Regulation of the degradative pathways from 4-toluenesulphonate and 4-toluenecarboxylate to protocatechuate in *Comamonas testosteroni* T-2

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***Comamonas testosteroni* T-2 was grown in salts medium containing intermediates of the established, inducible degradative pathway(s) for 4-toluenesulphonate/4-toluenecarboxylate. The specific activity or, if appropriate, the specific expression of pathway enzymes or their components was constant throughout growth and decreased only slowly in the stationary phase. It was found that the 4-toluenesulphonate methyl-monooxygenase system and 4-sulphobenzyl alcohol dehydrogenase (with 4-sulphobenzaldehyde dehydrogenase) were always co-induced, with similar ratios of their activities during growth with 4-toluenesulphonate, 4-toluenecarboxylate and 4-sulphobenzoate. We presume these enzymes to be co-expressed from one regulatory unit. The ratio of activities of the terephthalate 1,2-dioxygenase system to those of (1*R*,2*S*)-dihydroxy-1,4-dicarboxy-3,5-cyclohexadiene dehydrogenase was also constant, and present only during growth with 4-toluenecarboxylate or terephthalate. We presume these two enzymes to be co-expressed from a different regulatory unit. The oxygenase component of 4-sulphobenzoate 3,4-dioxygenase (PSBDOS) was expressed at high levels in most growth conditions examined, the exception being with 4-toluenecarboxylate as carbon source. However, no expression of a specific reductase activity linked to synthesis of the oxygenase of PSBDOS could be detected. The PSBDOS was thus active *in vivo* solely under conditions where the 4-toluenesulphonate methyl-monooxygenase system was also present, whose reductase is active with the oxygenase of the 4-sulphobenzoate 3,4-dioxygenase system *in vitro*, and, apparently, *in vivo*. The synthesis of PSBDOS is thus under the control of a third regulatory unit.**

Keywords: *Comamonas testosteroni*, regulation of aromatic catabolism, oxygenase systems, *p*-toluenesulphonate, *p*-toluate

INTRODUCTION

Comamonas testosteroni T-2 utilizes 4-toluenesulphonate or its non-sulphonated analogue, 4-toluenecarboxylate (*p*-

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Abbreviations: DCDDH, (1*R*,2*S*)-dihydroxy-1,4-dicarboxy-3,5-cyclohexadiene dehydrogenase; SOLDH, 4-sulphobenzyl alcohol dehydrogenase; SYDDH, 4-sulphobenzaldehyde dehydrogenase; PC450, protocatechuate 4,5-dioxygenase; PSBDOS, *p*-sulphobenzoate 3,4-dioxygenase system; TERDOS, terephthalate 1,2-dioxygenase system; TSMOS, 4-toluenesulphonate methyl-monooxygenase system.

toluate) as sole source of carbon and energy for growth (Fig. 1) (Locher *et al.*, 1989, 1991c; Thurnheer *et al.*, 1986). Toluenesulphonate, at least, is taken up by a specific and inducible transport system (Locher *et al.*, 1993), and the catabolic enzymes are inducible (Locher *et al.*, 1989, 1991c). In the cell, metabolism of toluenesulphonate and toluate is initiated by the same enzyme, the 4-toluenesulphonate methyl-monooxygenase system (TSMOS), which consists of a monomeric reductase (component B) and a homomultimeric oxygenase (component M), to yield 4-sulphobenzyl alcohol and 4-carboxybenzyl alcohol, respectively (Locher *et al.*, 1991b). 4-Sulphobenzylalcohol dehydrogenase, SOLDH, oxi-

Fig. 1. Transport and degradation of toluenesulphonate and toluate in *C. testosteroni* T-2, and the regulatory units deduced from this work (R1–R4). The regulation of the synthesis of specific uptake systems for toluenesulphonate (Locher *et al.*, 1993) and terephthalate (this work), and of a putative transport system for sulphobenzoate, is as yet undefined. Enzyme abbreviations are defined on the title page; substrates are abbreviated as follows: COL, 4-carboxybenzyl alcohol; CYD, 4-carboxybenzaldehyde; DCD, (1*R*,2*S*)-dihydroxy-1,4-dicarboxy-3,5-cyclohexadiene; PC, protococatechuate; PSB, 4-sulphobenzoate; SOL, 4-sulphobenzyl alcohol; SYD, 4-sulphobenzaldehyde; TC, 4-toluenecarboxylate; TER, terephthalate; TS, 4-toluenesulphonate.

dizes sulphobenzyl alcohol and carboxybenzyl alcohol to the corresponding aldehydes, 4-sulphobenzaldehyde and 4-carboxybenzaldehyde, respectively. The aldehydes are oxidized by 4-sulphobenzaldehyde dehydrogenase, SYDDH, to 4-sulphobenzoate and terephthalate (Locher *et al.*, 1989, 1991c). Sulphobenzoate is the substrate for the two-component 4-sulphobenzoate 3,4-dioxygenase system, PSBDO, which yields protococatechuate (Locher *et al.*, 1991a). PSBDO consists of a homomultimeric oxygenase (component A) presumably coupled to the uncharacterized reductase C; the system, however, is active and was characterized with the reductase of TSMOS (component B) (Locher *et al.*, 1991a, b). Protocatechuate is further metabolized via the 4,5 *meta*-cleavage pathway (Locher *et al.*, 1989). Terephthalate is oxygenated to the diene-diol (1*R*,2*S*)-dihydroxy-1,4-dicarboxy-3,5-cyclohexadiene by the multicomponent terephthalate 1,2-dioxygenase system, TERDOS, which consists of a heteromultimeric oxygenase (component Z) and partially characterized reductase component(s) (Schläfli *et al.*, 1994). The diene-diol is oxidized to protococatechuate by a (1*R*,2*S*)-dihydroxy-1,4-dicarboxy-3,5-cyclohexadiene dehydrogenase, DCDDH (Saller *et al.*, 1995).

We now report on the patterns of induction of components of the pathways degrading toluenesulphonate and toluate in *C. testosteroni* T-2.

METHODS

Materials. 4-Sulphobenzoate was from Aldrich. The sources of other chemicals, and of materials for protein purification and characterization, and for immunological experiments, are given elsewhere (Locher *et al.*, 1989; Schläfli *et al.*, 1994; Thurnheer *et al.*, 1986).

Growth of the organism and preparation of cell-free extracts.

C. testosteroni strain T-2 (DSM 6577) was grown in batch culture (portions of 1 l in 5 l flasks) in mineral salts medium containing 6 mM (aromatic substrate) or 10 mM (succinate) carbon source, essentially as described previously (Locher *et al.*, 1989; Thurnheer *et al.*, 1986); the medium was adjusted to pH 7.2 for sulphonated aromatics, to pH 6.8 for terephthalate, and to pH 7.0 for other substrates. For experiments with whole cells, portions of 20 ml were taken at intervals from the cultures. Reproducible activities could be measured when the cells were immediately chilled in an ice-water slurry and centrifuged at 4 °C for 20 min at 5000 *g*. The cells were washed, suspended in 0.5–1 ml 20 mM phosphate buffer, pH 7.0 (buffer A), and stored on ice. Oxygen uptake rates remained stable for at least 5 h. Cells

for the preparation of extracts were harvested in the late exponential growth phase (approx. 200 mg protein l⁻¹) and stored frozen as described previously (Locher *et al.*, 1991a). Cells were disrupted in a French pressure cell, and crude extract, in buffer A containing 1 mM dithiothreitol, was prepared as detailed elsewhere (Locher *et al.*, 1991a).

Analytical methods. The concentrations of aromatic compounds were determined by HPLC on reversed-phase columns (Thurnheer *et al.*, 1986); UV spectra were recorded in a diode array detector. Protein concentration in cell extracts was measured by the method of Bradford (1976) with bovine serum albumin as standard. The protein content of cell suspensions was assayed by a Lowry method (Kennedy & Fewson, 1968) with bovine serum albumin as standard. SDS-PAGE (Laemmli, 1970) was used to monitor the protein patterns of crude extracts and to separate oxygenase components for the affinity purification of antibodies (see below). Proteins in gels were visualized with Coomassie brilliant blue (Laemmli, 1970; Locher *et al.*, 1991a). Low-range prestained SDS-PAGE molecular mass standards were used (Bio-Rad), consisting of phosphorylase *b* (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa). Immunological detection of oxygenase components in separated proteins was done after proteins were blotted from SDS-PAGE onto nitrocellulose membranes. Blotting was at 4 °C in a Mini Trans-Blot cell (Bio-Rad) at 100 V (250 mA) for 1 h or at 30 V (40 mA) overnight, as detailed elsewhere (Schläfli *et al.*, 1994).

Enzyme assays. Oxygen uptake rates of resting cells (pathway activities; see Results for explanation) were measured at 30 °C in a Clark-type oxygen electrode with a 1 ml reaction vessel. Washed cell suspension (10–50 µl) was added to 0.5 ml of buffer A, equilibrated for 2 min, and the reaction was started by the addition of 600 nmol substrate. TSMOS was assayed as 4-ethylbenzoate-dependent oxygen uptake (Locher *et al.*, 1991b). TERDOS was assayed as pyridine-2,5-dicarboxylate-dependent oxygen uptake (Schläfli *et al.*, 1994). The activity of PSBDOS was calculated from oxygen uptake rates with sulphobenzoate, corrected for the presence of protocatechuate 4,5-dioxygenase (PC45O) (Locher *et al.*, 1991a). Unless otherwise indicated, all oxygenase assays contained (in 0.5 ml) 1 mg protein and activities were not optimized for the concentration of enzyme components (see Results for explanation). SOLDH was measured spectrophotometrically as the back reaction, i.e. the carboxybenzaldehyde-dependent oxidation of NADH (Locher *et al.*, 1991c). DCDDH was measured spectrophotometrically as diene-diol-dependent generation of NADH (Schläfli *et al.*, 1994). Dehydrogenase assays contained 50–500 µg protein. Components of oxygenases were purified as described elsewhere (Locher *et al.*, 1991a, b; Schläfli *et al.*, 1994).

Production and purification of specific antisera. All animal experiments were done according to the guidelines of the Schweizer Bundesamt für Veterinärwesen and approved by the Kantonale Aufsichtskommission für Tierversuche. Polyclonal antisera were raised in female New Zealand White rabbits against oxygenase M and reductase B of TSMOS, oxygenase A of PSBDOS and oxygenase Z of TERDOS (Schläfli *et al.*, 1994). Useful dilutions for the crude antisera were 1:100 for anti-B, 1:1000 for anti-M and anti-Z, respectively, and more than 1:10000 for anti-A. The crude antisera were purified by preincubation with the appropriate antigens separated by SDS-PAGE and blotted on to nitrocellulose membranes (Harlow & Lane, 1988). The purified antisera were used at dilutions of 1:25 (1:50 for anti-A). All affinity-purified antisera were highly specific: there was essentially no reaction (under standard

conditions) of any of the three antisera raised against an oxygenase component (anti-A, anti-M or anti-Zα) with any of the other two oxygenase components. Antiserum specific for component (reductase B) of TSMOS did not react with any other protein in any extract of *C. testosteroni* T-2 tested.

RESULTS

Growth kinetics

C. testosteroni T-2 grows exponentially in toluene-sulphonate-salts medium ($\mu = 0.37 \text{ h}^{-1}$), and the more rapid growth with toluenesulphonate than with sulphobenzoate has been interpreted as the explanation for transient excretion of sulphobenzoate in toluene-sulphonate-salts medium (Table 1). No other excreted intermediate is detected, and growth is directly proportional to the total aromatic substrate utilized (Locher *et al.*, 1989). *C. testosteroni* T-2 grows in sulphobenzyl alcohol-salts and in sulphobenzaldehyde-salts medium, but no growth kinetics could be examined due to the scarcity of these compounds (C. Malli & A. M. Cook, unpublished). Sulphobenzoate is utilized without detectable excretion of intermediates (Locher *et al.*, 1989). *C. testosteroni* T-2 grew in toluate-salts medium with the lowest growth rate of any compound tested (Table 1). Nonetheless, considerable transient accumulation of carboxybenzyl alcohol and terephthalate was observed. When the organism was grown in carboxybenzyl alcohol-salts medium, terephthalate was excreted transiently (Table 1). *C. testosteroni* T-2 in carboxybenzaldehyde-salts medium excreted carboxybenzyl alcohol and terephthalate transiently (Fig. 2). In contrast, terephthalate was utilized without detectable excretion of intermediates. In all cases there was mass balance, the growth yield being about 5 g protein per mol C (cf. Cook, 1987).

Enzyme assays

The specific activity of a multicomponent oxygenase is usually difficult to measure in cell extracts (e.g. Bünz & Cook, 1993; Locher *et al.*, 1991a; Tan *et al.*, 1994; Thurnheer *et al.*, 1990), a problem that might be avoided by measuring the activities in whole cells (Tan *et al.*, 1994). In our case, however, the substrate-dependent oxygen uptake rate of cells represents not only the activity of the specific oxygenative enzyme but also the transport of the substrate into the cell (Locher *et al.*, 1993), and, in the case of toluenesulphonate (and TSMOS) for example, two additional oxygenases (PSBDOS, PC45O) which oxygenate pathway intermediates (Locher *et al.*, 1989), and possibly also respiration. We were unable to eliminate these problems by using substrate analogues [4-ethylbenzoate for TSMOS and pyridine-2,5-dicarboxylate for TERDOS (Locher *et al.*, 1991b; Schläfli *et al.*, 1994)], because neither compound was biotransformed by appropriately induced whole cells, presumably because of a permeability barrier. Oxygen uptake rates of washed cells were thus regarded in this work as pathway activities, and not as oxygenase activities.

For reasons which should become clear in the following

Table 1. Some growth characteristics of *C. testosteroni* T-2

The data are representative of three or more batch cultures with, initially, 6 mM substrate (10 mM for succinate). Substantial lag phases (14–20 h) were observed routinely when toluate-salts medium was inoculated from actively growing precultures in toluate-salts medium; other media supported almost immediate growth after homologous transfer.

Substrate	Growth rate (μ , h ⁻¹)	Metabolites detected during growth (mM)*		
		Carboxybenzyl alcohol	Terephthalate	Sulphobenzoate
Toluenesulphonate†	0.37	ND	ND	1.5
Sulphobenzoate†	0.30	ND	ND	ND
Toluate	0.20	1.9	2.0	ND
Carboxybenzyl alcohol	0.50	—	0.5	ND
Carboxybenzaldehyde	0.24	1.1	3.4	ND
Terephthalate	0.37	ND	ND	ND
Succinate	0.77	ND	ND	ND

ND, Not detected.

* Maximal concentration of excreted intermediate during growth.

† Published data (Locher *et al.*, 1989).

Fig. 2. Growth of *C. testosteroni* T-2 in 6 mM carboxybenzaldehyde-salts medium as a differential plot of substrate utilization as a function of growth. ■, Sum of the concentrations of substrate and excreted metabolites; ◆, carboxybenzyl alcohol; ○, carboxybenzaldehyde; ◇, terephthalate.

Results, we chose not to measure activities of each multi-component oxygenase in cell extracts in a linearized assay (cf. Tan *et al.*, 1994), but to use the older method of doing all assays at the arbitrarily set protein concentration of 2 mg ml⁻¹ (Geary *et al.*, 1990). The data obtained may therefore not represent *in vivo* conditions but they allow, together with the immunoblots, an unambiguous characterization of the different extracts.

SOLDH, PC45O and DCDDH, whose assays present no problem (Locher *et al.*, 1991c; Schläfli *et al.*, 1994), were measured in appropriately diluted crude extracts. SYDDH was assayed but not quantified, because it was unstable in cell extracts.

Pathway activities, enzyme activities and their induction

The pathway activities for e.g. toluenesulphonate degradation and sulphobenzoate degradation in toluenesulphonate cultures and for terephthalate degradation in terephthalate cultures were essentially stable throughout growth and decreased only slowly in the stationary phase (not shown). Typical values for pathway activities are given in Table 2. The half-life of pathway activities in the stationary phase was about 50 h (not shown).

Oxygen uptake with protocatechuate was detected in all cells grown with aromatic compounds (Table 2). The inducible nature of these pathways was supported by the absence of activities in succinate-grown cells, and was largely confirmed (see below), when enzyme activities were measured.

One of the highest pathway specific activities observed was for toluenesulphonate in toluenesulphonate-grown cells (Table 2). The specific activity for sulphobenzoate in these cells was much lower, which we presume to cause a build-up of that compound in the cell followed by the excretion of sulphobenzoate in toluenesulphonate cultures (Tables 1 and 2). Activity for toluate and terephthalate was also observed (Table 2). The pathway specific activity for sulphobenzoate in sulphobenzoate-grown cells was lower than that in toluenesulphonate-grown cells; the ratio of specific activities toluenesulphonate:sulphobenzoate was similar to that in toluenesulphonate-grown cells. Minimal activity with toluate but none with terephthalate was observed in these cells (Table 2).

The highest pathway specific activity was for toluate in toluate-grown cells (Table 2), with the specific activity for terephthalate lower; we presume this to cause a build-up

Table 2. Oxygen uptake rates of whole cells with different pathway intermediates as assays of the pathway activities in cells of *C. testosteroni* T-2 grown with different substrates

The data are representative of cells from three growth experiments.

Growth substrate	Oxygen uptake rates [mkat (kg protein) ⁻¹] of washed cells to which were added				
	Toluene-sulphonate	Sulphobenzoate	Toluate	Terephthalate	Protocatechuate
Toluenesulphonate	7.1	3.4	4.5	1.1	5.0
Sulphobenzoate	4.2	2.3	1.0	< 0.1	3.7
Toluate	1.6	< 0.1	8.6	5.3	3.5
Terephthalate	< 0.1	0.4	< 0.1	4.4	4.0
Protocatechuate	< 0.1	< 0.1	< 0.1	< 0.1	5.1
Succinate	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

Table 3. Induction of enzymes in *C. testosteroni* T-2

The data are representative of three or more independent measurements.

Growth substrate	Enzyme (system) activities [mkat (kg protein) ⁻¹] in crude extracts of <i>C. testosteroni</i> T-2				
	TSMOS	SOLDH	PSBDOS	TERDOS	DCDDH
Toluenesulphonate	4.0	152	0.4	0.1	3.5
Sulphobenzoate	0.5	45.2	0.2	ND	1.8
Toluate	1.5	94	ND	0.35	13
Terephthalate	ND	5.6	ND	0.54	9.9
Succinate	ND	0.8	ND	ND	ND

ND, Not detected. Oxygen uptake rates of up to 0.05 mkat (kg protein)⁻¹ were observed in these extracts, but HPLC revealed no transformation of substrate.

of terephthalate in the cell followed by the excretion of that compound during growth in toluate-salts medium (Table 1, Fig. 2), though the pathway activities do not correspond to the growth rates (Table 1). The toluate-grown cells failed to oxygenate sulphobenzoate and had a low activity with toluenesulphonate. The terephthalate-grown cells had a pathway activity effectively only with terephthalate; there was no activity for toluate or toluenesulphonate, and a minimal activity with sulphobenzoate (Table 2).

No activity of TSMOS, PSBDOS, TERDOS or DCDDH (Table 3) was found in extracts of succinate-grown cells, and only traces of SOLDH (Table 3) and PC450 (Locher *et al.*, 1989) were detected. All activities thus appeared to be inducible.

Toluenesulphonate and toluate both induced TSMOS and SOLDH at very high levels, though activities in extracts of toluenesulphonate-grown cells were two- to threefold higher. Significant but lower levels of TSMOS and SOLDH were also observed in extracts from sulphobenzoate-grown cells, whereas very low activities of

SOLDH only were found in extracts of terephthalate-grown cells. The ratio of activities TSMOS:SOLDH was constant at about 1:60 in active extracts. SYDDH was detected only in those extracts containing significant levels of TSMOS and SOLDH. Extracts of cells grown in carboxybenzaldehyde contained active TSMOS and SOLDH (not shown). It would appear that the enzymes TSMOS, SOLDH and SYDDH share one pattern of induction.

Extracts of toluenesulphonate-grown cells contained the highest level of measurable PSBDOS activity [0.4 mkat (kg protein)⁻¹; Table 3]. The activity in extracts of sulphobenzoate-grown cells was only 0.2 mkat (kg protein)⁻¹, whereas extracts of toluate- and terephthalate-grown cells were inactive. The level of activity was independent of that of TSMOS (1:2.5, 1:10 and < 1:25 in extracts of sulphobenzoate-, toluenesulphonate- and toluate-grown cells, respectively; Table 3) and of that of TERDOS (4:1 and < 1:18 in extracts of toluenesulphonate- and terephthalate-grown cells, respectively). PSBDOS is thus regulated independently of both TSMOS and of TERDOS.

Fig. 3. Composite Western blots on nitrocellulose of extracts from *C. testosteroni* T-2 grown with different carbon sources and separated by SDS-PAGE. The same amount of protein (5 µg) was separated in each lane. Extracts were from cells grown with toluenesulphonate (lane 1), sulphobenzoate (lane 2), toluate (lane 3), terephthalate (lane 4) and succinate (lane 5). The blotted extracts were incubated with antisera specific for oxygenase A from PSBDOS (row A), oxygenase M from TSMOS (row M), reductase B from TSMOS (row B), and the α -subunit of oxygenase Z from TERDOS (row Z α). The image is a digitized composite; only those parts of the immunoblots which showed a significant reaction with the antisera are represented.

The most active TERDOS was found in extracts of terephthalate-grown cells, with somewhat lower levels in those of toluate-grown cells (Table 3). Activity could also be detected in extracts of toluenesulphonate-grown cells, corresponding to weak activity detected previously (Locher *et al.*, 1991c), with traces in extracts of sulphobenzoate-grown cells too (no transformation of substrate). The ratio of the activities of TERDOS:DCDDH in all active extracts was about 1:25. We thus consider TERDOS and DCDDH to belong to a further regulatory unit.

Expression of oxygenase components

Extracts of cells grown with the five standard carbon sources were separated by SDS-PAGE. Four different patterns were observed (not shown), with extracts of toluenesulphonate- and sulphobenzoate-grown cells sharing a pattern. The expression of oxygenase components B, M, A and Z was investigated using specific antisera (Fig. 3). Immunodetection of proteins in blotted extracts confirmed that components B (reductase) and M (oxygenase) of TSMOS were present only in extracts of toluenesulphonate-, toluate- and sulphobenzoate-grown cells. Signals with anti-Z α (the large oxygenase subunit of TERDOS) (and with anti-Z β , not shown) were readily visible in extracts of toluate- and terephthalate-grown cells, whereas the extract of toluenesulphonate-grown cells gave a weak signal (invisible in Fig. 3) and the extract of sulphobenzoate-grown cells none. Oxygenase A, in contrast, was detected in all samples, except the extract of toluate-grown cells. Thus even the extract of succinate-grown cells contained immunologically detectable amounts of oxygenase A, though no activity was found under the standard conditions (Tables 2 and 3). The presence of oxygenase A in protocatechuate-grown cells was not examined.

When extracts of terephthalate-grown or succinate-grown cells were incubated with added reductase B, PSBDOS activity was detected. The oxygenase component A, and not some cross-reacting protein, is thus present in succinate-grown and in terephthalate-grown cells.

DISCUSSION

Stability of multicomponent oxygenases

The loss of activity of multicomponent oxygenases in the stationary phase is considered normal, whether observed directly (Bünz & Cook, 1993; Junker *et al.*, 1994a, b; Moodie *et al.*, 1990; Sauber *et al.*, 1977; Thurnheer *et al.*, 1986; Wackett, 1990) or deduced from a comment on the correct timing for harvesting cells to purify proteins (Ensley *et al.*, 1982; Geary *et al.*, 1990; Pilkington & Dalton, 1990; Tonge *et al.*, 1977; Yamaguchi & Fujisawa, 1978). Our laboratory's initial experience with *C. testosteroni* T-2 seemed to be similar (Locher *et al.*, 1991a). After optimizing the growth conditions and refining the assay procedure for whole cells, we were surprised to find constant pathway activities throughout exponential growth (cf. Bünz & Cook, 1993; Junker *et al.*, 1994b) and high stability of these activities after growth (cf. Bünz & Cook, 1993; Junker *et al.*, 1994b; Moodie *et al.*, 1990). This behaviour of *C. testosteroni* T-2 is not only unusual, but it appears to be both metabolically expensive, given the large amounts of protein involved (often 4–10% of soluble protein per oxygenase component) (Locher *et al.*, 1991a, b; Schläfli *et al.*, 1994) and potentially harmful, in that the combination of reductase and oxygenase will tend to yield active oxygen species which may damage the cell. In this latter argument, a degradation of the reductase at least (Moodie *et al.*, 1990) would reduce this risk. We do not know how *C. testosteroni* T-2 maintains these activities. The generally short half-life of bacterial mRNA implies that the enzymes themselves are very stable in the cell. It would be interesting to see whether oxygenase systems in general are stable in this organism, or whether the effect is limited to TSMOS, TERDOS and PSBDOS.

Convergent and divergent pathways

Reviews of the degradation of aromatic compounds stress the convergence of pathways to yield the relatively few compounds subject to ring cleavage (cf. Dagley, 1975; Fewson, 1981; Stanier & Ornston, 1973), typified by the convergence of the metabolism of sulphobenzoate and terephthalate at protocatechuate, with subsequent ring cleavage of protocatechuate (Fig. 1). In *C. testosteroni* T-2, however, we also observe the less usual divergence of metabolism, caused by compounds sharing a common pathway and then requiring different reactions to allow entry to the common *meta* pathway (Fig. 1). The enzymes involved in the divergence are obviously required in large amounts in the cell, representing several percent of the cellular soluble protein (Locher *et al.*, 1991a; Schläfli *et al.*, 1994). Presumably for reasons of economy with protein synthesis, the organism regulates the degradative pathway in separate units, which we term regulatory unit R1 for

sidechain manipulation, regulatory unit R2 for removal of a carboxy substituent and preparation for ring cleavage, regulatory unit R3 for removal of a sulphonyl substituent and preparation for ring cleavage, and regulatory unit R4 comprising ring cleavage and possibly further reactions of the *meta* pathway (Fig. 1).

Regulatory unit R1

We consider this regulatory unit to consist of three catalytic activities, TSMOS (components B and M), SOLDH and SYDDH. These three enzymes were always synthesized (or absent) as a group (Table 3, Fig. 3). Where measurable, there was a constant ratio of specific activities (TSMOS:SOLDH; Table 3), and the presence of TSMOS and SOLDH during growth in carboxybenzaldehyde-salts medium confirmed the joint regulation. We do not yet know whether the transport of toluenesulphonate (Locher *et al.*, 1993) into the cell is co-regulated with the metabolic enzymes or what role transport, whose presence is deduced from the putative permeability barrier to ethylbenzoate, plays in the metabolism of toluate (cf. Harwood *et al.*, 1994).

Oxidation of the methyl sidechain of an aromatic compound results from a common set of degradative reactions (Assinder & Williams, 1990; Gibson & Subramanian, 1984; Locher *et al.*, 1991c; Mahajan *et al.*, 1994; Whited & Gibson, 1991), though the similarity of the chemistry need not indicate similarity of the enzymes (Locher *et al.*, 1991c). The regulation of the sidechain-oxidizing enzymes of TOL plasmid pWW0 is well characterized at the molecular level. The structural genes for the mono-oxygenase oxygenase (*xyiM*) and reductase components (*xyiA*), the alcohol dehydrogenase (*xyiB*) and the aldehyde dehydrogenase (*xyiC*), together with a gene of unknown function (*xyiN*), are clustered in one operon (*xyiCMABN*) (Harayama *et al.*, 1986). As with *C. testosteroni* T-2, the three enzyme activities are co-regulated. Transcription of the operon from the σ^{54} promoter *P_H* is regulated by the positive regulator XylR, which is activated by the presence of upper-pathway substrates/intermediates (Assinder & Williams, 1990). In contrast to the situation in *C. testosteroni* T-2, strong transcription from *P_H* only occurs in the late exponential growth phase (Hugouvieux-Cotte-Pattat *et al.*, 1990; Lorenzo *et al.*, 1993). Nothing is known about the stability of the enzymes in the stationary growth phase or the regulatory proteins which control the growth-phase-dependent regulation.

Regulatory unit R2

We consider this regulatory unit to consist of two enzymes, TERDOS and DCDDH. These two enzymes were always expressed (or absent) as a group with comparable ratios of activity, where both enzymes could be quantified (Table 3). The known structural genes of ring-activating multicomponent non-haem iron dioxygenase systems and the corresponding diene-diol dehydrogenases are clustered (Bergeron *et al.*, 1994;

Denome *et al.*, 1993; Harayama *et al.*, 1991; Hofer *et al.*, 1993; Neidle *et al.*, 1987; Tan & Fong, 1993; Zylstra & Gibson, 1989) and co-regulation of the two enzymes appears to be common. We anticipate a similar arrangement for TERDOS and DCDDH in *C. testosteroni* T-2.

We found evidence, in the form of an apparent permeability barrier to a substrate analogue, for the specific uptake of terephthalate by *C. testosteroni* T-2. The nature of the transport system and the regulation of its expression remain unclear.

Regulatory unit R3

This regulatory unit is presumed to consist of at least the oxygenase (component A) of PSBDOS. This dioxygenase system was initially described as a two-component system, consisting of the components A (oxygenase) and B (reductase); a reductase C was observed but not characterized (Locher *et al.*, 1991a). Our results confirm the subsequent suggestion of Locher *et al.* (1991b), that the expression of reductase B is controlled by regulatory unit R1 as a component of TSMOS. However, we found no evidence for reductase C; in contrast, under growth conditions where PSBDOS activity was needed, regulatory unit R1 was expressed, presumably to complement oxygenase A of PSBDOS with reductase B from TSMOS *in vivo*. It is unusual that two components of an oxygenase are in different regulatory units (van der Meer *et al.*, 1992), and the observation of two reductases for the dibenzofuran dioxygenase system (Bünz & Cook, 1993) was attributed to two constitutive pathways. It will be interesting to explore whether PSBDOS is a new acquisition in strain T-2, whether a mutation since isolation of the organism has led to loss of reductase C, whether reductase B is a better reductive unit for PSBDOS than reductase C, or whether some other explanation is required.

A fourth regulatory unit (Table 2, Fig. 1) is presumed to comprise the ring-cleaving enzyme, PC45O, and possibly the whole of the *meta* cleavage pathway of *C. testosteroni* T-2 (cf. Assinder & Williams, 1990; Doten *et al.*, 1987).

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