pTDN1, A Catabolic Plasmid Involved in Aromatic Amine Catabolism in *Pseudomonas putida* mt-2

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(Received 30 March 1987)

The ability of *Pseudomonas putida* mt-2 strain UCC2 to grow with aniline, *m*- and *p*-toluidine and *m*-toluate (the Tdn⁺ phenotype) is plasmid encoded. Strain UCC2 contains two plasmids of which pUCC2 is a deleted derivative of the TOL plasmid pWW0 (Worsey & Williams, 1975) and can be lost from the strain with no effect on the Tdn⁺ phenotype. The second plasmid in strain UCC2, pTDN1, harbours genes involved in the Tdn⁺ phenotype and is conjugative. The catechol 2,3-dioxygenase (C23O) structural gene resident on pTDN1 has been cloned into the broad host range vector pKT231. The relative specific activity towards substituted catechols of C23O expressed in the cloned fragment in *Escherichia coli* is similar to that expressed in *P. putida* strain UCC2.

INTRODUCTION

Anilines and ring-substituted anilines are major products of herbicide metabolism in the soil (Cripps & Roberts, 1978) and, as potential environmental pollutants, their biodegradation is of considerable interest. Several bacteria capable of utilizing anilines or toluidines (methylanilines) as sole sources of carbon and energy have been isolated (Aoki *et al.*, 1983, 1984; Kaminski *et al.*, 1983; Helm & Reber, 1979; Appel *et al.*, 1984; Raabe *et al.*, 1984). Preliminary evidence for plasmid involvement in aromatic amine catabolism has been shown for *Pseudomonas* sp. strain CIT1 (Anson & Mackinnon, 1984) and *Pseudomonas* strain JL1 (Latorre *et al.*, 1984).

Recently, a derivative of *Pseudomonas putida* mt-2 (pWW0) has been described that is capable of utilizing aniline, and *m*- and *p*-toluidine as sole sources of carbon and nitrogen (McClure & Venables, 1986). This strain of *P. putida* mt-2, designated strain UCC2, metabolized these substrates by oxidative deamination followed by dissimilation via an inducible *meta*-cleavage pathway. Strain UCC2 was shown to contain two plasmids, a deleted derivative of the TOL plasmid pWW0, pUCC2, and a novel plasmid designated pTDN1. Strain UCC2 was also found to lose the Tdn⁺ phenotype, readily and irreversibly, during subculture on benzoate or other non-selective carbon sources. In all such Tdn⁺ derivatives, pUCC2 appeared unaltered, whereas pTDN1 had invariably been lost or undergone deletion. It was assumed therefore, that pTDN1 encoded the Tdn⁺ phenotype, and that pUCC2 made no contribution to it. In the present study, we validate this assumption by the isolation of derivatives of strain UCC2 in which pUCC2 has been lost and pTDN1 retained, and also by cloning part of the Tdn pathway.

METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Batch cultures for assay of catechol 2,3-dioxygenase (C23O) were prepared as previously described (McClure & Venables, 1986).

Preparation of cell extracts. Crude extracts for C23O assay were prepared by resuspending cell pellets in 0.1 M-potassium phosphate buffer pH 7.5 with 10% (v/v) acetone. The samples were sonicated for five periods of 40 s at

Abbreviation: C23O, catechol 2,3-dioxygenase (EC 1.13.11.2).

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Genotype/phenotype	Reference
<i>P. aeruginosa</i> PU21	R2	Ilv ⁻ Leu ⁻ Cb Sm Su	Bayley <i>et al.</i> (1979)
<i>E. coli</i> SK1592	-	<i>endA gal hsdR4</i> <i>hsdM⁺ sbcB15 thi T1^r</i>	Kushner (1978)
<i>P. putida</i> mt-2			
PaW8	pWW0-8	Mxy ⁻ Mtol ⁻	Williams & Murray (1974)
KT2442	-	<i>hsdR1 hsdM⁺ Rif</i>	Franklin <i>et al.</i> (1981)
UCC2	pTDN1, pUCC2	Tdn ⁺ , Mxy ⁻	McClure & Venables (1986)
UCC2-2	pTDN1	Tdn ⁺ , Mxy ⁻	This study

* Phenotype abbreviations: Ilv and Leu denote requirement for isoleucine-valine and leucine respectively; Cb, Sm, Su, and Rif denote resistance to carbenicillin, streptomycin, sulphonamide and rifampicin respectively; Mxy⁻ and Mtol⁻ denote inability to grow on *m*-xylene and *m*-toluate respectively; Tdn⁺ denotes ability to grow on aniline, *m*- and *p*-toluidine and *m*-toluate.

0 °C in an MSE Soniprep 50. After removal of cell debris by centrifugation at 100000 *g* for 60 min at 4 °C, the supernatant was used for assay.

Enzyme assay. C23O was assayed as described by Sala-Trepat & Evans (1971), using catechol, 3-methylcatechol and 4-methylcatechol as assay substrates.

Conjugation. Plate filter matings were done as described by McClure & Venables (1986).

Plasmid isolation. Plasmid DNA was isolated by the method of Wheatcroft & Williams (1981) for large plasmids, and by the 'cleared lysate' method (Guerry *et al.*, 1974) for vector DNA. Rapid screening of plasmid DNA in transformants was done using the method of Holmes & Quigley (1981). Restriction endonuclease digestion and gel electrophoresis were done as previously described (McClure & Venables, 1986).

Cloning of the C23O gene. The gene encoding the C23O enzyme in strain UCC2 was cloned following ligation and transformation by standard procedures and as described (McClure, 1986).

RESULTS

Elimination of pUCC2 from strain UCC2 was accomplished by introduction of the resistance plasmid R2 which is incompatible with pWW0 (Bayley *et al.*, 1979) and therefore also with pUCC2. Plasmid R2 was transferred from *P. aeruginosa* PU21 to strain UCC2 and transconjugants selected on aniline minimal agar plates containing streptomycin (1 mg ml⁻¹). One such transconjugant was subjected to growth at elevated temperature (37 °C) or in the presence of acridine orange or mitomycin C (up to 30 µg ml⁻¹) in an attempt to cure this strain of R2. Growth in aniline minimal medium at elevated temperature (37 °C) was the only treatment which resulted in detectable loss of streptomycin resistance (encoded by R2), at a level of 1 % after 50 generations.

Plasmid DNA was prepared from one of the Tdn⁺Sm^s derivatives isolated above (strain UCC2-2) and subjected to endonuclease digestion with *Eco*RI, *Hind*III, and *Xho*I. The restriction profiles obtained were compared to those of plasmid DNA isolated from strain UCC2 and pWW0-8, which is identical to pUCC2 (Fig. 1). The restriction profiles of plasmid DNA from strain UCC2-2 (tracks 4, 7 and 10) are consistent with the conclusion that this strain carries a single plasmid, pTDN1. The restriction profiles of plasmid DNA from strain UCC2 (tracks 3, 6 and 9) represent fragments arising from both pTDN1 and pUCC2.

Transfer of the Tdn⁺ phenotype from strain UCC2-2

The Tdn⁺ phenotype was transferable from strain UCC2-2, the derivative containing only pTDN1, to *P. putida* KT2442 at a similar frequency to that from the parent strain UCC2 (> 1 × 10⁻⁴ transconjugants per donor cell). One such transconjugant contained a single plasmid indistinguishable from pTDN1 on the basis of endonuclease restriction profile (data not shown). Therefore, pTDN1 is a conjugative plasmid capable of mediating its own transfer in the presence or absence of pUCC2.

Fig. 1. Restriction digests of plasmid DNA from UCC2, UCC2-2 and pWW0-8. Lane 1, λ HindIII standard; lanes 2, 5 and 8, pWW0-8; lanes 3, 6 and 9, UCC2 plasmid DNA; lanes 4, 7 and 10, UCC2-2 plasmid DNA.

Table 2. Relative C23O activity in cell free extracts of *E. coli* SK1592(pNCM1) and strain UCC2

Strain/growth substrate	Assay substrate ...	Percentage activity of C23O*		
		Catechol	3-Methylcatechol	4-Methylcatechol
<i>E. coli</i> SK1592(pNCM1)				
Acetate		100 (1770)	200	41
Acetate + <i>m</i> -toluate		100 (1830)	197	43
Acetate + <i>p</i> -toluidine		100 (1450)	220	44
<i>P. putida</i> mt-2 strain UCC2				
<i>p</i> -Toluidine		100 (951)	180	40

* Percentage of activity with catechol, specific activity given in parentheses as milliunits (mg protein)⁻¹. One unit of activity is the amount of enzyme required to convert 1 μ mol substrate min⁻¹. Specific activities given are the mean of at least three independent determinations.

Cloning of the C23O gene from strain UCC2

Plasmid DNA isolated from strain UCC2 was digested with *Xho*I and ligated to the broad host range vector pKT231 (Bagdasarian *et al.*, 1981) cleaved with the same enzyme. Following ligation overnight, the ligation mix was used to transform *Escherichia coli* SK1592 and transformants selected were sprayed with 100 mM-catechol to detect colonies expressing C23O activity. Such colonies immediately turn yellow due to the conversion of catechol to 2-hydroxymuconic semialdehyde. One such colony was purified and was shown to contain an insert into the kanamycin resistance gene of the vector, corresponding to the 7.6 kb *Xho*I fragment of pTDN1. This plasmid is designated pNCM1.

The specific enzyme activity of C23O was estimated in cell free extracts prepared from batch cultures of *E. coli* SK1592(pNCM1) grown with acetate, acetate + *m*-toluate, and acetate + *p*-toluidine as carbon sources (Table 2). High C23O activity was observed in cell free extracts of *E.*

coli SK1592(pNCM1) irrespective of the growth substrate. This suggests that transcription occurred from the active kanamycin resistance gene promoter of pKT231, as *Pseudomonas* promoters have been shown to function to a negligible extent in *E. coli* (Franklin *et al.*, 1981). The ratio of activity of C23O of *E. coli* SK1592(pNCM1) towards catechol, 4-methylcatechol and 3-methylcatechol is similar to that observed with strain UCC2, and differs from the C23O specified by the TOL plasmid pWW0 (McClure & Venables, 1986).

DISCUSSION

In this study we provide conclusive evidence that a novel plasmid pTDN1 is involved in the ability of *P. putida* mt-2 strain UCC2 to grow on aniline, *m*- and *p*-toluidine and *m*-toluate. The gene encoding the *meta*-cleavage enzyme C23O is situated on a 7.6 kb *Xho*I fragment of pTDN1. The relative activity of C23O towards catechol and 3- and 4-methylcatechol in *E. coli* SK1592(pNCM1) is similar to that of the enzyme in strain UCC2. It is also interesting to note the similarity in substrate specificity to the C23O II enzyme encoded on pWW15, which has been shown to contain two non-homologous C23O genes (Keil *et al.*, 1985).

The majority of Tdn⁻ derivatives of strain UCC2 examined have undergone deletion from pTDN1 rather than loss of the complete plasmid (McClure, 1986; unpublished results). The high frequency with which these events occur suggests that pTDN1 contains structural features which result in ready deletion of specific, though not unique, segments of pTDN1. Both pRA500 of *P. putida* and the TOL plasmid pWW20 of *P. putida* MT20 undergo a variety of non-specific deletions giving rise to cured derivatives (Jain *et al.*, 1984; Pickup & Williams, 1982).

The isolation of strain UCC2-2 containing only pTDN1 will greatly facilitate further detailed investigation and characterization of aromatic amine catabolism in *P. putida* mt-2. The origin of pTDN1 is presently under investigation. Loss of pUCC2 has no effect on the ability of strain UCC2 to grow with aniline, *m*- or *p*-toluidine or *m*-toluate and therefore does not appear to play a role in the catabolism of these substrates.

We are grateful to Professor P. A. Williams for supplying *P. aeruginosa* PU21(R2) and *P. putida* PaW8(pWW0-8).

REFERENCES

- ANSON, J. G. & MACKINNON, G. (1984). Novel *Pseudomonas* plasmid involved in aniline degradation. *Applied and Environmental Microbiology* **48**, 868-869.
- AOKI, K., SHINKE, R. & NISHIRA, H. (1983). Metabolism of aniline by *Rhodococcus erythropolis* AN-13. *Agricultural and Biological Chemistry* **47**, 1611-1616.
- AOKI, K., OHTSUKA, K., SHINKE, R. & NISHIRA, H. (1984). Rapid biodegradation of aniline by *Frateuria* species ANA-18 and its aniline metabolism. *Agricultural and Biological Chemistry* **48**, 865-872.
- APPEL, M., RAABE, T. & LINGENS, F. (1984). Degradation of *o*-toluidine by *Rhodococcus rhodochrous*. *FEMS Microbiology Letters* **24**, 123-126.
- BAGDASARIAN, M., LURZ, R., RUECKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF 1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237-247.
- 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.
- BAYLEY, S. A., MORRIS, D. W. & BRODA, P. (1979). The relationship of degradative and resistance plasmids of *Pseudomonas* belonging to the same incompatibility group. *Nature, London* **280**, 338-339.
- CRIPPS, R. E. & ROBERTS, T. R. (1978). Microbial degradation of herbicides. In *Pesticide Microbiology*, pp. 669-730. Edited by J. R. Hill & S. J. L. Wright. London: Academic Press.
- 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 genes for the entire regulated, aromatic ring *meta*-cleavage pathway. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 7458-7462.
- GUERRY, P., LEBLANC, D. J. & FALKOW, S. (1973). General method for the isolation of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **116**, 1064-1066.
- HELM, V. & REBER, H. (1979). Investigation of the regulation of aniline utilisation in *Pseudomonas multivorans* strain AN1. *European Journal of Applied Microbiology and Biotechnology* **7**, 191-199.
- HOLMES, D. S. & QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193-197.
- JAIN, R. K., BAYLY, R. C. & SKURRAY, R. A. (1984). Characterization and physical analysis of a 3,5-xyleneol degradative plasmid in *Pseudomonas putida*. *Journal of General Microbiology* **130**, 3019-3028.

- KAMINSKI, U., JANKE, D., PRAUSER, H. & FRITCHE W. (1983). Degradation of aniline and monochloroaniline by *Rhodococcus* sp. AN 117 and a pseudomonad: a comparative study. *Zeitschrift für allgemeine Mikrobiologie* **23**, 235–246.
- KUSHNER, S. R. (1978). An improved method for transformation of *Escherichia coli* with ColE1 derived plasmids. In *Genetic Engineering*, pp. 17–23. Edited by H. B. Boyer & S. Nicosia. Amsterdam: Elsevier.
- LATORRE, J., REINEKE, W. & KNACKMUS, H. J. (1984). Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. *Archives of Microbiology* **140**, 159–165.
- MCCLURE, N. C. (1986). *Genetic and biochemical studies of aromatic amine catabolism by Pseudomonas species*. PhD thesis, University of Wales.
- MCCLURE, N. C. & VENABLES W. A. (1986). Adaptation of *Pseudomonas putida* mt-2 to growth on aromatic amines. *Journal of General Microbiology* **132**, 2209–2218.
- 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.
- RAABE, T., APPEL, M. & LINGENS, F. (1984). Degradation of *p*-toluidine by *Pseudomonas testosteroni*. *FEMS Microbiology Letters* **25**, 61–64.
- SALA-TREPAT, J. M. & EVANS, W. C. (1971). The meta-cleavage of catechol by *Azotobacter* species: the 4-oxalocrotonate pathway. *European Journal of Biochemistry* **20**, 400–413.
- 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.
- WORSEY, M. J. & WILLIAMS, P. A. (1975). Metabolism of toluene and the xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. *Journal of Bacteriology* **124**, 7–13.