

Regulation of the Plasmid-specified Naphthalene Catabolic Pathway of *Pseudomonas putida*

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The regulation of the catabolic pathway for the degradation of naphthalene specified by the plasmids NAH, pND140 and pND160 was studied using plasmid-borne regulatory mutants in a *Pseudomonas putida* host strain. Growth of strains harbouring the parent plasmids in the presence of salicylate resulted in induction of selected enzymes involved in the conversion of naphthalene to catechol (naphthalene oxygenase, salicylaldehyde dehydrogenase and salicylate hydroxylase) and enzymes of the *meta*-cleavage pathway (catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde hydrolase and 2-hydroxymuconic semialdehyde dehydrogenase). Partial induction was also observed for all the NAH-encoded enzymes assayed when using *m*-toluate as the inducing compound, over a genetic block. Mutants were obtained for each plasmid where the three enzymes of the *meta*-cleavage pathway were produced constitutively suggesting that the enzymes of the *meta*-cleavage pathway belong to one operon. In these mutants, enzymes involved in the conversion of naphthalene to catechol were not produced constitutively but remained inducible during growth on salicylate indicating that these enzymes belong to a separate operon or operons.

INTRODUCTION

The pathway for the degradation of naphthalene found in several pseudomonads is outlined in Fig. 1. Naphthalene is degraded to catechol, and all three plasmids studied in this work, NAH (Dunn & Gunsalus, 1973) and pND140 and pND160 (Dunn *et al.*, 1980), encode the *meta*-cleavage pathway for the subsequent metabolism of catechol. In some other naphthalene-utilizing strains which possess this same pathway no evidence has been obtained to implicate plasmids. Many of the naphthalene-utilizing strains which have the *meta*-cleavage pathway for catechol breakdown also have the *ortho*-cleavage pathway. In strains which have both pathways for the degradation of catechol, the route of catechol metabolism depends on the relative induction of each pathway (Davies & Evans, 1964; Catterall *et al.*, 1971 *a, b*; Jerina *et al.*, 1971; Barnsley, 1976 *a, b*).

There are at present few published results concerning the regulation of the plasmid-encoded naphthalene catabolic pathway. It has been reported that in *Pseudomonas putida* strain PpG7 (ATCC 17485), which harbours the NAH plasmid, salicylate is the inducer of the enzymes which convert naphthalene to catechol and of the enzymes of the *meta*-cleavage pathway for catechol (Barnsley, 1975, 1976 *a*). The possibility was suggested that all the enzymes involved in the catabolism of naphthalene are regulated as a unit in strain PpG7.

The present paper describes aspects of the regulation of the naphthalene catabolic pathways encoded by plasmids NAH, pND140 and pND160 and describes properties of some regulatory mutants of these plasmids.

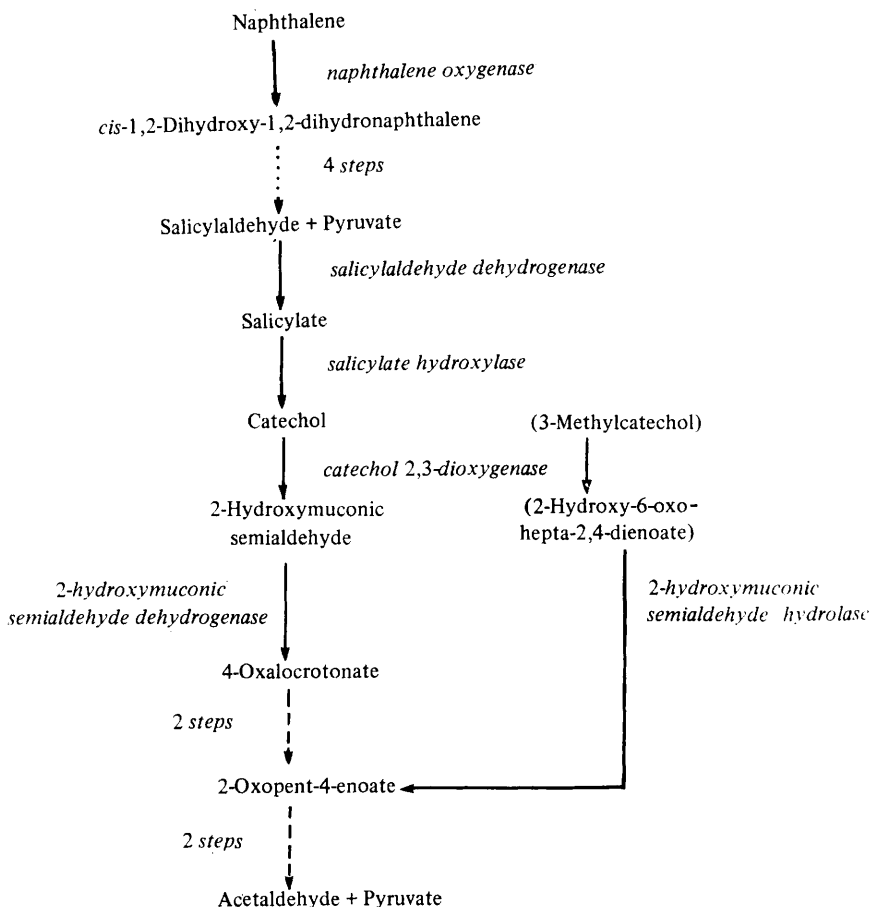


Fig. 1. Outline of the plasmid-encoded naphthalene catabolic pathway. The plasmid-encoded catechol 2,3-dioxygenase has a broad specificity and, in addition to the conversion of catechol to 2-hydroxymuconic semialdehyde, it is able to convert 3-methylcatechol to 2-hydroxy-6-oxohepta-2,4-dienoate. These two compounds are then degraded by different mechanisms within the *meta*-cleavage pathway.

METHODS

Bacterial strains and plasmids. The bacteria and plasmids, listed in Table 1, were handled as previously described (Dunn & Gunsalus, 1973; Austen & Dunn, 1977*a*; White & Dunn, 1977; Dunn *et al.*, 1980).

Isolation of spontaneous plasmid mutants. Six large colonies of PP1-3(pND140) and PP1-3(pND160) from PAS plus benzoate growth response plates (Dunn *et al.*, 1980) were purified by single colony isolation on benzoate and then tested for growth on several aromatic substrates (see Table 2). All had gained the ability to grow well on benzoate and phenol but still could not grow on *m*-toluate. One mutant derived from pND140 and one from pND160 were retained for further study and were designated pND141 and pND161, respectively.

Culture conditions for organisms used in enzyme assays. The minimal salts medium was PAS (Chakrabarty, 1972). Cultures were grown overnight at 30 °C with shaking in PAS plus 10 mM-acetate plus 5 mM-succinate and then added as a 25 % (v/v) inoculum to PAS plus 10 mM-acetate plus 5 mM inducer or PAS plus 15 mM-acetate plus 5 mM inducer (induction over a genetic block) or PAS plus 15 mM-acetate (no inducer). The latter cultures were incubated at 30 °C with shaking for 3.5 h prior to assay of catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase and for 6 h prior to assay of naphthalene oxygenase, salicylaldehyde dehydrogenase, salicylate hydroxylase and catechol 2,3-dioxygenase. These induction conditions were chosen following a study of primary growth substrates, inducers and induction periods. No diauxic growth curves or catabolite repression of the enzymes studied in the naphthalene catabolic pathway were observed when using acetate as the primary growth substrate.

Table 1. *Bacterial strains and plasmids*

<i>Strains of Pseudomonas putida*</i>		
PP1-2	wild-type	Wong & Dunn (1974)
PP1-3	<i>ben-1</i>	Wong & Dunn (1974)
PP1-10	<i>ben-2</i>	Austen & Dunn (1977a)
<i>Catabolic plasmids</i>		
NAH	Dunn & Gunsalus (1973)	
pND110	NAH control mutant (Austen & Dunn, 1977b)	
pND111	NAH control mutant (Austen & Dunn, 1977b)	
pND140	New plasmid specifying naphthalene degradation (Dunn <i>et al.</i> , 1980)	
pND141	pND140 control mutant	
pND160	New plasmid specifying naphthalene degradation (Dunn <i>et al.</i> , 1980)	
pND161	pND160 control mutant	
TOL	Williams & Murray (1974); Wong & Dunn (1974)	

* The parental bacterial host strain (PP1-2) can convert phenol and benzoate to catechol independently and catechol is then degraded by the *ortho*-cleavage pathway. The enzyme system responsible for conversion of benzoate to catechol is also able to convert *m*-toluate to 3-methylcatechol; however, 3-methylcatechol cannot be further metabolized through the *ortho*-cleavage pathway (Austen & Dunn, 1977a). Strain PP1-3 is defective in catechol 1,2-dioxygenase, the first enzyme of the *ortho*-cleavage pathway; PP1-10 is defective in the conversion of benzoate to catechol.

Preparation of extracts used in assays of salicylaldehyde dehydrogenase, salicylate hydroxylase and catechol 2,3-dioxygenase. Bacteria were harvested by centrifuging at 2 °C, washed twice with cold 50 mM-sodium phosphate buffer (pH 7.0) and resuspended at a concentration of about 0.1 g wet wt ml⁻¹ in similar buffer. This suspension (5 ml) was disrupted at 2 °C using three 1 min treatments with a Bronwill Sonic Oscillator model BIOIV using the needle probe at the LO 80 setting. Cell debris was removed by centrifuging at 39000 g for 1 h at 4 °C (Barnsley, 1975).

Preparation of extracts used in assays of enzymes of the meta-cleavage pathway for catechol. Bacteria were harvested by centrifuging at 2 °C, washed twice with cold 0.85 % (w/v) KCl, resuspended in 4 ml cold 50 mM-phosphate buffer (pH 7.5) containing acetone (10 %, v/v), and disrupted by sonication at 2 °C using three 1 min treatments as above. Cell debris was removed by centrifuging at 12000 g for 10 min at 2 °C (Austen & Dunn, 1977a).

Preparation of 2-hydroxymuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoate. Catechol 2,3-dioxygenase was purified (as far as the first crystallization) from benzoate-induced PP1-3 (TOL) using the method described by Nozaki *et al.* (1963). A sample (0.2 ml) of the purified catechol 2,3-dioxygenase in 0.05 mM-phosphate buffer (pH 7.5) containing acetone (10 %, v/v) was added to 3 µmol catechol or 3-methylcatechol and 280 µmol sodium phosphate buffer (pH 7.0) in a total volume of 3 ml. The solutions were shaken intermittently at ambient temperature for 10 min and then kept in ice and used the same day. The 0.2 ml sample of catechol 2,3-dioxygenase contained sufficient activity to oxidize 20 µmol catechol min⁻¹.

NAD glycohydrolase treatment. Extract (1 ml) was incubated for 1 h with 3.5 mg NAD glycohydrolase (Sigma) at ambient temperature and then centrifuged at 5000 g for 5 min at 4 °C. The supernatant was kept in ice and used the same day. This method is similar to that used by Sala-Trepat *et al.* (1972).

Protein and dry weight determinations. Protein was estimated using the Lowry method with bovine serum albumin as the standard. Dry weights were determined by evaporating samples (5 ml) of the washed whole-cell suspension to dryness in a hot air oven at 105 °C and correcting for buffer residue weights.

Enzyme assays. All spectrophotometric assays were done at 20 °C using silica cuvettes (1 cm light path) in a recording spectrophotometer. All extracts and cell suspensions were used during the day on which they were prepared.

Naphthalene oxygenase was measured spectrophotometrically (Shamsuzzaman & Barnsley, 1974a) using whole cells. The absorption coefficient of naphthalene at 276 nm used in calculations of reaction rates was 4510 l mol⁻¹ cm⁻¹ (not corrected for the presence of salicylate). The specific activities presented are therefore minimum values. One unit of activity is defined as that which converts 1 µmol naphthalene min⁻¹.

Salicylaldehyde dehydrogenase was assayed according to the method of Shamsuzzaman & Barnsley (1974b). One unit of enzyme activity is defined as that which reduces 1 µmol NAD min⁻¹.

Salicylate hydroxylase (EC 1.14.13.1) was assayed according to the method of Barnsley (1975). One unit of enzyme activity is defined as that which oxidizes 1 µmol NADH min⁻¹.

Catechol 2,3-dioxygenase (EC 1.13.11.2) was determined by measuring the rate of increase in absorbance at 375 nm due to conversion of catechol to 2-hydroxymuconic semialdehyde (Nozaki *et al.*, 1963). The assay mixture contained 560 μmol Tris buffer (pH 8.5), 0.1 μmol catechol and extract in a total volume of 3 ml. Conversion of 1 μmol catechol to 2-hydroxymuconic semialdehyde at pH 8.5 causes an increase in A_{375} of 12.4 (Austen & Dunn, 1977a). One unit of enzyme activity is defined as that which converts 1 μmol catechol min^{-1} .

2-Hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase were assayed at pH 8.0 which is optimal for activity of the NAH-encoded enzymes. 2-Hydroxymuconic semialdehyde hydrolase activity was determined at 388 nm using 2-hydroxy-6-oxohepta-2,4-dienoate (the catechol 2,3-dioxygenase ring fission product of 3-methylcatechol) as substrate according to the method of Murray & Williams (1974). 2-Hydroxymuconic semialdehyde dehydrogenase activity was determined by subtracting the rate of disappearance of 2-hydroxymuconic semialdehyde in an assay mixture which contained an extract treated with NAD glycohydrolase from the rate in an assay mixture which contained extract supplemented with NAD. The rate of 2-hydroxymuconic semialdehyde disappearance was determined by measuring the rate of decrease in A_{375} . The former assay mixture contained 280 μmol sodium phosphate buffer (pH 8.0), 0.1 μmol 2-hydroxymuconic semialdehyde and extract treated with NAD glycohydrolase in a total volume of 3 ml. The latter assay mixture contained 280 μmol sodium phosphate buffer (pH 8.0), 0.1 μmol 2-hydroxymuconic semialdehyde, 1 μmol NAD and extract in a total volume of 3 ml (Sala-Trepat *et al.*, 1972). Conversion of 1 μmol 2-hydroxy-6-oxohepta-2,4-dienoate and 2-hydroxymuconic semialdehyde causes decreases in A_{388} and A_{375} of 7.1 and 11.7, respectively. Units of enzyme activity are defined as the conversion of 1 μmol substrate min^{-1} .

RESULTS AND DISCUSSION

Plasmid-encoded mutations were used to study regulation within the naphthalene catabolic pathways encoded by NAH, pND140 and pND160. The isolation of two mutants of the NAH plasmid, pND110 and pND111, has been described by Austen & Dunn (1977b). Plasmid pND110 specifies a catechol 2,3-dioxygenase with an altered inducer specificity and has lost the ability to utilize naphthalene. Plasmid pND111 encodes a catechol 2,3-dioxygenase which appears to be produced constitutively.

Growth responses for PP1-2, PP1-3 and PP1-10 (Table 2) are in accordance with the strain characteristics described in Table 1. Strain PP1-3 harbouring the regulatory mutant pND111 acquired the ability to grow well on phenol and benzoate and to give improved growth on *m*-toluate; PP1-3(pND110) acquired the last two growth phenotypes. When growth is observed on phenol, benzoate or *m*-toluate in the absence of a functional *ortho*-cleavage pathway, the growth phenotypes are composite phenotypes; the host cell converts each compound to the corresponding catechol and then these are degraded via the plasmid-encoded *meta*-cleavage pathway. Consequently PP1-10 harbouring these plasmids is unable to grow on benzoate or *m*-toluate and this can be useful as it permits the use of benzoate and *m*-toluate as inducers, over a genetic block. It also eliminates toxicity problems when a strain can convert benzoate or *m*-toluate to the corresponding catechol but cannot further catabolize these compounds.

The growth responses associated with residence of plasmids pND140 and pND160 in both PP1-3 and PP1-10 and mutant plasmids pND141 and pND161 in PP1-3 are given in Table 2. Plasmid-associated growth on benzoate, *m*-toluate and phenol required the use of the host cell-encoded ability to convert these substrates to catechol or 3-methylcatechol (Dunn *et al.*, 1980). Comparison with the growth responses of PP1-3(pND140) and PP1-3(pND160) indicated that PP1-3(pND141) and PP1-3(pND161) had gained the ability to grow well on benzoate and phenol but, surprisingly, not on *m*-toluate. Assay of the pND141 and pND161-encoded catechol 2,3-dioxygenases indicated that the enzyme was produced constitutively and did not require the presence of aromatic inducers (Table 3). That is, the pND141 and pND161 mutants appeared to be similar to pND111.

The observation that a single mutation causes a change in the regulation of several enzymes simultaneously suggests that the genes which specify these enzymes may share a control element. Therefore, selected NAH, pND110, pND111, pND140, pND141, pND160

Table 2. Growth responses of *P. putida* strains harbouring naphthalene catabolic plasmids

Incubation was for 3 d at 30 °C. Responses were rated as follows: 4+, colonies about 2 mm diam.; 3+, 1 mm; 2+, 0.5 mm; +, 0.1 mm; —, no growth. L indicates that large colonies, at least 0.5 mm diam., appeared after 2 to 5 d incubation at a frequency of about 10^{-7} on benzoate or phenol or after about 8 d on *m*-toluate. B indicates a black or brown coloration of the medium after about 2 d incubation.

Bacterial and plasmid strain	Growth response on substrate					
	Naphthalene (vapour phase)	Salicyl-aldehyde (2.5 mm)	Salicylate (10 mm)	Benzoate (10 mm)	<i>m</i> -Toluate (10 mm)	Phenol (2.5 mm)
PP1-2	—	—	—	4+	—(B)	3+
PP1-3	—	—	—	—(B)	—(B)	—(B)
PP1-10	—	—	—	—	—	3+
PP1-3(NAH)	4+	+	4+	—(B)	+	—(B,L)
PP1-10(NAH)	4+	+	4+	—	—	3+
PP1-3(pND110)	—	—	4+	3+(B)	2+	—(B,L)
PP1-10(pND110)	—	—	4+	—	—	3+
PP1-3(pND111)	4+	+	4+	3+(B)	2+	2+
PP1-10(pND111)	4+	+	4+	—	—	3+
PP1-3(pND140)	4+	+	4+	—(B,L)	—(B)	—(B,L)
PP1-10(pND140)	4+	+	4+	—	—	3+
PP1-3(pND141)	4+	+	4+	4+	—(B,L)	3+
PP1-3(pND160)	4+	+	4+	—(B,L)	—(B)	—(B,L)
PP1-10(pND160)	4+	+	4+	—	—	3+
PP1-3(pND161)	4+	+	4+	4+	—(B,L)	3+

and pND161-encoded naphthalene catabolic pathway enzymes were assayed in order to determine the overall regulatory changes that had arisen as a result of the mutations. Naphthalene oxygenase, salicylaldehyde dehydrogenase and salicylate hydroxylase were chosen as representatives of the enzymes which convert naphthalene to catechol. Catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase were chosen as representatives of the *meta*-cleavage pathway enzymes (Table 3).

Growth in the presence of salicylate induced each of the six enzymes in PP1-10(NAH). Benzoate did not elicit any significant increase in the constitutive activity of each enzyme but *m*-toluate appeared to cause partial induction of each enzyme. In other words, the NAH-specified naphthalene oxygenase, salicylaldehyde dehydrogenase, salicylate hydroxylase and initial *meta*-cleavage pathway enzymes have similar inducer specificities. This result suggested that these enzymes share a regulatory gene.

The observation that PP1-10(pND110) lacked detectable naphthalene oxygenase and salicylaldehyde dehydrogenase activities explains the inability of PP1-10(pND110) to grow on naphthalene (Table 2). Because no naphthalene-utilizing revertants of PP1-10(pND110) could be obtained it is possible that the absence of these activities was the result of a deletion in the NAH plasmid DNA. Comparison with NAH-specified enzyme assay results indicates that the pND110-specified catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase all exhibited similar altered inducer specificity. The similarity and simultaneity of these inducer specificity alterations suggests that these enzymes are controlled by one regulatory gene. It appears that salicylate hydroxylase is controlled by the same regulatory gene but since the induction ratio is low compared to the other three enzymes, this enzyme may belong to a different operon.

The pND111-specified catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase were constitutive and non-inducible in PP1-10(pND111). In contrast, the pND111-specified naphthalene oxygenase,

Table 3. *Activities of selected enzymes specified by NAH, pND110, pND111, pND140, pND141, pND160 and pND161 after growth in the presence of various inducers*

Enzyme activities are expressed as munits (mg protein)⁻¹, except those for naphthalene oxygenase which are expressed as munits (mg dry wt)⁻¹. Values given in parentheses are activities expressed as a percentage of the rate obtained with salicylate as inducer; NA indicates that the relative activity could not be calculated. Activities presented for the first four enzymes were the result of a 6 h induction period; a 3.5 h induction period was used for the remaining three enzymes.

Bacterial and plasmid strain	Inducer	Enzyme activity after 6 h induction				Enzyme activity after 3.5 h induction			
		Naphthalene oxygenase	Salicylaldehyde dehydrogenase	Salicylate hydroxylase	Catechol 2,3-dioxygenase	Catechol 2,3-dioxygenase	2-Hydroxymuconic semialdehyde dehydrogenase	2-Hydroxymuconic semialdehyde hydrolase	
PP1-10(NAH)	None	1 (5)	10 (3)	1 (1)	10 (2)	10 (3)	1 (4)	< 0.5 (NA)	
	Salicylate	21 (100)	400 (100)	119 (100)	600 (100)	365 (100)	27 (100)	5 (100)	
	Benzoate	1 (5)	16 (4)	4 (3)	10 (2)	10 (3)	1 (4)	< 0.5 (NA)	
	<i>m</i> -Toluate	7 (33)	80 (20)	23 (19)	235 (39)	170 (47)	14 (52)	2 (40)	
PP1-10(pND110)	None	< 1	< 10	3 (2)	15 (1)	30 (5)	1 (3)	< 0.5 (NA)	
	Salicylate	< 1	< 10	162 (100)	1300 (100)	620 (100)	31 (100)	8 (100)	
	Benzoate	< 1	< 10	50 (31)	1100 (85)	555 (90)	25 (81)	6 (75)	
	<i>m</i> -Toluate	< 1	< 10	45 (28)	900 (69)	530 (86)	29 (94)	6 (75)	
PP1-10(pND111)	None	1 (5)	13 (3)	2 (2)	1000 (71)	630 (87)	35 (95)	8 (100)	
	Salicylate	22 (100)	450 (100)	137 (100)	1400 (100)	725 (100)	37 (100)	8 (100)	
	Benzoate	3 (14)	30 (7)	1 (1)	1200 (86)	620 (86)	35 (95)	7 (88)	
	<i>m</i> -Toluate	6 (27)	90 (20)	32 (23)	1300 (93)	770 (106)	40 (108)	7 (88)	
PP1-10(pND140)	None	< 1 (NA)	< 10 (NA)	< 1 (NA)	40 (7)	35 (12)	5 (10)	< 0.5 (NA)	
	Salicylate	25 (100)	317 (100)	25 (100)	605 (100)	290 (100)	48 (100)	8 (100)	
	Benzoate	< 1 (NA)	< 10 (NA)	< 1 (NA)	40 (7)	35 (12)	5 (10)	< 0.5 (NA)	
	<i>m</i> -Toluate	< 1 (NA)	< 10 (NA)	< 1 (NA)	55 (9)	30 (10)	4 (8)	< 0.5 (NA)	
PP1-3(pND141)	None	< 1 (NA)	< 10 (NA)	< 1 (NA)	520 (102)	410 (103)	50 (91)	7 (70)	
	Salicylate	22 (100)	328 (100)	23 (100)	510 (100)	400 (100)	55 (100)	10 (100)	
	Benzoate	< 1 (NA)	< 10 (NA)	< 1 (NA)	540 (106)	380 (95)	48 (87)	7 (70)	
	<i>m</i> -Toluate	< 1 (NA)	< 10 (NA)	< 1 (NA)	490 (96)	450 (113)	55 (100)	8 (80)	
PP1-10(pND160)	None	< 1 (NA)	< 10 (NA)	< 1 (NA)	50 (7)	30 (11)	5 (11)	< 0.5 (NA)	
	Salicylate	20 (100)	310 (100)	21 (100)	670 (100)	280 (100)	44 (100)	7 (100)	
	Benzoate	< 1 (NA)	< 10 (NA)	< 1 (NA)	60 (9)	30 (11)	4 (9)	< 0.5 (NA)	
	<i>m</i> -Toluate	< 1 (NA)	< 10 (NA)	< 1 (NA)	50 (7)	35 (13)	3 (7)	< 0.5 (NA)	
PP1-3(pND161)	None	< 1 (NA)	< 10 (NA)	< 1 (NA)	540 (101)	385 (103)	43 (108)	6 (67)	
	Salicylate	26 (100)	355 (100)	30 (100)	535 (100)	375 (100)	40 (100)	9 (100)	
	Benzoate	< 1 (NA)	< 10 (NA)	< 1 (NA)	520 (96)	385 (103)	45 (113)	6 (67)	
	<i>m</i> -Toluate	< 1 (NA)	< 10 (NA)	< 1 (NA)	525 (98)	395 (105)	40 (100)	7 (78)	

salicylaldehyde dehydrogenase and salicylate hydroxylase did not show any alteration in regulation. They were still inducible in PP1-10(pND111) and exhibited approximately the same inducer specificities and induction ratios as the corresponding NAH-specified enzymes. These observations suggest that the genes specifying the initial *meta*-cleavage pathway enzymes belong to one operon. The NAH genes which encode the naphthalene catabolic pathway enzymes thus appear to comprise at least two operons, namely the genes specifying naphthalene conversion to catechol and the genes specifying the *meta*-cleavage pathway.

The pND140 and pND160-encoded naphthalene oxygenase, salicylaldehyde dehydrogenase, salicylate hydroxylase and initial *meta*-cleavage pathway enzymes exhibited similar inducer specificities. This suggests that the enzymes share a regulatory gene. Unlike the NAH-encoded system, *m*-toluate appeared unable to cause partial induction of the enzymes assayed.

The mutations in pND141 and pND161 resulted in increased constitutive activity of catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase. The first two enzymes showed similar induction ratios in PP1-10(pND140) and in PP1-10(pND160) extracts. The 2-hydroxymuconic semialdehyde hydrolase induction ratios were not calculated because of the lack of detectable activity after growth in the absence of inducer. These results suggest that the pND140 and pND160-encoded *meta*-cleavage pathway enzymes belong to one operon. The pND140 and pND160-specified enzymes which convert naphthalene to catechol were not constitutive in PP1-3(pND141) and PP1-3(pND161) indicating that they belong to a separate operon (or operons) from the enzymes of the *meta*-cleavage pathway.

In summary, these results suggest that the NAH, pND140 and pND160 genes which specify the naphthalene catabolic pathway comprise at least two operons: the genes specifying the conversion of naphthalene to catechol, and the genes specifying catechol degradation.

The inability of PP1-3(pND141) and PP1-3(pND161) to grow on *m*-toluate was unexpected. The host cell PP1-3 can convert *m*-toluate to 3-methylcatechol and the plasmid-encoded *meta*-cleavage pathway is produced constitutively. 3-Methylcatechol is degraded through the hydrolase branch of the *meta*-cleavage pathway. Constitutive activities of 2-hydroxymuconic semialdehyde hydrolase appear to be quite adequate to permit growth when compared to the enzyme activities produced by NAH, pND110 and pND111 which permit PP1-3 harbouring these plasmids to grow on *m*-toluate. Use of *m*-toluate as an inducing agent for PP1-3(pND141) and PP1-3(pND161) did not inhibit or prevent induction of any of the enzymes assayed but it is evident that growth on *m*-toluate could only be obtained as the result of an additional mutation in either PP1-3(pND141) or PP1-3(pND161) (Table 2).

The relative ease with which mutants exhibiting altered control of the naphthalene catabolic pathway could be isolated suggested a reason for the different regulation of naphthalene catabolism reported by Williams *et al.* (1975) and Barnsley (1975, 1976*a*) in *Pseudomonas* P_G (NCIB 9816). Williams *et al.* (1975) reported that in this strain growth in the presence of naphthalene induced the enzymes which convert naphthalene to catechol and the enzymes of the *meta*-cleavage pathway. Growth in the presence of salicylate resulted in induction of salicylate hydroxylase and the *ortho*-cleavage pathway enzymes. Barnsley (1975, 1976*a*) reported that in a strain which was also supplied as NCIB 9816 growth in the presence of naphthalene, salicylate or a salicylate analogue induced the enzymes which convert naphthalene to catechol but not the *meta*-cleavage pathway enzymes. Catechol was degraded through the *ortho*-cleavage pathway in this strain. Barnsley (1976*a*) also found that in *Pseudomonas* P_G (obtained from P. A. Williams) growth in the presence of naphthalene, salicylate or a salicylate analogue induced the enzymes which convert naphthalene to catechol. However, high constitutive activities of the *meta*-cleavage pathway enzymes were not obtained in the absence of any aromatic inducers. These activities were much higher

than the corresponding activities observed by Barnsley (1976*a*) in NCIB 9816 and by Williams *et al.* (1975) in *Pseudomonas* P_G. In other words, the three strains which were originally supplied as NCIB 9816 showed different regulation of naphthalene catabolism.

It is possible that regulation of naphthalene catabolism in these strains is similar to that of the NAH-specified pathway and that in each strain a mutation has altered control of the pathway.

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