The Purification and Properties of Two Isofunctional 2-Hydroxy-6-oxohepta-2,4-dienoate Hydrolases from *Alcaligenes eutrophus* Strain 345

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Two 2-hydroxy-6-oxohepta-2,4-dienoate (HOD) hydrolases have been purified to homogeneity from *Alcaligenes eutrophus* ATCC 17707. One, HODHII, is encoded by the TOL-like plasmid, pRA1000, and the other, HODHI, is chromosomally-encoded. HODHI and HODHII have an M_r of 104×10^3 and 116×10^3 , respectively, and both are dissociated by SDS into three subunits of equal size which are not linked by disulphide bonds. Both hydrolases are active against a similar range of substrates formed by the action of catechol 2,3-oxygenases, generally being more active against substrates formed from 3-alkyl- rather than 4-alkyl-substituted catechols. HODHI and HODHII had similar K_m values for HOD, but HODHI had a significantly lower K_m value for, and a higher activity against, 2-hydroxymuconic semialdehyde than HODHII. It was shown by immunodiffusion studies that the two hydrolases were immunologically distinct and that HODHI had no common antigenic determinants with HOD hydrolases from the *Pseudomonas putida* strains NCIB 9865, NCIB 10015 and mt-2; HODHII gave a line of identity with antiserum to the hydrolase from *P. putida* NCIB 9865. A comparison of the physico-chemical properties of HODHI and HODHII with the same properties of the HOD hydrolases from the three *P. putida* strains demonstrated several similarities between the five HOD hydrolases which have been purified.

INTRODUCTION

In our studies on the degradation of *m*-toluate and phenol by *Alcaligenes eutrophus* strain 345 (ATCC 17707) via *meta*-cleavage pathways, we reported that the 2-hydroxymuconic semialdehyde hydrolase, HMSHII, elicited during growth on *m*-toluate, had different substrate specificities from the corresponding enzyme, HMSHI, when the growth substrate was phenol (Hughes *et al.*, 1984*b*). We also reported that HMSHII was encoded on an 85 kb plasmid, pRA1000, whereas HMSHI was chromosomally-encoded.

Bayly & DiBerardino (1978) reported the purification and properties of hydrolases carrying out the same functions as HMSHI and II, from two different isolates of *Pseudomonas putida* biotype A, and more recently, Duggleby & Williams (1986) have reported the isolation and characterization of a plasmid-encoded hydrolase from *P. putida* mt-2, which carries the 117 kb TOL plasmid, pWW0. We now report the purification of HMSHI and HMSHII and present a comparison of some of their properties with those of the isofunctional hydrolases previously reported. For the reasons given by Duggleby & Williams (1986), viz. that 'the physiologically most important substrate' for such hydrolases is 2-hydroxy-6-oxohepta-2,4-dienoate (HOD), we shall refer to the hydrolases HMSHI and HMSHII as HODHI and HODHII, respectively.

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Abbreviations: HMS, 2-hydroxymuconic semialdehyde; HOD, 2-hydroxy-6-oxohepta-2,4-dienoate; HODH, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase.

METHODS

Organisms, method of cultivation and preparation of cell extracts. *A. eutrophus* strain 345 (ATCC 17707) was obtained from P. J. Chapman, University of Minnesota, and was maintained as described by Hughes *et al.* (1984*a*); some of its metabolic properties have been described (Hughes *et al.*, 1984*a, b*). To obtain cells from which HODHII was to be purified, strain 345 was grown in the presence of *m*-toluate. For the purification of HODHI, a derivative of strain 345, RA1012, which had been cured of the plasmid pRA1000 (Hughes *et al.*, 1984*a*) was grown in the presence of phenol. Cells were grown and cell extracts were prepared as described previously (Bayly & DiBerardino, 1978), except that DL-lactate (20 mM) replaced fumarate as the basal carbon source. The concentration of protein in cell extracts and fractions was determined by the method of Lowry.

Physicochemical and immunological examination of purified hydrolase preparations. M_r values of the native enzymes were determined from sedimentation equilibrium data essentially as described by Yphantis (1964). Analytical polyacrylamide disc gel electrophoresis (PAGE) was by the method of Davis (1964) using a 7.5% (w/v) gel with 2.5% (w/v) cross-linker at pH 8.9. SDS-PAGE was done as described by Weber & Osborn (1969), using 10% gels. The following standards were used to determine subunit size: bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (25000), all from Boehringer, and lysozyme (14400) from Sigma. Reduced and non-reduced samples for SDS-PAGE were prepared as described by Tack *et al.* (1972). Amino acid analyses of the purified enzymes were done on samples (1–2 mg protein) which were prepared and analysed as described by Bayly & DiBerardino (1978); duplicate analyses were not done. Heat stability and immunological studies were done as described by Bayly & DiBerardino (1978).

Determination of K_m values for HODHI and II against HOD and 2-hydroxymuconic semialdehyde (HMS) were calculated from Lineweaver–Burk plots. Enzymes were assayed in triplicate at substrate concentrations ranging from 70 to 2 μ M for HOD and from 170 to 3 μ M for HMS; at least 15 different concentrations of substrate were used over each range. The relative activity against different substrates was determined at pH 7.6 using substrate concentrations of approximately 70 μ M.

Enzyme assays. All assays for activity of the hydrolases against the *meta*-cleavage products of catechol and some of its homologues were done in a final reaction volume of 3 ml and at 23 °C. The reaction mixture contained 33 mM-KH₂PO₄ (pH 7.6), approximately 0.2 μ mol substrate (prepared as described by Bayly & DiBerardino 1978), and enzyme. The specific activities were calculated using the molar absorption coefficients reported by Bayly *et al.* (1966) and Bayly & DiBerardino (1978). The unit of activity is defined as μ mol substrate min⁻¹ (mg protein)⁻¹.

Chemicals. 3,4-Dimethylcatechol and 3-isopropylcatechol were gifts from P. J. Chapman and Coalite and Chemical Products, Bolsover, UK, respectively. Catechol, and 3-methyl- and 4-methylcatechol were from Aldrich. All catechols were purified by vacuum sublimation before use.

Purification of HODHI and HODHII. The procedures used for both enzymes were essentially the same and, with the exception of the last step, differed only in such details as the volume of samples applied to columns, the volume of fractions collected and the flow rate of eluants. All steps were done at 2–4 °C. For HODHI, approximately 500 g wet weight of cells was used to prepare the crude cell extract and, for HODHII, approximately 270 g wet weight of cells was used.

Step 1. Cell extracts were brought to either 30% saturation (HODHI) or 20% saturation (HODHII) by the slow addition of solid (NH₄)₂SO₄ and, after equilibration for 30 min, the precipitated protein was removed by centrifugation at 20000 *g* for 15 min. The supernatant solution was brought to 50% saturation (HODHI) and 40% saturation (HODHII) with (NH₄)₂SO₄ and, after equilibration, the precipitate was collected by centrifugation and dissolved in 0.05 M-Tris/HCl buffer, pH 7.8. The solutions were dialysed against the same buffer until free of ammonium ions as shown by testing with Nessler's reagent.

Step 2. The dialysed solutions were applied to DEAE-cellulose columns (5 × 50 cm) which had been previously equilibrated with 0.05 M-Tris/HCl, pH 7.8; the protein was eluted with 2 l of a linear gradient of 0–1.0 M-NaCl in the same buffer. Pooled active enzyme fractions were dialysed against 0.05 M-Tris/HCl, pH 7.8.

Step 3. The dialysed enzyme fractions were applied to DEAE-cellulose columns (2.5 × 30 cm) equilibrated as above and the protein was eluted with 800 ml of a linear gradient of 0–0.5 M-NaCl in the same buffer.

Step 4. Fractions with activity of at least 20% of that in the most active fraction were pooled, concentrated to 5 ml by ultrafiltration (PM-30 Amicon membrane), applied to a Sephadex G-200 column (2.6 × 60 cm) and eluted with 0.05 M-Tris/HCl, pH 8.2.

Step 5. Pooled fractions, selected as in step 4, were dialysed against 0.5 M-Tris/HCl, pH 8.2, applied to a DEAE-Sephacrose column (2.5 × 30 cm) and the protein was eluted with 500 ml of a linear gradient of 0–1.0 M-NaCl in the same buffer.

Step 6. For HODHI, the pooled fractions were concentrated to 5 ml as above, applied to a Sephadex G-150 column (2.6 × 60 cm) previously equilibrated with 0.05 M-Tris/HCl, pH 7.8 and the protein was eluted with the same buffer.

For HODHII, the pooled fractions from step 5 were dialysed against 0.05 M-Tris/HCl, pH 7.5, applied to a

DEAE-cellulose column (2.5 × 30 cm) equilibrated with the same buffer and the protein was eluted with a linear gradient of 0.0–3 M-NaCl in the same buffer.

Fractions in which the elution profile for protein was coincident with enzyme activity were examined by PAGE and the fractions were pooled and stored at 4 °C. Samples of the pooled fractions to be used for amino acid analysis and for the production of antisera were dialysed to remove salts and then lyophilized. Examinations for substrate specificity, K_m values, pH optima, inhibition/activation studies, M_r and subunit structure were completed within 7 d of step 6.

RESULTS

A summary of the purification procedures and the yields obtained for HODHI and HODHII is shown in Table 1.

PAGE of fraction 86, step 6, which contained the peak activity of HODHI, showed a major and a minor protein band (Fig. 1*a*). A densitometer tracing of this gel showed that the major band constituted about 93% of the protein. The same pattern was obtained with fractions 78–94 (step 6) and these fractions were pooled. Elution of the protein bands from an unstained gel showed the main band to be active against HOD whereas the minor band was inactive. Sedimentation equilibrium analysis of the pooled fractions showed HODHI to have an M_r of 104×10^3 . SDS-PAGE showed a major band corresponding to an M_r of 34.4×10^3 , which was unaltered when the protein was reduced with 2-mercaptoethanol before electrophoresis. HODHI therefore appears to be a trimer of subunits of identical size which are not linked by disulphide bonds.

The peak of activity of HODHII was in fraction 63, step 6; PAGE of this fraction showed five protein bands (Fig. 1*b*); the same pattern was observed with each of fractions 60–76 from step 6, which were pooled and used for all further procedures.

Segments from each of three unstained gels of fraction 63 were cut out as indicated in Fig. 1*b*; the protein was eluted from each segment with 0.1 ml 0.05 M-Tris/HCl, pH 7.5, and assayed against HOD, HMS and 2-hydroxy-5-methylmuconic semialdehyde. The eluate from each segment of gel had relative activities against those substrates in the ratio 100:14:5, respectively, which is approximately the same as that for fraction 63 before PAGE and also for crude extract of strain 345 grown in the presence of *m*-toluate (Hughes *et al.*, 1984*b*). On this basis it seems that none of the protein bands from fraction 63 correspond to HODHI as the ratio of activities of HODHI against the same substrates is quite different (see Table 3).

Sedimentation equilibrium analysis showed HODHII to have an M_r of 116×10^3 . SDS-PAGE of HODHII showed a single protein band corresponding to an M_r of 36.7×10^3 ; HODHII therefore appears to be a trimer of identically sized subunits. There was no difference in the electrophoretic mobility of the protein before and after reduction with 2-mercaptoethanol, which indicated that, like those of HODHI, the subunits of HODHII are not linked by disulphide bonds.

Even though multiple bands were obtained from PAGE of HODHII the finding of only a single protein band following SDS-PAGE indicates that HODHII had been purified to homogeneity. It is probable that the multiple bands observed were due to dissociation or aggregation of the protein during electrophoresis, especially as bands eluted from each of the three segments of the gel (Fig 1*b*) all showed the same enzyme activity as the sample before electrophoresis. Keat & Hopper (1978) reported a similar observation with *p*-cresol methylhydroxylase purified from *P. putida* NCIB 9869, where all three bands obtained on PAGE had enzyme activity. A maleylpyruvate hydrolase purified from *P. alcaligenes* by Bayly *et al.* (1980) also behaved similarly.

Immunological specificity

Antisera prepared against each purified hydrolase were examined by Ouchterlony double diffusion against both purified enzymes. A single precipitin band was formed between each and its homologous antiserum. No precipitin bands were observed when the antiserum to HODHI was tested against purified HODHII (and vice versa) and against each of the purified HOD hydrolases from *P. putida* NCIB 10015 and NCIB 9865, or against a crude extract of *m*-toluate-

Table 1. *Summary of the procedures used in the purification of HODHI and HODHII from A. eutrophus 345*

Step	Procedure	Total volume (ml)	Total protein (mg)	Specific activity*	Total activity (units)	Purification (fold)	Recovery (%)
HODHI							
	Crude extract†	1150	52500	0.18	9450	—	100
1	Ammonium sulphate (30–50%)	570	19900	0.19	3780	1.1	40
2	DEAE-cellulose; 0–1.0 M-NaCl-0.05 M-Tris (pH 7.8)	355	2020	0.81	1640	4.5	17
3	DEAE-cellulose; 0–0.5 M-NaCl-0.05 M-Tris (pH 7.8)	16.5	1630	0.64	1040	3.55	11
4	Sephadex G-200; 0.05 M-Tris (pH 8.2)	4	272	3.52	957	19.5	10
5	DEAE-Sepharose; 0–1.0 M-NaCl-0.05 M-Tris (pH 8.2)	1.3	112	5.15	577	28.6	6
6	Sephadex G-150; 0.05 M-Tris (pH 7.8)	11.5	30	17.4	522	96.6	5.5
HODHII							
	Crude extract‡	1000	27200	0.26	7080	—	100
1	Ammonium sulphate (20–40%)	260	8840	0.67	5920	2.6	84
2	DEAE-cellulose; 0–1.0 M-NaCl-0.05 M-Tris (pH 7.8)	260	2940	1.7	5000	6.5	71
3	DEAE-cellulose; 0–0.5 M-NaCl-0.05 M-Tris (pH 7.8)	25	1260	3.97	5000	15.2	71
4	Sephadex G-200; 0.05 M-Tris (pH 8.2)	39	162	10.4	1680	40	24
5	DEAE-Sepharose; 0–1.0 M-NaCl-0.05 M-Tris (pH 8.2)	14.5	113	10.8	1220	41.5	17
6	DEAE-cellulose; 0–0.3 M-NaCl-0.05 M-Tris (pH 7.5)	1.5	30	10.1	303	39	4.2

* HODH activity was determined against HOD; specific activity is expressed as $\mu\text{mol substrate min}^{-1} (\text{mg protein})^{-1}$.

† Phenol-grown strain RA1012.

‡ *m*-Toluate-grown strain 345.

grown *P. putida* mt-2. These results indicated that HODHI shared no common antigenic determinants with either its isofunctional enzyme, HODHII, or the other hydrolases tested.

When the antiserum to HODHII was tested against the purified hydrolase from *P. putida* NCIB 10015 and a crude extract of *m*-toluate-grown *P. putida* mt-2, no precipitin bands were detected. However each of HODHII, the purified hydrolase from *P. putida* NCIB 9865, and crude extract of *m*-toluate-grown *P. putida* mt-2 all gave a line of identity with the antiserum to purified hydrolase of *P. putida* NCIB 9865.

Amino acid composition

The amino acid compositions of HODHI and HODHII (Table 2) show a high degree of similarity, differing significantly only in the number of valine residues per mole. The low cysteine values in both enzymes are in agreement with the observation that the subunits of these enzymes are not linked by disulphide bonds. The same unidentified amino acid was detected in similar amounts in both hydrolases.

Heat stability and pH optima

Both HODHI and HODHII were similar in their sensitivity to heat. No activity of HODHII was present after treatment for 2 min at 45 °C and HODHI was inactivated after 4 min at the same temperature.

Both enzymes showed maximal activity at pH 8.6 and had a similar profile of activity over the

Fig. 1. Polyacrylamide gel electrophoresis of (a) HODHI and (b) HODHII. (1) PAGE, (2) SDS-PAGE, non-reduced, (3) SDS-PAGE, reduced. The arrows indicate those segments which were cut out of corresponding unstained gels and whose relative activities were tested against substrates (i)–(iii) listed in Table 3.

Table 2. Amino acid compositions of HODHI and HODHII from *A. eutrophus*

Amino acid	HODHI		HODHII	
	Percentage composition [g (100g protein) ⁻¹]	Residues per 34000g*	Percentage composition [g (100g protein) ⁻¹]	Residues per 37000g*
Cysteine	0.8	2	0.6	2
Aspartic acid	10.2	30	10.2	28
Threonine	4.5	13	4.3	12
Serine	5.9	17	4.7	13
Glutamic acid	8.1	24	9.2	25
Glycine	10.8	32	9.9	28
Alanine	12.9	38	12.4	34
Valine	8.8	26	5.8	16
Methionine	0	0	0	0
Isoleucine	4.4	13	6.1	12
Leucine	10.7	31	11.6	32
Tyrosine	0	0	0	0
Phenylalanine	3.3	10	3.8	10
Histidine	0	0	0	0
Lysine	3.1	9	2.0	6
Arginine	6.5	19	7.3	20
Proline	5.4	16	5.8	16
Unidentified amino acid	4.1	—	5.3	—

* Equivalent to residues per mole of subunit, given to the nearest integer.

pH range 5.0–10.0. In both cases there was a marked decrease in activity above pH 9.0. At pH 7.6, at which assays were routinely done, the activity of HODHI and II was about 75–80% of that at pH 8.6.

Table 3. Comparison of properties of HODHs from strains of *A. eutrophus* and *P. putida*

	<i>A. eutrophus</i> RA1012 (HODHI)	<i>A. eutrophus</i> 345 (HODHII)	<i>P. putida</i> * NCIB 10015	<i>P. putida</i> * NCIB 9865	<i>P. putida</i> mt-2†
M_r	104×10^3	116×10^3	118×10^3	100×10^3	65×10^3
Number and M_r of subunits	3; 34.4×10^3	3; 36.7×10^3	4; 27.7×10^3	4; 25×10^3	2; 32×10^3
Relative activity versus‡:					
(i) 2-Hydroxy-6-oxohepta-2,4-dienoate (HOD)	100	100	100	100	100
(ii) 2-Hydroxymuconic semialdehyde (HMS)	31	12	7	3	6.9
(iii) 2-Hydroxy-5-methylmuconic semialdehyde	3	5	3	1	3.2
(iv) 2-Hydroxy-7-methyl-6-oxoocta-2,4-dienoate	149	0	20	5	ND
(v) 2-Hydroxy-5-methyl-6-oxohepta-2,4-dienoate	0	41	14	67	ND
K_m (M) versus:					
(i) 2-Hydroxy-6-oxohepta-2,4-dienoate (HOD)	5.8×10^{-5}	1.2×10^{-6}	6.7×10^{-6}	2.7×10^{-5}	3.6×10^{-5}
(ii) 2-Hydroxymuconic semialdehyde (HMS)	3.6×10^{-4}	6.2×10^{-2}	ND	ND	3×10^{-5}

ND, Not determined.

* Data from Bayly & DiBerardino (1978).

† Data from Duggleby & Williams (1986).

‡ (i)–(v) are the products obtained by the action of catechol 2,3-oxygenase on 3-methylcatechol, catechol, 4-methylcatechol, 3-isopropylcatechol and 3,4-dimethylcatechol respectively.

Kinetic properties

The comparative rates of hydrolysis of the *meta*-cleavage products of catechol and some alkyl-substituted catechols by HODHI and HODHII, and the K_m values of these enzymes, are shown in Table 3. Relative to HOD (the *meta*-cleavage product of 3-methylcatechol), HMS was attacked by HODHI at about three times the rate of HODHII. There was a marked difference between the K_m of HODHII for the *meta*-cleavage products of catechol and 3-methylcatechol, whereas, with HODHI, the K_m values for these substrates were much closer. Significant differences were also found when the *meta*-cleavage products of 3-isopropylcatechol and 3,4-dimethylcatechol were used as substrates (Table 3).

Inhibition/stimulation of hydrolase activity

The effect of different cations, a chelating agent and a thiol-specific agent on the activity of HODHI and HODHII against HOD was examined after the purified enzymes had been incubated at 20 °C for 30 min with the compounds at a final concentration of 5 mM. Both hydrolases were substantially inactivated (90–100%) by Zn^{2+} and Cu^{2+} , and HODHI was inactivated to a much greater extent (90%) by *p*-chloromercuribenzoate than was HODHII (35%). The greater sensitivity of HODHI to the latter reagent may indicate either a greater availability of the thiol groups of the enzyme to react with that compound than is the case with HODHII or that HODHII possesses a relatively labile thiol group that has been modified during the purification processes. The activity of HODHI was decreased by about 50% in the presence of Mn^{2+} whereas HODHII activity was stimulated to a level about 50% higher when Mn^{2+} was present. EDTA showed no significant stimulatory or inhibitory effect. No differences in the above results were obtained when the hydrolases were dialysed for 24 h against 0.05 M-Tris/HCl pH 8.0 containing 0.05 M-EDTA before being tested.

DISCUSSION

The two hydrolases, HODHI and HODHII, purified from *A. eutrophus* strain 345 are similar to one another in several respects, but also show important differences.

HODHI and HODHII have similar M_r values and both appear to be trimers whose subunits are of similar size and are not linked by disulphide bonds. They have similar pH profiles, are inactivated by heat at similar temperatures, and their overall amino acid compositions differ significantly only in the larger number of valine residues present in HODHI. Immunodiffusion experiments showed that the two hydrolases are immunologically distinct and they therefore cannot share any common antigenic determinants on their surfaces.

There is, however, a considerable difference in their kinetic properties as shown by the higher catalytic activity and greater K_m of HODHI against HMS as compared to HODHII. This is consistent with the report of Hughes *et al.* (1984*a, b*) that strain 345 metabolizes phenol exclusively via a hydrolytic cleavage of HMS and not by the action of an aldehyde dehydrogenase as has been shown in some *P. putida* strains (Murray *et al.*, 1972; Sala-Trepat *et al.*, 1972; Wigmore *et al.*, 1974) and in *A. eutrophus* strain 335 (ATCC 17697) (Hughes & Bayly, 1983).

Some properties of other isofunctional HODHs from three strains of *P. putida* have been reported. Bayly & DiBerardino (1978) isolated hydrolases from each of *P. putida* NCIB 10015 and *P. putida* NCIB 9865, and Duggleby & Williams (1986) purified a hydrolase from *P. putida* mt-2. The latter authors have made comparisons of some of the properties of the *P. putida* hydrolases and Table 3 summarizes some properties of the hydrolases now reported from *A. eutrophus* and those from the *P. putida* strains. It should be noted that the comparative activities against different substrates in *P. putida* mt-2 (Table 3) are calculated from V_{max} values whereas the remaining data are derived from assays in which the substrate concentrations in some cases may have been below saturation levels. With the exception of the enzyme from *P. putida* mt-2, M_r values of the enzymes are similar and, while the subunit M_r values of all five hydrolases lie in the range $25\text{--}37 \times 10^3$, the apparent number of subunits varies from two to four.

Marked differences are apparent in the relative activity of the enzymes against the same substrates, which may reflect that the strains have evolved to enhance their catalytic activities towards compounds which they may encounter as metabolites arising from different growth substrates. The high activity of HODHI towards the *meta*-cleavage product of 3-isopropylcatechol stands out from the other results. This compound, 2-hydroxy-7-methyl-6-oxoocta-2,4-dienoate, would be an intermediate if 3-isopropylbenzene was metabolized via 3-isopropylcatechol as was suggested by Gibson & Subramanian (1984), but *A. eutrophus* RA1012 has not been tested for growth on 3-isopropylbenzene. The K_m values for the hydrolases tested show similar preferential binding capacities for the same substrates except for HODHII to HMS. This compound would not be a metabolite when *m*-toluate was metabolized by *A. eutrophus* 345 and hence there would have been no pressure for HODHII to have evolved to attack that compound.

There is some immunological cross-reactivity between the various hydrolases. Duggleby & Williams (1986) reported a cross-reaction between antiserum to the hydrolase from *P. putida* mt-2 and crude extract of phenol-induced *P. putida* NCIB 10015. In this study, although antisera to HODHII did not react with a crude extract of the *m*-toluate-grown *P. putida* mt-2, both purified HODHII and a crude extract of the latter strain gave a line of identity with antisera to the hydrolase purified from *P. putida* NCIB 9865.

The comparative information available on the properties of the five HODHs now purified show several points of similarity between the enzymes. However, on the evidence at present available, it is not possible to draw definite conclusions as to their evolutionary relatedness and, as Duggleby & Williams (1986) have stated, further such evidence 'is much more likely to come from molecular comparisons of the coding DNA ...'.

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REFERENCES

- BAYLY, R. C. & DiBERARDINO, D. (1978). Purification and properties of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase from two strains of *Pseudomonas putida*. *Journal of Bacteriology* **134**, 30-37.
- BAYLY, R. C., DAGLEY, S. & GIBSON, D. T. (1966). The metabolism of cresols by species of *Pseudomonas*. *Biochemical Journal* **101**, 293-301.
- BAYLY, R. C., CHAPMAN, P. J., DAGLEY, S. & DiBERARDINO, D. (1980). Purification and some properties of maleylpyruvate hydrolase and fumarylpyruvate hydrolase from *Pseudomonas alcaligenes*. *Journal of Bacteriology* **143**, 70-77.
- DAVIS, B. J. (1964). Disc electrophoresis. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404-427.
- DUGGLEBY, C. J. & WILLIAMS, P. A. (1986). Purification and some properties of the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (2-hydroxymuconic semialdehyde hydrolase) encoded by the TOL plasmid pWW0 from *Pseudomonas putida* mt-2. *Journal of General Microbiology* **132**, 717-726.
- GIBSON, D. T. & SUBRAMANIAN, V. (1984). Microbial degradation of aromatic hydrocarbons. In *Microbial Degradation of Organic Compounds*, pp. 181-252. Edited by D. T. Gibson. New York: Marcel Dekker.
- HUGHES, E. J. L. & BAYLY, R. C. (1983). Control of catechol meta-cleavage pathway in *Alcaligenes eutrophus*. *Journal of Bacteriology* **154**, 1363-1370.
- HUGHES, E. J. L., BAYLY, R. C. & SKURRAY, R. A. (1984a). Characterization of a TOL-like plasmid from *Alcaligenes eutrophus* which controls the expression of a chromosomally encoded *p*-cresol pathway. *Journal of Bacteriology* **158**, 73-78.
- HUGHES, E. J. L., BAYLY, R. C. & SKURRAY, R. A. (1984b). Evidence for isofunctional enzymes in the degradation of phenol, *m*- and *p*-toluate and *p*-cresol via catechol meta-cleavage pathways in *Alcaligenes eutrophus*. *Journal of Bacteriology* **158**, 79-83.
- KEAT, M. J. & HOPPER, D. J. (1978). *p*-Cresol and 3,5-xenol methylhydroxylases in *Pseudomonas putida* NCIB 9869. *Biochemical Journal* **175**, 649-658.
- MURRAY, K., DUGGLEBY, C. J., SALA-TREPAT, J.-M. & WILLIAMS, P. A. (1972). The metabolism of benzoate and the methylbenzoates via the *meta* cleavage pathway by *Pseudomonas arvilla* mt-2. *European Journal of Biochemistry* **28**, 301-310.
- SALA-TREPAT, J.-M., MURRAY, K. & WILLIAMS, P. A. (1972). The metabolic divergence in the *meta* cleavage of catechols by *Pseudomonas putida* NCIB 10015. Physiological significance and evolutionary implications. *European Journal of Biochemistry* **28**, 347-356.
- TACK, B. F., CHAPMAN, P. J. & DAGLEY, S. (1972). Purification and properties of 4-hydroxy-4-methyl-2-oxoglutarate aldolase. *Journal of Biological Chemistry* **247**, 6444-6449.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406-4412.
- WIGMORE, G. J., BAYLY, R. C. & DiBERARDINO, D. (1974). *Pseudomonas putida* mutants defective in the metabolism of the products of *meta* fission of catechol and its methyl analogues. *Journal of Bacteriology* **120**, 31-37.
- YPHANTIS, D. A. (1964). Equilibrium ultracentrifugation of dilute solutions. *Biochemistry* **3**, 297-317.