New derivatives of TOL plasmid pWW0

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(Received 1 May 1992; revised 5 February 1993; accepted 20 April 1993)

Two new segregants, PPW1-1 and PPW161-1, of *Pseudomonas putida* were isolated from the stock cultures PaW85(pWW0) and PaW85(pWW0-161). Strain PPW1-1 had lost its ability to grow on *m*-xylene but was able to grow on *m*-toluate. A deletion of the left-hand of transposon Tn4651, including the *upper*-operon genes, had taken place in plasmid pWW0^{mut1}, isolated from strain PPW1-1. Additional deletions were observed in pWW0^{mut1} after 'benzoate-curing' (plasmids pWW0^{mut15}, pWW0^{mut19}, pWW0^{mut27}). The genes of the *upper*-operon and beginning of the *meta*-operon were deleted from pWW0-161^{mut1}, isolated from strain PPW161-1. Despite this deletion, cells of PPW161-1 grew on all normal TOL plasmid substrates. The Tol⁺ phenotype was stable in cells of PPW161-1 growing on benzoate. We propose that this is because in cells of strain PPW161-1 the catabolic genes deleted from pWW0-161^{mut1} were integrated into the chromosome at the site where the (chromosomally encoded) *ortho*-pathway genes are located, resulting in the inability of the cells to use this pathway.

Introduction

The 115 kb TOL plasmid pWW0 is one of the best studied catabolic plasmids (Williams & Murray, 1974; Worsey & Williams, 1975; Assinder & Williams, 1990; Burlage *et al.*, 1989). It determines the ability of *Pseudomonas putida* to degrade xylenes, toluene and related hydrocarbons.

Toluene degrading (*xyl*) genes (Fig. 1) of TOL plasmid pWW0 are organized as two catabolic operons, the *upper*- and *meta*-operons, which are positively regulated by *xylR* and *xylS* (Ramos *et al.*, 1987). They occupy a 39 kb region of the plasmid and are included in the transposon Tn4651 (56 kb), which is itself inserted into the transposon Tn4653 (70 kb) (Tsuda & Iino, 1988) (see Fig. 2). Recombination between direct 1.4 kb repeats leads to deletion of catabolic genes from the plasmid (Meulien *et al.*, 1981). The deletion of these catabolic genes gives rise to the cryptic transposon Tn4652 (17 kb) from Tn4651. Such Tol[−] derivatives are well known and have the same structure as plasmid pWW0-8 (Bayley *et*

al., 1977; Downing *et al.*, 1979). Tol[−] mutants are frequently observed in cultures of *P. putida* growing on benzoate, an intermediate of toluene degradation. This is known as 'benzoate-curing'. Cells that have lost the catabolic part of the TOL plasmid or the TOL plasmid itself grow on benzoate using the chromosomally encoded *ortho*-pathway, and oxidize catechol via an intradiol fission reaction catalysed by catechol 1,2-dioxygenase (Williams & Murray, 1974).

The integration of TOL degradative genes into the chromosome or into other plasmids has often been observed (Jeenes & Williams, 1982; Sinclair *et al.*, 1986; Sinclair & Holloway, 1991). Usually, the genes have been transferred by transposition of Tn4651 or Tn4653. However, in some cases the insertions do not coincide with the terminal inverted repeats of the transposons (Lehrbach *et al.*, 1982). Mobilization of plasmid-encoded genes from the chromosome back to the plasmid has also been observed (Mäe & Heinaru, 1991).

In this paper, we describe two Tol⁺ segregants, PPW161-1 and PPW1-1, which were isolated from stock cultures of PaW85(pWW0-161) and PaW85(pWW0), and from which deletion of the plasmid-encoded catabolic genes had taken place.

Methods

Bacterial strains and plasmids. The *Pseudomonas putida* strains and plasmids used in this study are listed in Table 1, with indications of their characteristics and genealogy.

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Fig. 1. TOL-plasmid-encoded metabolic pathway for degradation of toluene and xylenes (adapted from Harayama & Rekik, 1990). Toluene ($R = R' = H$), *m*-xylene ($R = H$, $R' = CH_3$) and *p*-xylene ($R = CH_3$, $R' = H$) serve as growth substrates. Numbered chemical intermediates (only for *m*-xylene metabolism) (1) *m*-xylene; (2) *m*-methylbenzyl alcohol; (3) *m*-methylbenzaldehyde; (4) *m*-toluate; (5) 1,2-dihydroxy-3-methylcyclohexa-3,5-diene-1-carboxylate; (6) 3-methylcatechol; (7) 2-hydroxy-6-oxohepta-2,4-dienoate; (8) 2-oxopent-4-ene-1,6-dioate; (9) 2-oxohex-4-ene-1,6-dioate; (10) acetate; (11) 2-oxopent-4-enoate; (12) 4-hydroxy-2-oxovalerate; (13) acetaldehyde; (14) pyruvate. Enzyme abbreviations: XO, xylene monooxygenase (multicomponent); BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate 1,2-dioxygenase (multicomponent);

Media and growth conditions. The strains of *P. putida* were grown at 30 °C on Luria broth (LB) (Lennox, 1955) as complete medium and on M9 (Adams, 1959) as minimal medium with addition of trace elements (Bauchop & Elsdon, 1960). Either *m*-toluate, benzoate or succinate (5 mM) was used as the single carbon source. *m*-Xylene was placed on the bottom of an inverted plate. Carbenicillin (4 mg ml⁻¹), streptomycin (1 mg ml⁻¹) and tryptophan (80 µg ml⁻¹) were added to the media as required.

Growth rate determination. Growth rates ($\mu_{\max}$) were determined from the increase in OD₅₄₀ during exponential growth in batch cultures.

DNA purification and manipulations. The TOL plasmid and its derivatives were extracted using CsCl gradients (Maniatis *et al.*, 1982). For rapid plasmid detection the method of Kado & Liu (1981) was used. Restriction endonuclease digestion of plasmid DNA, agarose gel electrophoresis, DNA transfer to nitrocellulose filters, preparation of ³²P-labelled DNA by nick-translation and DNA-DNA hybridization were all carried out according to Maniatis *et al.* (1982). Transformation of *P. putida* cells was carried out according to the method of Bagdasarian & Timmis (1982). Conjugational experiments in LB were carried out by the method described by Williams & Murray (1974).

Catechol 2,3-dioxygenase. Catechol 2,3-dioxygenase activity was measured as described by Feist & Hegeman (1969). Protein concentration was determined by the method of Bradford (1976).

Study of plasmid stability during growth on benzoate. Culture growth, cell number measurements and the detection of clones carrying TOL plasmids were carried out as in Williams *et al.* (1988).

Results

General characteristics of isolated strains

Segregants of strains PaW85(pWW0) and PaW85(pWW0-161) were isolated from stock cultures subcultured on *m*-toluate-containing minimal medium, and were designated PPW1-1 and PPW161-1 respectively. The plasmids pWW0^{mut1} (85.6 kb) and pWW0-161^{mut1} (95 kb) isolated from these strains were smaller than the original TOL plasmids indicating that deletions had occurred.

The two segregant strains PPW1-1 and PPW161-1 grew on *m*-toluate and benzoate and expressed catechol 2,3-dioxygenase (a *meta*-pathway enzyme) activity. *m*-Xylene, an *upper*-pathway substrate, was utilized only by PPW161-1. PPW161-1 grew more slowly on benzoate ($\mu_{\max}$ 0.48 h⁻¹) than the parent strain PaW85(pWW0) or PPW1-1. The $\mu_{\max}$ value for both PaW85(pWW0) and PPW1-1 was 0.63 h⁻¹.

DHCDH, 1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase; C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxy-6-oxohepta-2,4-dienoate semialdehyde dehydrogenase; HMSH, 2-hydroxy-6-oxohepta-2,4-dienoate semialdehyde hydrolase; 4OT, 4-oxalocrotonate tautomerase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-hydroxypent-2,4-dienoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. *xylA* to *xylZ* designate the structural genes for catabolic enzymes.

Table 1. *Pseudomonas putida* strains

mXyl⁺, mTol⁺, Ben⁺, ability to grow on *m*-xylene, *m*-toluate, benzoate as sole carbon sources, respectively; Cb^r, Sm^r, resistance to carbenicillin and streptomycin; Trp⁻, requirement for tryptophan.

Strain	Plasmid	Characteristics	Notes/reference	Source
PaW1	pWW0	mXyl ⁺ mTol ⁺	Williams & Murray (1974)	Gifts of P. Broda (UMIST, Manchester, UK)
PaW161	pWW0-161	mXyl ⁺ mTol ⁺ Cb ^r (Tn401)	Plasmid pWW0::Tn401; Franklin <i>et al.</i> (1981)	
PaW8	pWW0-8	mXyl ⁻ mTol ⁻	Bayley <i>et al.</i> (1977)	
PaW85		mXyl ⁻ mTol ⁻ (Tn4652)	Bayley <i>et al.</i> (1977)	
PpG1400		mXyl ⁻ mTol ⁻ Trp ⁻ RecA ⁻	Bagdasarian & Timmis (1982)	L. Kasak (Tartu University)
IRJA4		mXyl ⁻ mTol ⁻ Ben ⁻ Trp ⁻ Sm ^r	Mutant obtained from PaW85	
PPW1-1	pWW0 ^{mut1}	mXyl ⁻ mTol ⁺	Segregant derived from PaW85(pWW0) on <i>m</i> -toluate	This study
PPW1-15	pWW0 ^{mut15}	mXyl ⁻ mTol ⁻	'Benzoate-cured' segregant of PPW1-1	
PPW1-19	pWW0 ^{mut19}	mXyl ⁻ mTol ⁻	'Benzoate-cured' segregant of PPW1-1	
PPW1-27	pWW0 ^{mut27}	mXyl ⁻ mTol ⁻	'Benzoate-cured' segregant of PPW1-1	
PPW161-1	pWW0-161 ^{mut1}	mXyl ⁺ mTol ⁺ Cb ^r (Tn401)	Segregant derived from PaW85(pWW0-161) on <i>m</i> -toluate	

Table 2. Catechol 2,3-dioxygenase activity in cell extracts of *Pseudomonas putida* strains containing pWW0 or its derivatives

Strain	Growth substrate*	Catechol 2,3-dioxygenase activity [U (mg protein) ⁻¹]
PPW161-1	<i>m</i> -Xylene	2.56
	Benzoate	1.13
	<i>m</i> -Toluate	0.95
	LB	0.31
PPW1-1	<i>m</i> -Xylene	1.09
	Benzoate	0.77
	<i>m</i> -Toluate	1.76
	LB	0.10
PaW85(pWW0)	<i>m</i> -Xylene	2.46
	Benzoate	0.98
	<i>m</i> -Toluate	1.57
	LB	0.08
PaW85(pWW0-8)	Benzoate	< 0.005
	LB	< 0.005
Transformant PaW85(pWW0-161 ^{mut1})	Benzoate	0.84
	LB	0.49

* LB, Luria broth.

Catechol 2,3-dioxygenase was induced in PPW1-1 and PPW161-1 by growth on all three substrates (Table 2). The data obtained indicated that the *meta*-pathway was functional in both strains.

Growth of PPW161-1 on benzoate resulted in stable maintenance of the pWW0-161^{mut1} plasmid: appearance of cells with the mTol⁻ phenotype was not observed even

after batch cultivation for 116 generations. However, instability of the Tol⁺ phenotype, similar to PaW85(pWW0) was observed in PPW1-1 after batch culture using benzoate as the carbon source. mTol⁻ segregants appeared within 87 generations. Three of the Tol⁻ segregants, PPW1-19, PPW1-27 and PPW1-15, were investigated further. They carried plasmids pWW0^{mut19}, pWW0^{mut27} and pWW0^{mut15}, of size 67.4 kb, 75.35 kb and 60 kb respectively.

Molecular organization of pWW0 derivatives

Restriction analysis of pWW0-161^{mut1}, pWW0^{mut1}, pWW0^{mut19}, pWW0^{mut27} and pWW0^{mut15} was carried out using endonucleases *Eco*RI and *Xho*I. The homology between the above plasmids and the catabolic part of pWW0 was determined. Cloned fragments of plasmid pWW0 (17 kb transposon Tn4652, *meta*-operon including the regulatory genes *xylS* and *xylR*, and a fragment including the left-hand of Tn4652 and the start of the *upper*-operon) were used as hybridization probes. Restriction analysis and hybridization patterns of *Eco*RI restriction fragments of pWW0-161^{mut1} with the above probes revealed that the genes of the *upper*-operon and the operator-promoter part of the *meta*-operon were absent. The limits of the deletion in pWW0-161^{mut1} are shown in Fig. 2. In the case of pWW0^{mut1}, the *upper*-operon and the left-hand of Tn4652 were deleted (Fig. 2).

Strains PPW1-19, PPW1-27 and PPW1-15 were selected as mTol⁻ segregants of PPW1-1 cells during growth on benzoate. The plasmids isolated from them had no homology with the *meta*-operon nor with the *xylR* and

Fig. 2. (a) TOL plasmid pWW0 and its *EcoRI* and *XhoI* restriction map. The *upper*- and *meta*-operon, the regulatory genes *xylR* and *xylS* and transposon limits are shown. The 1.4 kb repeated sequences are marked by arrows. The insertion site for Tn401 in pWW0-161 is shown. (b) pWW0 plasmid derivatives that appeared during growth of *P. putida* PaW85(pWW0) on *m*-toluate or benzoate. The thick lines indicate the pWW0 plasmid DNA which remained intact in the derivatives observed; the thin lines indicate the regions deleted. The sites of possible Tn4652 insertion from the chromosome into pWW0^{mut1} plasmid leading to the formation of pWW0^{mut15}, pWW0^{mut19} and pWW0^{mut27} are denoted by triangles. The black boxes indicate the left- (LH) and right- (RH) hands of the 17 kb transposon Tn4652.

xylS regulatory genes, evidence of complete deletion of the catabolic genes from the plasmids. But unlike the parent plasmid pWW0^{mut1}, strong homology with the complete 17 kb transposon Tn4652 was observed. Recovery of the complete transposon Tn4652 from pWW0^{mut19}, pWW0^{mut27} and pWW0^{mut15} may be the result of its transposition from the chromosome. A copy of Tn4652 has been located in the chromosome of cells originating from strain PaW85 (Meulien & Broda, 1982). Transposition of Tn4652 to different sites of pWW0^{mut1} and subsequent intraplasmidic recombination between two copies of the right-hand of Tn4652 could have led to the total loss of TOL catabolic genes (*meta*-operon) in these segregants. This mechanism also explains the absence of fragments ED, EV and part of EE in pWW0^{mut27}. A new E(I+E) (4.85 kb) fragment was formed which was homologous to the 17 kb Tn4652 (Fig. 2).

In the case of pWW0^{mut19}, Tn4652 was inserted in the ES fragment. A new fragment E(I+S) (5.4 kb) was detected (Fig. 2). For pWW0^{mut15}, transposition of Tn4652 into the EC fragment could be proposed. After recombination, a new fragment E(I+C) (5.6 kb) homologous to Tn4652 was formed (Fig. 2).

Transfer of plasmid-specific characteristics

To determine whether the active catabolic genes were physically present on pWW0-161^{mut1}, transformation of pWW0-161^{mut1} into strains PaW85 and PpG1400 was carried out. The transformants did not grow on medium with *m*-xylene indicating the absence of the *upper*-pathway. Weak growth of PaW85(pWW0-161^{mut1}) on *m*-toluate was accompanied by accumulation of black pigment in the medium. The PpG1400(pWW0-161^{mut1}) transformants did not grow on *m*-toluate. All transformants turned yellow when spread with catechol (100 mM), an indication of the presence of active catechol 2,3-dioxygenase. The activity of catechol 2,3-dioxygenase in the benzoate induced transformant PaW85(pWW0-161^{mut1}) was comparable with that measured for the other strains carrying TOL plasmids (see Table 2). However, unlike other strains, PaW85(pWW0-161^{mut1}) also had high catechol 2,3-dioxygenase activity in uninduced cells.

The above data confirmed that pWW0-161^{mut1} had no *upper*-pathway and that the *meta*-pathway was incomplete. Taking into account (i) the presence of catechol 2,3-dioxygenase activity in the transformant

PaW85(pWW0-161^{mut1}), (ii) the fact that these cells were unable to utilize *m*-toluate, (iii) our data on restriction analysis and (iv) the arrangement of the *upper*- and *meta*-pathway genes (Harayama *et al.*, 1989; Harayama & Rekik, 1990), we concluded that the genes of the *upper*-pathway and part of the *meta*-pathway (up to gene *xylZ*) were absent from plasmid pWW0-161^{mut1}. The catechol 2,3-dioxygenase located in the remaining part of the *meta*-operon was constitutively expressed (see Table 2).

pWW0-161^{mut1} was not stably maintained in the transformant PaW85(pWW0-161^{mut1}) in prolonged batch culture on benzoate containing media. The appearance of cells lacking catechol 2,3-dioxygenase activity was observed. No plasmidless cells were observed, however. Electrophoresis of plasmid DNA showed that in cells lacking catechol 2,3-dioxygenase activity two types of deletion had occurred. This should be considered unequivocal evidence of the genetic instability of pWW0-161^{mut1} in cells growing on benzoate. Therefore the stability of pWW0-161^{mut1} observed in strain PPW161-1 growing on benzoate could not be explained by the peculiarities of the plasmid genetic structure. The disparity of the phenotype of strain PPW161-1 and the structure of pWW0-161^{mut1} suggested that the *upper*-pathway and the beginning of the *meta*-pathway, including the operator–promoter part and the *xylXY* genes are located in the chromosome in strain PPW161-1. The stability of pWW0-161^{mut1} observed in PPW161-1 cells growing on benzoate could be explained by disturbance of the normal expression of the *ortho*-pathway genes as a result of the insertion of plasmid catabolic genes into the chromosome. The absence of an active *ortho*-pathway for benzoate degradation in the cells might prevent the loss of TOL plasmid encoded genes of the *meta*-pathway from the cells.

Strain IRJA4, a Ben[−] mutant, was used as a recipient in conjugation experiments. As a result of conjugation of IRJA4 with strain PPW1-1 transconjugants with the phenotype mXyl[−]mTol⁺Ben⁺Trp[−]Cb^sSm^r appeared. Conjugation with strain PPW161-1 gave rise to transconjugants with the phenotype mXyl⁺mTol⁺Ben⁺Trp[−]Cb^sSm^r. All transconjugants therefore preserved the phenotype of the donor cells and also possessed catechol 2,3-dioxygenase activity. The sizes of plasmids separated from the transconjugants were the same as those of donor cells.

Results of conjugation experiments with strain PPW161-1 showed that the TOL catabolic genes deleted from plasmid pWW0-161^{mut1} had been preserved in the cells and could be transferred to recipient cells with pWW0-161^{mut1}. It should be stressed that the transconjugants carried only the pWW0-161^{mut1} plasmid of original size where the *upper*-pathway and some of the *meta*-pathway genes were deleted.

Recovery of genes of the *upper*-operon on pWW0-161^{mut1} was observed during cultivation of strain PPW161-1 on minimal medium containing a high concentration of benzoate (4.5 g l^{−1}) plus *m*-toluate (0.5 g l^{−1}). A 140 kb plasmid was isolated from these cells. Homology with the *upper*-pathway genes was detected in this plasmid. Thus the TOL catabolic genes deleted from pWW0-161^{mut1} and preserved in the cells of PPW161-1 could be integrated back into a plasmid in some cases.

A separate conjugation experiment was carried out with a RecA[−] recipient strain PpG1400. The RecA[−] transconjugants which arose from mating PPW161-1 with strain PpG1400 had the phenotype mXyl[−]mTol[−]Ben⁺Trp[−]Cb^r and carried pWW0-161^{mut1}, indicating that in this case the genes for utilization of *m*-xylene and *m*-toluate were not transferred. No additional plasmids were observed.

Discussion

Alterations in the structure of the TOL plasmid pWW0 resulting in transposon mutagenesis or spontaneous deletions have been described in the literature. Most of these are so-called legitimate deletions. Their end-points coincide with the ends of transposons Tn4651 and Tn4653 or occur between direct 1.4 kb repeats flanking the catabolic region of pWW0. However, several exceptions have been observed where the deletion end-points do not coincide with those of the transposons (Lehrbach *et al.*, 1982; Lebens & Williams, 1985). We observed two new 'illegitimate' deletions that had occurred in pWW0. The structures of pWW0 derivatives detected by us indicate that partial loss of catabolic genes from the plasmid and even deletion of part of Tn4652 can occur.

We propose that pWW0-161^{mut1} appeared as a result of the deletion of the *upper*-operon and operator–promoter part of the *meta*-pathway including genes *xylXY*. In spite of this deletion, cells of strain PPW161-1 could grow on all the usual TOL plasmid substrates. No additional plasmids were detected in PPW161-1 cells. Although it is likely that the catabolic genes deleted from the plasmid were integrated into the chromosome, we have, as yet, no direct evidence of this. Examples of functional complementation of operons located on plasmid and chromosome or on two different plasmids have been described previously (Kunz & Chapman, 1981; Shaw & Williams, 1988). In the present case, complementation occurred not only between separately located TOL plasmid catabolic operons but also between genes of the *meta*-pathway. It is very likely that such relocation of the *meta*-pathway was the reason for the slower growth rate of strain PPW161-1 in comparison with that of PaW85(pWW0) on benzoate.

The unusual stability of the TOL plasmid in strain

PPW161-1 growing on benzoate could not be explained by the structure of pWW0-161^{mut1}. Rather, this stability was probably caused by insertion of the plasmid catabolic genes into the chromosome at a site where the *ortho*-pathway genes are located, resulting in the inability of the cells to use the *ortho*-pathway.

Insertion of TOL plasmid genes into the chromosome might occur by a mechanism of homologous recombination. This is a reversible process and the genes involved can be mobilized from the chromosome and transferred during conjugation. After transfer, the TOL genes can be inserted back into the chromosome.

Mobilization of genes located in the chromosome during conjugational transfer has also been observed previously. For example, the genes of the CAM (camphor degradation) plasmid are preferentially located in the chromosome, and the presence of pWW0 or pWW0-8 in cells increases the frequency of transfer of CAM genes (Mäe *et al.*, 1991). However, it is not clear why catabolic genes deleted from the TOL plasmid appear to be re-inserted into the chromosome rather than remaining in the plasmid after conjugation.

The absence of the *meta*-pathway promoter on the TOL plasmid in strain PPW161-1 did not influence the normal induction and activity of the *meta*-pathway genes remaining on pWW0-161^{mut1}. The regulatory role of both ends of Tn4652 in expression of phenol catabolic (*phe*) genes was demonstrated by Kivisaar *et al.* (1989). Plasmid pWW0-161^{mut1} carried genes of the *meta*-pathway, between the right- and left-hands of Tn4652 in the absence of the original promoter. In cells carrying pWW0-161^{mut1}, catechol 2,3-dioxygenase was constitutively expressed at a high level, and we propose that Tn4652 acted as a second-order constitutive promoter.

The cryptic transposon Tn4652 belongs to the class II transposons (Tsuda *et al.*, 1989). Tn4652 transposition involves co-integrant formation by transposon-specific transposase and its subsequent resolution. We propose that the appearance of additional deletions and recovery of the complete transposon Tn4652 in pWW0^{mut15}, pWW0^{mut19} and pWW0^{mut27} after growth of PPW1-1 cells on benzoate is the result of transposition of Tn4652 located in the chromosome into different sites of pWW0^{mut1}. As a result of this transposition, two copies of the right-hand of Tn4652 should appear on the plasmid. However, subsequent recombination between these directly repeated sequences may have led to deletion of the remaining *meta*-pathway catabolic genes which were located between the duplicated right-hands of Tn4652. The resulting Tol⁻ cells had a growth advantage on benzoate due to degradation of benzoate via the chromosomally encoded *ortho*-pathway.

Our results indicate the effective adaptability of *Pseudomonas putida* and its plasmids to different external

conditions, and the great diversity of mechanisms of genetic variability that make this possible.

We thank L. Kasak, M. Kivisaar and V. Kõiv for cloned fragments used in hybridization assay.

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