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Combined chromosomal and plasmid encoded control for the degradation of phenol in *Pseudomonas putida*

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SUMMARY

The TOL(M1) metabolic plasmid was transferred from *Pseudomonas arvilla* mt-2 to a mutant of *Pseudomonas putida*. Although neither the donor nor the recipient could grow on phenol, the transconjugants could grow slowly on this carbon source. In these transconjugants phenol was converted to catechol by chromosomal encoded phenol hydroxylase followed by degradation of catechol by low uninduced levels of the plasmid encoded catechol *meta* cleavage pathway. A mutant, which grew well on phenol, was isolated from one of the transconjugants and it was found that phenol could now act as an inducer for the *meta* cleavage pathway.

1. INTRODUCTION

A detailed understanding of the genetic basis of the degradation of the simple aromatic compounds is of considerable importance as these structures form the basis of a variety of substituted aromatic compounds, some of which are fairly resistant to biodegradation. A transmissible plasmid which codes for the degradation of the simple aromatic compounds, benzoate, *m*-toluate and *p*-toluate, has been reported recently (Williams & Murray, 1974; Wong & Dunn, 1974). This plasmid has been referred to as the TOL plasmid (Williams & Murray, 1974) and the BEN/TOL plasmid (Wong & Dunn, 1974). Henceforth we shall use the simpler designation of TOL. It is possible to transfer this plasmid into strains which are well characterized both genetically and biochemically, and this permits a detailed study of induction patterns of plasmid encoded enzyme systems by making use of a combined genetic and biochemical approach. For this purpose we used derivatives of a strain of *P. putida* (PP1–2) described previously (Wong & Dunn, 1974).

Strain PP1–2 can utilize either phenol or benzoate as a sole source of carbon and energy. Each compound is converted to catechol and degraded through the catechol *ortho* cleavage pathway. The alternate pathway for the degradation of catechol, the *meta* cleavage pathway, does not appear to be present in this strain as it was not possible to detect the first enzyme in this pathway (Dunn & Gunsalus, 1973). Strain PP1–3 (a mutant of PP1–2) is unable to grow on either benzoate or phenol as sole source of carbon and energy and is defective in catechol-1,2-oxygenase, the first enzyme in the *ortho* cleavage pathway (Wong & Dunn, 1974). Although it is

unable to grow on phenol or benzoate PP1–3 turned the media black when incubated with either phenol or benzoate. This colouration is considered to be due to the enzymatic conversion of either benzoate or phenol to catechol, followed by chemical oxidation of catechol. The TOL(M1) plasmid can be readily transferred into PP1–3 from *P. arvilla* mt-2 by selecting for growth on *m*-toluate. Neither the original plasmid bearing strain of *P. arvilla* mt-2 or PP1–3 can grow on phenol, but PP1–3 carrying the M1 plasmid can do so.

This paper reports a detailed genetic and biochemical analysis of the chromosomal and plasmid encoded properties which contribute to this growth phenotype. In addition the isolation of a mutant plasmid with an alteration in the metabolic control for the breakdown of phenol is reported.

2. MATERIALS AND METHODS

The bacterial strains used are described in Table 1. The techniques that were described by Wong & Dunn (1974) include mutagenesis with *N*-methyl-N′-nitro-

Table 1. *Bacterial strains*

Strain no.	Genotype	Derivation	Reference
PP1–2	wt	—	Wong & Dunn (1974)
PP1–3	*ben-1*	NG mutagenesis of PP1–2	Wong & Dunn (1974)
PP1–18	*ben-1*, *phl-1*	NG mutagenesis of PP1–3	—
PP1–19	*phl-2*	NG mutagenesis of PP1–2	—
PP1–23	*ben-1*, *met-2*	NG mutagenesis of PP1–3	—
PP1–24	*ben-1*, *met-2*, *pfs-3*, *str-2*	Spontaneous resistance mutations in PP1–23	—
PP1–3(M1)	*ben-1*, M1	Conjugation (PArl-6 × PP1–3)	—
PP1–3(M1–1)	*ben-1*, M1–1	Spontaneous mutation of PP1–3 (M1) to good growth on phenol	—
PP1–24(M1)	*ben-1*, *met-2*, *pfs-3*, *str-2*,M1	Conjugation (PP1–3(M1) × PP1–24	—
PP1–24(M1–1)	*ben-1*, *met-2*, *pfs-3*, *str-2*, M1–1	Conjugation (PP1–3(M1–1) × PP1–24)	—
PP1–19(M1)	*phl-2*, M1	Conjugation (PP1–24(M1) × PP1–19)	—
PP1–19(M1–1)	*phl-2*, M1–1	Conjugation (PP1–24(M1–1) × PP1–19)	—
PP1–18(M1)	*ben-1*, *phl-1*, M1	Conjugation (PP1–24(M1) × PP1–18)	—
PP1–18(M1–1)	*ben-1*, *phl-1*, M1–1	Conjugation (PP1–24(M1–1) × PP1–18)	—
PAr1–1(MI)*	wt	—	Wong & Dunn (1974)
PAr1–5(M1)	*met-1*, M1	NG mutagenesis of PAr1-1(M1)	—
PAr1–6(M1)	*met-1*, *tra-1*, M1	NG mutagenesis of PAr1–5	

Abbreviations: ben, benzoate; M1, the TOL plasmid; M1-1, a mutant TOL plasmid; met, methionine; NG, *N*-methyl-*N*-nitro-N^1-nitrosoguanidine.

* PArl–1, *P. arvilla* mt–2(ATCC 23973); pfs, resistance to phage pf 16; phl, phenol; str, resistance to streptomycin; tra, increased transfer frequency of M1; wt, wild type.

N-nitrosoguanidine, curing with mitomycin C, conjugation using auxotrophic contraselection and extraction and assay of catechol-1,2-oxygenase and catechol-2,3-oxygenase. Conjugation using streptomycin contraselection involved the use of donor strains which were sensitive to streptomycin with recipients made resistant to 250 μg/ml streptomycin. Conjugation was performed in the same way as for auxotrophic contraselection experiments except that the culture was plated on the appropriate selective media supplemented with 250 μg/ml streptomycin. For enzyme induction studies, cells were grown in 10 mM sodium acetate to determine uninduced enzyme levels or, when necessary, to provide a utilizable growth substrate. The method used to assay phenol hydroxylase was as described by Sala-Trepat, Murray & Williams (1972).

3. RESULTS

(i) *Growth responses on phenol*

The M1 plasmid was transferred by conjugation from PArl–6(M1) to PP1–3 selecting for growth on *m*-toluate and using auxotrophic contraselection. Fifty recombinant colonies were purified and growth responses compared with the parental strains on minimal agar plates containing either phenol or *m*-toluate. Since all recombinant colonies gave identical growth responses one was chosen as being representative of this class and called PP1–3(M1) (Table 2).

Table 2. *Growth responses on phenol and* m*-toluate minimal agar plates*

	Carbon source	
Strain no.	*m*-Toluate (10 mM)	Phenol (2·5 mM)
PAr1–6(M1)	+ + + +	−
PP1–2	− (B)	+ + + +
PP1–3	− (B)	− (B)
PP1–3(M1)	+ + + +	+ (L & S)

Growth responses are represented as: +, slight growth through to + + + + for good growth; −, no growth; B, black colouration of the medium; L & S, large and small colonies.

As expected, the strains bearing the M1 plasmid grew well on *m*-toluate. Neither PP1–2 nor PP1–3 grew on *m*-toluate although a black colouration of the medium was observed in each case due to the conversion of *m*-toluate to 3-methylcatechol by benzoate hydroxylase, followed by chemical oxidation of the 3-methylcatechol. Although PP1–2 has a functional catechol *ortho* cleavage pathway, 3-methylcatechol cannot be metabolized by this pathway. PP1–2 grew well on phenol as, in this strain, phenol is degraded through the *ortho* cleavage pathway. PP1–3 is unable to grow on phenol as catechol-1,2-oxygenase is defective in this mutant. Therefore neither the donor strain PAr1–6(M1) nor the recipient strain PP1–3 can grow by utilizing phenol as the sole source of carbon and energy whereas PP1–3(M1), grew slowly, and a few large colonies appeared. The large colonies appeared at a frequency of approximately 10^{-7}. Twenty large colonies were purified and all grew

as well on benzoate and *m*-toluate, as did the parent strain PP1–3(M1), illustrating that the M1 plasmid was still present. Comparative growth studies were carried out with PP1–3(M1) and one of the mutants that grew well on phenol. The mutant chosen was named PP1–3(M1–1) as it was shown later that this phenotype arose from a mutation carried on the M1 plasmid. Growth responses of these two strains were examined in both liquid and solid media.

Table 3. *Growth responses with various concentrations of phenol on minimal agar plates*

	Phenol concentration (mM)			
Strain no.	2·5	5·0	7·5	10·0
PP1–2	+ + + +	+ + + +	+ +	–
PP1–3(M1)	+ (L & S)	+ (L & S)	N.D.	N.D.
PP1–3(M1–1)	+ + + +	+ + + +	+ + +	+ +

Abbreviations used to represent growth responses are listed in Table 2; N.D., not determined.

The results in Table 3 illustrate that the parent *P. putida* strain PP1–2 can grow on solid media with concentrations up to 7·5 mM phenol. PP1–3(M1) can grow slowly up to 5 mM phenol and in each case a few large colonies appeared. PP1–3(M1–1) grew well on phenol with concentrations up to 10 mM.

Mean generation times were determined in liquid culture using 2·5 mM phenol and for PP1–2 was found to be 1·5 h, for PP1–3(M1) approximately 20 h. and for PP1–3(M1–1) was 2 h. Although PP1–3(M1–1) could grow on plates with a higher concentration of phenol than could PP1–2, the mean generation time was longer in liquid culture. Since a marked difference was apparent when comparing the growth of PP1–3(M1) and PP1–3(M1–1) on phenol, a detailed study was undertaken to determine the genetic and biochemical basis of phenol degradation in these two strains.

(ii) *Comparative studies on PP1–3(M1) and PP1–3(M1–1)*

(*a*) *Plasmid characteristics*

To determine whether the M1 plasmid was still transmissible from PP1–3(M1) and PP1–3(M1–1), a conjugation experiment was carried out using PP1–24 as recipient. Selection was made for growth on *m*-toluate using the streptomycin contraselection procedure. In each case plasmid transfer was detected at a frequency of approximately 10^{-6} per donor cell. One recombinant colony from each cross was purified and used as the plasmid donor strain in later experiments. The recombinants were named PP1–24(M1) and PP1–24(M1–1) respectively.

To determine if the plasmids could still be lost, the frequency of both spontaneous and mitomycin C induced curing was determined. The plasmid was lost spontaneously from PP1–3(M1) at a frequency of 1 % and from PP1–3(M1–1) at a frequency of less than 0·1 %. Following growth in the presence of 5 μg/ml of

mitomycin C the plasmid was lost at a frequency of 4% from PP1–3(M1) and 0·6% from PP1–3(M1–1). One mitomycin C cured organism was retained for each strain and these were named PP1–3(M1)$^-$ and PP1–3(M1–1)$^-$.

(*b*) *Genetic basis for the conversion of phenol to catechol*

Although strain PP1–3 is blocked in the first enzyme of the catechol *ortho* cleavage pathway, it is able to convert phenol to catechol. It is possible that the phenol growth phenotype of PP1–3(M1) and PP1–3(M1–1) is due in part to the chromosomal genes for enzymes converting phenol to catechol. To investigate this, mutants of PP1–2 and PP1–3 were isolated which were defective in phenol hydroxylase. Following mutagenesis of PP1–2 with NG the desired mutant was isolated by screening for an organism able to grow on benzoate but not on phenol. It was necessary to screen 4000 clones to find the desired mutant (PP1–19). As well as being unable to grow on phenol, no black colouration was produced when the mutant was inoculated into a phenol medium and no phenol hydroxylase activity could be detected. A similar method was used to isolate a mutant of PP1–3 which produced a black colouration on benzoate medium but formed no black colouration when plated on phenol medium. This mutant was designated PP1–18 and to obtain this mutant it was necessary to screen 6000 clones. A valid enzyme assay to verify the absence of phenol hydroxylase could not be carried out with PP1–18 since it was not possible to assay this enzyme in the parent strain, PP1–3. To assay phenol hydroxylase in PP1–3 it was necessary to induce the enzyme by growth on 10 mM sodium acetate plus 2·5 mM phenol. Under these conditions a black colouration developed in the medium which was due, presumably to the enzymatic conversion of phenol to catechol followed by chemical oxidation of catechol. Attempts to detect phenol hydroxylase in this culture were unsuccessful and this may be due to inhibition caused by the accumulation of catechol or its oxidation products. To test this phenol hydroxylase was induced in PP1–2 then, just prior to assay, 1·25 mM catechol was added to the culture and incubated with shaking for 10 min at 30°. Following this treatment no phenol hydroxylase activity could be detected. It was assumed therefore that the inability to detect phenol hydroxylase in PP1–3 was due to product inhibition (or inactivation) of the enzyme.

Following the isolation of PP1–19 and PP1–18, the M1 and the M1–1 plasmids were introduced into each strain using both PP1–24(M1) and PP1–24(M1–1) as donor strains. Auxotrophic contraselection was used to select against the donor strains and selection for transfer was achieved by plating on *m*-toluate plates. Transconjugants from each cross were purified and tested for growth on phenol and it was found that they were all unable to grow on this carbon source. This observation indicated that the conversion of phenol to catechol in PP1–3(M1) and PP1–3(M1–1) was in fact contributed by genetic information encoded by the bacterial chromosome.

(*c*) *Genetic basis for the catabolism of catechol*

At this point it was not known whether the improved growth on phenol of PP1–3(M1–1) was due to a mutation on the bacterial chromosome or due to a

mutation on the plasmid. To determine the origin of this mutation, the plasmids were transferred from PP1–24(M1) and PP1–24(M1–1) into the original PP1–3 and the two mitomycin C cured strains, namely PP1–3(M1)$^-$ and PP1–3(M1–1)$^-$. Using auxotrophic contraselection and selecting for growth on *m*-toluate, the transfer frequency in all crosses was approximately 10^{-5} per donor cell. In the three crosses, all transconjugants derived from the donor PP1–24(M1) had the same phenotype as PP1–3(M1) in that they grew poorly on phenol and large colonies appeared at a low frequency. On the other hand, all transconjugants derived from the donor PP1–24(M1–1) grew well on phenol and had the phenotype of PP1–3(M1–1). This illustrates that the phenol growth phenotype in PP1–3-(M1–1) is associated with a plasmid borne mutation and not associated with a mutation in the bacterial host cell.

(*d*) *Induction of the plasmid borne catechol* meta *cleavage pathway*

Since the ability of PP1–3(M1–1) to grow well on phenol has been shown to be due to a mutation on the M1 plasmid it should be possible to detect a biochemical difference between the parent plasmid (M1) and the mutant plasmid (M1–1) in

Table 4. *Induction of catechol-2,3-oxygenase*

Growth substrate and inducer	Strain No. PP1-3(M1)	PP1-3(M1-1)	PP1-18(M1-1)
	Specific activity of catechol-2,3-oxygenase.		
Acetate (10 mM)	0·2	0·2	0·2
Acetate (10 mM) +*m*-toluate (5 mM)	2·0	1·9	2·0
m-Toluate (5 mM)	2·1	2·0	2·1
Acetate (10 mM) +phenol (2·5 mM)	0·2	1·7	1·6
Phenol (2·5 mM)	0·2	1·6	N.D.

No catechol-1,2-oxygenase was detected under any conditions; N.D., not determined; activity of catechol-2,3-oxygenase is expressed as micromoles of 2-hydroxy-muconic semi-aldehyde produced per min. per milligram of protein based on on $E_{mM}^{375\,nm} = 30$ (Feist & Hegeman, 1969).

relation to the phenol growth phenotype. It appeared likely that there had been selection for a mutant with an alteration in the control of phenol degradation and the induction of the plasmid borne catechol *meta* cleavage pathway in PP1–3(M1) was compared with PP1–3(M1–1). PP1–18(M1–1) was included to determine whether phenol could induce the *meta* cleavage pathway although it could not be converted to catechol. As an indication of the induction of the plasmid borne *meta* cleavage pathway the first enzyme (catechol-2,3-oxygenase) in the sequence was assayed (Table 4).

The results in Table 4 indicate that significant uninduced levels of catechol-2,3-oxygenase could be detected in all strains assayed. In either the presence or absence of acetate, *m*-toluate resulted in a 10-fold induction of catechol-2,3-

oxygenase. In PP1–3(M1) the presence of phenol did not increase the enzyme level whereas in the strains bearing the mutant plasmid, phenol produced approximately an 8-fold increase. The fact that induction occurred in spite of the genetic block in PP1–18(M1–1) suggested that the induction was due to phenol itself rather than a metabolite. These results illustrate that the ability of PP1–3(M1–1) to grow better on phenol than PP1–3(M1), arises from a plasmid borne mutation whereby phenol can act as an inducer of the catechol *meta* cleavage pathway.

4. DISCUSSION

The strain PP1–3(M1) is able to grow slowly on phenol as the sole source of carbon and energy although neither the *P. arvilla* mt-2 donor strain nor the PP1–3 recipient could grow on this substrate. This growth phenotype arose because phenol was converted to catechol by phenol hydroxylase which is encoded by the host cell chromosome. The catechol formed was further degraded by low uninduced levels of the catechol *meta* cleavage pathway.

Mutants of PP1–3(M1) which grew very well on phenol could be readily isolated. This mutation in PP1–3(M1–1) was shown to be located on the plasmid and the basis of the mutation was that phenol could now act as an inducer of the plasmid encoded *meta* cleavage pathway. In addition, the plasmid M1–1 was more stable than its parent M1 when each was compared in the host cell PP1–3. Other characteristics of the M1 and M1–1 plasmids were similar.

To date three metabolic plasmids have been reported which code for the degradation of aromatic compounds. These include the TOL plasmid referred to in this paper, the SAL plasmid (Chakrabarty 1972) and the NAH plasmid (Dunn & Gunsalus 1973). None of these plasmids have been reported to code for the complete breakdown of phenol but with the isolation of the mutant plasmid reported in this work it should now be possible to construct strains which are better able to degrade this compound. For strain construction of this type the primary strain would need to have an active phenol hydroxylase and be fairly resistant to the toxic effects of phenol. In relation to this certain phenol degrading strains contain phenol hydroxylase and also have both the catechol *ortho* and *meta* cleavage pathways within one strain (Feist & Hegeman 1969). More rapid degradation of phenol may be achieved by the introduction of the mutant TOL plasmid into strains of this type.

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