Degradation of *p*-toluic acid (*p*-toluenecarboxylic acid) and *p*-toluenesulphonic acid via oxygenation of the methyl sidechain is initiated by the same set of enzymes in *Comamonas testosteroni* T-2

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Comamonas testosteroni T-2 utilizes *p*-toluate (TC, 4-toluenecarboxylate) as sole source of carbon and energy for growth. Cells grown in TC-salts medium oxygenated terephthalate (PcB, 4-carboxybenzoate) and contained protocatechuate 4,5-dioxygenase but no detectable (methyl)catechol dioxygenase. The intermediates 4-carboxybenzyl alcohol (COL), 4-carboxybenzaldehyde (CYD) and PcB were detected during the metabolism of TC. A TC methyl-monooxygenase system, a COL dehydrogenase and a CYD dehydrogenase were detected, analogous to the known degradative pathway and enzymes for 4-toluenesulphonate (TS) to 4-sulphobenzoate (PSB) (Locher *et al.*, *Journal of General Microbiology* 135, 1969–1978, 1989). Genetic evidence indicated that the steps from TS to PSB and from TC to PcB were catalysed by the same enzymes. This hypothesis was substantiated by purifying or separating the appropriate enzymes from cells grown in TS-salts and TC-salts media. The behaviour of pairs of enzymes was effectively identical in all chromatographic and catalytic properties that were compared. The data support the existence of a novel pathway for the degradation of TC, with the same initial pathway enzymes being used to metabolize TS.

Introduction

Oxygenation of a methyl sidechain of a methyl-aromatic compound, followed by two oxidative steps to the carboxylate, is a standard and widespread, but not universal metabolic sequence to initiate a bacterial degradative pathway (Dagley, 1971, 1988) (Fig. 1, lines 1, 2 and 6). It is not then surprising that *Comamonas testosteroni* T-2, which degrades 4-toluenesulphonate (TS), should use sidechain oxygenation and oxidation to initiate the pathway (Locher *et al.*, 1989) in place of oxygenation of the aromatic ring, another standard reaction (Fig. 1, lines 1, 2, 3, 4, 5 and 8). Ring oxygenation has now been confirmed quantitatively

(Thurnheer *et al.*, 1990) as the initial reaction in the degradation of TS by another organism. The metabolism of TS in *C. testosteroni* T-2 proceeds via the TS monooxygenase system (TSMOS) to the corresponding alcohol (SOL), which is oxidized in two steps via the aldehyde (SYD) to 4-sulphobenzoate (PSB). PSB is subject to concomitant dioxygenation and desulphonation, followed by *meta* ring-cleavage (Locher *et al.*, 1989, 1991*a, b*) (Fig. 1, line 4).

The carboxy analogue of TS, *p*-toluate (TC, 4-toluene carboxylate), in contrast, is only known to be degraded via ring-oxygenative systems, typified by the reactions encoded by the TOL plasmid pWW0 (Burlage *et al.*, 1989; Gibson, 1988; Assinder & Williams, 1990) (Fig. 1, line 3). No substantiated report of the degradation of TC via protocatechuate is known; the known pathways for *o*- and *m*-toluate are also via ring oxygenation (Engelberts *et al.*, 1989; Gibson, 1988).

We now report that TC is degraded via PcB in *C. testosteroni* T-2, and that the same set of enzymes is used to degrade TS to PSB and TC to PcB.

Abbreviations: TS, 4-toluenesulphonate; SOL, 4-sulphobenzyl alcohol; SYD, 4-sulphobenzaldehyde; PSB, 4-sulphobenzoate; TC, *p*-toluate (4-toluenecarboxylate); COL, 4-carboxybenzyl alcohol; CYD, 4-carboxybenzaldehyde; PcB, 4-carboxybenzoate (terephthalate); TSMOS, 4-toluenesulphonate methyl-monooxygenase system; TCMOS, *p*-toluate methyl-monooxygenase system; PSBDOS, 4-sulphobenzoate dioxygenase system.

Fig. 1. Methyl monooxygenases, ring oxygenations and methyl hydroxylases as initial reactions in some bacterial degradative pathways for methyl-aromatic compounds. The 'parent molecule' is a methyl-aromatic compound which is metabolized via ring oxygenation (to the right) or via an attack on the methyl group (to the left). If no arrow is shown, no reaction is known. Line 4 is adapted from Locher *et al.* (1989, 1991a, b) and Thurnheer *et al.* (1990); other pathways are adapted from reviews: lines 1, 2 and 3 (Gibson, 1988; Gibson & Subramanian, 1984), line 5 (Ribbons, 1988), lines 6, 7 and 8 (Bayly *et al.*, 1988; Hopper, 1988).

Methods

Materials. COL was purchased from Sigma and Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid)] from Fluka. SYD was purified from a commercial preparation and SOL was prepared enzymically from SYD (Locher *et al.*, 1989): each was collected after separation by HPLC (100 mM-acetic acid brought to pH 4.5 with NH_4OH) and dried in a rotary evaporator. The sources of other chemicals and of the materials for protein purification are given elsewhere (Locher *et al.*, 1989, 1991a, b; Thurnheer *et al.*, 1986).

Analytical methods. The apparatus was indicated by Locher *et al.* (1991a), except for the thermospray HPLC-MS, which consisted of an HPLC system (Applied Chromatography Systems model 350/04), a Vestec VT 201 MS and a Teknivent Vector 1 data system (Dr I. A. S. Lewis, King's College London, UK). Aromatic compounds were determined by HPLC after isocratic separation on reversed phase columns: UV-spectra were recorded in a diode array detector (Locher *et al.*, 1989). The sample for analysis by HPLC-MS was pre-separated in a volatile mobile phase (100 mM-ammonium acetate, see above) and examined by HPLC-MS in the same mobile phase. Protein concentration was measured by the method of Bradford (1976), with bovine serum albumin as standard.

SDS-PAGE (Laemmli, 1970) was used to monitor protein purifications and to estimate M_r under denaturing conditions: proteins were stained with Coomassie Brilliant Blue (Locher *et al.*, 1991a). The N-terminal amino acid sequences were determined with protein bands blotted from SDS-PAGE on to polyvinylidene difluoride membranes and subjected to Edman degradation (Locher *et al.*, 1991a, b). The M_r of native enzymes was estimated by gel filtration (Locher *et al.*, 1991a).

Organisms, growth media and the preparation of cell-free extracts. *C. testosteroni* T-2 (Thurnheer *et al.*, 1986; Locher *et al.*, 1989) was grown in TS-, TC- or succinate-salts medium (Locher *et al.*, 1989). Cells for enzyme purification were grown as described by Locher *et al.* (1991b): when appropriate, TC was substituted for TS. Cells were ruptured in a French pressure cell and crude extracts were prepared as described previously (Locher *et al.*, 1991a). The stability of TSMOS is given elsewhere (Locher *et al.*, 1991b). The dehydrogenases in crude extract (15 mg protein ml^{-1} and containing 1 mM-dithiothreitol) were stable for several weeks at -20°C and for several days at 4°C ; diluted samples at 4°C rapidly lost activity (85–100% in 2 d), and this loss was more rapid in the presence of dithiothreitol.

A spontaneous, stable, rifampicin-resistant mutant of *C. testosteroni* T-2 was derived and designated strain T-2R. Two strains of *Escherichia coli* HB101 were used. Strain SH1041 contained pUCD623, the suicide vector for Tn4431 (Shaw & Kado, 1987). Strain SH1021 contained pRK2013, a Mob⁺ helper plasmid (Ditta *et al.*, 1980). YEPG medium (yeast extract, peptone and glucose) was defined by Sayler *et al.* (1985).

Generation and classification of transposon mutants. The transposon vector pUCD623 was mobilized into *C. testosteroni* T-2R by triparental surface mating with a third strain carrying pRK2013 (Towner & Vivian, 1976). Exconjugants were selected by growth on YEPG agar containing rifampicin (100 $\mu\text{g ml}^{-1}$) and tetracycline (12.5 $\mu\text{g ml}^{-1}$). Tetracycline-resistant mutants of strain T-2R (putative transposon mutants) were screened in microtitre plates for the release of sulphite from TS (the reaction of the wild-type) by the transient development of yellow colour with 250 μM -Ellman's reagent (Johnston *et al.*, 1975) on YEPG agar containing 5 mM-TS. Transposon mutants that failed to give a colour change were tested for growth in TS-salts medium, and some grew slowly. The remaining transposon mutants were grown in 10 ml YEPG medium containing 5 mM-TS and tetracycline (12.5 $\mu\text{g ml}^{-1}$), harvested, washed and resuspended in TS-salts medium for 24 h. The medium was then examined by HPLC for the transformation of TS.

Enzyme assays. The activity of the two-component 4-toluene-sulphonate methyl-monooxygenase system (TSMOS, consisting of reductase B and oxygenase M), and the analogous TCMOS was assayed as oxygen uptake essentially as described elsewhere (Locher *et al.*, 1991b), except that TS (or TC) was used as the substrate. Reductase(s) B was identified as TSMOS (TCMOS) activity when combined with component(s) M; the reductase was assayed routinely at 25°C as reduction of cytochrome *c* (Locher *et al.*, 1991a).

SOL dehydrogenase and SYD dehydrogenase were originally assayed discontinuously by HPLC (Locher *et al.*, 1989), which was unsuitable for monitoring enzyme purifications. Continuous spectrophotometric (340 nm) assays were developed and optimized by monitoring the reactions by HPLC. SOL dehydrogenase was measured as the back-reaction from SYD, which was more readily available, the pH being chosen to optimize the yield of SOL and to minimize SYD dehydrogenase activity. The reaction mixture contained (in 1.0 ml) 50 μmol Tris/HCl, pH 7.5, 600 nmol NADH, 300 nmol SYD and 50 to 500 μg protein, with which the reaction at 30°C was started: the reference cuvette contained (in 1.0 ml) 50 μmol Tris/HCl, pH 7.5, 300 nmol NADH and 300 nmol SYD. The reaction rate was constant for 3 min, during which time effectively stoichiometric amounts (>95%) of SOL were formed. SYD dehydrogenase was measured in the forward reaction and the pH was chosen to optimize the yield of PSB and to minimize SOL dehydrogenase activity. The reaction mixture contained (in 1.0 ml) 50 μmol Tris/HCl, pH 9.0, 600 nmol NAD⁺, 30 nmol SYD and 25 to 250 μg protein, with which the reaction at 30°C was started. The reaction rate was constant for 1 min, during which time essentially stoichiometric amounts (>90%) of PSB were formed. COL dehydrogenase and CYD dehydrogenase were measured in analogous reactions.

Enzyme purifications. Crude extract was treated with protamine sulphate to remove nucleic acids (Locher *et al.*, 1991a). Reductase(s) B and oxygenase(s) M were purified via a common anion-exchange step (Mono Q, Fig. 3) followed by individual hydrophobic-interaction chromatography (phenyl-Superose) and gel filtration as described elsewhere (Locher *et al.*, 1990a, b). The purification or separation of the SOL (COL) dehydrogenase and the SYD (CYD) dehydrogenase involved the same techniques. The anion exchanger, with or without the dithiothreitol necessary for TSMOS, also separated the dehydrogenases. Pooled and concentrated samples of SOL (COL) dehydrogenase were separated on the hydrophobic interaction column with an initial mobile phase of 20 mM-potassium phosphate containing 0.6 M- $(\text{NH}_4)_2\text{SO}_4$, pH 7.5 and eluted in a decreasing gradient of $(\text{NH}_4)_2\text{SO}_4$. The mobile phase in the subsequent gel-filtration chromatography of SOL (COL) dehydrogenase was 20 mM-potassium phosphate containing 100 mM- Na_2SO_4 , pH 7.5. Pooled and concentrated fractions of SYD (CYD) dehydrogenase were separated on the hydrophobic-interaction column with an initial mobile phase of 20 mM-potassium phosphate, pH 6.8, and eluted in an increasing gradient to 45% (v/v) ethylene glycol. Alternatively, material was examined by gel-filtration chromatography with a mobile phase of 50 mM-potassium phosphate containing 150 mM- Na_2SO_4 , pH 6.8.

Results

Degradative pathway of p-toluate in *C. testosteroni* T-2

Whole cells (not shown) or extracts (Fig. 2) of *C. testosteroni* T-2 grown in TC-salts medium degraded TC with the transient accumulation of two intermediates, which were tentatively identified as COL and PcB by co-

Fig. 2. Degradation of TC via COL and PcB by extracts of TC-grown cells of *C. testosteroni* T-2. The reaction mixture (3 ml initial vol., shaken at 30 °C) contained 150 µmol potassium phosphate buffer, pH 6.8, 3 µmol NADH, 400 nmol FeSO₄, 17 mg protein and 3 µmol TC, with which the reaction was started. Samples were taken at intervals for analysis by HPLC. ■, TC; ▲, COL; ●, PcB.

Table 1. Chromatographic behaviour and data from UV spectra of TC, of pathway intermediates in the degradation of TC, and of reference compounds

Samples were separated by HPLC on reversed-phase columns with a mobile phase of 52% (v/v) methanol in 40 mM-potassium phosphate buffer, pH 2.2.

Substance	Source and catalyst	Retention time (min)	Data from UV spectra		
			Max. (nm)	Min. (nm)	Max.* (nm.)
TC	Standard	9.1	198	214	238 ^a
COL	Standard	4.2	199	213	235 ^a
COL	TC, crude extract†	4.2	199	213	235 ^a
COL	CYD, enzyme‡	4.2	202	213	235 ^a
CYD	Standard	5.1	201	218	252 ^a
CYD	COL, enzyme‡	5.1	198	217	251 ^a
PcB	Standard	4.9	196	212	241 ^b
PcB	TC, crude extract†	4.9	197	212	241 ^b
PcB	CYD, enzyme§	4.9	196	212	241 ^b

* *a*, There is a shoulder at a longer wavelength; *b*, at a longer wavelength there is a weak maximum, which we did not define.

† This material was a transient intermediate.

‡ Purified COL dehydrogenase was used at pH 9.0, either in the forward- or in the back-reaction: the forward reaction was not favoured.

§ CYD dehydrogenase after separation by anion exchange chromatography.

chromatography with authentic material and by UV-spectra (Table 1). The identity of COL was confirmed by mass spectrometry (Locher *et al.*, 1991*a*); the identity of PcB was confirmed by thermospray HPLC-MS, where the molecular ion ($M^+ + 1 = 167$) was observed. Each

Table 2. Specific activities of enzymes involved in the degradation of TC in extracts of *C. testosteroni* T-2 grown in succinate-salts medium or TC-salts medium

Reaction	Specific activity [mkat (kg protein) ⁻¹] in extracts from cells grown with:	
	Succinate	TC
TC oxygenation*	<0.1	1.4
COL dehydrogenase	1	81
CYD dehydrogenase	1	11
PcB oxygenation*	<0.1	3.3
Protocatechuate oxygenation	0.5	5.0
Catechol oxygenation	<0.1	<0.1
4-Methylcatechol oxygenation	<0.1	<0.1
<i>p</i> -Cresol oxygenation	<0.1	<0.1

* The substrate is subject to transformation to intermediates which are also oxygenated (cf. Figs 1 and 2), so the values represent the activities of more than one oxygenase.

intermediate of the pathway TC to COL to CYD to PcB was obtained from its putative precursor (Table 1), and the necessary enzyme activities could be assayed (Table 2), so we consider the pathway to be established. This pathway was absent in succinate-grown cells (Table 2), and is thus inducible, as is the analogous pathway for TS to PSB in this organism (Locher *et al.*, 1989). PcB was oxygenated in extracts of TC-grown cells and the presumed product of re-aromatization, protocatechuate, was subject to 4,5-dioxygenation (Table 1).

Analysis of mutants of strain T-2

Extracts of cells grown in TC-salts medium could also convert TS to PSB, and extracts of TS-grown cells could convert TC to PcB. It was unclear whether strain T-2 contained two independent sets of enzymes catalysing similar reactions (TS to PSB and TC to PcB) and also active on the corresponding substrate in the other pathway, or whether there was only one set of enzymes. Analyses of the three available classes of transposon-insertion mutants impaired in growth on TS-salts agar supported the latter hypothesis. Most mutants (53 of 55) unable to grow on TS-salts agar also failed to grow on TC-salts agar. Mutants which grew slowly on TS-salts agar could mostly (11 of 12) grow on TC-salts agar. And most mutants (5 of 6), blocked in the metabolism of PSB but able to convert TS to PSB, were able to utilize TC for growth. These growth properties and the largely concomitant loss of the ability to transform TC and TS to their carboxylated derivatives suggested a shared pathway for sidechain oxygenation and oxidation of TS and TC, but specific enzymes for oxygenation of PSB or PcB to protocatechuate (see Fig. 5).

Fig. 3. Anion-exchange chromatography of extracts of *C. testosteroni* T-2 grown in TS-salts or TC-salts medium and the location of the activities of the monooxygenase and the two dehydrogenases. Cell extract (150 to 400 mg) from TC-grown (a) or TS-grown cells (b) was treated with protamine sulphate to remove nucleic acids, and loaded on a Mono Q column. Relevant data from four chromatograms are shown, because the mobile phase for the separation of the dehydrogenases was 25 mM-potassium phosphate buffer, pH 7.0 (fraction nos < 20), whereas the components of the monooxygenase system(s) (TSMOS, TCMOS) were separated in the same buffer including 1 mM-dithiothreitol (fraction nos > 20). SOL and COL dehydrogenases (I, i; ■) or SYD and CYD dehydrogenases (II, ii; □) were located with the spectrophotometric assay. The two components of TSMOS (TCMOS) were located by the coupled oxygen uptake assay. Component B, the reductase (IV, iv; ○), was assayed in the presence of crude component M (oxygenase) (fractions 23 to 25). The oxygenase component (III, iii; ●) was measured in the presence of crude reductase from fraction 31. The salt gradient (---) and the absorbance due to protein (A_{280} ; —) are indicated.

Separation and purification of enzymes

Extracts of TS-grown and TC-grown cells were subjected to anion-exchange chromatography (Fig. 3a, b). The three biochemical reactions from TS to PSB could be separated into four protein peaks, which represented the reductase (IV) and oxygenase (III) components of TSMOS, a single SOL dehydrogenase (I) and a single SYD dehydrogenase (II). Similarly, the reactions converting TC to PCB were separated into four protein peaks, which represented the reductase (iv) and oxygenase (iii) components of 'TC monooxygenase', a single 'COL dehydrogenase' (i) and a single 'CYD dehydrogenase' (ii). The corresponding activities (e.g. I and i) had identical chromatographic behaviour, which supported there being one set of enzymes for both sets of substrates.

The co-identity of the corresponding proteins from the two culture media was confirmed by further chromatographic properties (Table 3). Each pair of samples demonstrated essentially identical behaviour on hydrophobic-interaction chromatography, gel-filtration chromatography (i.e. native M_r), and, at least for I, III and IV, on SDS-PAGE (Fig. 4). Correspondingly, the pairs of samples had identical substrate ranges and identical

relative activities with the appropriate substrates (Table 3). Purified SOL dehydrogenase was active effectively only with NADH; rates with NADPH were 50-fold lower. SYD dehydrogenase, examined after anion-exchange chromatography, was more active with NAD⁺ (100%) than with NADP⁺ (12%).

The N-terminal amino acid sequence of TSMOS oxygenase from the TC-salts medium was Met-Phe-Ile-Ile-Asn-Asn-X-Tyr-Val-(Ala)-Ala-Ala. This is identical with the published sequence of the N-terminus of the corresponding protein from TS-grown cells (cf. Locher *et al.*, 1991b). This direct comparison of these molecules at the molecular level reconfirms the identity established in Table 3.

C. testosteroni NCIB 8893 (Dagley & Patel, 1957), which degrades *p*-cresol via a methyl hydroxylation (Hopper, 1988), did not grow in TC-salts medium, so the pathway of toluate degradation from strain T-2 was absent from NCIB 8893; extracts of TS-grown strain T-2 did not degrade *p*-cresol (Table 2). The type strain of *C. testosteroni*, DSM 50244, did not grow in TC-salts medium, or in TS-, PSB- or PCB-salts media. So the pathway in Fig. 5 is probably rare in *Comamonas testosteroni*.

Fig. 4. SDS-PAGE of crude extracts of *C. testosteroni* T-2 grown in TS-salts or TC-salts medium, and of separated or purified enzymes. The samples derived from TS-grown cells (to the left of a given pair of lanes) and TC-grown cells were separated on 12% (w/v, acrylamide) gels. Lanes 1 and 2, crude extract; 3 and 4, component M (oxygenase) of TSMOS; 5 and 6, component B (reductase) of TSMOS; 8 and 9, SOL dehydrogenase. The M_r markers are in lanes 7 and 10.

Table 3. Comparison of properties of enzymes from *C. testosteroni* T-2 grown in salts medium containing TS or TC

Enzyme (with appropriate growth substrate)	Gradient concn (mm)*		M_r		Relative activity towards different substrates:†		
	Ion-exchange chromatography (gradient Na_2SO_4)	HIC chromatography (gradient $(\text{NH}_4)_2\text{SO}_4$)	Gel filtration	SDS-PAGE	TC:TS	DCIP:cyt. c	CYD:SYD
Monooxygenase (TS)	35	275	152000	42000	1:1:1		
Monooxygenase (TC)	35	260	145000	42000	1:1:1		
Reductase (TS)	60	150	39000	36000		0.6:1	
Reductase (TC)	55	150	40000	36000		0.6:1	
Alcohol dehydrogenase (TS)	0	45	62000	30000			0.7:1‡
Alcohol dehydrogenase (TC)	0	45	65000	30000			0.7:1‡
Aldehyde dehydrogenase§ (TS)	25	0 + EG	180000	34000			1.2:1
Aldehyde dehydrogenase§ (TC)	25	0 + EG	192000				1.0:1

* The data refer to the salt concentrations at which the appropriate protein was eluted from the ion-exchange column (Mono Q) and the hydrophobic interaction column (HIC). The term EG refers to the 20% (v/v) ethylene glycol required to elute the aldehyde dehydrogenase from the column. The data represent means of at least two determinations.

† In addition to our usual abbreviations, DCIP refers to 2,6-dichlorophenol indophenol and cyt. c refers to cytochrome c. With the exception of the aldehyde dehydrogenase, protein of the highest purity was used.

‡ Five further compounds were tested as substrates for the alcohol dehydrogenase: benzaldehyde, *p*-hydroxybenzaldehyde, *p*-methoxybenzaldehyde and the *ortho*-isomers of CYD and SYD. None was a substrate.

§ The aldehyde dehydrogenase was unstable, and survived only two separatory columns, so material from the ion exchanger could be examined with either the HIC or the gel-filtration column, but not with both. This relatively hydrophobic protein tended to be lost on concentration, probably on the filter membrane: after recognizing this problem, activity could always be correlated with the presence of a band near M_r 34000 on SDS-PAGE gels.

|| Four further compounds were tested as substrates for the aldehyde dehydrogenase: benzaldehyde, *p*-methoxybenzaldehyde and the *ortho*-isomers of CYD and SYD. The relative activities of the enzyme from TS-grown or TC-grown cells for those substrates, relative to the activity for SYD, was 0.6:0.4:0.0:1 or 0.5:0.3:0.0:1. The enzyme preparation used was from the ion-exchange column.

Discussion

The pathway of degradation of TC via PcB (Fig. 5) is proven by observing all the necessary intermediates (Table 1), by observing the required enzyme activities (Table 2) and by isolation or separation of those enzymes (Table 3). We presume the degradation of PcB to proceed via dioxygenation to a dihydrodiol, reduction to protocatechuate and then *meta* ring-cleavage (Fig. 5). An apparently two-component PcB dioxygenase system has

been observed in PcB-grown cells of strain T-2 (H. R. Schläfli & A. M. Cook, unpublished). The formation of the PcB dihydrodiol and a dihydrodiol dehydrogenase have been demonstrated in a strain of *Pseudomonas putida* (M. E. Ruppen, personal communication), and we have evidence for their presence in strain T-2 (H. R. Schläfli & A. M. Cook, unpublished). The *meta* cleavage of protocatechuate, observed in TS-grown cells (Locher *et al.*, 1989), was also observed in TC-grown cells (Table 2).

Fig. 5. Shared pathway of degradation of TS and TC to the level of the dibasic acid, and the steps to protocatechuate.

The unusual nature of the pathway from TC to PcB lies not in the reactions catalysed (see Introduction), however rarely the enzymes are purified (Locher *et al.*, 1991*b*; Chalmers *et al.*, 1990; Shaw & Harayama, 1990); rather, the novelty lies in the relative location of the different substituents on the aromatic ring. Whereas the oxygenation of a methyl group, positioned *meta* to a carboxy group, is known in a threefold substituted ring (Fig. 1, line 6; cf. line 8), there appears to be no example of the conversion to the carboxy group of a methyl group that is positioned *para* to a carboxy group (Fig. 1).

The reaction sequence involving oxygenation of an aromatic methyl group followed by two dehydrogenases to give an aromatic carboxy group is widespread (Fig. 1), as are relatively wide substrate ranges in those enzymes tested (Fewson, 1981). *C. testosteroni* T-2 can use largely the same set of enzymes to degrade the sulphonated (xenobiotic) compound TS, and its carboxy analogue, TC: only one additional catalytic enzyme, the desulphonative dioxygenation of PSB (Locher *et al.*, 1991*a*), is required to degrade the xenobiotic compound. What is still poorly understood is the nature of the transport system(s) that must be necessary (Thurnheer *et al.*, 1990; Groenewegen *et al.*, 1990) to bring, e.g., TC or TS from the growth medium into contact with the degradative enzymes.

The small amount of information available on the methyl-monooxygenases [TS/TC methyl-monooxygenase (Locher *et al.*, 1991*b*); toluene/xylene methyl-monooxygenase (Suzuki *et al.*, 1991)] illustrates the diversity possible in apparently similar degradative pathways. The M_r values of the corresponding components (two) in each system are similar, but the corresponding N-terminal amino acid sequences are different; both reductases are soluble, but the TOL-plasmid-encoded oxygenase is obviously membrane bound, whereas that from strain T-2 is soluble. Our limited information on SOL and SYD dehydrogenases extends this diversity.

SYD dehydrogenase, with its low subunit M_r (34 000) and apparently hexameric structure (Table 3), shows no superficial similarity to the recently purified di- or tetrameric aromatic aldehyde dehydrogenases (subunit M_r about 55 000) (Shaw & Harayama, 1990; Chalmers *et al.*, 1990), or even to the tetrameric *o*-CYD dehydrogenase (subunit M_r 41 000) in *Alcaligenes* sp., which is inactive with *p*-CYD (Kiyohara *et al.*, 1981), just as our SYD dehydrogenase is inactive with *o*-CYD (Table 3). Analogously, SOL dehydrogenase has an M_r (30 000) different from those of the known aromatic alcohol dehydrogenases (M_r 40 000; Shaw & Harayama, 1990; Chalmers *et al.*, 1991) and of other enzymes in the class of zinc-dependent, long-chain alcohol dehydrogenases (Wales & Fewson, 1991). The M_r value corresponds more to the bacterial short-chain alcohol dehydrogenases and not to other known groups of alcohol dehydrogenases (cf. Wales & Fewson, 1991). In related work, S. W. Hooper (unpublished) demonstrated the absence of hybridization between TOL (pWW0) upper-pathway genes and total DNA from strain T-2. Similarly, R. M. Chalmers and C. A. Fewson (personal communication) found that Western-blotting experiments failed to reveal any cross-reactions when denatured SOL dehydrogenase and SYD dehydrogenase (in crude extracts and partially purified) were probed with antisera against benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from *Acinetobacter calcoaceticus* or the corresponding TOL-plasmid-encoded enzymes from *Pseudomonas putida*. Similarly of substrates and chemical reaction do not necessarily mean high levels of identity between enzymes.

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