not absolutely essential lies in this right-hand region.

These results reinforce others from this laboratory which show the facility with which degradative plasmid structures can change under different nutritional conditions (Jeenes *et al.*, 1982; Jeenes & Williams, 1982). The loss of DNA from pWW20 appears to be more like that from the xylanol degradative plasmid (Hopper & Kemp, 1980; Wheatcroft & Williams, 1981), which readily undergoes a variety of non-specific deletions during growth on non-selective media to give a xylanol-negative phenotype, than the very specific excision undergone by pWW0 on benzoate selection (Bayley *et al.*, 1977). However, the high frequency of occurrence of B3 segregants, comparable after benzoate selection with plasmid-free segregants (Worsey & Williams, 1977), suggests a high degree of structural instability which might well be the result of the presence of particular DNA elements which facilitate the observed deletions.

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