Regulation of the Enzymes of the *meta*-Cleavage Pathway of *Pseudomonas putida*: The Regulon is Composed of Two Operons

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SUMMARY

In *Pseudomonas putida* NCIB10015 the gene determining phenol hydroxylase is in a separate operon from the genes determining catechol 2,3-oxygenase and subsequent enzymes of the *meta*-cleavage pathway.

In the wild-type strain, non-coordinate induction of phenol hydroxylase and catechol 2,3-oxygenase occurred when catechol, phenol or isomers of cresol were used as inducers. Phenol hydroxylase was repressed to a greater extent than catechol 2,3-oxygenase when induced by phenol in the presence of either benzoate or acetate. In a mutant deficient in catechol 2,3-oxygenase, 3- or 4-methylcatechol repressed formation of phenol hydroxylase but not of enzymes functioning later in the pathway.

Four mutants had an altered regulatory element that resulted in different effects on the expression of phenol hydroxylase and catechol 2,3-oxygenase genes when isomers of cresol were used as inducers.

Repression of enzyme induction by acetate, benzoate or 3- or 4-methylcatechol showed that catechol 2,3-oxygenase and subsequent *meta*-cleavage pathway genes form part of the same operon. Benzoate or 3- or 4-methylcatechol repressed phenol hydroxylase but none of the other enzymes in the pathway, while acetate coordinately repressed catechol 2,3-oxygenase and subsequent enzymes.

INTRODUCTION

Species of the genus *Pseudomonas* exhibit an extensive nutritional versatility in regard to sources of carbon (Stanier, Palleroni & Doudoroff, 1966) and many of the catabolic sequences for dissimilation of these compounds by various species of the genus have been elucidated (Dagley, 1972). However, the regulation of many of these pathways is still unknown and detailed studies at the genetic level are restricted to three systems, namely the *hut*, *catBC* and *ami* loci (Wheelis, 1975).

The degradative sequence for dissimilation of phenol and the isomers of cresol by *Pseudomonas putida* NCIB10015 (strain U) has been elucidated from biochemical (Bayly, Dagley & Gibson, 1966; Bayly & Dagley, 1969; Sala-Trepat, Murray & Williams, 1972) and genetic studies (Bayly & Wigmore, 1973; Wigmore, Bayly & Di Berardino, 1974). The regulation of enzymes of the *meta*-cleavage pathway has not been studied extensively. Feist & Hegeman (1969) showed that phenol and the isomers of cresol are the functional inducers of the enzymes of the *meta*-cleavage pathway since non-metabolizable phenol analogues induced phenol hydroxylase and other enzymes of the pathway and phenol

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Table 1. *Growth of wild-type strain and mutants with aromatic compounds as sole sources of carbon*

Strains were inoculated on to solid basal medium and into liquid basal medium containing different carbon compounds (2.5 mM), and incubated at 30 °C for 24 h. Unless indicated otherwise, growth on both types of medium was the same.

Strain	Carbon source				
	Phenol	<i>o</i> -Cresol	<i>m</i> -Cresol	<i>p</i> -Cresol	Benzoate
PsUo	+	+	+	+	+
PsUD1	+	—	—	—	+
PsUo7	+	+	+	+	—
PsU5/R11	+	—	—	—	+
PsUL2	+	+	—	—	+
PsUL12 series					
(solid)	+	+	+	—	+
(liquid)	+	±	±	—	+

* Growth as a result of *ortho*-fission of catechol.

+, Growth; —, no growth; ±, growth after lag phase, which varied for different mutants of the PsUL12 series.

induced the other enzymes in a phenol hydroxylase-defective mutant. Wigmore & Bayly (1974) showed that it was probable that a single regulatory protein mediates the inductive control since, in a mutant strain with altered effector specificity, the expression of all enzymes of the *meta*-cleavage pathway was affected equally, suggesting that the *meta*-cleavage enzymes exist as a single regulon as defined by Maas & Clark (1964). We now report evidence suggesting that this regulon is composed of two separately regulated operons.

METHODS

Media and growth conditions. The basal mineral salts medium of Hegeman (1966) was used with appropriate additions of sources of carbon. Phenol, catechol, or isomers of cresol were added at 2.5 mM. Fumarate, benzoate, succinate, acetate and pyruvate were each added to give concentrations of 10 mM, unless otherwise specified. The final pH of the medium was 7.4. Solid media were prepared by the addition of 1.2 % (w/v) Oxoid agar. Organisms were grown and suspensions and extracts were prepared as described by Bayly & Wigmore (1973).

Isolation of mutants. The wild-type strain *P. putida* NCIB10015 is designated PsUo. The growth substrate specificities of strain PsUo, and mutant strains derived from it, are listed in Table 1. Mutants PsUD1 and PsU5/R11, defective in catechol 2,3-oxygenase activity, have been described previously (Bayly & Wigmore, 1973). Mutant PsUL2, previously reported by Wigmore & Bayly (1974), has a regulatory element of altered specificity involved in the induction of *meta*-cleavage pathway enzymes. Mutant PsUo7 is blocked in the benzoate oxidase system which converts benzoate to catechol (Reiner, 1971); it was selected by the penicillin/D-cycloserine method as described by Wigmore *et al.* (1974). The selective carbon source (SCS) was catechol (2.5 mM) and the contra-selective carbon source (CSCS) was benzoate (2.5 mM). After treatment with ethyl methanesulphonate (EMS) the bacteria were grown in basal medium plus fumarate before the first exposure to the CSCS. Mutants PsUL12, PsUL14, PsUL16, and PsUL18 were selected by the same method except that the SCS was *m*-cresol (2.5 mM) and the CSCS was *p*-cresol (2.5 mM); these are referred to collectively as the PsUL12 series of mutants. Before EMS-mutagenized cells were first

exposed to the CSCS they were grown on basal medium containing phenol. These mutants grew poorly on *m*-cresol and hence the enrichment must have been due solely to the bactericidal effects of the antibiotics in the presence of the CSCS, *p*-cresol. Mutants were lyophilized soon after isolation and maintained for use as described by Kemp & Hegeman (1968).

Enzyme assays. The following enzymes were assayed by published procedures: catechol 2,3-oxygenase (EC 1.13.11.2), 2-hydroxymuconic semialdehyde hydrolase and NAD⁺-dependent aldehyde dehydrogenase (Bayly & Wigmore, 1973); 4-hydroxy-2-oxovalerate aldolase (Feist & Hegeman, 1969) and 2-oxopent-4-enoate hydratase (Collinsworth, Chapman & Dagley, 1973).

Oxygen uptake by washed suspensions. A Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge) connected to a 10 mV Speedomax recorder was used. The reaction vessel contained 290 μ mol phosphate buffer pH 7.4 saturated with air at 30 °C and cell suspension in a total volume of 3 ml. After determination of endogenous uptake of oxygen, 100 μ l of substrate (10 μ mol ml⁻¹) was added to the reaction vessel. At 30 °C the concentration of oxygen in the air-saturated buffer was taken to be 0.26 mM.

Detection and estimation of catechol and 3- and 4-methylcatechols in culture supernatant fluids. To 2.9 ml of sample (containing 3 to 50 nmol catechol ml⁻¹ and previously adjusted to pH 7.6) was added 0.1 ml (2 mg protein) of heat-treated (55 °C; 5 min) extract of phenol-grown cells of strain PsUo. When the reaction was complete, the absorption spectrum of the products of ring-cleavage of the catechol was determined, and the catechol derivative in the sample was identified and its concentration was determined using the spectral data of Bayly *et al.* (1966).

Estimation of protein. Protein in extracts was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Reagents. Catechol and 3- and 4-methylcatechols were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A., and were purified by vacuum sublimation. Other reagents were obtained from commercial sources and used without further treatment.

RESULTS

Phenol hydroxylase and catechol 2,3-oxygenase in strain PsUo

Induction of phenol hydroxylase and catechol 2,3-oxygenase by phenol, catechol or isomers of cresol was studied in strain PsUo growing on either fumarate, acetate, pyruvate or succinate as carbon source. Synthesis of the enzymes was non-coordinate (Fig. 1). After growth in the presence of catechol, no phenol hydroxylase activity was detected although significant levels of catechol 2,3-oxygenase were induced. The cresols tended to induce a lower ratio of phenol hydroxylase to catechol 2,3-oxygenase than did phenol.

Induction of both phenol hydroxylase and catechol 2,3-oxygenase was repressed by acetate. The repressive effect on phenol hydroxylase was greater than that on the oxygenase, especially at higher acetate concentrations (Table 2).

When strain PsUo growing in basal medium containing fumarate was exposed to a mixture of phenol and benzoate (2.5 mM), induction of catechol 2,3-oxygenase was unaffected but no phenol hydroxylase was detected. Growth was unaffected by adding benzoate, and substrate levels of benzoate (300 nmol ml⁻¹) did not affect phenol hydroxylase activity in whole cells. The specific activity of catechol 2,3-oxygenase of strain PsUo grown in the presence of catechol was unchanged by the presence of 2.5 mM-benzoate. Mutant PsUo7 grew on phenol as a sole source of carbon with a doubling time of 90 min but was unable to

Fig. 1. Induction of phenol hydroxylase and catechol 2,3-oxygenase by different inducers. To strain PsUo growing exponentially in basal medium containing fumarate (10 mM) was added either phenol (●), catechol (○), or *o*-, *m*- or *p*-cresol (Δ) to a final concentration of 2.5 mM. After 2 h further incubation, extracts were assayed for phenol hydroxylase and catechol 2,3-oxygenase activities as described in Methods. In some experiments, succinate (10 mM) or acetate (10 mM) was used instead of fumarate.

Table 2. *Effect of acetate on induction of phenol hydroxylase and catechol 2,3-oxygenase*

Strain PsUo was grown to mid-exponential phase in basal medium containing 5 mM-acetate and then phenol (2.5 mM) and various amounts of acetate were added to each flask. After 2 h further incubation, enzyme activities in cell suspensions were assayed as described in Methods. Results are expressed as the percentage activity relative to the activities of phenol hydroxylase and catechol 2,3-oxygenase produced with 2.5 mM added acetate [190 and 382 $\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$ respectively].

Acetate added (mM)	Phenol hydroxylase activity	Catechol 2,3-oxygenase activity
2.5	100	100
10	46	83
20	23	46
50	< 2	4.7

grow on benzoate (10 mM) plus phenol, thus demonstrating the physiological significance of the repression of phenol hydroxylase by benzoate. The wild-type strain grew on both media with doubling times of 73 and 75 min respectively.

Repression by monomethylcatechols

When either 3- or 4-methylcatechol (2.5 mM) was added with phenol to a culture of mutant PsUD1 growing on fumarate, phenol hydroxylase was repressed to approximately 50 % of the level when phenol was added alone. The addition of 3- or 4-methylcatechol at 0.1 mM did not repress phenol hydroxylase but at 1 mM these monomethylcatechols repressed phenol hydroxylase by approximately 25 %. Growth of the culture was unaffected by the addition of either of the monomethylcatechols.

The effect of the monomethylcatechols on induction of catechol 2,3-oxygenase could not be tested in mutant PsUD1 which is defective in this enzyme. However, they did not repress

2-hydroxymuconic semialdehyde hydrolase, aldehyde dehydrogenase or 2-oxopent-4-enoate hydratase, all enzymes acting later in the *meta*-cleavage degradation of phenol.

Genetic evidence for non-coordinate expression of phenol hydroxylase and catechol 2,3-oxygenase

The four mutants of the PsUL12 series, when tested by replica plating, were able to grow on all of the phenolic compounds tested except *p*-cresol. After the replica plates containing *p*-cresol had been incubated overnight, each mutant produced an orange-brown coloration of the medium at the site of inoculation; the appearance was identical to that of mutant PsUD1 and the extragenically suppressed revertant PsU5/R11 (both defective in catechol 2,3-oxygenase) when these mutants were grown in the presence of *p*-cresol. This suggests either that mutants of the PsUL12 series lack catechol 2,3-oxygenase activity when grown in the presence of *p*-cresol or that any catechol 2,3-oxygenase present is unable to use 4-methylcatechol as substrate.

All four mutants in the PsUL12 series accumulated coloured products during growth in the presence of the isomers of cresol. After 4 h incubation in fumarate basal medium containing any isomer of cresol, the medium was blue-grey, and by 6 h it was red-purple. All these mutants grew at the same rate as the wild type in the presence of each phenolic compound when fumarate was present and also when phenol was the sole carbon source. Mutants PsUL12 and PsUL14 showed no growth after 15 h incubation when either *o*- or *m*-cresol was the sole carbon source, but grew after 24 h incubation. Mutants PsUL16 and PsUL18 grew at the expense of either *o*- or *m*-cresol after a 9 to 10 h lag, whereas the wild type grew after a 4 to 5 h lag.

The coloured products accumulated during the growth of mutants of the PsUL12 series in liquid media in the presence of the isomers of cresol may be a consequence of the accumulation of monomethylcatechols. Catechol forms a blue complex with metallic ions, especially iron (Wheelis & Stanier, 1970), and the colour change to orange-brown may be due to quinone formation (Ribbons, 1970). Patel & Gibson (1974) identified 1,2-naphthoquinone as a non-enzymic autoxidation product of 1,2-dihydroxynaphthalene. Catechol could not be detected after growth of mutants of the PsUL12 series in the presence of phenol. The levels of monomethylcatechols accumulated were less than 10 % of the level accumulated by mutant PsUD1 (defective in catechol 2,3-oxygenase) under the same growth conditions.

The accumulation of monomethylcatechols during growth in the presence of the isomers of cresol indicates that the genetic defect in mutants of the PsUL12 series must be exerted at the level of catechol 2,3-oxygenase expression or function. The effect, as well as being conditional in that different behaviour was observed after growth on solid media and in liquid media containing either *o*- or *m*-cresol (Table 1), must also involve either substrate or effector specificity, depending on whether the altered gene determined either the catechol 2,3-oxygenase protein or a regulatory determinant respectively. The substrate specificity of the catechol 2,3-oxygenase induced by phenol in strain PsU0 and mutant PsUL12 was unchanged as the ratio of enzymic activities determined with catechol or 3- or 4-methylcatechol as substrate was identical in the two strains.

However, in all mutants of series PsUL12, induction for 2 h with either *o*- or *m*-cresol resulted in an 80 to 90 % reduction of the specific enzymic activity compared with the wild-type strain and when *p*-cresol was used as inducer the reduction was 95 to 97 % (Table 3). The percentage reduction in specific enzymic activity was independent of the substrate used for assay. The observations suggest that the behaviour of mutants of the PsUL12 series is a

Table 3. *Specific activity of catechol 2,3-oxygenase in extracts of PsUo and mutants of the PsUL12 series after induction with isomers of cresol (2.5 mM) during growth on basal medium containing fumarate (10 mM)*

Activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Inducer	Substrate	Strain				
		PsUo	PsUL12	PsUL14	PsUL16	PsUL18
<i>o</i> -Cresol	Catechol	90	20	10	20	10
	3-Methylcatechol	110	20	10	20	10
	4-Methylcatechol	410	80	50	90	40
<i>m</i> -Cresol	Catechol	120	30	20	30	20
	3-Methylcatechol	110	20	20	20	10
	4-Methylcatechol	390	120	70	120	60
<i>p</i> -Cresol	Catechol	60	3	4	3	3
	3-Methylcatechol	60	2	2	2	2
	4-Methylcatechol	160	9	10	6	6

Table 4. *Oxidation of phenol by suspensions of intact cells of strain PsUo and mutants of the PsUL12 series after induction with isomers of cresol (2.5 mM) during growth on basal medium containing fumarate (10 mM)*

Rates of oxidation are expressed as $\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$.

Inducer	Strain				
	PsUo	PsUL12	PsUL14	PsUL16	PsUL18
<i>o</i> -Cresol	56	56	36	42	24
<i>m</i> -Cresol	70	65	57	73	35
<i>p</i> -Cresol	46	17	12	11	14

consequence of an altered effector specificity which results in reduced levels of catechol 2,3-oxygenase after induction with any of the isomers of cresol.

The low induced levels of catechol 2,3-oxygenase in mutants of PsUL12 series (Table 3) were consistent with the small amounts of monomethylcatechols that they accumulated compared with the amounts accumulated by mutant PsUD1, which lacked detectable catechol 2,3-oxygenase activity (Bayly & Wigmore, 1973).

Wigmore *et al.* (1974) demonstrated coordinately lowered phenol hydroxylase and catechol 2,3-oxygenase activities when the mutant PsUL2 was induced with either *m*- or *p*-cresol. The activities were about 10 % and 1 % of wild-type levels after induction with *m*- and *p*-cresol respectively. Growth of mutant PsUL2 in the presence of any isomer of cresol as carbon source did not result in accumulation of either 3- or 4-methylcatechol under the conditions of growth tested, suggesting that in mutants of the PsUL12 series there was also a non-coordinate lowering of phenol hydroxylase and catechol 2,3-oxygenase activities.

Phenol hydroxylase activity of each of the mutants of the PsUL12 series was determined after induction by isomers of cresol (Table 4). Although the rates of oxygen uptake were reduced when mutant PsUL18 was induced with either *o*- or *m*-cresol, mutants PsUL12, PsUL14 and PsUL16 had phenol hydroxylase activities similar to the wild type after induction by *o*- or *m*-cresol. After induction by *p*-cresol, the phenol hydroxylase activities of all mutants of the PsUL12 series was about 30 to 40 % of the wild-type level (Table 4) while catechol 2,3-oxygenase activities were reduced to about 5 % of the wild-type level (Table 3).

Table 5. *Repression of some meta-cleavage pathway enzymes by acetate*

Strain PsUo was grown to mid-exponential phase in basal medium containing 5 mM-acetate and then phenol (2.5 mM) and various amounts of acetate were added to each flask. After 2 h further incubation, enzyme activities in extracts were assayed as described in Methods. Results are expressed as the percentage repression of enzymic activity taking the value at 2.5 mM added acetate as zero repression.

Acetate added (mM)	Catechol 2,3-oxygenase	Hydrolase	Hydrolase + Dehydrogenase	Hydratase
10	14	18	15	25
20	54	50	27	71
50	92	88	91	97

Thus the mutants of the PsUL12 series apparently have an altered regulatory element resulting in the differential effects on the expression of the phenol hydroxylase and catechol 2,3-oxygenase genes.

Catechol 2,3-oxygenase and subsequent enzymes are part of a single regulatory element

Benzoate and the monomethylcatechols repressed phenol hydroxylase in strain PsUo and mutant PsUD1 respectively but did not repress other enzymes of the *meta*-cleavage pathway. Various concentrations of acetate added with the inducer repressed, probably coordinately, all the enzymes assayed (Table 5).

DISCUSSION

Biochemical studies of the wild-type strain PsUo and derived mutants suggest that the phenol hydroxylase gene is part of a different operon from that containing the genes determining the other enzymes of the *meta*-cleavage pathway.

The observation that catechol induced significant levels of enzymes of the *meta*-cleavage pathway in strain PsUo is consistent with the observations of Bayly (1969) and Murray & Williams (1974), but different from that of Feist & Hegeman (1969). Despite numerous attempts, induction of phenol hydroxylase by catechol has not been demonstrated for strain PsUo grown in batch culture with fumarate as sole carbon source. This is in contrast to the detection, by Murray & Williams (1974), of phenol hydroxylase in catechol-grown organisms of the same strain. This discrepancy may be due to the concentration of catechol present during induction since significant levels of phenol hydroxylase have been observed in wild-type and mutant derivatives of strain PsUo grown in continuous culture in fumarate basal medium containing 2.5 mM-catechol (D. I. McKenzie, unpublished observations), whereas the initial concentration present in batch culture would be reduced during growth of the culture.

Under all conditions of induction of strain PsUo so far tested, including induction with catechol, catechol 2,3-oxygenase and all subsequent enzymes of the *meta*-cleavage pathway appear to be expressed coordinately (Bayly & Sexton, unpublished observations) and hence the genes coding for them may exist as part of a single operon. High acetate concentrations coordinately repressed catechol 2,3-oxygenase and subsequent enzymes. During growth of conditional catechol 2,3-oxygenase-constitutive derivatives of strain PsUo on solid media with various non-aromatic compounds as carbon source, catechol 2,3-oxygenase and subsequent enzymes were synthesized but phenol hydroxylase was not formed (Wigmore, Di Berardino & Bayly, 1977). Studies of a polarity mutant, PsU5, described by Bayly & Wigmore (1973), indicate that the genes coding for all enzymes required for the *meta*-

bolism of 2-hydroxy-muconic semialdehyde to acetaldehyde and pyruvate are contiguous and are transcribed as a polycistronic message (Wigmore & Bayly, 1977). It is assumed that the 2,3-oxygenase gene transcript is part of this polycistronic message and that it must be located at the operator-proximal end (Wigmore & Bayly, 1977).

Hence the genes determining the enzymes for the catabolism of phenol and the isomers of cresol in strain PsUo exist as two operons. One operon specifies only phenol hydroxylase whereas the second codes for catechol 2,3-oxygenase and all subsequent enzymes of the *meta*-cleavage pathway. It is highly probable that the operons constitute a single regulon. The phenolic substrate apparently serves directly as the effector molecule for the induction of all *meta*-cleavage pathway enzymes, as strains defective in phenol hydroxylase due to a structural gene mutation form wild-type levels of all other enzymes of the pathway when induced with phenol or an isomer of cresol (Feist & Hegeman, 1969; Sala-Trepat *et al.*, 1972; Wigmore, 1975). Studies of induction of the *meta*-cleavage pathway with non-metabolizable phenol analogues also support this view (Feist & Hegeman, 1969). The influence of the effector molecule is thought to be mediated by a single regulatory protein. In mutant strain PsUL2, the effector specificity of this regulatory protein has been altered so that 10 % or 1 to 2 % of wild-type levels of all *meta*-cleavage pathway enzymes are induced by *m*-cresol or *p*-cresol respectively (Wigmore & Bayly, 1974).

Mutants of the PsUL12 series exhibit a conditional effect in that different behaviour was observed after growth on solid media and in liquid media containing either *o*- or *m*-cresol. As yet we have no explanation of this phenomenon although the effect could be mediated by the regulatory protein. Similar behaviour was observed in conditional constitutive producers of catechol 2,3-oxygenase (Wigmore *et al.*, 1977) and Bayly (1969) demonstrated the influence of growth conditions on the induction of both catechol 1,2-oxygenase and catechol 2,3-oxygenase in strain PsUo.

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