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Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas arvilla* mt-2

By C. L. WONG and N. W. DUNN

School of Biological Technology, University of New South Wales
P.O. Box 1, Kensington, New South Wales, 2033, Australia

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SUMMARY

Pseudomonas arvilla mt-2 (ATCC 23073) has been shown to harbour a transmissible plasmid which codes for the degradation of benzoate and *m*-toluate. Plasmid-borne genetic information codes for the conversion of these compounds to catechol then the assimilation of catechol via the *meta* cleavage pathway.

1. INTRODUCTION

Two major pathways have been demonstrated which result in the degradation of catechol, the *ortho* pathway (Ornston & Stanier, 1964) and the *meta* pathway (Dagley *et al.* 1964; Evans, 1963). These two pathways have been shown to co-exist in the same bacterial cell, the relative induction of each pathway being dependent on the growth substrate (Feist & Hegeman, 1969). Recent studies in two Pseudomonads have illustrated that the genetic information for the degradation of naphthalene (Dunn & Gunsalus, 1973) and salicylate (Chakrabarty, 1972) to catechol and the initial enzymes of the *meta* pathway are plasmid borne. The *ortho* pathway is also present in these two strains and appears to be encoded by chromosomal genetic material. These results suggest that other strains carrying both the *ortho* and *meta* pathways should be studied critically in an attempt to detect new plasmids. One such strain worthy of this study is *Pseudomonas arvilla* mt-2 which can grow at the expense of benzoate, *m*- and *p*-toluate and which carries both the *ortho* and *meta* catechol cleavage pathways (Murray *et al.* 1972; Nakazawa & Yokoto, 1973). The majority of Pseudomonads can utilize benzoate as their sole source of carbon and energy (Stanier, Palleroni & Doudoroff, 1966). Therefore a biochemically and preferably genetically characterized strain was required for use as recipient for possible plasmids from *P. arvilla* mt-2. The recipient strain chosen for this purpose was PPl-2 (PpG572) which contains the catechol *ortho* cleavage pathway but not the *meta* cleavage pathway (Dunn & Gunsalus, 1973) and for which a transduction system is available (Gunsalus *et al.* 1968).

This paper reports the construction of appropriate recipient strains and their use for the identification and preliminary characterization of a transmissible plasmid in *P. arvilla* mt-2.

2. MATERIALS AND METHODS

The bacterial strains used are described in Table 1. Media, maintenance of stock cultures and growth conditions have been outlined previously (Gunsalus *et al.* 1968; Dunn & Gunsalus, 1973). The genetic techniques of mutagenesis, curing with mitomycin C and conjugation (using the technique of auxotrophic contraselection to prevent growth of the donor) were described by Dunn & Gunsalus (1973). Techniques for induction and assay of catechol-1,2-oxygenase and

Table 1. *Bacterial strains*

Stock No.	Genotype	Derivation	Reference
PPl-1 (PpGl)	wt, CAM	—	Rheinwald *et al.* (1973)
PPl-2 (PpG572)	wt	Spontaneous loss of CAM plasmid from PPl-1	Rheinwald *et al.* (1973)
PPl-3	*ben-1*	NG mutagenesis of PPl-2	—
PPl-4	*ben-1*, BEN/TOL	Conjugation (PArl-2 × PPl-3)	—
PPl-5	*ben-1*	Spontaneous loss of BEN/TOL plasmid from PPl-4	—
PPl-6	*ben-1*, BEN/TOL	Transduction (pf16.PPl-4 × PPl-3)	—
PPl-7	*ben-1*	Spontaneous loss of BEN/TOL from PPl-6	—
PPl-8	*met-1*	NG mutagenesis of PPl-2	—
PArl-1	wt, BEN/TOL	—	Murray *et al.* (1972)
PArl-2	*his-1*, BEN/TOL	NG mutagenesis of PArl-1	—
PArl-3	wt	Spontaneous loss of BEN/TOL from PArl-1	—
PArl-4	wt, BEN/TOL	Conjugation (PArl-2 × PArl-3)	—

P. arvilla mt-2 was a gift from Dr John Harrison.

Abbreviations: *ben*, benzoate; BEN/TOL, plasmid coding for the degradation of benzoate and m-toluate; CAM, plasmid coding for the degradation of camphor; *his*, histidine; *met*, methionine; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PArl-1, *P. arvilla* mt-2; wt, wild type.

Phage sensitivity and transduction studies were carried out using pf16 (Gunsalus *et al.* 1968).

catechol-2,3-oxygenase have also been outlined previously (Dunn & Gunsalus, 1973). The activity of catechol-1,2-oxygenase is expressed as the micromoles of *cis, cis*-muconate produced per minute per milligram of protein based on $\epsilon_{\mathrm{nM}}^{260\,\mathrm{nm}} = 17$ (Feist & Hegeman, 1969). Catechol-2,3-oxygenase activity is expressed as the micromoles of 2-hydroxy-muconic semialdehyde produced per milligram of protein based on $\epsilon_{\mathrm{nM}}^{375\,\mathrm{nm}} = 30$ (Feist & Hegeman, 1969).

3. RESULTS

(i) *Construction of strains*

(*a*) *Isolation of a donor strain from PArl-1*

A suitable method for the conjugational transfer of metabolic plasmids uses auxotrophic contraselection against the donor strain. Therefore a histidine requiring auxotroph was isolated from PArl-1 following NG mutagenesis. This mutant was designated PArl-2 and grew well on either benzoate or *m*-toluate.

(*b*) *Isolation of a recipient strain from PPl-2*

Since PPl-2 contains a functional *ortho* cleavage pathway for the degradation of both phenol and benzoate an attempt was made to isolate a mutant defective in catechol-1,2-oxygenase. A NG-mutagenized culture of PPl-2 was plated for single colonies on Vogel–Bonner minimal medium, and then replicated onto a minimal salt medium (PAS) containing benzoate as the sole source of carbon. In all, 5000 single colonies were tested and one mutant (PPl-3) was chosen which would not grow at the expense of either phenol or benzoate. In each case the medium turned black, indicative of accumulation and chemical oxidation of catechol. Enzyme assays illustrated that catechol-1,2 oxygenase was absent from this mutant (see below).

(*c*) *Isolation of a recipient strain from PArl-1*

During the course of genetic manipulation of PArl-1 it was noted that occasional organisms could be isolated spontaneously which failed to grow on *m*-toluate but were still able to utilize benzoate. Nakazawa & Yokota (1973) reported a similar group of organisms which, unlike the parent, degraded benzoate via the *ortho* pathway rather than the *meta* pathway. In fact in this class of organism the *meta* cleavage pathway could not be detected by these workers. One organism from this spontaneous group was designated PArl-3 and used, in addition to PPl-3, as a conjugational recipient.

(ii) *Genetic transfer from PArl-2 by conjugation*

An attempt was made to obtain conjugational transfer selecting for conjugants which grew on either benzoate or *m*-toluate. Genetic transfer was obtained and it was noted that conjugants appeared at the same frequency (10^{-5} per donor cell) when using PPl-3 as recipient irrespective of whether selection was made for benzoate or *m*-toluate. One hundred benzoate utilizing conjugants were tested and all utilized *m*-toluate, likewise 100 *m*-toluate utilizing clones utilized benzoate. All of these conjugants were sensitive to the phage pf16 as was the recipient PPl-3. The donor strain is resistant to this phage. One of the clones which utilized both benzoate and the *m*-toluate was designated PPl-4 and utilized in additional studies.

When using PArl-3 as recipient, conjugants were also obtained but at a slightly higher frequency (10^{-4} per donor cell). One of these clones was called PArl-4.

(iii) *Plasmid characterization*

(*a*) *Mitocmycin C curing*

PPl-4 was tested to determine the frequencies of spontaneous and mitomycin C induced loss of the ability to utilize both benzoate and *m*-toluate. In addition, PArl-1 and PArl-4 were tested for spontaneous loss of the ability to utilize *m*-toluate. The results, given in Table 2, show that the ability to utilize both benzoate and *m*-toluate was lost simultaneously from PPl-4. In all, fifty such clones were tested and one was chosen and designated PPl-5. In addition it was possible to obtain derivatives of PArl-1 and PArl-4, which could not utilize *m*-toluate.

At this stage of the investigation, studies on PArl derivatives were discontinued and all additional work was performed with the PPl derivatives.

Table 2. *Curing of the ability to utilize benzoate and* m-*toluate*

Bacterial strain and treatment	Percentage cells cured	Phenotype of cured cells
PPl-4 (spontaneous)	2	Ben −, *m*-Tol −
PPl-4 (5 μg/ml mitomycin C)	4	Ben −, *m*-Tol −
PArl-1 (spontaneous)	2	Ben +, *m*-Tol −
PArl-4 (spontaneous)	2	Ben +, *m*-Tol −
*PPl-6 (spontaneous)	2	Ben −, *m*-Tol −
*PPl-6 (5 μg/ml mitomycin C)	4	Ben −, *m*-Tol −

* PPl-6 was obtained by transduction – see section (iii)(*b*).

(*b*) *Transductional transfer of the ability to utilize benzoate and* m-*toluate*

Since the previous results suggest that the ability to utilize benzoate and *m*-toluate is plasmid-borne, it is possible that the genetic information for this pathway is clustered and can be transferred by transduction. The results of pf16-mediated transductions are given in Table 3. For comparative purposes a methionine-requiring mutant was also used as transductional recipient, selection being made for prototrophy. The results illustrate that the ability to utilize either benzoate or *m*-toluate can be transferred by transduction. One hundred benzoate utilizing transductants were tested and found to utilize *m*-toluate and *vice versa*. One such strain was designated PPl-6. Additional tests with these transductants illustrated that, as with the conjugants, the ability to utilize both benzoate and *m*-toluate could be lost spontaneously or at an increased frequency following growth in the presence of mitomycin C (Table 2). One of these spontaneously cured strains was designated PPl-7.

(*c*) *Biochemical characterization of strains*

The previous work indicates that PArl-1 carried a transmissible plasmid which codes for the degradation of benzoate and *m*-toluate. To determine whether these compounds are degraded via the *ortho* or *meta* cleavage pathway the initial enzyme in each pathway, namely catechol-1,2-oxygenase and catechol 2,3-oxygenase, was

Table 3. *Transductional transfer of ability to utilize benzoate and* m-*toluate*

Transducing preparation	Recipient	Selection	Transduction frequency per phage particle
pf16.PPl-4	PPl-3	Ben+	5×10^{-7}
pf16.PPl-4	PPl-3	*m*-Tol+	4×10^{-7}
pf16.PPl-4	PPl-8	Met+	6×10^{-7}

Table 4. *Specific activity of catechol-1,2- and catechol-2,3-oxygenase in all strains derived from PPl-2*

	Inducing substrate					
	None		Benzoate		*m*-Toluate	
Strain no.	1,2	2,3	1,2	2,3	1,2	2,3
PPl-2	0	0	1·2	0	0	0
PPl-3, PPl-5 and PPl-7	0	0	0	0	0	0
PPl-4, and PPl-6	0	0	0	3·2	0	3·3

All strains were grown in succinate (5 mM) containing 5 mM of the inducing compound where applicable; 1,2, specific activity of catechol-1,2-oxygenase; 2,3, specific activity of catechol-2,3-oxygenase. Since the specific activities of catechol-2,3-oxygenase were so high in these strains the validity of these results was tested under conditions that gave complete inactivation of any catechol-2,3-oxygenase (Nakazawa & Yokota, 1973).

assayed (Table 4). These results demonstrate the blockage of the *ortho* cleavage pathway at the catechol-1,2-oxygenase step in PPl-3. As expected it was not possible to detect any catechol 2,3-oxygenase in this organism. Following transfer (by either conjugation or transduction) of the genetic information coding for benzoate and *m*-toluate degradation into PPl-3, high levels of catechol-2,3-oxygenase were detected. Catechol-1,2-oxygenase activity could not be detected. When this growth phenotype was lost either spontaneously or following mitomycin C treatment, catechol-2,3-oxygenase could no longer be detected and these strains appeared to be identical to their parent PPl-3. These results illustrate that it is the *meta* pathway which is plasmid-borne in these strains.

4. DISCUSSION

These results illustrate that *P. arvilla* mt-2 (PArl-1) harbours a transmissible plasmid which codes for the degradation of both *m*-toluate and benzoate. Following transfer of this plasmid into the biochemically defined PPl-3 it was possible to demonstrate that plasmid-borne genetic information coded for conversion to catechol and also the degradation of catechol via the *meta* pathway. Spontaneous or mitomycin C curing of the plasmid could be readily demonstrated. It should be noted at this point that the ability to convert benzoate to catechol need not be plasmid-borne, although this phenotypic property was associated with plasmid

movement in this system. The primary recipient (PPl-3) was derived from PPl-2, which can utilize benzoate via the catechol *ortho* cleavage pathway. Although the mutant PPl-3 is defective in catechol-1,2-oxygenase this strain can still convert benzoate to catechol. This phenotype may result therefore from a composite pathway derived from recipient chromosomal genes in combination with the plasmid-borne catechol *meta* cleavage pathway. Experiments are under way to clarify this point.

The mutant P100 reported by Nakazawa & Yokota (1973) in *P. arvilla* mt-2 can be readily explained by assuming the absence of the plasmid described in this paper.

The demonstration of this plasmid is also interesting in view of the work reported by Murray *et al.* (1972) where alternative pathways have been demonstrated for the breakdown of 2-hydroxymuconic semialdehyde by the *meta* pathway. Re-examination of this situation, taking advantage of the genetic manipulation that this plasmid allows, may facilitate determination of the genetic location of these alternative pathways.

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