

Evidence for a transmissible catabolic plasmid in *Pseudomonas putida* encoding the degradation of *p*-cresol via the protocatechuate *ortho* cleavage pathway

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SUMMARY

Evidence is presented that a strain of *Pseudomonas putida* harbours a catabolic plasmid which encodes for the degradation of *p*-cresol through the protocatechuate *ortho* cleavage pathway. This plasmid can transfer giving approximately 10^{-3} transconjugants per donor cell, can be cured with mitomycin C, belongs to the P-9 plasmid incompatibility group and can be transduced with the bacteriophage pf16.

1. INTRODUCTION

A number of catabolic plasmids have been reported which encode the *meta* cleavage pathway for the degradation of catechol, 3- and 4-methylcatechol (Chakrabarty, 1972; Dunn & Gunsalus, 1973; Williams & Murray, 1974; Wong & Dunn, 1974; Williams & Worsey, 1976). However, this is only one of the variety of cleavage pathways responsible for the degradation of substituted dihydric aromatic compounds (Chapman, 1972; Dagley, 1975). This paper reports the identification of a transmissible catabolic plasmid from a strain of *Pseudomonas putida* which encodes the degradation of *p*-cresol via the protocatechuate *ortho* cleavage pathway.

2. MATERIALS AND METHODS

Biological material is listed in Table 1. The host strains in which this work was conducted were PP1-2 and its mutant derivatives. PP1-2 is obtained by curing the CAM plasmid from ATCC 17453 strain which is believed to be closely related to ATCC 17452. Ornston (1966) characterized the protocatechuate *ortho* cleavage pathway of ATCC 17452 and also found that ATCC 17453 determines this same pathway (personal communication). Neither strain grows on *p*-hydroxybenzoate and each strain encodes all enzymes of the protocatechuate *ortho* cleavage pathway except for the enzyme β -carboxy-*cis,cis*-muconate lactonase (Fig. 1). In our studies when ATCC 17452 and PP1-2 and its mutant derivatives are plated onto *p*-hydroxybenzoate plates mutants allowing slow growth on *p*-hydroxybenzoate appear at a frequency of approximately 10^{-8} /cell plated.

Table 1. *Biological material*

Bacterial strains		
Stock no.	Genotype	Derivation and/or reference
PP1-2	wt	Wong & Dunn (1974)
PP1-8	<i>met-1</i>	Wong & Dunn (1974)
PP1-25(CAM)	<i>trp-1</i>	PpG273 in Dunn & Gunsalus (1973)
PP1-24	<i>ben-1, met-2</i> <i>pfs-3, str-2</i>	Wong & Dunn (1976)
PAr1-6(TOL)	<i>met-1, itf-1</i>	Austen & Dunn (1977)
PP7-1(pND50)	wt	Obtained as NCIB9866,
PP7-2(pND50)	<i>trp-1</i>	NG mutagenesis of PP7-1 (pND50)
ATCC 12633	wt	<i>P. putida</i> biotype A, strain 90 Stanier <i>et al.</i> (1966)
ATCC 17485	wt	<i>P. putida</i> biotype A, strain 111 Stanier <i>et al.</i> (1966)
ATCC 17503	wt	<i>P. aeruginosa</i> , ATCC 17503 or 1C Stanier <i>et al.</i> (1966) or PAO1 (Haas & Holloway, 1976)
ATCC 17498	wt	<i>P. fluorescens</i> biotype A, strain 126 Stanier <i>et al.</i> (1966)
ATCC 17633	wt	<i>P. putida</i> biotype A, strain 266 Stanier <i>et al.</i> (1966)
NRRL B769	wt	<i>P. indoloxidans</i>
Plasmids		
Plasmid no.	Characteristics or reference	
TOL	Williams & Murray (1974); Wong & Dunn (1974)	
CAM	Rheinwald, Chakrabarty & Gunsalus (1973)	
pND50	Catabolic plasmid encoding degradation of <i>p</i> -cresol by the protocatechuate <i>ortho</i> cleavage pathway	

ben, Benzoate; *met*, methionine; *pfs*, resistance to phage pf16; *str*, resistance to streptomycin; *itf*, increased transfer frequency of resident plasmid; *trp*, tryptophan; wt, wild type.

That PP1-2 and its mutant derivatives have a defective protocatechuate *ortho* cleavage pathway should be kept in mind when introducing pND50 and studying growth on *p*-hydroxybenzoate. To verify that the *p*-hydroxybenzoate growth phenotype for both transconjugants and transductants were associated with the plasmid pND50, the plasmid was routinely cured with mitomycin C or TOL. The cured strains were then tested to ensure that the host cell was unable to grow on *p*-hydroxybenzoate and that revertants to growth on that carbon source could be obtained at a low frequency. When it is stated in the text, that the phenotype of PP1-2 derivatives was *p*-hydroxybenzoate negative this means that the *p*-hydroxybenzoate growth phenotype was as for the wild type PP1-2. Neither PP1-2 nor the *p*-hydroxybenzoate utilizing mutants were able to utilize *p*-cresol as the sole source of carbon and energy. PP1-2 does carry a functional catechol *ortho* cleavage pathway but no catechol *meta* cleavage pathway (Austen & Dunn, 1977).

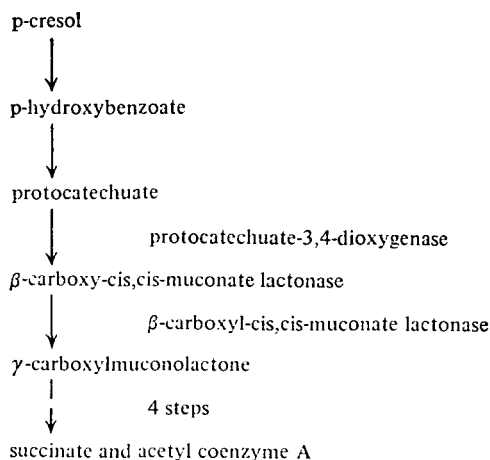


Fig. 1. Outline of pathway for the degradation of *p*-cresol via the protocatechuate *ortho* cleavage pathway.

The basic growth medium consisted of PAS salts medium (Chakrabarty, 1972). Methods used were as follows: mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (Fargie & Holloway, 1965); plasmid transfer by conjugation (Austen & Dunn, 1977); mitomycin C curing (Rheinwald, Chakrabarty & Gunsalus, 1973) and transduction with bacteriophage pf16 (White & Dunn, 1977).

For enzyme assays, cultures were grown overnight at 30 °C in a reciprocal shaker in PAS + 10 mM acetate + 5 mM succinate. A 25% inoculum was then added to PAS + 10 mM acetate + 2.5 mM inducing compound and incubated for 4 h at 30 °C on a reciprocal shaker. In the absence of inducer the acetate concentration was increased to 15 mM. Cells from 200 ml cultures were harvested by centrifugation at 2 °C, washed once with 40 ml of 0.02 M phosphate buffer, pH 7.0, then resuspended in 4 ml of the same buffer. Cells were disrupted by sonication and cell debris was removed by centrifugation. Assays of protocatechuate-3,4-dioxygenase (E.C. 1.13.11.3) were carried out by the method of Gibson (1971). One unit of enzyme activity is defined as the amount which oxidizes 1.0 μ mol of protocatechuate per minute. For specific activity determinations, protein was estimated using Folin-Ciocalteu reagent (Lowry *et al.* 1951) with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

A strain of *P. putida*, stocked as NCIB 9866, was obtained from the NCIB culture collection. It was believed to be that studied by Chapman & Hopper (1968) and Hopper (1976). The strain studied by those workers utilized *p*-cresol and 2,4-xyleneol as sole sources of carbon and energy. However, the strain we obtained differed from the authentic NCIB 9866 in being able to degrade only *p*-cresol and not 2,4-xyleneol. A second culture obtained from the NCIB collection was also unable to utilize 2,4-xyleneol. Since genetic evidence is presented in this paper that a trans-

missible plasmid (pND50), encoding the degradation of *p*-cresol, is harboured by this strain, for convenience we shall call it PP7-1(pND50) from the outset.

PP7-1(pND50) was mutagenized with NG and a *p*-cresol-utilizing tryptophan-requiring auxotroph, PP7-2(pND50), was isolated. This auxotroph was used as the donor strain with PP1-2 and PP1-8 as recipients, and using auxotrophic counter-selection selection was made for growth on *p*-cresol. In each case presumptive transconjugants were obtained at a frequency of approximately 10^{-3} per donor cell. All transconjugants were sensitive to the phage pf16, grew well on *p*-hydroxybenzoate and those derived from PP1-8 were methionine auxotrophs. Studies were undertaken to determine the genetic basis of these newly acquired catabolic properties.

Following growth of PP1-2(pND50) in the presence of 25 μ g/ml mitomycin C, 5% of the colonies tested had lost the ability to utilize *p*-cresol and *p*-hydroxybenzoate whilst still able to grow on PAS + 10 mM succinate. When 1000 colonies were tested after growth in the absence of mitomycin C, none had lost the ability to degrade either *p*-cresol or *p*-hydroxybenzoate. To study the effect of introducing other catabolic plasmids on the maintenance of pND50, the TOL plasmid was introduced by conjugation from PAr1-6(TOL) and the CAM plasmid from PP1-25 (CAM). TOL transferred into both PP1-2 and PP1-2(pND50) at a frequency of approximately 10^{-4} transconjugants per donor cell. Twenty transconjugants from the PP1-2(pND50) cross were purified by streaking three times for single colonies on the *p*-toluate selection medium. When these transconjugants were tested for the ability to grow on *p*-cresol and *p*-hydroxybenzoate all had lost those growth responses characteristic of PP1-2(pND50) and acquired those of PP1-2 (TOL). This suggested that the ability to degrade *p*-cresol and *p*-hydroxybenzoate was encoded by a plasmid incompatible with TOL. To ensure that TOL was not interfering with the expression of the *p*-cresol growth phenotype, TOL was cured spontaneously from some of the transconjugants; such cured strains remained unable to utilize *p*-cresol and *p*-hydroxybenzoate. This plasmid incompatibility with TOL places it in the P-9 incompatibility group (White & Dunn, 1978).

CAM transferred into both PP1-2 and PP1-2(pND50) at a frequency of approximately 10^{-2} transconjugants per donor cell. Twenty transconjugants from the PP1-2(pND50) cross were purified by streaking three times for single colonies on the camphor selection medium. All these transconjugants retained the ability to grow on *p*-cresol and *p*-hydroxybenzoate suggesting compatibility of pND50 with the CAM plasmid which belongs to the P-2 incompatibility group.

A phage preparation of pf16 grown on PP1-2(pND50) was used as the transducing preparation and selection was made for transduction of the ability to utilize *p*-cresol with PP1-2 as recipient. Transductants were obtained at a frequency of approximately 10^{-9} per phage particle; these were also able to utilize *p*-hydroxybenzoate. Three transductants were used as donor strains, with PP1-24 as the recipient strain. *p*-Cresol- and *p*-hydroxybenzoate-utilizing, methionine-requiring transconjugants were obtained at a frequency of approximately 10^{-4} per donor

cell using streptomycin contra-selection. This suggested close linkage between the catabolic characteristics and transfer ability.

Using PP1-8(pND50) as donor, the ability of pND50 to transfer to some other strains was tested. Transconjugants were obtained at a frequency of approximately 10^{-3} per donor cell when using ATCC 12633, ATCC 17485 (from which the NAH plasmid had been cured), and ATCC 17503 (PAO1) grown at 43 °C; approximately 10^{-7} when using PAO1 grown at 37 °C; and less than 10^{-8} when using ATCC 17498, ATCC 17633 (cured of the OCT plasmid) and NRRL B769. The low frequency of transfer to PAO1 grown at 37 °C compared to that when grown at 43 °C indicates that pND50 harboured in PP1-8 is susceptible to the strain-specific restriction system of PAO1 as is TOL (Austen & Dunn, 1977). PAO1 harbouring pND50 was found to be sensitive to the sex specific phage PR4 as is PAO1 harbouring the TOL catabolic plasmid (White & Dunn, 1978). Curing of pND50 from PAO1 can be followed by loss of the *p*-cresol growth phenotype with a concomitant loss in PR4 sensitivity.

Transfer of pND50 always resulted in coinheritance of the ability to utilize *p*-cresol (which was selected) and *p*-hydroxybenzoate. In all likelihood *p*-cresol is converted to *p*-hydroxybenzoate and then to protocatechuate as has been reported for the authentic NCIB 9866 (Chapman & Hopper, 1968; Hopper, 1976). Tests were carried out to determine whether protocatechuate was then degraded by either the *ortho* or *meta* cleavage systems. The initial enzyme in the *ortho* cleavage pathway is protocatechuate-3,4-dioxygenase whereas that for the *meta* cleavage pathway is protocatechuate-4,5-dioxygenase. Following growth of PP1-2(pND50) on acetate, on acetate + *p*-cresol, or on acetate + *p*-hydroxybenzoate, specific activities of 0.05, 4.5 and 4.8 respectively were obtained for protocatechuate-3,4-dioxygenase. No activity was detected for protocatechuate-4,5-dioxygenase using the method of Gibson (1971). Although the host strain PP1-2 encodes a protocatechuate-3,4-dioxygenase (L. N. Ornston, personal communication) this enzyme could not be detected when using growth and induction conditions for PP1-2 identical to those used for PP1-2(pND50). By analogy with the work of Chapman & Hopper (1968) and Ornston (1966) the pathway for degradation of *p*-cresol by the protocatechuate pathway is outlined in Fig. 1. The alternative pathway through which *p*-cresol can be degraded by some strains, the catechol *meta* cleavage pathway (Wigmore, Bayly & Di Berardino, 1974), could not be detected in PP1-2(pND50). In addition to the studies on PP1-2(pND50), the introduction of pND50 into PP1-2 by transduction was also accompanied by the appearance of an inducible protocatechuate-3,4-dioxygenase. Specific activities similar to those presented for PP1-2(pND50) were obtained. Curing of the plasmid from these strains was accompanied by a corresponding loss of this inducible system. This therefore confirms the presence of a transmissible plasmid which encodes the conversion of *p*-cresol through the protocatechuate *ortho* cleavage pathway.

This interpretation of the results should be viewed carefully in relation to the host strain in which this study was undertaken. The enzyme system which converts *p*-cresol to *p*-hydroxybenzoate, based on growth phenotypes, appears to be absent

from PP1-2 and the β -carboxy-*cis,cis*-muconate lactonase is absent from PP1-2 (L. N. Ornston, personal communication). It would appear then that the plasmid encodes at least these two enzyme systems. All other enzymes of the protocatechuate *ortho* cleavage pathway are encoded by the host cell although, as with protocatechuate-3,4-dioxygenase, they may not be detectable following induction conditions used in this study. Although we think it highly likely that the newly identified plasmid encodes the full pathway, clarification of this point will require the location of a suitable host strain completely lacking all enzymes of the protocatechuate *ortho* cleavage pathway or the use of an alternative method to distinguish host cell encoded enzymes from those encoded by the plasmid system.

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