Physical map of the aromatic amine and *m*-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: location of a unique *meta*-cleavage pathway

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A restriction endonuclease map was derived for the aromatic amine and *m*-toluate catabolic plasmid pTDN1 present in *Pseudomonas putida* UCC22, a derivative of *P. putida* mt-2. The plasmid is 79 ± 1 kbp in size and can be divided into a restriction-site-deficient region of 51 ± 1 kbp and a restriction-site-profuse region of 28 kbp which begins and ends with directly repeating sequences of at least 2 kbp in length. A mutant plasmid isolated after growth of the host on benzoate had lost the restriction-profuse region by a straightforward recombinational loss retaining one copy of the direct repeat. Analysis of clones, deletion and Tn5 insertion mutants strongly suggested that the *meta*-cleavage pathway of pTDN1 was situated in the region readily deleted. The catechol 2,3-dioxygenase (C23O) gene of pTDN1 showed no hybridization or restriction homology to previously described C23O genes of TOL plasmids pWW0 and pWW15. In addition, there was little homology between intact pTDN1, pWW0 and pWW15, suggesting the presence of a unique *meta*-cleavage pathway. We also demonstrated that pTDN1 did not originate from *P. putida* mt-2 chromosome.

Introduction

Anilines and ring-substituted anilines are major products of herbicide metabolism in the soil. Isolation and construction of bacterial strains for the biodegradation of these potential environmental pollutants is of significant interest.

Plasmid-encoded degradation of aniline was first reported by Anson & Mackinnon (1984) but genetical and physical characterization of the plasmid was not done. The only other aromatic-amine-degrading plasmid was reported by McClure & Venables (1986). This latter plasmid was discovered after the adaptation of *Pseudomonas putida* mt-2 (ATCC 33015) to growth on the aromatic amines aniline and *m*- and *p*-toluidine. *P. putida* mt-2 carries TOL plasmid pWW0 which encodes the degradation of toluene and *m*- and *p*-xylene via benzoate, *m*-toluate and *p*-toluate respectively (Williams & Murray, 1974; Worsey & Williams, 1975). The adapted strain

UCC2 contained a derivative of pWW0 deleted of its catabolic genes, plus a novel plasmid designated pTDN1. Additionally, UCC2 retained the ability to grow on *m*-toluate and coupled with aromatic amine degradation this was termed the Tdn⁺ phenotype (McClure & Venables, 1986). Data from oxygen uptake experiments, release of ammonia by UCC2 incubated with aniline or *m*- or *p*-toluidine, identification of *meta*-cleavage products and induction profiles of the relevant *meta*-cleavage enzymes indicated a pathway analogous to the TOL *meta*-cleavage pathway incorporating a toluidine oxygenase (TO) (Fig. 1; McClure & Venables, 1986). Subsequently, the deleted derivative of pWW0 was removed with no loss of phenotype to give strain UCC2-2; pTDN1 was found to be conjugative, yielding a Tdn⁺ phenotype in transconjugants, and a catechol 2,3-dioxygenase (C23O) gene was cloned from pTDN1 and its expression investigated (McClure & Venables, 1987). pTDN1 showed no restriction homology to pWW0 and it was proposed that its appearance during the adaptation of strain mt-2 may have been due to its emergence from a cryptic chromosomally integrated state.

This paper describes a complete restriction map for pTDN1 and the results of growth on benzoate, which produces a Tdn⁺ phenotype resulting from a recombinational

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Abbreviations: C23O, catechol 2,3-dioxygenase; DHCDH, dihydroxycyclohexadiene carboxylate dehydrogenase; TO, toluidine oxygenase.

Fig. 1. Proposed pathway of aromatic amine and *m*-toluate dissimilation by *P. putida* UCC22. R = NH₂, R¹ = H and R² = H, aniline; R = NH₂, R¹ = CH₃ and R² = H, *m*-toluidine; R = NH₂, R¹ = H and R² = CH₃, *p*-toluidine; R = COOH, R¹ = H and R² = H, benzoate; R = COOH, R¹ = CH₃ and R² = H, *m*-toluate. TO, toluidine oxygenase; DHCDH, dihydroxycyclohexadiene carboxylate dehydrogenase; C23O, catechol 2,3-dioxygenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; 4OT, 4-oxalocrotonate tautomerase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxo-4-pentenoate hydratase; HOA, 2-oxo-4-hydroxypentenoate aldolase.

tional loss of plasmid DNA between two directly repeating regions approximately 24 kbp apart. We also further characterized the C23O gene of pTDN1 which appears to form part of a unique *meta*-cleavage pathway for the degradation of *m*-toluate, benzoate and aromatic amines. Additionally, we demonstrate that pTDN1 did not arise from the chromosome of strain mt-2 and is in no way derived from pWW0; we speculate on the origin of this plasmid.

Methods

Bacterial strains and plasmids. The *Pseudomonas putida* and *Escherichia coli* strains and the plasmids used and constructed during the course of this investigation are detailed in Table 1.

Media and culture conditions. Nutrient agar and nutrient broth were prepared according to the manufacturers' instructions (Difco). Solid and liquid minimal media were prepared with previously described supplements (Eaton & Ribbons, 1982). To ensure maintenance of recombinant plasmids streptomycin (25 µg ml⁻¹), for pKT231 derivatives, and ampicillin (100 µg ml⁻¹), for pHG327 and pBR322 derivatives, were added to media. Transposon insertion mutants were maintained with kanamycin (25 µg ml⁻¹), for Tn5, and rifampicin (100 µg ml⁻¹) for *P. putida* KT2442 background. Retention of pNJ5000 was achieved by addition of tetracycline (10 µg ml⁻¹). Carbon source was at 5 mM concentration in minimal media, pTDN1-containing strains being maintained on *p*-toluidine and aniline and TOL⁺ strains on *m*-toluate.

Chromosomal DNA extraction. *P. putida* strains were incubated overnight, with shaking, in 200 ml of minimal medium containing 10 mM-acetate and 0.05% (w/v) Casamino acids. Cells were collected by centrifugation at 6000 r.p.m. for 20 min and the pellet resuspended in 30 ml 25% (w/v) sucrose prepared in 50 mM-Tris/HCl, pH 8. Cell

suspension (15 ml) was then mixed with 1 ml lysozyme (5 mg ml⁻¹) and left at room temperature for 2–3 min; 2.5 ml 250 mM-EDTA (pH 8) was added and the suspension mixed carefully and left a further 2–3 min; 2.5 ml SDS (20%, w/v, in 50 mM-Tris/HCl, pH 8) was added, carefully mixed, and left for 2–3 min. An equal volume of a mixture of 50% (v/v) phenol in chloroform was added and the lysate immediately centrifuged at 15000 r.p.m. for 30 min. As much of the upper aqueous phase as possible was carefully removed using a wide outlet plastic pipette. CsCl was carefully dissolved in an appropriate volume of extract and chromosomal DNA purified by density-gradient centrifugation at 40000 r.p.m. (Sorvall TV-865B rotor) for 48 h. After ethidium bromide extraction and suitable dialysis of the DNA the result was a concentrated (up to 1 µg µl⁻¹) high-molecular-mass sample suitable for restriction digestion.

Plasmid DNA extraction and manipulation. Presumptive Tn5 insertion mutants were screened using the procedure of Birnboim & Doly (1979); detailed analysis was done on plasmid DNA extracted by the procedure of Wheatcroft & Williams (1981). Screening for the presence of recombinant plasmids was done according to Holmes & Quigley (1981) and vector and cloned DNA prepared in quantity by CsCl/ethidium bromide density-gradient centrifugation of cleared lysates (Guerry *et al.*, 1973). Restriction endonuclease digestion and ligation with T4 ligase were done according to the manufacturer's (NBL) instructions. *E. coli* strains were transformed by standard procedures (Cohen *et al.*, 1972) and transformants selected on media containing an appropriate antibiotic for vector selection.

Production of transposon insertion mutants. *E. coli* ED2196 containing pLG221 was conjugated on a 0.45 µm Millipore filter with UCC22. Tdn⁺Km^r transconjugants were selected. pLG221 is not stably maintained outside enteric bacteria (Boulnois *et al.*, 1985), therefore transconjugants were derivatives of UCC22 with Tn5 in the chromosome or pTDN1. A selection of transconjugants were rapidly screened by direct plate conjugation with *P. putida* KT2442 selecting for transfer of Km^r to reveal the sites of Tn5 insertion. pTDN1 transfers to KT2442 at a frequency > 1 × 10⁻⁴ per donor. Twelve donors which yielded transconjugants at an appreciably lower rate than

Table 1. *Bacterial strains and plasmids*

Abbreviations: Tdn⁺, ability to grow on aromatic amines and *m*-toluate; Lac⁺, growth on lactose; Rif, rifampicin; Tc, tetracycline; Sm, streptomycin; Km, kanamycin; Ap, ampicillin; C23O, catechol 2,3-dioxygenase. For designations of restriction fragments (XC, HB etc.) see Table 2 and Fig. 2.

Strain	Plasmid	Notes and reference
<i>P. putida</i> mt-2		
UCC22	pTDN1	Formerly UCC2-2 (McClure & Venables, 1987) Tdn ⁺ (McClure & Venables, 1986)
UCC23	pTDN1-3	Tdn ⁻ deleted derivative isolated after growth on benzoate (this study)
KT2440	—	Franklin <i>et al.</i> (1981)
KT2442	—	KT2440 Rif ^r (Franklin <i>et al.</i> , 1981)
PaW1	pWW0	TOL ⁺ (Murray <i>et al.</i> , 1972; Williams & Murray, 1974)
PaW130	—	TOL ⁻ Rif ^r (Keil <i>et al.</i> , 1985b).
<i>P. putida</i>		
MT15	pWW15	TOL ⁺ (Keil & Williams, 1985)
<i>E. coli</i>		
SK1592	—	Kushner (1978)
	pTDN1-1000	C23O ⁺ formerly pNCM1. XB of pTDN1 cloned into pKT231 (McClure & Venables, 1987)
C600	—	Bachmann (1987)
	pNJ5000	Tc ^r unstable derivative of RP4 (Grinter, 1983)
	pKT231	Sm ^r Km ^r (Bagdasarian <i>et al.</i> , 1981)
	pTDN1-1002	XC of pTDN1 in pKT231 (this study)
	pTDN1-1004	XD of pTDN1 in pKT231 (this study)
	pTDN1-1006	XE of pTDN1 in pKT231 (this study)
	pTDN1-1014	HB of pTDN1 in pKT231 (this study)
	pTDN1-1015	HB of pTDN1 in opposite orientation to pTDN1-1014 (this study)
	pTDN1-1016	HC of pTDN1 in pKT231 (this study)
	pTDN1-1020	EC of pTDN1 in pKT231 (this study)
JM107	—	Lac ⁻ host strain for transformation involving pHG327 (Yanisch-Perron <i>et al.</i> , 1985)
	pHG327	Lac ⁺ Ap ^r (Stewart <i>et al.</i> , 1986)
	pTDN1-1010	HD of pTDN1 in pHG327 (this study)
	pTDN1-1011	HD of pTDN1 in opposite orientation to pTDN1-1010 (this study)
	pTDN1-1018	pTDN1-1011 digested with <i>Sst</i> I and religated to leave a 2.1 kbp <i>Sst</i> I– <i>Hind</i> III fragment derived from HD (this study)
	pTDN1-1012	HE of pTDN1 in pHG327 (this study)
PM191	—	C600 <i>recA</i> (Keil <i>et al.</i> , 1985a)
	pWW15-3151	Xh (C23OI) fragment of pWW15 in pKT230 (Keil <i>et al.</i> , 1985a)
	pWW15-3161	Bj (C23OII) fragment of pWW15 in pBR322 (Keil <i>et al.</i> , 1985a)
ED2196	—	Gasson & Willetts (1977)
	pLG221	Tn5 donor Km ^r (Boulnois <i>et al.</i> , 1985)

this ($<10^{-6}$ per donor) were retained and assumed to contain Tn5 in the chromosome due to the low rate of transfer. Such donors yield a variety of pTDN1::Tn5 insertion mutants on conjugation, whereas donors with Tn5 already present in pTDN1 give identical transconjugants.

DNA–DNA hybridization. Restriction fragments were separated by agarose gel electrophoresis and transferred by Southern blotting (Southern, 1975) to Biotodyne filter membranes (Pall Ultrafine Filtration Corp.). Radiolabelled probes were prepared by nick-translation incorporating [α -³²P]dCTP using a preparative kit (BRL–Gibco) and probe purified by elution through Sephadex G-50. Hybridizations were done using a PR800 Hybride-Ease Hybridization Chamber (Hoefer Scientific Instruments) at 65 °C using the manufacturer's protocol. Hybridization stringency was controlled by using low ($2 \times$ SSPE, 1%, w/v, SDS), moderate ($0.5 \times$ SSPE, 1%, w/v, SDS) and high ($0.1 \times$ SSPE, 1%, w/v, SDS) stringency washes consecutively for 30 min, each at 65 °C. ($2 \times$ SSPE is 300 mM-NaCl, 20 mM-NaH₂PO₄, 2 mM-Na₂EDTA, pH 7.4). Where homology of pTDN1 and its C23O gene to TOL plasmids, C23OI and C23OII and chromosomal DNA was investigated only a low stringency wash was used. For all other hybridizations low, moderate and high stringency washes were used. Autoradiography was done using Kodak XRP film; film and filter were

sandwiched between two Phillips intensifying screens in a Kodak cartridge at -70 °C.

Catechol spray test. This was done as described by Franklin *et al.* (1981).

Mobilization using pNJ5000. Triparental conjugations were done on 0.45 μ m Millipore membranes involving *E. coli* C600(pNJ5000), *P. putida* KT2442 and C600 containing pTDN1-1014 or pTDN1-1015. After incubation, mixtures were resuspended in 0.95% (w/v) NaCl and plated to aniline minimal medium supplemented with rifampicin (100 μ g ml⁻¹).

Results

Restriction map of pTDN1

A complete restriction map is given in Fig. 2, and fragment sizes for the enzymes *Hind*III, *Xho*I, *Eco*RI, *Bam*HI, *Sal*I and *Sma*I, in Table 2. A combination of

Fig. 2. Restriction endonuclease map of pTDN1 and positioning of a C23O gene. Arrows indicate the known extent of the repeated regions. For recombinant plasmids pTDN1-1010, pTDN1-1011 and pTDN1-1018 the closed bar indicates vector pHG327 whilst the open bar is the extent of inserted pTDN1; the broken arrow indicates the direction of transcription from the vector operator/promoter. The positions of Tn5 insertion for pTDN1-543 (▼) and for pTDN1-511 (▽) are indicated. Restriction enzyme abbreviations: H, *HindIII*; X, *XhoI*; E, *EcoRI*; B, *BamHI*; Sa, *SalI*; S, *SmaI*; Ss, *SstI*; Si, *SinI*; Bs, *BstEII*; Pv, *PvuII*; P, *PstI*.

Table 2. *pTDN1* restriction fragments

Enzyme ...	Designation and size (kbp)					
	<i>HindIII</i>	<i>XhoI</i>	<i>EcoRI</i>	<i>BamHI</i>	<i>SalI</i>	<i>SmaI</i>
	HA	XA	EA	BA	SaA	SA
	(51 ± 1)	(59 ± 1)	(51 ± 1)	(52 ± 1)	(52 ± 1)	(62 ± 1)
	HB	XB	EB	BB	SaB	SB
	(12.65)	(7.95)	(19.85)	(9.8)	(13.25)	(8.3)
	HC	XC	EC	BC	SaC	SC
	(9.3)	(6.9)	(3.3)	(7.0)	(5.4)	(3.5)
	HD	XD	ED	BD	SaD	SD
	(3.65)	(3.2)	(2.8)	(5.0)	(4.5)	(2.3)
	HE(2)	XE	EE(2)	BE	SaE	SE
	(1.1)	(1.5)	(0.85)	(4.7)	(2.7)	(1.35)
		XF			SaF	SF
		(0.45)			(0.9)	(1.1)
						SG
						(0.9)

approaches ensures its integrity: (a) single and double digestion of the native plasmid; (b) cloning of fragments HB, HC, HD, HE, XC, XD, XE and EC, determination of their restriction profiles, and hybridization to variously digested pTDN1; (c) restriction digestion of a variety of deletion mutants and pTDN1::Tn5 insertion mutants.

The region otherwise devoid of restriction enzyme sites contains a single *SimI* site (Fig. 2). pTDN1-543 has Tn5 inserted approximately 8.85 kbp into HA. By digestion of this mutant with *SimI*, and double-digestion

involving *SimI* and the other six enzymes, the region is effectively cut into three fragments. Allowing for the contribution of Tn5 (Jorgensen *et al.*, 1979) the region was accurately sized.

Restriction site profuse region is bounded by direct repeats

As the restriction map of pTDN1 was constructed analysis revealed that the HE clone (pTDN1-1012) and also the EC clone (pTDN1-1020) hybridized with fragments mapped several kilobases apart on the intact pTDN1. Fig. 3 shows examples of such hybridization.

Fig. 3. Demonstration of repeated regions within plasmid pTDN1 and the position of Tn5 insertion in pTDN1-543. A: lane 1, bacteriophage λ DNA digested with *HindIII/EcoRI*; 2, pTDN1 *XhoI*; 3, pTDN1 *HindIII*; 4, pTDN1 *BamHI*; 5, pTDN1 *SalI*; 6, pTDN1 *EcoRI*. B, autoradiogram of the Southern blot of A probed with ^{32}P -labelled pTDN1-1012 (bearing HE of pTDN1). C: lane 1, λ *HindIII/EcoRI*; 2, pTDN1 *EcoRI*; 3, pTDN1 *SalI*; 4, pTDN1 *HindIII*; 5, pTDN1-543 *HindIII*. D, autoradiogram of the Southern blot of C probed with ^{32}P -labelled pTDN1-1020 (bearing EC of pTDN1).

Where HE was used as a probe, fragments XA (lane B2), HE (lane B3), BA and BE (lane B4), SaA and SaC (lane B5) and EA, EB and EE (lane B6) all showed hybridization. Where EC was the probe, fragments EA and EC (lane D2), SaA, SaC, SaD and SaF (lane D3) and HA and HC (lane D4) hybridized. Mapping data and hybridization suggested initially that the repeated region involved all of HE and at least part of EC up to and including SaF. Therefore, we could predict the presence of two HE (HE_L and HE_R), two EE (EE_L and EE_R) and possibly two SaF fragments (Fig. 2). pTDN1-1002 (containing XC) revealed no hybridization with HA, XA, BA or EA (data not presented); therefore the repeated region ends before the first *Xho*I site anticlockwise of HE_L . Cloned EC as probe revealed the relative position of Tn5 insertion in HA of pTDN1-543. With regard to Fig. 2 Tn5 could be inserted 8.85 kbp clockwise of HE_L or the same distance anti-clockwise of HE_R . However, the novel fragment between HE and the first *Hind*III site of Tn5 will only hybridize with EC if it is situated clockwise of HE_L , as it contains a region of homology with EC. Fig. 3 reveals that the novel fragment did not hybridize (lane D5), there only being equivalent hybridization to HC apparent in the adjacent lane.

Therefore, Tn5 is situated anticlockwise of HE_R in pTDN1-543.

Growth of UCC22 (which carries pTDN1) on benzoate minimal medium resulted in rapid loss of the ability to utilize *m*-toluate and aromatic amines, Tdn⁻ cells remaining white when sprayed with catechol. When such 'benzoate-cured' derivatives were analysed they were all found to contain an identical deleted pTDN1 plasmid which retained HA and HE and EA and EE. A representative isolate was retained and termed UCC23 containing pTDN1-3. Equivalent concentrations of pTDN1 and pTDN1-3 were hybridized against a mixture of the HC clone (pTDN1-1016) and the HE clone (pTDN1-1012) (Fig. 4). Computer-aided image analysis of the resulting autoradiograph revealed approximately twice the density and area of hybridization for HE (lane B3) and EE (lane B5) of pTDN1 when compared to equivalent pTDN1-3 bands (lanes B2 and B4). Hybridization also revealed the absence of SaF from pTDN1-3 (lane B6). Therefore, an absolute boundary for the repeated region is given by L terminating before XC and R starting in SaF and the distribution of restriction sites suggest it is a direct repeat. The deletion seen in pTDN1-3 is a direct recombinational loss of approxi-

Fig. 4. Demonstration that deletion in pTDN1-3 occurs between direct repeats, one copy of the repeated region being retained. A: lane 1, λ *Hind*III/*Eco*RI; 2, pTDN1-3 *Hind*III; 3, pTDN1 *Hind*III; 4, pTDN1-3 *Eco*RI; 5, pTDN1 *Eco*RI; 6, pTDN1-3 *Sal*I; 7, pTDN1 *Sal*I. B, autoradiogram of the Southern blot of A probed with ³²P-labelled pTDN1-1012 (bearing HE of pTDN1) and pTDN1-1016 (bearing HC of pTDN1).

mately 26 kbp with one copy of the direct repeat being retained.

Location of catabolic genes

We knew from a previous study (McClure & Venables, 1987) that XB contained a C23O gene. HD is within XB (Fig. 2). pTDN1-1010 and pTDN1-1011 were constructed containing HD in opposite orientations. Transformation of either of these constructs into *E. coli* JM107 resulted in transformants which showed C23O activity as adjudged by the catechol spray test. pTDN1-1010 was digested with *Sst*I and religated to give pTDN1-1018 which also exhibited C23O activity in *E. coli* JM107. The Tn5 insert in pTDN1-511 is a further 0.2 kbp downstream of the *Sst*I site utilized in the construction of pTDN1-1018, but the plasmid still confers an active C23O. This locates the C23O gene to a 1.9 kbp region of HD (Fig. 2). A fine restriction endonuclease map of HD revealed no restriction site homology to previously described clones bearing C23O genes (Chatfield & Williams, 1986).

We have cloned HB in both orientations to give pTDN1-1014 and pTDN1-1015. When these two plasmids were mobilized into *P. putida* KT2442 using pNJ5000, pTDN1-1014 conferred the ability to grow on aniline but not *m*-toluate or *p*-toluidine whereas pTDN1-1015 failed to confer the ability to grow on any of these substrates. The products of TO and dihydroxycyclohexa-

diene carboxylate dehydrogenase (DHCDH) activity on aniline is catechol which may be further metabolized via a chromosomal *ortho*-cleavage pathway in *P. putida* KT2442. This suggests the presence of at least these two genes on HB expressed only in an orientation reading from the HE_L end of HB.

Fifteen pTDN1::Tn5 mutants were obtained in *P. putida* KT2442, and showed the phenotypes indicated in Fig. 5. All insertions which affected *m*-toluate and aromatic amine degradation were situated within a 15 kbp section of the region deleted in pTDN1-3. Most insertions caused loss of all phenotypes and from our knowledge of pathways coded on pTDN1 and *P. putida* KT2442 chromosome we can suggest possible explanations. The inserts in HB are likely to be in and around TO and any polar effect on subsequent *meta*-pathway genes could explain complete loss of phenotype. Mutations affecting positive regulators of the pathway could cause complete loss of phenotype; thus the group of inserts in HC could be regulatory. pTDN1-511 in KT2442 confers weak growth on *p*-toluidine and *m*-toluate and normal growth on aniline. This probably indicates fully functional TO and DHCDH genes (situated on HB); the product of the enzymes encoded by these genes can be further metabolized via the chromosomal *ortho*-cleavage pathway. The gene order for the *meta*-cleavage pathway on pTDN1 may be different to that found on TOL plasmids as there is a gap of at least 2 kbp between the accurately mapped position of the C23O on HD and the TO/DHCDH functions on HB.

Fig. 5. pTDN1::Tn5 mutants of *P. putida* KT2442. The mapped position of insertion of Tn5 for plasmids pTDN1-51 to pTDN1-549 along with the relevant phenotype of the host strain is indicated. An open bar indicates a normal phenotype while a hatched bar indicates weak growth on the substrate. A closed bar indicates no growth on the substrate although some of these mutants show high reversion frequencies ($>10^{-6}$ per cell).

Origin of pTDN1

To test the hypothesis that pTDN1 originated from the chromosome of *P. putida* mt-2, and to check for any homology with TOL plasmids, the following hybridizations were done. Initially, pTDN1-1000 (pNCM1), known to contain a functional C23O gene derived from pTDN1, was used as a radiolabelled probe against *Xho*I-digested chromosomal DNA from UCC23 and two isogenically related strains KT2440 and PaW130, derived from *P. putida* mt-2. Additionally, pTDN1-1000 was probed against plasmids carrying previously characterized C23O genes, pWW15-3151 (C23OI), pWW15-3161 (C23OII), and pWW15, the parent plasmid of these C23O genes. In no case was hybridization detectable (data not presented). Intact pTDN1 was then probed against digested pWW0, pWW15 and chromosomal DNA from UCC23, KT2440 and PaW130 (Fig. 6). Some hybridization was evident with *Xho*IE (XE, lane B3) and *Hind*III A (HA, lane B4) fragments of pWW0 and Xh (lane B5) and *Bam*HI h/i (Bh/i, lane B6) of pWW15, but only after prolonged (14 d) autoradiograph exposure, suggesting weak homology. We attribute smeared background hybridization involving chromosomal digests (lanes B7, 8 and 9) to slight chromosomal contamination of the probe DNA, evident only on prolonged exposure. We conclude that pTDN1 did not originate from the chromosome of *P. putida* mt-2.

Discussion

A complete restriction map of plasmid pTDN1 has been obtained, and its size has been determined as 79 ± 1 kbp. The restriction sites are localized to a region of about 28 kbp, the extremities of which comprise repeated nucleotide sequences in direct orientation. This region also appears to contain the degradative genes of pTDN1. The region of about 51 kbp which lies outside the direct repeats is devoid of restriction sites for the enzymes *Hind*III, *Xho*I, *Eco*RI, *Bam*HI, *Sal*I and *Sma*I, and also for a further nine hexanucleotide-recognizing enzymes which we have not used for accurate mapping. Similarly, Hooper *et al.* (1989) have described a 4-chlorobiphenyl catabolic plasmid of 53 kbp with most restriction enzyme sites clustered in a 5 kbp region. It has been suggested that clustering of restriction enzyme sites in regions which determine phenotype, and scarcity in regions which determine replication and self-transfer, reflect evolution to broad host-range, and restriction-site-rich regions represent recently acquired genes (Meyer *et al.*, 1977; Meyer & Shapiro, 1980). Several of our pTDN1::Tn5 derivatives transfer, replicate and show catabolic activity in *E. coli* but the full breadth of pTDN1 host-range has not been determined.

It is likely that the catabolic genes required for aromatic amine and *m*-toluate degradation are located in the region of pTDN1 between the direct repeats. This

Fig. 6. DNA-DNA hybridization homology of radiolabelled pTDN1 with pWW0, pWW15, and chromosomal DNA of UCC23, KT2440 and PaW130. A: lane 1, λ *Hind*III; 2, pTDN1 *Xho*I; 3, pWW0 *Xho*I; 4, pWW0 *Hind*III; 5, pWW15 *Xho*I; 6, pWW15 *Bam*HI; 7, UCC23 *Xho*I; 8, KT2440 *Xho*I; 9, PaW130 *Xho*I. B, autoradiogram of the Southern blot of A probed with ³²P-labelled pTDN1.

conclusion is supported by several observations: (i) deletions of the region between the repeats, as in pTDN1-3, causes complete loss of degradative ability; (ii) all Tn5 insertions which produce loss of degradative ability are located in this region; (iii) the C23O, TO and DHCDH genes have been cloned on fragments from this region.

The distribution of Tn5 insertions in pTDN1 is instructive. The method used for Tn5 mutagenesis was not selective for loss of Tdn⁺ phenotype, but was selective for retention of replication and conjugal transfer. As all but one of the insertions obtained were in the catabolic region (representing only one-third of the plasmid) it can be inferred that the bulk of the non-catabolic region is occupied by replication and transfer functions.

Previous studies have indicated, on the basis of genetic homology and substrate specificity, that C23O enzymes can be categorized as either of two types, C23OI and C23OII, though the former category can be subdivided on the basis of some differences in internal restriction sites (Keil *et al.*, 1985a; Chatfield & Williams, 1986; Harayama *et al.*, 1987; Ghosal *et al.*, 1987). The substrate specificity of the C23O from pTDN1 is similar to that of C23OII (McClure & Venables, 1987), but its lack of restriction and hybridization homology suggest it is unrelated to either C23OI or C23OII. Although the C23O proteins from pTDN1 and pWW0 are tetramers of similar molecular mass only four of the first 36 amino acid residues so far sequenced correspond (S. Chapman, personal communication; Nakai *et al.*, 1983). We are currently determining the nucleotide sequence of the C23O gene from pTDN1.

Biochemical evidence indicates that the *meta*-cleavage pathway of pTDN1 comprises enzymes isofunctional to those of TOL plasmids (McClure & Venables, 1986). There is strong evidence to support the theory of a common ancestry for the *meta*-pathways of a variety of TOL plasmids (Keil *et al.*, 1985b; Shaw & Williams, 1988) and NAH plasmids (Farrell & Chakrabarty, 1979; Lehrbach *et al.*, 1983; Cane & Williams, 1986; Assinder & Williams, 1988). Within catabolic-coding regions there is preservation of gene order, and restriction and hybridization homology. In view of this, it is surprising that there is little homology between the *meta*-cleavage pathway genes of pTDN1 and those of the TOL plasmids pWW15 and pWW0. Our results show only weak hybridization of pTDN1 with the XE and HA fragments of pWW0. XE is within HA and carries genes *xylG*, *xylF*, *xylJ* and *xylI* (Franklin *et al.*, 1983; Harayama *et al.*, 1984) and presumably one or more of these genes have some homology with an analogous region of pTDN1. The same results and conclusions apply in the case of hybridizations between pTDN1 and pWW15.

The presence in pTDN1 of direct repeat sequences which flank the region containing the catabolic genes, and the deletion of this region during growth on benzoate, parallels the situation in the TOL plasmid pWW0 (Bayley *et al.*, 1977; Meulien *et al.*, 1981). Deletions between homologous sequences have been shown to occur within other catabolic plasmids: in an isopropylbenzene-degrading plasmid pRE4 (Eaton & Timmis, 1986), and in a 3-chlorobenzoate-degrading plasmid pBR60 (Wyndham & Straus, 1988), although in both cases the sequences are less-well-characterized than in pWW0. pBR60 was isolated from *Alcaligenes* sp. BR60 and shows marked instability, readily yielding mutants which no longer utilize 3-chlorobenzoate. The loss of phenotype has been correlated to a 14 kbp deletion in pBR60 which occurs between two directly repeating regions (Wyndham *et al.*, 1988). The direct repeats of pBR60 show remarkable restriction homology to those of pTDN1. This is in contrast to the 1.4 kbp, direct repeats of pWW0 which show no restriction or hybridization homology to pTDN1. The direct repeats of pTDN1 are also considerably larger than this: the minimum size is dictated by a *SaI* site to the left and a *HindIII* site to the right, with regard to Fig. 2, with the strong hybridization of EC to EA suggesting that left-hand termination is well into SaF, but actually finishes before the second *SaI* site. Taken together, these data indicate a length in excess of 2 kbp. Most IS elements thus far described are less than 2 kbp in size (Kleckner, 1981; Iida *et al.*, 1983; Grindley, 1985). However, Barsomian & Lessie (1986) and Gaffney & Lessie (1987) describe rearrangements of pTGL1 and pTGL6 in *P. cepacia* mediated by several IS elements, one of which, IS408, is 2.7 kbp in size. IS elements may promote replicon fusion which can occur due to the formation of a cointegrate during transposition (Shapiro, 1979). The entire donor replicon is inserted into the target and the transposon itself is duplicated. This cointegrate is subsequently resolved by recombination to give one copy of the element in the donor and recipient (Grindley, 1985). However, if resolution does not occur then a cointegrate consisting of donor/recipient bounded by two copies of the IS element can persist. Such cointegrates, bearing an additional copy of the IS element, were seen by Barsomian & Lessie (1986). Tn4652, a 17 kbp transposon derived from TOL plasmid pWW0, also forms such cointegrates (Tsuda & Iino, 1987) and resolution was found to be weak when compared to that of Tn3 (De La Cruz & Grinstead, 1982) such that 90–95% of cells still carried a cointegrate after 1 week of culture. It is possible that pTDN1 arose by a similar cointegrate event mediated by a transposon/insertion element.

The origin of pTDN1 remains obscure. However,

hybridization studies with the chromosome of UCC23 and related strains have yielded some clues. Chromosomal digests of UCC23 always had a profile identical to those of PaW130 and KT2440. Therefore, the original bacterial isolate bearing pTDN1 was derived from PaW1. We have tried to repeat the adaptation of McClure & Venables (1986) using *p*-toluidine and aniline at 1, 2 and 5 mM concentrations, and PaW1 from various sources as well as derivatives PaW8, KT2440 and PaW130, without success. We conclude that pTDN1 arose from an external source during the original adaptation or was present in some form in the original PaW1 culture used. This fact does not detract from the nature of pTDN1 which is clearly a uniquely described plasmid.

The following paper (Saint & Venables, 1990) describes factors affecting the stability of the Tdn phenotype in various *P. putida* hosts bearing pTDN1.

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