Loss of Tdn catabolic genes by deletion from and curing of plasmid pTDN1 in *Pseudomonas putida*: rate and mode of loss are substrate and pH dependent

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The ability to degrade aromatic amines and *m*-toluate (Tdn⁺ phenotype), encoded by plasmid pTDN1, was lost from *Pseudomonas putida* hosts after subculture in benzoate, succinate, acetate and glucose minimal medium, the fastest rate of loss occurring where benzoate was the substrate. Tdn[−] cells had either lost the entire pTDN1 plasmid or suffered a recombinational deletion of a specific 26 kbp region. Proportional increase of Tdn[−] cells resulted from their growth-rate advantage, and additionally, where benzoate was the substrate, from its metabolism via the chromosomal *ortho*-cleavage pathway incorporating a short lag phase. The ratio of whole plasmid loss to deletion was substrate and pH dependent. Deletion of catabolic genes was not required for loss of pTDN1 but by comparison was a prerequisite for loss of TOL plasmid pWW0. It appeared that *m*-toluate and benzoate were channelled via chromosomally encoded benzoate oxygenase and dihydroxycyclohexadiene carboxylate dehydrogenase prior to pTDN1 encoded *meta*-cleavage.

Introduction

There have been several studies on the ability of various TOL plasmids to be 'cured' by growth on benzoate minimal medium. A common feature of TOL plasmids appears to be the ability to encode the dissimilation of *m*- and *p*-toluates, and benzoate via the same suite of enzymes involving a catechol 2,3-dioxygenase (C23O) *meta*-cleavage reaction (Fig. 1). The *Pseudomonas* hosts of these TOL plasmids possess an alternative chromosomally encoded route for benzoate metabolism which proceeds via the catechol 1,2-dioxygenase *ortho*-cleavage reaction. The plasmid pathway is induced by benzoate whilst the chromosomal pathway is induced by one of its intermediate products, *cis,cis*-muconate. This control system usually results in benzoate being metabolized via the plasmid-encoded pathway, as insufficient *cis,cis*-muconate accumulates to fully induce the chromosomal pathway. However, where the plasmid pathway is non-

functional, cells growing by the chromosomal pathway become amplified due to faster growth rate on the substrate (Nakazawa & Yokota, 1973; Williams & Murray, 1974). Such amplified cells are plasmid 'cured' or have suffered plasmid deletions (Williams & Worsey, 1976; Worsey & Williams, 1977; Kunz & Chapman, 1981; Pickup & Williams, 1982; Pickup *et al.*, 1983; Keil & Williams, 1985). The period of amplification varies considerably and occurs faster with TOL plasmids present in *Pseudomonas* MT14, MT15 and MT20 than with the archetypal TOL plasmid pWW0 of *Pseudomonas putida* mt-2 (PaW1) (Williams & Worsey, 1976).

pTDN1 confers on its host the ability to metabolize the aromatic amines aniline, *m*- and *p*-toluidine and also *m*-toluate, known collectively as the Tdn⁺ phenotype (McClure & Venables, 1986). The proposed pathway of dissimilation is TOL-like in that aromatic amines and *m*-toluate are channelled down a *meta*-cleavage pathway bearing isofunctional enzymes (Fig. 1). The wild-type isolate UCC22 bearing pTDN1 is a derivative of *P. putida* mt-2 devoid of pWW0 but bearing a functional chromosomal *ortho*-cleavage pathway [McClure and Venables, 1986, 1987; Saint *et al.*, 1990 (accompanying paper)]. The Tdn⁺ phenotype was found to be unstable after subculture on benzoate minimal medium and Tdn[−] cells showed no toluidine oxygenase, C23O, 2-hydroxy-

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Abbreviations: C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxy-muconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; NA, nutrient agar; NB, nutrient broth.

Fig. 1. Isofunctional steps of aromatic degradation encoded by pTDN1 and TOL (pWW0). Abbreviations: C23O, catechol 2,3-dioxygenase; C12O, catechol 1,2-dioxygenase; 2-HMS, 2-hydroxymuconic semialdehyde.

muconic semialdehyde dehydrogenase (HMSD) or 2-hydroxymuconic semialdehyde hydrolase (HMSH) activities (McClure & Venables, 1986). It seems reasonable to assume that benzoate can be metabolized via the plasmid-encoded pathway in strain UCC22.

MT14, MT15 and MT20 produce several classes of phenotypically distinguishable TOL-deletion mutants (Worsey & Williams, 1977; Pickup & Williams, 1982; Keil & Williams, 1985) and it has been suggested that such TOL plasmids have regions of homology which act as 'hotspots' for recombinational deletion events (Williams *et al.*, 1988). In the case of pWW0 growth of the host on benzoate was found to highlight two effects, either complete plasmid loss or deletion of a specific 39 kbp region bearing all the catabolic genes (Bayley *et al.*, 1977). This region is bounded by two direct repeats, 1.4 kbp in size (Meulien *et al.*, 1981). Deletion could therefore be ascribed to direct recombinational loss and recently it was shown that this occurs more frequently than plasmid loss (Williams *et al.*, 1988). Similarly, we have found that the majority of UCC22 Tdn⁻ cells, produced by growth on benzoate, contain a deleted derivative of pTDN1, a specific 26 kbp deletion occurring between two direct repeats, and it is likely that the region lost contains all the Tdn genes (Saint *et al.*, 1990).

Recent work has suggested different roles for benzoate in the production of cured and deleted TOL derivatives. Keshavarz *et al.* (1985) found that after prolonged chemostat culture on benzoate of *P. putida* PPK1, carrying a non-conjugative TOL plasmid, a switch to *m*-toluate allowed recovery of plasmid-containing cells. They suggested that benzoate was affecting plasmid partitioning but its effect was not absolute, thereby allowing a residual plasmid-containing population to be maintained. Clarke & Laverack (1984) found that plating strains containing TOL or naphthalene plasmids on high concentrations of benzoate yielded a high proportion of 'cured' colonies. Stephens & Dalton (1987) also attribute a direct effect to benzoate and other weak lipophilic acids, possibly by disrupting the cell membrane and affecting plasmid partitioning. More recently, they have suggested that benzoate selectively inhibits the growth of plasmid-containing cells (Stephens & Dalton, 1988). Williams *et al.* (1988) presented good evidence that for *P. putida* mt-2 at least, the differential growth-rate theory is sufficient to explain benzoate 'curing'. They did experiments with wild-type and transposon-tagged pWW0 and found that growth at low pH and on other weakly lipophilic acids had no 'curing' effect. Also, benzoate did not cause loss of pWW60-1, a naphthalene plasmid from the same incompatibility group as pWW0,

which might be expected to be affected equally if benzoate acted on plasmid replication or partition.

This paper describes an investigation of the factors affecting stability of the Tdn phenotype in various *P. putida* hosts containing pTDN1; in some cases we compared the stability of TOL plasmid pWW0 under the same conditions. We also monitored the ratio of whole plasmid loss to plasmid deletion under different conditions.

Methods

Strains and plasmids. *Pseudomonas putida* and *Escherichia coli* strains and plasmids are listed in Table 1.

Curing experiments. Liquid and solid minimal media were prepared as described by Eaton & Ribbons (1982); the pH was adjusted to 6.4 by excluding the appropriate amount of NaOH. The sodium salts of benzoate, succinate and acetate were used and all carbon sources were used at 5 mM concentration except acetate (10 mM). Tryptophan (100 µg ml⁻¹) was added routinely to minimal media for convenience and standardization of experiments, after first demonstrating that its presence did not affect curing rates. Routinely, starter cultures and selective subcultures were incubated overnight, with shaking, at 30 °C, except where pTDN1- or pTDN1-543-containing strains were grown on *m*-toluate or benzoate, when they were incubated for up to 48 h. Starting cultures were from single colonies inoculated into *m*-toluate minimal medium to ensure no curing prior to selective subculture. Starter culture integrity was monitored by plating suitable dilutions onto nutrient agar (NA) and spraying with 100 mM-catechol. Wild-type cells (Tdn⁺ Ben⁺ or Mtol⁺ Ben⁺) turned yellow due to the production of 2-hydroxymuconic semialdehyde from catechol by the plasmid-

encoded C23O; strains lacking C23O (Tdn⁻ Ben⁺ or Mtol⁻ Ben⁺) remained white. In the case of pWW0-encoded C23O activity an intense yellow colour was produced after about 1 min; with pTDN1- and pTDN1-543-containing strains colour took 3–4 min to fully develop and was less intense. A 0.1 ml volume of a 10⁻² dilution of starter culture in 0.95% (w/v) sterile NaCl was inoculated into 5 ml of appropriate medium. After incubation 0.1 ml samples of 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilutions in 0.95% (w/v) sterile NaCl were plated onto NA for screening by catechol spray test or estimation of retention of Km^r. Colony counts were done concomitantly to estimate generation number. At least 1000 colonies were monitored per subculture. A 0.1 ml volume of a 10⁻² dilution was used to reinoculate fresh minimal medium and the process repeated. Where UCC543 was monitored for retention of Km^r, colonies were sprayed with catechol and 50 white colonies replicated to NA and NA plus 25 µg kanamycin ml⁻¹. Where UCC5431 was monitored 100 colonies were taken from NA and replicated to NA and NA plus 25 µg kanamycin ml⁻¹.

Transfer of pTDN1 and pWW0 to a Rec⁻ host. An overnight L-broth culture of PpG1400 was plated onto NA plus 100 µg rifampicin ml⁻¹. A Rec⁻ (UV^s) Trp⁻ Rif^r mutant was retained and termed UCC30. Conjugations were done as described in the accompanying paper (Saint *et al.*, 1990) using PaW1 and UCC22 as donors and selecting for transconjugants on minimal medium containing tryptophan and rifampicin plus *m*-toluate or aniline respectively. Both conjugations gave a transfer frequency of approximately 5 × 10⁻⁴ per donor. Two transconjugants having the required phenotypes were retained and termed UCC31(pTDN1) and UCC32(pWW0).

Production of UCC52. UCC52 was one of twelve UCC22 derivatives bearing Tn5 in the chromosome used for producing pTDN1::Tn5 mutations (Saint *et al.*, 1990).

Plasmid isolation and analysis. Plasmid DNA was extracted by the procedure of Wheatcroft & Williams (1981); restriction analysis was done as previously described by Saint *et al.* (1990).

Table 1. *Bacterial strains and plasmids*

Abbreviations: Tdn⁺, ability to grow on aromatic amines and *m*-toluate, Ben⁺, growth on benzoate; Mtol⁺, growth on *m*-toluate. Km, kanamycin; Rif, rifampicin; Rec⁻, recombination deficient. C23O, catechol 2,3-dioxygenase. HA, *Hind*III-derived restriction fragment A of pTDN1.

Strain	Plasmid	Notes and reference
<i>P. putida</i> mt-2		
UCC22	pTDN1	Tdn ⁺ Ben ⁺ (Saint <i>et al.</i> , 1990)
UCC23	pTDN1-3	Tdn ⁻ Ben ⁺ produced by growth on benzoate (Saint <i>et al.</i> , 1990)
UCC52	pTDN1	Tdn ⁺ Ben ⁺ Km ^r . No benzoate metabolism via chromosomal <i>ortho</i> -pathway due to Tn5 insertion (this paper)
UCC521	pTDN1-3	Tdn ⁻ Ben ⁻ Km ^r produced by growth on succinate (this paper)
KT2442		Rif ^r derivative of KT2440 (Franklin <i>et al.</i> , 1981)
UCC24	pTDN1	pTDN1 in KT2442 (McClure and Venables, 1987)
UCC543	pTDN1-543	Tdn ⁺ Ben ⁺ Km ^r Tn5 insertion in HA of pTDN1 in KT2442 (Saint <i>et al.</i> , 1990)
UCC5431	pTDN1-5431	Tdn ⁻ Ben ⁺ Km ^r derivative of pTDN1-543 (this paper)
PaW1	pWW0	Mtol ⁺ Ben ⁺ (Williams & Murray, 1974)
PpG1400		Rec ⁻ Trp ⁻ (Hermann <i>et al.</i> , 1979)
UCC30		Rif ^r derivative of PpG1400 (this paper)
UCC31	pTDN1	Tdn ⁺ Ben ⁺ UCC30 (this paper)
UCC32	pWW0	Mtol ⁺ Ben ⁺ UCC30 (this paper)
<i>E. coli</i>		
C600	pNMB1	pSUP5011 Mob replaced by pTDN1 derived C23O (gift of N. C. McClure)

Results

Stability of the Tdn phenotype under varying growth and pH conditions

Wild-type strain UCC22 and UCC24, both Tdn⁺, and PaW1, Mtol⁺, were subcultured on benzoate minimal medium and nutrient broth (NB) to compare the stability of Tdn and Mtol phenotypes. Growth of UCC22 and UCC24 on benzoate minimal medium resulted in rapid loss of the Tdn phenotype and repeat experiments showed that after 20–25 generations fewer than 0.1% of cells were Tdn⁺, which was the limit of detection (Fig. 2). There was a low level of Tdn phenotype loss on NB which showed some oscillation, seen most clearly with UCC24 (Fig. 2). This suggests that although loss occurred it did not confer a large growth advantage in this medium. Loss of Mtol phenotype by PaW1 was much slower in benzoate minimal medium compared to loss of Tdn phenotype by UCC22 and UCC24, and in repeat experiments rates of loss were less consistent than seen with the Tdn phenotype. No loss of Mtol phenotype from PaW1 was detectable after NB subculture.

Plasmids were extracted from 12 Tdn[−] isolates from each growth survey. In every case isolates contained an identical deleted derivative of pTDN1 equivalent to pTDN1-3 (Saint *et al.*, 1990), in which *Hind*III derived restriction fragments carrying catabolic genes were lost, whilst HA and one copy of HE were retained (Fig. 3, lane 2). Also, Tdn[−] cells containing similarly deleted pTDN1 derivatives were produced after growth and subculture of UCC22 and UCC24 on succinate, acetate and glucose minimal medium. This prompted a thorough investigation of the factors involved in loss of the Tdn phenotype on various media.

Fig. 2. Loss of Tdn⁺ phenotype from UCC22 and UCC24 and loss of Mtol⁺ phenotype from PaW1 after subculture on NB and benzoate minimal medium. ▲, UCC24; ●, UCC22; ■, PaW1 after subculture on benzoate minimal medium; △, UCC24; ○, UCC22; □, PaW1 after subculture on NB.

Fig. 3. Restriction digestion of pTDN1 and pTDN1-543 and their deleted derivatives with *Hind*III. Lane 1, pTDN1; 2, pTDN1-3; 3, pTDN1-543; 4, pTDN1-5431; 5, λ DNA digested with *Hind*III and *Eco*RI. The arrow indicates the novel 3.5 kbp fragment derived from Tn5 present in pTDN1-543 and pTDN1-5431.

As plasmid-deleted and plasmid-cured Tdn[−] cells can only be distinguished by DNA isolation, a time-consuming process, further studies on the modes of loss of Tdn phenotype on various substrates were done using UCC543 bearing pTDN1-543. pTDN1-543 bears a Tn5 insertion in HA of pTDN1 (Saint *et al.*, 1990) outside the region deleted to give pTDN1-3. Therefore, where pTDN1-3-like deletion derivatives of pTDN1-543 arise Tn5 is retained and cells are Tdn[−] Km^r; where the whole plasmid, and therefore Tn5 is lost, cells become Tdn[−] Km^s.

Loss of the Tdn phenotype from UCC543 was monitored after growth and subculture on benzoate, glucose, succinate and acetate minimal medium at pH 6.8 (Fig. 4a) and pH 6.4 (Fig. 4b). Concomitantly, the percentage of Tdn[−] cells retaining Km^r at each subculture was determined, revealing the ratio of plasmid-cured to plasmid-deleted cells (Fig. 5). There was little difference in the rates of loss of Tdn phenotype at pH 6.8 or pH 6.4 on all substrates and the kinetics of phenotype loss were similar to the wild-type UCC22 bearing pTDN1, indicating that the presence of Tn5 in pTDN1 does not affect stability. The ratio of deletion to whole plasmid loss was substrate and pH dependent. Total plasmid loss was more rapid at pH 6.8 on all substrates (Fig. 5a). For example, the rate of loss of Tdn phenotype in succinate minimal medium was similar at either pH such that after 50 generations no Tdn⁺ cells were

Fig. 4. Loss of Tdn^+ phenotype after subculture of UCC543 at pH 6.8 (a) and pH 6.4 (b) in minimal medium supplemented with: ●, benzoate; ■, succinate; ▲, acetate; ○, glucose.

detectable (Fig. 4), but at pH 6.4 70% of such cells were Km^r (plasmid deleted) whereas at pH 6.8 only 26% were Km^r . Growth on acetate minimal medium at either pH value gave the highest level of whole plasmid loss. Tdn^-Km^s and Tdn^-Km^r isolates (twelve from each experiment) were examined for plasmid content. Tdn^-Km^s isolates contained no plasmid DNA whereas Tdn^-Km^r isolates contained a deleted derivative of *pTDN1-543* (Fig. 3, lanes 3 and 4) still bearing the characteristic 3.5 kbp novel fragment derived from *Tn5*. A Tdn^-Km^r isolate (UCC5431) was retained in order to investigate the stability of the deleted plasmid. This isolate was grown in benzoate, succinate and acetate minimal medium and loss of the Km^r marker was monitored. Loss was most evident where acetate was the substrate, reaching >99% after 84 generations (Fig. 6). After 92 generations, 73% of succinate-grown cells were Km^r and after 91 generations, 34% of benzoate-grown cells were Km^r (Fig. 6). Plasmid analysis revealed that Km^r cells always contained *pTDN1-5431* and Km^s cells contained no plasmid. Growth on NB produced some

Fig. 5. Proportion of plasmid-deleted (Km^+) to plasmid-cured (Km^s) Tdn^- UCC543 cells after subculture at pH 6.8 (a) and pH 6.4 (b) in minimal medium supplemented with: ●, benzoate; ■, succinate; ▲, acetate; ○, glucose. □, NB (pH 6.8).

loss of Km^r but this was not detected until 50 generations of growth, and reached only 1% after >75 generations.

UCC52 is defective in the benzoate ortho-cleavage pathway

After subculture of *Tn5* insertion mutant UCC52 on benzoate minimal medium and plating of appropriate dilutions to minimal medium containing 5 mM-*m*-toluate and 0.5 mM-succinate all colonies retained the ability to grow on *m*-toluate. An inactive *meta*-cleavage pathway, produced by plasmid deletion or loss, allows conversion of *m*-toluate to 3-methylcatechol only by the chromosomal benzoate oxidation gene products, but accumulation of black oxidation products from such a conversion were not seen.

However, when stability studies were done on the UCC52 Tdn phenotype after subculture in NB, succinate, acetate, benzoate and glucose minimal medium (Fig. 7), loss of phenotype correlated well with that seen in

Fig. 6

Fig. 7

Fig. 6. Loss of pTDN1-5431 from UCC5431 after subculture on minimal medium (pH 6.8) containing: ●, benzoate; ■, succinate; ▲, acetate. □, NB (pH 6.8).

Fig. 7. Loss of Tdn⁺ phenotype from UCC52 after subculture on minimal medium containing: ●, benzoate; ■, succinate; ▲, acetate; ○, glucose. □, NB (pH 6.8).

UCC22 (Fig. 2) and UCC24 (Fig. 4a) with the exception of benzoate. Growth on benzoate minimal medium was followed for a maximum of 92 generations but loss of Tdn phenotype was never seen. This demonstrated that Tn5 insertion had resulted in a benzoate-specific stability of pTDN1, and therefore the Tdn phenotype. Plasmid extraction revealed that Tdn⁻ isolates of UCC52 contained a plasmid equivalent to pTDN1-3 (Fig. 3, lane 2). One such isolate was retained and termed UCC521. Tdn⁻ isolates were always Ben⁻, strongly suggesting that the Tn5 insertion had affected the chromosomally encoded benzoate *ortho*-cleavage pathway. UCC521 was used to confirm this hypothesis. Spectrophotometric analysis (data not shown) revealed the following: UCC521 accumulated 3-methylcatechol from *m*-toluate and catechol from benzoate and *E. coli* containing pNMB1, bearing the cloned C23O gene from pTDN1, converted accumulated 3-methylcatechol to 2-hydroxy-6-oxohepta-2,4-dienoate and catechol to 2-hydroxy-muconic semialdehydes. Conversion of benzoate to catechol was detectable only after a 10 h incubation of UCC521 with the substrate; subsequently, during a 2 h period, benzoate completely disappeared from the medium. Buildup of 3-methylcatechol and its oxidation products was slow, media progressively darkening after 24 h.

Stability of Tdn and Mtol phenotypes in a Rec⁻ host

Rec⁻ strains UCC31 and UCC32 were subcultured on benzoate minimal medium at pH 6.8 and pH 6.4 and succinate minimal medium at pH 6.8. No loss of Mtol

phenotype was seen for UCC32 on any of these media after 90 generations of growth. For UCC31, rates of loss of Tdn phenotype were significantly reduced (Fig. 8). For example, after 20 generations of growth on succinate minimal medium, 73% of UCC24 cells were Tdn⁻ (Fig. 4a) whereas only 11% of UCC31 cells were Tdn⁻ (Fig. 8). However, at this stage of growth a similar percentage of Tdn⁻ UCC24 cells had completely lost pTDN1 (Fig. 5a). After growth for 24 generations on benzoate 36% of UCC31 cells were still Tdn⁺ (Fig. 8), whereas at the same stage no Tdn⁺ cells were detectable with UCC22 (Fig. 2) or UCC24 (Fig. 4a). Tdn⁻ derivatives of UCC31 were screened for the presence of plasmid DNA but it was not detectable in isolates obtained after <20 generations,

Fig. 8. Plasmid curing of pTDN1 from Rec⁻ UCC31 in minimal medium supplemented with: ●, benzoate (pH 6.8); ○, benzoate (pH 6.4); ■, succinate (pH 6.8). □, NB (pH 6.8).

indicating there was no recombinational loss of Tdn phenotype. It was noticeable that reduction of pH affected whole plasmid loss especially in the initial 40 generations. Loss of Tdn phenotype, and therefore plasmid, accelerated slowly at pH 6.4 but the point at which 100% loss was approached was similar at both pH 6.8 and pH 6.4 (Fig. 8). This effect was seen when whole plasmid loss was followed in benzoate minimal medium at pH 6.8 and pH 6.4 with UCC24 (Fig. 5*a, b*). There was, however, a difference between the overall rates of plasmid loss from UCC24 and UCC31 in that loss from UCC31 on benzoate and succinate minimal medium approached 100% at an earlier stage than UCC24, even though loss of Tdn phenotype occurs more rapidly in this strain. This suggests that selective pressure to lose pTDN1 is alleviated by deletion of the catabolic genes, a conclusion which is also supported by the lower rate of loss observed with the deleted plasmid pTDN1-5431 (Fig. 6).

Presence of Tdn function confers a growth-rate disadvantage

When agar containing succinate minimal medium was used to screen for loss of Tdn phenotype after 2 d incubation, colonies which turned yellow when sprayed with catechol (Tdn⁺) were approximately half the size of colonies which remained white (Tdn⁻). This suggested that Tdn⁻ cells possessed a significant growth-rate advantage and this might be detectable by standard growth-rate experiments. (Table 2). It was not clear whether KT2442 had a significant growth rate advantage

over UCC23 (bearing deleted plasmid pTDN1-3) but both these strains appeared to have a growth-rate advantage over UCC22 (bearing pTDN1) on benzoate, succinate and glucose minimal medium and UCC23 showed a growth-rate advantage over UCC22 on acetate minimal medium. In the case of UCC30 the presence of pTDN1 (UCC31) reduced the growth rate of the host strain on succinate minimal medium significantly and more than halved the growth rate on benzoate minimal medium. However, the large difference in growth rate seen between UCC23 and UCC22 and UCC30 and UCC31 on benzoate minimal medium was probably also attributable to the variation of efficiency in benzoate metabolism by the chromosomal *ortho*- and plasmid *meta*-cleavage pathways. Lowering the pH to 6.4 also lowered growth rates of UCC22 on benzoate, glucose and acetate minimal medium and UCC31 on benzoate minimal medium. This suggests that in the case of pTDN1 a faster growth rate results in greater levels of whole plasmid loss (Fig. 5, Fig. 8).

There are two possible explanations why no loss of Mtol phenotype is seen after subculture of UCC32 in benzoate: that the growth rate of UCC32 on benzoate is equal to or in excess of UCC30, thereby conferring no growth-rate advantage on plasmid-cured cells, or that recombinational deletion is a prerequisite for whole plasmid loss. The growth rate of UCC32 on benzoate minimal medium is less than that of UCC30 (Table 2), so the second explanation appears to be the case.

No evidence was found for the inhibition of growth in the presence of benzoate of cells containing pTDN1 or the deleted derivative pTDN1-3 (Table 2). KT2442 and

Table 2. *Effects of the presence of pWW0, pTDN1 or pTDN1-3 and the pH of the growth medium on the growth rates of P. putida strains*

All substrates were at 5 mM concentration except acetate (10 mM). Values are means of two independent experiments. ND, Not determined.

Strain	pH	Growth rate ($\Delta OD_{600} h^{-1}$) on:					
		Benzoate	Succinate	Glucose	Acetate	<i>m</i> -Toluate	Aniline
UCC30*	6.8	0.693	0.721	ND	ND	ND	ND
UCC31 (pTDN1)*	6.8	0.340	0.506	ND	ND	ND	ND
	6.4	0.292	ND	ND	ND	ND	ND
UCC32 (pWW0)*	6.8	0.521	ND	ND	ND	ND	ND
PaW1 (pWW0)	6.8	0.641	ND	ND	ND	ND	ND
UCC22 (pTDN1)	6.8	0.546	0.796	0.641	0.619	0.130	0.162
	6.4	0.505	0.796	0.597	0.554	ND	ND
UCC23 (pTDN1-3)†	6.8	0.721	0.875	0.666	0.693	ND	ND
		(ND)	(0.875)	(0.753)	(0.641)	ND	ND
UCC52 (pTDN1)†	6.8	0.462	(0.753)	ND	(0.641)	0.092	0.187
KT2442	6.8	0.721	0.924	0.693	0.597	ND	ND

* Cells grown in the presence of 100 μg tryptophan ml^{-1} .

† Values in parentheses are from experiments in which cells were grown in the presence of 5 mM-benzoate as well as the substrate indicated.

UCC30 entered exponential growth after 3 h in benzoate minimal medium, PaW1 and UCC32 after 5 h and UCC52, UCC22 and UCC31 after approximately 9 h. UCC52 and UCC22 took approximately 24 h to reach exponential phase in aniline or *m*-toluate minimal medium and growth rates for both substrates were slow. These long lag phases did not appear to be associated with enzyme induction since when cells were subcultured to fresh medium the pattern was repeated.

Exponential growth of UCC52, UCC22 and UCC31 on benzoate minimal medium started after a similar time period to the chromosomally encoded disappearance of benzoate from media inoculated with UCC521. There was an accumulation of the oxidation products of 3-methylcatechol at the onset of exponential growth of UCC22 and UCC52 on *m*-toluate, which coincided with an identical accumulation seen in *m*-toluate minimal medium inoculated with UCC521. This suggests that the chromosomally encoded benzoate oxygenase and dihydroxycyclohexadiene carboxylate dehydrogenase are responsible for the initial steps of benzoate and *m*-toluate dissimilation in *P. putida* strains containing pTDN1.

Discussion

Cells bearing pTDN1 rapidly lost the Tdn phenotype after growth in minimal medium on a variety of substrates. Loss of the Tdn phenotype occurred by two mechanisms, either deletion of a 26 kbp section bearing the catabolic genes or by complete plasmid loss. We attribute proportional increase of Tdn⁻ cells after growth on succinate, acetate and glucose minimal medium to their demonstrable growth-rate advantage. Such instability on a range of substrates has not previously been demonstrated for catabolic plasmids. Loss of the Tdn phenotype after growth on benzoate minimal medium was extremely rapid and we attribute this to three factors. Plasmid-deleted or cured cells in the presence of benzoate have a dual growth-rate advantage. Firstly, there is a reduction in metabolic load, i.e. the same growth advantage seen in succinate, glucose and acetate minimal medium. Secondly, such cells express the *ortho*-cleavage pathway of benzoate utilization which bestows a further growth-rate advantage. These two factors together may explain why there is such a large growth rate difference between UCC22 and UCC23 and UCC30 and UCC31 on benzoate minimal medium (Table 2). Thirdly, the long lag phases of cells expressing the pTDN1-encoded *meta*-cleavage pathway for benzoate utilization allows plasmid-deleted and cured cells to become established during the early phases of culture. This can clearly be seen by comparing growth of UCC22

in benzoate minimal medium when the initial inoculant was aniline-grown cells, all containing functional pTDN1, with those of NB-grown cells, where a small percentage of inoculant cells are plasmid-cured/deleted. From a NB starter, UCC22 entered exponential growth after approximately 6 h and exhibited a similar growth rate to UCC23. This reflects the rapid establishment of plasmid-deleted and cured cells in the culture. There would no doubt be variation in this lag phase in repeated experiments as each NB starter culture would show variation in numbers of plasmid-cured/deleted cells. This also highlights the importance of commencing experiments with standard inocula. Repeat experiments using inocula prepared on *m*-toluate minimal medium gave reproducible rates of Tdn phenotype loss. Stephens & Dalton (1987) found that rates of curing of TOL plasmid pWW15 were not reproducible on certain substrates though it is not clear whether the nature of the inoculum was a contributory factor to this result. Clearly, using non-selective inocula can affect curing rates appreciably.

When PaW1 was subcultured in NB, no Mtol⁻ segregants were identified. However, when pTDN1-containing strains were treated likewise Tdn⁻ clones were readily detected (Fig. 2). This reflects relative unselected levels of loss of the two phenotypes when cells are under no metabolic pressure. Williams *et al.* (1988) suggested that for PaW1 such segregants were present at as high a frequency as 10⁻²–10⁻³. Our data suggests such segregants are in fact present at frequencies <10⁻³, as we screened at least 1000 colonies per subculture. Only a small percentage of Tdn⁻ UCC543 cells were Km^s after NB subculture (Fig. 5a), indicating that under non-selective conditions deletion occurs more frequently than whole plasmid loss. The greater frequency of deletion detected for pTDN1 when compared to pWW0 may be due to its direct repeats, the 'hotspots' for recombinational loss, being longer and closer together than those of pWW0.

The long lag phase of cells bearing pTDN1 in benzoate minimal medium raises an interesting question concerning the expression of the chromosomal *ortho*-cleavage pathway in such cells. During this long lag phase one would expect the *ortho*-cleavage pathway to become fully induced but this does not happen. The growth kinetics of UCC22 and UCC52 are comparable on benzoate minimal medium, which is direct evidence for an inoperative *ortho*-cleavage pathway in UCC22 equivalent to the situation in UCC52. Additionally, if the *ortho*-cleavage pathway normally operated in UCC22 then loss of Tdn phenotype would be reduced to a level equivalent to those seen during growth on succinate, acetate and glucose minimal medium, as there would be no additional *ortho*- versus *meta*-cleavage growth-rate advantage. This suggests that the *ortho*-cleavage pathway is

repressed in the presence of complete pTDN1. If such repression occurs it is not clear how this affects the chromosomally encoded benzoate oxygenase and dihydroxycyclohexadiene carboxylate dehydrogenase as little is known concerning the normal induction of these enzymes. Our results suggest that in cells bearing pTDN1 both benzoate and *m*-toluate are initially metabolized via these enzymes and their products subsequently channelled down the plasmid-encoded *meta*-cleavage pathway. Conclusive proof that these two enzymes are required for such conversions could be gained by transfer of pTDN1 to a benzoate-oxygenase-deficient mutant of *P. putida* mt-2 such as that described by Cuskey & Sprenkle (1988).

There was no evidence for a direct effect of benzoate on plasmid loss as proposed for certain TOL plasmids (Stephens and Dalton, 1987, 1988). The presence of benzoate did not inhibit growth of plasmid-containing cells (Table 2) and undissociated benzoate (at pH 6.4) was not a more effective curing agent (Fig. 4*b*). It appeared that for pTDN1, the rates at which whole plasmid loss or plasmid deletion occurred were governed by the type of substrate and the pH of growth. Both deletion and whole plasmid loss appeared to occur concomitantly, with the former occurring more rapidly (Fig. 5). Deletion is not a prerequisite for whole plasmid loss as revealed by curing studies with UCC31 (Fig. 8). When cell populations were 100% Tdn⁻, loss of Km^r still occurred (Figs 4 and 5); also, UCC5431 exhibited loss of Km^r after subculture (Fig. 6) indicating loss of deleted plasmid. Total loss of plasmid was most pronounced after growth of the host on acetate minimal medium (Figs 5 and 6). One can envisage a metabolic advantage to cells which have completely lost a large catabolic plasmid and this is likely to be most pronounced with a substrate such as acetate that has a low energy yield. Plasmid loss was greater in minimal medium on all substrates at pH 6.8 than at pH 6.4 (Fig. 5). This increase in plasmid loss as neutral pH is approached has been found in other studies. Curing of R-plasmid TP181 from *E. coli* using various phenolic compounds was critically affected by the pH of the growth medium (Lakshmi *et al.*, 1987, 1989) with curing found to be most efficient at pH 7–7.2. Such alterations in pH undoubtedly affect cell viability and therefore growth rate (Table 2). Faulty plasmid partitioning is likely to be more prevalent amongst more rapidly dividing cells at pH 6.8.

Stephens & Dalton (1987) found that for TOL plasmid pWW15 deletions always occurred prior to plasmid loss. In the case of TOL plasmid pWW0 Williams *et al.* (1988) showed that plasmid deletion occurred far more frequently than plasmid loss after subculture of the host on benzoate minimal medium. We have demonstrated that deletion of the 39 kbp region from pWW0 appears

to be a prerequisite for plasmid loss as Rec⁻ UCC32 did not yield plasmid-cured cells after subculture on benzoate minimal medium. Williams *et al.* (1988) found that a deleted derivative of pWW0 was stably maintained after subculture on benzoate minimal medium. This suggests that whole plasmid loss occurs as a direct result of recombination and does not occur subsequent to this event. In the case of pTDN1, clearly recombination is not required for whole plasmid loss.

We have demonstrated a complex set of factors which determine stability of Tdn catabolic genes on pTDN1. Clearly, therefore, the stability of different TOL plasmids and plasmids such as pTDN1, bearing isofunctional catabolic genes to TOL plasmids, are governed by different factors. This is because the nature of the replicon bearing the catabolic genes is the major influence on their stability. Therefore, it is not possible to put forward a single theory which accounts for the proportional increase of plasmid-deleted and cured cells under various growth conditions.

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