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Isolation of Mutants with Altered Metabolic Control of the NAH Plasmid-encoded Catechol *Meta*-cleavage Pathway

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Abstract

Two types of mutants which displayed altered regulation of the NAH catabolic plasmid-encoded catechol *meta*-cleavage pathway were isolated in *Pseudomonas putida*. Altered metabolic control was indicated by assay of catechol 2,3-dioxygenase. In one type of mutant the catechol 2,3-dioxygenase was synthesized constitutively. In the other type the range of carbon sources which induce the catechol 2,3-dioxygenase was increased.

Introduction

The catabolism of organic chemicals by microorganisms has received considerable attention. In particular there has been interest in the application of microorganisms to the degradation or detoxification of natural and synthetic organic compounds which may cause pollution problems or which may provide novel sources of carbon. In any detailed study of the biodegradation of such compounds it is essential to understand the metabolic regulation which is operative in the degradative pathways. If metabolic control is understood it should be possible to obtain optimal biodegradation of a particular compound; however, the understanding of metabolic control of the degradation of a range of compounds is limited. This situation has arisen because little use has been made of mutagenesis to facilitate the identification of inducing compounds and genetic exchange has not generally been available amongst bacterial strains with interesting catabolic properties. Added complications have been the presence of more than one enzyme which is active on specific compounds and the presence of alternative pathways for biodegradation of one compound.

One of the catabolic systems which has received much attention is the catechol *meta*-cleavage pathway. Ribbons (1970) showed that growth on phenol and the methylphenols (cresols) caused synthesis of the enzymes of the catechol *meta*-cleavage pathway in a strain of *Pseudomonas aeruginosa*. Likewise, Murray *et al.* (1972) illustrated that induction occurs during growth of *P. arvilla* mt-2 on benzoate and methylbenzoates (toluates). To identify the inducer of the phenol catabolic pathway, Feist and Hegeman (1969) and Ribbons (1970) isolated *P. putida* and *P. aeruginosa* mutants respectively which were blocked at phenol 2-monooxygenase (EC 1.14.13.7) and then studied induction over this block. Under these conditions it was shown that phenol is an inducer in each situation. Wigmore and Bayly (1974) have reported a mutant in which control of the *meta*-cleavage pathway was affected. In this mutant the ability of *p*-cresol to induce the *meta* pathway was lost although other phenolic compounds still caused induction.

Recent work has illustrated that the catabolism of some aromatic compounds is encoded by transmissible plasmids. The NAH (Dunn and Gunsalus 1973), SAL (Chakrabarty 1972) and TOL (Williams and Murray 1974; Wong and Dunn 1974) plasmids encode the catechol *meta*-cleavage pathway. These plasmids facilitate use of genetic manipulation in the study of catabolic pathways. This was exemplified in work reported by Wong and Dunn (1976). A strain of *P. putida* with a blocked catechol *ortho*-cleavage pathway was constructed by mutation. Transfer of the TOL plasmid into this strain permitted very slow growth on phenol. This was due to the conversion of phenol into catechol by the host-cell-encoded phenol 2-monooxygenase and subsequent catabolism of catechol by low uninduced levels of the plasmid-encoded catechol *meta*-cleavage pathway. A spontaneous mutant in which phenol induced the plasmid-encoded pathway was easily isolated. This general approach of strain construction and plasmid manipulation permits the isolation of regulatory mutants and enables the nature and location of such mutations to be established.

This paper reports the construction of host cell and plasmid combinations which facilitate the isolation of mutants with altered metabolic control of the NAH-encoded catechol *meta*-cleavage pathway.

Materials and Methods

The bacterial strains, plasmids and plasmid mutants used in this study are listed in Table 1. References for general procedures and media are as reported by White and Dunn (1977).

Table 1. Bacterial strains and plasmids[A]

Bacterial strains		
Strain No.	Genotype	Reference
PP1-2	*wt*	Wong and Dunn (1974)
PP1-3	*ben*-1	Wong and Dunn (1974)
PP1-10	*ben*-2	Austen and Dunn (1977)
PP1-18	*ben*-1, *phl*-1	Wong and Dunn (1976)
PP1-24	*ben*-1, *met*-2, *pfs*-3, *str*-2	Wong and Dunn (1976)
PAr1-6 (TOL)	*met*-1, *tra*-1, (TOL)	Wong and Dunn (1976)
PP2-4 (NAH)	*leu*-1, *tra*-1, (NAH)	Austen and Dunn (1977)
Catabolic plasmids		
Designation		Characteristics or reference
NAH		Austen and Dunn (1977)
TOL		Austen and Dunn (1977)
pND110		NAH plasmid-encoded regulatory mutant
pND111		NAH plasmid-encoded constitutive mutant

[A] Abbreviations: ben, benzoate; leu, leucine; met, methionine; NAH, plasmid encoding the degradation of naphthalene; pfs, resistance to pf16; phl, phenol; PP, *P. putida;* PAr, *P. arvilla;* str, resistance to streptomycin; TOL, plasmid encoding the degradation of toluate; tra, increased transfer frequency of resident plasmid; wt, wild type.

Growth of organisms, preparation of cell-free extracts, estimation of protein and assay of catechol 2,3-dioxygenase (EC 1.13.11.2) at pH 8·5 are as reported by Austen and Dunn (1977). In all assays the primary growth substrate was 10 mM acetate. All inducers were added to a concentration of 5 mM except phenol which was added to a concentration of 2·5 mM. One unit of catechol 2,3-dioxygenase activity is defined as that which oxidizes 1·0 μmol of catechol per minute.

The method used for plasmid transfer by conjugation is as reported by Austen and Dunn (1977). When donor strains were auxotrophic, auxotrophic contraselection was employed to select against the donor strain. Streptomycin contraselection was employed against prototrophic donors. If recipient strains were auxotrophic then the appropriate amino acid was incorporated into the selection medium. The method of mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NG) was as reported by Dunn and Gunsalus (1973).

Results

Isolation of Mutants

(i) *Induction of the NAH-encoded catechol 2,3-dioxygenase*

The NAH plasmid encodes the degradation of both naphthalene and salicylate. After introduction of NAH into PP1-3 [functional benzoate 1,2-dioxygenase (EC 1.13.99.2) and phenol 2-monooxygenase but defective catechol 1,2-dioxygenase], PP1-3 (NAH) has the additional genetic information and hence the potential to utilize *m*-toluate, benzoate, and phenol. The host cell is able to convert these substrates into the corresponding catechol compounds which could be further catabolized via the plasmid-encoded catechol *meta*-cleavage pathway. However, this strain is unable to grow on either phenol or benzoate but can grow slowly on *m*-toluate (Austen and Dunn 1977). It is possible that the inability to grow on phenol and benzoate arises because (1) neither compound causes significant induction of the enzymes of the catechol *meta*-cleavage pathway or (2) rapid conversion, which results in the accumulation and chemical oxidation of catechol, causes toxicity problems. To clarify this situation induction of catechol 2,3-dioxygenase of the NAH-encoded catechol *meta*-cleavage pathway was studied.

Table 2. Induction of catechol 2,3-dioxygenase encoded by the NAH plasmid

Strain	Inducer	Activity[A]	Strain	Inducer	Activity[A]
PP1-3 (NAH)	None	10	PP1-10 (NAH)	Benzoate	10
	Salicylate	395		*p*-Toluate	10
	m-Toluate	190		Phenol	5
	Benzoate	0	PP1-18 (NAH)	None	15
	p-Toluate	25		Salicylate	460
	Phenol	0		*m*-Toluate	180
PP1-10 (NAH)	None	10		Benzoate	0
	Salicylate	365		*p*-Toluate	25
	m-Toluate	170		Phenol	5

[A] Activity of catechol 2,3-dioxygenase expressed as milli-units per milligram protein.

The NAH plasmid was transferred into PP1-3, PP1-18 (functional benzoate 1,2-dioxygenase but defective phenol 2-monooxygenase and catechol 1,2-dioxygenase) and PP1-10 (functional phenol 2-monooxygenase and catechol 1,2-dioxygenase but defective benzoate 1,2-dioxygenase). For comparative purposes growth responses of these strains are listed in Table 5. Catechol 2,3-dioxygenase was assayed following growth of these strains on acetate (15 mM acetate and no inducer) and acetate plus the following inducers: salicylate, *m*-toluate, *p*-toluate, benzoate, and phenol. The results of three separate experiments were used to calculate the specific enzyme activities which are given in Table 2.

Salicylate caused the highest levels of induction whereas *m*-toluate caused intermediate levels of induction. Induction over the genetic block in PP1-10 (NAH) indicates that *m*-toluate caused the observed induction. Benzoate, phenol and *p*-toluate did not cause significant induction when compared to the uninduced enzyme level. However, even this low uninduced level could not be detected when phenol or benzoate were converted into catechol in strains where the catechol could not be further utilized. In all such flasks the media turned black due to the accumulation and chemical oxidation of catechol. In those strains in which the host-cell-encoded conversion by phenol 2-monoxygenase and benzoate 1,2-dioxygenase had been eliminated, low uninduced levels of catechol 2,3-dioxygenase were once again observed. *p*-Toluate is not converted into 4-methylcatechol by the host cell (Austen and Dunn 1977), therefore no toxicity problems were observed in relation to this inducer. Function of the catechol *ortho* cleavage pathway in PP1-10 (NAH) did not appear to significantly affect induction of catechol 2,3-dioxygenase when compared to PP1-3 (NAH) and PP1-18 (NAH) which were defective in this pathway.

(ii) *Mutant selection*

The toxicity of catechol and/or its oxidation products may cause problems in the selection of mutants with altered regulatory control in the catechol *meta*-cleavage pathway. In theory, it should have been possible to plate directly onto PAS (minimal salts) medium supplemented with *m*-toluate, benzoate or phenol in order to isolate the desired mutants. However, to select the required rare mutant it was necessary to plate high cell numbers (10^9 cells per plate) onto the solid selection plates. This approach resulted in a rapid coloration of the selection medium; the intensity of the coloration, by visual observation, was benzoate > phenol > *m*-toluate.

An attempt to isolate spontaneous NAH mutants was made by plating PP1-3 (NAH) onto PAS + phenol (2·5 mM) and PAS + benzoate (2·5 and 5·0 mM) selection plates. NG-induced mutants were selected by plating PP1-3 (NAH) onto PAS + *m*-toluate (5 mM). Mutants able to grow on phenol appeared at a frequency of approximately 10^{-7} per cell plated. Six of the mutants were purified and retained for further study. Large colonies appeared on the *m*-toluate plates at 10^{-7} per cell plated and could be clearly distinguished from the parent colonies which grew slowly. The *m*-toluate-selected mutants displayed variable colony morphology on *m*-toluate but uniform morphology on nutrient agar. Considerable difficulty was encountered in obtaining a mutant with uniform morphology on *m*-toluate, but one isolate was obtained and retained for further study. No benzoate-selected mutants were observed probably because of the toxicity of catechol and its oxidation products. However, benzoate-utilizing clones were isolated during a study of the compatibility of the NAH and TOL plasmids.

Using benzoate for selection, TOL was transferred by conjugation from PAr1-6 (TOL) into PP1-3 (NAH). The selection plates became black due to the accumulation of catechol oxidation products, and transconjugants (approximately 10^{-7} per donor cell) appeared after only 7 days incubation and grew very slowly under these conditions. One hundred transconjugants were purified by streaking for single colonies on benzoate plates and when tested 96 were unable to grow on naphthalene, which indicated NAH plasmid segregation. Further tests showed that the other four colonies had a stable naphthalene growth phenotype. They were unable to grow on *p*-toluate but grew slowly on *m*-toluate and benzoate. A black coloration was observed around

the colonies during growth on the latter substrate. One of these colonies was chosen for a detailed study and was designated N+B+(B). Assuming that this phenotype was due to the presence of a plasmid, the most probable explanations were that (1) recombination between TOL and NAH had occurred, or (2) a mutant NAH plasmid had been selected.

Initially mitomycin C was used in an attempt to cure either the naphthalene or benzoate growth phenotype but this proved unsuccessful. However, 10% of the colonies tested on benzoate caused no black coloration of the medium during growth on benzoate. This new phenotype was designated N+B+(C). Additional curing experiments revealed that PP1-3 (N+B+(C)) could grow in the presence of nutrient broth supplemented with mitomycin C at 15 μg/ml whereas PP1-3 (N+B+(B)) could grow in the same medium with mitomycin C at only 5 μg/ml.

Table 3. Genetic location of the N+B+(B) and N+B+(C) phenotypes

Donor	Recipient	Phenotype of transconjugants[A]
PP1-24 (NAH)	PP1-3	PP1-3 (NAH)
	PP1-3 (N+B+(B))−	PP1-3 (NAH)
	PP1-3 (N+B+(C))−	PP1-3 (NAH)
PP1-24 (N+B+(B))	PP1-3	PP1-3 (N+B+(B))
	PP1-3 (N+B+(B))−	PP1-3 (N+B+(B))
	PP1-3 (N+B+(C))−	PP1-3 (N+B+(C))
PP1-24 (N+B+(C))	PP1-3	PP1-3 (N+B+(B))
	PP1-3 (N+B+(B))−	PP1-3 (N+B+(B))
	PP1-3 (N+B+(C))−	PP1-3 (N+B+(C))

[A] Strains which showed the N+B+(B) phenotype did not always cause black coloration of the medium. This was shown to be due to the age of the inoculum used. Black coloration was never observed with strains which showed the N+B+(C) phenotype irrespective of the age of the inoculum.

Since mitomycin C curing was not successful, forced segregation was attempted by the introduction of the TOL plasmid using *p*-toluate for selection. TOL transferred from PAr1-6 (TOL) into both PP1-3 (N+B+(B)) and PP1-3 (N+B+(C)) at frequencies of approximately 10^{-6} transconjugants per donor cell. Ten transconjugants from each cross were purified and growth tests showed that they had lost the ability to grow on naphthalene. Since TOL encodes the degradation of *m*-toluate and benzoate, segregation of these phenotypes could not be tested. Therefore TOL was cured spontaneously (approximately 1% of cells tested) from each strain and one cured colony was retained from each strain and called PP1-3 (N+B+(B))− and PP1-3 (N+B+(C))− respectively. These colonies were unable to utilize either benzoate or *m*-toluate and in fact had the same phenotypic properties as the parent strain PP1-3.

The plasmids harboured by PP1-3 (N+B+(B)) and PP1-3 (N+B+(C)) were transferred by conjugation into PP1-24 using a streptomycin contraselection and selecting for growth on naphthalene. Transconjugants were obtained at a frequency of 10^{-7} and 10^{-8} per donor cell respectively. To determine the difference between PP1-3 (N+B+(B)) and PP1-3 (N+B+(C)), the parent NAH plasmid and the

plasmids carried by PP1-24 (N+B+(B)) and PP1-24 (N+B+(C)) were transferred into PP1-3, PP1-3 (N+B+(B))− and PP1-3 (N+B+(C))−. The growth phenotypes of the resultant transconjugants were established (Table 3). Whether the plasmid was obtained originally from PP1-3 (N+B+(B)) or PP1-3 (N+B+(C)), the phenotypes of the transconjugants were the same for each recipient. Therefore the difference between the original PP1-3 (N+B+(B)) and PP1-3 (N+B+(C)) isolates appears to be in the host cell. The N+B+(C) phenotype was observed after introduction of either plasmid into PP1-3 (N+B+(C))−. The N+B+(B) phenotype was observed after introduction of either plasmid into PP1-3 or PP1-3 (N+B+(B))−. Therefore the ability to degrade benzoate appeared to be plasmid associated. This benzoate growth phenotype was not observed when the original NAH plasmid was introduced into any of the three recipients. These results suggested that the plasmid in both PP1-3 (N+B+(B)) and in PP1-3 (N+B+(C)) was the same plasmid. The plasmid in PP1-3 (N+B+(B)) was therefore retained for additional studies. It is important to note that all that has been shown so far is that the (N+B+(B) phenotype moves with the resident plasmid. Insufficient evidence is available to state whether this phenotype arises from a mutation of NAH or by recombination between NAH and TOL. This is clarified by the results presented later in the paper and so for convenience the N+B+(B) plasmid shall hereon be referred to as an NAH mutant plasmid.

In summary, three types of mutants were isolated: (1) those able to grow on phenol, (2) those better able to grow on *m*-toluate, and (3) those able to grow on benzoate (N+B+(B)).

Table 4. Growth responses of mutants on solid minimal agar medium

Incubation temperature was 30°C and incubation time was 3 days. Responses were rated as follows: 4+, colony size *c.* 2·0 mm in diameter; 3+, 1 mm; 2+, 0·5 mm; +, 0·1 mm; −, no growth. L indicates that large colonies, at least 0·5 mm in size, grew after 5 days at a frequency of *c.* 10^{-7}. B indicates a black coloration of the medium after *c.* 2 days

Strain(s)	Carbon source[A]					
	Nah	Sal	*m*-Tol	*p*-Tol	Ben	Phl
PP1-3	—	—	−(B)	—	−(B)	−(B)
PP1-3 (NAH)	4+	4+	+	—	−(B)	−(B, L)
PP1-3 (NAH–phl selected mutants)	4+	4+	2+	—	3+(B)	2+
PP1-3 (NAH–*m*-tol selected mutant)	—	4+	2+	—	3+(B)	−(B, L)
PP1-3 (NAH–ben selected mutant, N+B+(B))	4+	4+	2+	—	3+(B)	2+

[A] Abbreviations: Nah, naphthalene; Sal, salicylate; *m*-Tol, *m*-toluate; *p*-Tol, *p*-toluate; Ben, benzoate; Phl, phenol.

(iii) *Growth responses of the three types of mutants*

The growth phenotypes of the isolated mutants were determined (Table 4). It is apparent that the strain isolated on benzoate [PP1-3 (N+B+(B))] had the same phenotype as the six mutants that were isolated on phenol. They all grew on *m*-toluate, benzoate, and phenol. On the other hand, the strain isolated on *m*-toluate was quite different. This strain grew on both *m*-toluate and benzoate but not on phenol.

However, after further incubation large colonies grew on the latter carbon source. Unexpectedly, this mutant was unable to grow on naphthalene but had retained the ability to grow on salicylate. It was decided to study the *m*-toluate-selected mutant and the benzoate-selected mutant in more detail. The plasmids present in these two strains were named pND110 and pND111 respectively.

Table 5. Determination of the pND110 and pND111 encoded growth phenotypes on solid minimal agar
Incubation conditions and response ratings are as in Table 4

Strain	Carbon source[A]					
	Nah	Sal	*m*-Tol	*p*-Tol	Ben	Phl
PP1-3	—	—	−(B)	—	−(B)	−(B)
PP1-10	—	—	—	—	—	3+
PP1-18	—	—	−(B)	—	−(B)	—
PP1-3 (NAH)	4+	4+	+	—	−(B)	−(B, L)
PP1-10 (NAH)	4+	4+	—	—	—	3+
PP1-18 (NAH)	4+	4+	+	—	−(B)	—
PP1-3 (pND110)	—	4+	2+	—	3+(B)	−(B, L)
PP1-10 (pND110)	—	4+	—	—	—	3+
PP1-18 (pND110)	—	4+	2+	—	3+(B)	—
PP1-3 (pND111)	4+	4+	2+	—	3+(B)	2+
PP1-10 (pND111)	4+	4+	—	—	—	3+
PP1-18 (pND111)	4+	4+	2+	—	3+(B)	—

[A] Abbreviations as in Table 4.

Genetic Characterization of the Mutant Plasmids, pND110 and pND111

The host-cell- and plasmid-encoded contribution to the phenotypes associated with pND110 and pND111 plasmid residence were established by using PP1-3, PP1-10 and PP1-18 as host strains. The plasmid pND111 was introduced into these strains from the previously constructed PP1-24 (N+B+(B)) [now PP1-24 (pND111)] using auxotrophic contraselection. Similarly, pND110 was transferred from PP1-3 (pND110) into PP1-24 using streptomycin contraselection and then transferred into PP1-3, PP1-10 and PP1-18 using auxtrophic contraselection. Transconjugants were purified in all cases and the growth responses to a range of carbon sources were determined (Table 5). It is apparent that conversion of *m*-toluate and benzoate into 3-methylcatechol and catechol respectively in PP1-3 (pND110) and PP1-3 (pND111) is a host-cell-encoded phenotype as no growth was observed on these carbon sources in the absence of the host cell benzoate 1,2-dioxygenase [PP1-10 (pND110) and PP1-10 (pND111)]. Similarly, no growth was ever observed on phenol in the absence of a host-cell-encoded phenol 2-monooxygenase. Other growth responses were as expected from results reported previously in this paper and from results reported by Austen and Dunn (1977).

Earlier in this paper it was shown that pND111 plasmid could be forced to segregate by introduction of TOL using *p*-toluate for selection of transconjugants. A similar transfer was carried out using PAr1-6 (TOL) as donor and PP1-3 (pND110) as recipient and selecting for growth on *p*-toluate. Twenty transconjugants were purified by streaking for single colonies three times on the selection medium. Tests showed that 19 transconjugants had lost the ability to grow on salicylate and therefore illustrated the plasmid nature of pND110.

Biochemical Characterization of pND110 and pND111

PP1-3 (pND110) and PP1-3 (pND111) have growth phenotypes which differ from PP1-3 (NAH). Genetic studies reported in this paper indicate that the growth phenotype changes are the result of changes in the resident plasmid genotype and are not simply alterations in the host cell. It was anticipated that these changes should be reflected by assays of the catechol 2,3-dioxygenase following induction by different substrates.

Table 6. Induction of catechol 2,3-dioxygenase encoded by the mutant plasmids pND110 and pND111

Host	Inducer	Activity[A]		Host	Inducer	Activity[A]	
		pND110	pND111			pND110	pND111
PP1-3	None	30	700	PP1-10	None	30	630
	Salicylate	625	580		Salicylate	620	725
	m-Toluate	290	655		*m*-Toluate	530	770
	Benzoate	100	750		Benzoate	555	620
	p-Toluate	250	720		*p*-Toluate	350	650
	Phenol	15	485	PP1-18	None	30	570
					Salicylate	595	635
					Phenol	20	620

[A] Activity of catechol 2,3-dioxygenase expressed as milli-units per milligram protein.

Where possible induction over genetic blocks was determined and the results are given in Table 6. pND110 showed no significant induction when phenol was used as the inducer. However, benzoate, *m*-toluate and *p*-toluate caused synthesis of significantly increased levels of catechol 2,3-dioxygenase when compared with the parent NAH plasmid. This was evident in PP1-10 (pND110) induction studies, i.e. in the absence of a host-cell-encoded benzoate 1,2-dioxygenase. When PP1-3 was used as the host strain, lower enzyme levels were observed, probably because of toxicity problems. It is interesting to note that although *p*-toluate is not a growth substrate (Austen and Dunn 1977), it is also capable of inducing synthesis of catechol 2,3-dioxygenase in strains which harbour pND110.

pND111 showed a different induction pattern to pND110. In this case high levels of catechol 2,3-dioxygenase were produced in the absence of any aromatic inducers. It seemed therefore that this mutant was a constitutive mutant.

No evidence was obtained that TOL had contributed to the phenotype of pND111. The pH optimum of the catechol 2,3-dioxygenase was the same as that of the NAH plasmid-specified enzyme and the plasmid was unable to transfer into *Pseudomonas aeruginosa* (PA01) when this recipient was grown at 43°C which is a characteristic of the NAH, but not the TOL, plasmid (Austen and Dunn 1977). Finally, enzyme assay data obtained with one of the phenol-selected mutants were identical to those obtained with pND111, indicating that the same mutant types can appear in the absence of the TOL plasmid.

Discussion

When working with aromatic systems it is clear that care must be taken to avoid or reduce toxicity problems which may prevent the isolation of desired mutants. Direct selection of mutants on benzoate medium was not achieved; however, mutants

isolated on phenol and *m*-toluate could grow on benzoate. It was only in a conjugation experiment using benzoate selection medium that mutants could be isolated from benzoate plates. On these selection plates, growth was very slow but presumably the presence of TOL in either the donor or recipient was able to reduce the effective catechol concentration and permit growth.

The characterization of pND111 (N+B+(B)) and pND110 illustrated the advantage of working with plasmid systems. It is a relatively straightforward procedure to utilize the plasmid system, within a defined genetic background, to establish the genetic location and metabolic control in such mutants.

The genetic characterization of pND110 and pND111 revealed that growth on phenol, benzoate and *m*-toluate was a composite growth phenotype. The initial conversion into catechol or 3-methylcatechol was encoded by the host cell and then further catabolism was via the plasmid-encoded catechol *meta*-cleavage pathway.

The mutants isolated on phenol and pND111 gave the same growth responses, and comparison by assay of catechol 2,3-dioxygenase revealed that this enzyme was produced constitutively. In pND110 the range of compounds capable of inducing the catechol 2,3-dioxygenase was extended. It is apparent that *m*-toluate is a more effective inducer in this mutant than in the parent NAH plasmid. Benzoate and *p*-toluate are capable of causing high levels of induction whereas no such induction was observed in NAH. Although *p*-toluate caused induction, growth cannot occur on this substrate because neither the plasmid nor the host cell encodes information for the conversion of *p*-toluate to 4-methylcatechol (Austen and Dunn 1977). Introduction of pND110 into a host strain capable of this conversion would permit growth on this substrate as also would introduction of pND111. In the latter case no induction of the plasmid information would be required. Since induction in PP1-10 (pND110) occurs over a genetic block with benzoate and *m*-toluate, and over a natural block with *p*-toluate, it appears that these three substrates are actually acting as inducers. The appearance of large colonies, when PP1-3 (pND110) is plated on phenol, indicates that mutants to this growth phenotype can still be isolated readily.

This paper illustrates theoretically and then practically how control mutants in catabolic pathways can be isolated by utilizing catabolic plasmids. This takes advantage of the fact that plasmids are transmissible and that an appropriate host strain can be obtained. To isolate mutants it is necessary to find a host cell in which a substrate can be degraded to one of the intermediates in the plasmid-encoded pathway. It is a requirement that neither this compound nor any of the intermediates formed can cause significant induction of the plasmid-encoded enzyme system. It is also necessary that this compound be unable to support significant growth of the host cell. This may be achieved by a mutation which blocks the host cell pathway as was the case with PP1-3. Provided that toxicity of any of the intermediates does not inhibit growth, this system permits the direct isolation of control mutants. A reverse situation can also be envisaged whereby a strain can be constructed to enable the isolation of control mutants in the host cell genetic information. Studies aimed at this system are in progress.

This study also provides a method to increase catabolic range. Provided that the host strain has the required catabolic properties to support plasmid function there is the possibility that the introduction of a catabolic plasmid into a new host strain will result in the appearance of new phenotypes. In some cases inducible or constitu-

tive mutants will be required to facilitate use of the appropriate genetic information which would permit growth on the new substrate(s).

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