Degradative pathways for
p-toluenecarboxylate and p-toluenesulfonate
and their multicomponent oxygenases in
Comamonas testosteroni strains PSB-4 and T-2

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Three multicomponent oxygenases involved in the degradation of p-
toluenesulfonate and p-toluenecarboxylate and the regulation of their
synthesis have been examined in three strains (T-2, PSB-4 and TER-1) of
Comamonas testosteroni. Strain T-2 utilizes p-toluenesulfonate as a source
of carbon and energy for growth via p-sulfobenzoate and protocatechuate, and p-
toluenecarboxylate via terephthalate and protocatechuate, and has the
unusual property of requiring the reductase (TsaE) of the toluenesulfonate
methyl monoxygenase system (TsaMB) in an incompletely expressed
sulfobenzoate dioxygenase system (PsbAC) [Schlafli Oppenberg, H. R., Chen, G.,
independently isolated C. testosteroni PSB-4 utilized only sulfobenzoate and
terephthalate via protocatechuate. Mutant TER-1, derived from strain T-2,
utilized only terephthalate via protocatechuate. We detected no enzymes of
the pathway from toluenesulfonate to sulfobenzoate in strains
PSB-4 and TER-1, and confirmed by PCR and Southern blot analysis that the genes
(tsaMB) encoding toluenesulfonate monoxygenase were absent. We concluded that,
in strain PSB-4, the regulatory unit encoding the genes for the conversion of
toluenesulfonate to sulfobenzoate was missing, and that generation of mutant
TER-1 involved deletion of this regulatory unit and of the regulatory unit
encoding desulfonation of sulfobenzoate. The degradation of sulfobenzoate in
strain PSB-4 was catalysed by a fully inducible sulfobenzoate dioxygenase
system (PsbACPSB-4), which, after purification of the oxygenase component
(PsbAPSB-4), turned out to be indistinguishable from the corresponding
component from strain T-2 (PsbA). Reductase PsbCPSB-4, which we could
separate but not purify, was active with oxygenase PsbAPSB-4 and PsbAT2.
Oxygenase PsbAPSB-4 was shown by electron paramagnetic resonance
spectroscopy to contain a Rieske [2Fe-2S] centre. The enzyme system
oxygenating terephthalate was examined and the oxygenase component
purified and characterized. The oxygenase component in strains T-2 (and
mutant TER-1) and PSB-4 were indistinguishable. The reductase component,
which we separated but failed to purify, was active with the oxygenase from
all strains. Gains and losses of blocks of genes in evolution is discussed.

Keywords: Comamonas testosteroni, terephthalate dioxygenase system, p-sulfobenzoate
dioxygenase system, identical enzymes

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Abbreviations: PcaP, protocatechuate 4.5-dioxygenase; PsbAC, p-sulfobenzoate dioxygenase system comprising a reductase component (PsbC) and an
oxygenase component (PsbA); TerZap, terephthalate dioxygenase system comprising a reductase component (TerR) and a two-subunit oxygenase
component (TerZapZBC); TsaC, sulfobenzyl alcohol dehydrogenase; TsaD, sulfobenzaldehyde dehydrogenase; TsaMB, p-toluenesulfonate methyl mono-
oxynase system comprising a reductase component (TsaB) and an oxygenase component (TsaM).
INTRODUCTION

The degradation of p-toluensulfonate and p-toluene-carboxylate (p-tolu) to protocatechuate in *Comamonas testosteroni* T-2 proceeds via p-sulfobenzoate and terephthalate, respectively (Fig. 1). Most of the enzymes have been purified (Locher et al., 1991a, b, c; Saller et al., 1995; Schläflí et al., 1994), the physiology of their regulation examined (Schlafli Oppenberg et al., 1995) and the genes encoding the toluenesulfonate monoxygenase system sequenced (F. Junker & R. Kiewitz, unpublished).

The degradative pathway(s) for toluenesulfonate and toluene-carboxylate in strain T-2 is unusual in that, in addition to the characteristic convergence (e.g. Fewson, 1981; Stanier & Ornston, 1973) at the ring cleavage substrate, protocatechuate, the initial degradative enzymes only are shared, with the consequence that the pathway includes a divergence. In contrast to the divergence in the *meta* pathway encoded on the TOL plasmid (Assinder & Williams, 1990), the branches of the divergence (Fig. 1) are separately regulated (Schlafli Oppenberg et al., 1995). A further unusual phenomenon in strain T-2 is the sharing of a reductase (TsaB) between two class IA oxygenase systems [p-toluensulfonate methyl monoxygenase (TsaMB) and p-sulfobenzoate dioxygenase (PsbAC)] (cf. Batie et al., 1992), whereby the loss of a reductase (PsbC) over time is suspected (Schlafli Oppenberg et al., 1995). Concomitant with this sharing of a reductase, the oxygenase component, PsbA, of oxygenase system PsbAC is synthesized constitutively under most conditions (Schlafli Oppenberg et al., 1995).

A second, independently isolated organism, *C. testosteroni* PSB-4, is able to utilize sulfobenzoate, though not toluenesulfonate (Busse et al., 1992; Thurnheer et al., 1986). Given the similarities sometimes observed amongst multicomponent oxygenases of the same function from different organisms (Furukawa et al., 1993; Harayama et al., 1992), contrasted with the loss of one reductase component from strain T-2 (Schlafli Oppenberg et al., 1995), we decided to compare and contrast aromatic metabolism and its regulation in strains T-2 and PSB-4.

A third organism became available to us from work with strain T-2 in a chemostat. A. J. Tien & T. Egli (EAWAG, Switzerland, personal communication) obtained a mutant which metabolized neither toluenesulfonate nor sulfobenzoate, but retained the ability to utilize terephthalate, and we termed the mutant TER-1. We wanted to ascertain whether deletion or regulatory mutants were involved.

We now confirm that strain PSB-4 lacks the genes (*tsaMB*) for the TsaMB system and that there is high similarity between the PsbAC systems found in strains T-2 and PSB-4, and that there is high similarity among the components of terephthalate dioxygenase (TerZaDR) in all organisms tested. Mutant TER-1 seems to have arisen by deletion of genetic material.

METHODS

Materials. Polyclonal antibodies from rabbit serum were prepared previously (Schlafli et al., 1994; Schlafli Oppenberg et al., 1995). Chemicals were of the highest purity available commercially (Locher et al., 1989, 1991a; Saller et al., 1995; Schlafli et al., 1994, 1995; Schlafli Oppenberg et al., 1995). Materials and apparatus for the purification and characterization of proteins are described elsewhere (Locher et al., 1991a; Schlafli et al., 1994).

Organisms, growth and the preparation of cell-free extracts. *C. testosteroni* PSB-4 and *C. testosteroni* T-2 (DSM 6577) were isolated by Thurnheer et al. (1986) and identified by Busse et al. (1992). *C. testosteroni* TER-1 was isolated by A. J. Tien & T. Egli (personal communication). Cells were grown in mineral salts medium containing 6 mM sulfobenzoate or terephthalate as described elsewhere (Locher et al., 1991a; Schlafli et al., 1994). On occasion, medium was supplemented with sterile toluenesulfonate or sulfobenzoate to a final concentration of 3 mM.
Cultures were harvested at mid-exponential growth phase (about 160 mg protein l-1), and the cell pellets frozen at -20 °C or used immediately. Cell suspensions were disrupted in a French pressure cell and the supernatant fluid after centrifugation was used for the experiments (Locher et al., 1991a; Schläfi et al., 1994).

Analytical methods. Oxygenase system PsbAC or oxygenase system TerZaB+R was assayed as substrate-dependent oxygen uptake in a Clark-type oxygen electrode (Locher et al., 1991a; Schläfi et al., 1994). NADH reductase activity was assayed photometrically as NADH-dependent reduction of dichloro-phenolindophenol (DCPIP) or cytochrome c (Locher et al., 1991a). TsaC was assayed photometrically as the NADH-dependent reduction of carboxybenzaldehyde, and sulfobenzaldehyde dehydrogenase (TsaD) was assayed as the NADH-dependent oxidation of carboxybenzaldehyde (Locher et al., 1991c). Products of enzyme reactions were identified (co-chromatography) and quantified by reversed-phase HP1.C at 1991a).

OD546

UV spectra of separated reaction products were determined in a diode-array detector (Locher et al., 1991a). SDS-PAGE was used to determine M, values of proteins under denaturing conditions and to monitor protein purifications. Proteins in gels were visualized by staining with Coomassie brilliant blue (Laemmli, 1976) or with silver nitrate (Bloom et al., 1987). Proteins separated by SDS-PAGE were sometimes electroblotted onto nitrocellulose membranes (Schlæfi Oppenberg et al., 1995). Affinity-purified antisera raised against oxygenase component PsaA of oxygenase system PsaAC, against component TsaB of oxygenase system TsaMB, and against oxygenase subunit TerZa of oxygenase system TerZaB+R were used to detect cross-reacting proteins from C. testosteroni PsaAC in blotted extracts and in fractions of purified enzymes (Schlæfi Oppenberg et al., 1995). Reductase TsaB was prepared as described elsewhere (Locher et al., 1991a). Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as standard. Growth was assayed as OD660 (an OD660 of 1.0 represented 200 mg protein l-1). The N-terminal amino acid sequences were determined as described elsewhere (Schlæfi et al., 1994). EPR spectra of a component (about 2 mg in 0.3 ml) at X band were recorded with an EPR 300 instrument (Bruker) at 10 K as described elsewhere (Riester et al., 1989).

PCR-analysis of tsaMB and Southern blot hybridization. PCR reactions with total DNA of strain T-2, PSB-4 and TER-1 were done to assay for the presence of tsaMB genes encoding TsaMB (F. Junker & R. Kiewitz, unpublished). Total DNA from 100 ml batch cultures (200 mg protein l-1) of all three strains was prepared by the cetyltrimethylammonium bromide precipitation method (Ausubel et al., 1987). The PCR mixture (50 µl) contained 10 ng DNA, 200 µM each dNTP, 50 pmol each primer, 5 µl DMSO and 0.25 units SuperTaq (Stehelin & Ge) in the buffer supplied by the manufacturer. The PCR conditions were: 95 °C for 5 min, 45 °C for 30 s, 72 °C for 10 s, 94 °C for 30 s. The last three steps were repeated 30 times. The following primers (F. Junker & R. Kiewitz, unpublished) were used: 5' AAAAAATCTTGAGCCAGGT 3' (sense strand of tsaM) and 5'TTGAGCTTTTGTGTAATC 3' (antisense strand of tsaB). The size of the expected product was 386 bp. The PCR product or total DNA digested with PstI was separated on a 1.4% agarose gel. The DNA was blotted onto a Hybond-N nylon membrane (Amersham International) and hybridized with 5' end-labelled [32P]dATP oligonucleotide probes (primers for tsaM and tsaB used for PCR reaction) (Ausubel et al., 1987).

Purification of oxygenase system PsaAC from strain PSB-4 or T-2. Oxygenase system PsaAC, PsaAC, was purified from sulfate-grown cells. Crude extracts free of nucleic acids were chromatographed on an anion-exchange column (Mono Q HR) as described for oxygenase system PsaAC, (Locher et al., 1991a) except that a smaller column was used (10 × 100 mm). The oxygenase component was then purified by hydrophobic interaction chromatography (Locher et al., 1991a).

Purification of the oxygenase component TerZaB from strain PSB-4, T-2 or mutant TER-1. Oxygenase component TerZaB, TerZaB, or TerZaB, was purified from terphathalate-grown cells as described elsewhere (Schlæfi Oppenberg et al., 1995).

RESULTS

Growth and enzyme activities

We confirmed that strain PSB-4 utilized sulfobenzoate but not toluenesulfonate as a sole source of carbon and energy for growth (cf. Thurnheer et al., 1986), and observed that terphathalate but not toluene-carboxylate was utilized. In mutant TER-1, terphathalate was utilized as sole source of carbon and energy for growth, but toluenesulfonate, toluene-carboxylate and sulfobenzoate were not. Growth of strains PSB-4 and TER-1 in 10 mM succinate salts medium did not lead to significant synthesis of toluenesulfonate, sulfobenzoate or terphthalate oxygenating enzymes, whereas a basal level of the proteocatechuate 4,5-dioxygenase (PcaP) was observed in strains PSB-4 and T-2 (Table 1). Inducible oxygenation of sulfobenzoate and of protocatechuate or of terphthalate and protocatechuate was detected in strain PSB-4 (Table 1), whereas no detectable oxygenase system TsaMB, dehydrogenase TsaC (Table 1) or dehydrogenase TsaD (not shown) was found. The presence of the genes tsaMB (F. Junker & R. Kiewitz, unpublished) encoding TsaMB in T-2, PSB-4 and TER-1 was checked for by PCR (Fig. 2a). A product of the expected size, 386 bp, was observed in strain T-2 only. In addition, total DNA from each of the three strains was digested with PstI and analysed by Southern blot hybridizations. The oligonucleotides used for PCR were now used as probes, and each gave a signal at 2 kb (as did the PCR product) for T-2 only (Fig. 2b). We thus concluded that no gene encoding the oxygenation or subsequent oxidation of toluenesulfonate to sulfobenzoate (regulatory unit R1 in strain T-2; Fig. 1) is present in strain PSB-4 or in mutant TER-1.

The failure of mutant TER-1 to utilize sulfobenzoate might not mean loss of genes encoding a putative oxygenase system, PsaAC, but may reflect solely the lack of reductase component TsaB, which is needed for activity of oxygenase system PsaAC in the parent strain T-2 (see Introduction). However, the largely constitutive synthesis of oxygenase component PsaA, which was readily visualized on SDS-PAGE gels (Schlæfi Oppenberg et al., 1995; visible in Fig. 4a, lane 1 and Fig. 5a, lane 5), was not matched by a band of putative PsaB, in corresponding experiments with mutant TER-1 (not shown). Similarly, when extracts of mutant TER-1 were supplemented with reductase component TsaB, there was no oxygenation of sulfobenzoate.
Table 1. Induction of enzyme activities of crude extract in C. testosteroni T-2, PSB-4 and TER-1 as a function of the growth substrate

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth substrate</th>
<th>Enzyme specific activity [mkat (kg protein)$^{-1}$]*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TsaMB</td>
</tr>
<tr>
<td>T-2</td>
<td>Toluene sulfonate</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Toluene carboxylate</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Sulfo benzoate</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Terephthalate</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>ND‡</td>
</tr>
<tr>
<td>PSB-4</td>
<td>Toluene sulfonate</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Toluene carboxylate</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sulfo benzoate</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Terephthalate</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>ND</td>
</tr>
<tr>
<td>TER-1</td>
<td>Toluene sulfonate</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Toluene carboxylate</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sulfo benzoate</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Terephthalate</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>ND</td>
</tr>
</tbody>
</table>

* nd, not detected; +, not quantified; −, no growth.
† Data from Locher et al. (1983).
‡ Data from Schlüfi Oppenberg et al. (1995).

Fig. 2. Presence and absence of tsaMB encoding the components of methyl monooxygenase system TsaMB in three strains of C. testosteroni. (a) Agarose gel: total DNA of strain T-2, PSB-4 or TER-1 was used in a PCR reaction with primers of the genes tsaMB and total DNA was digested with PstI. The PCR product of 386 bp overlaps both genes. (b) Southern blot hybridization with probe for tsaM: the primer used for PCR was labelled and used as probe. Lanes 2 and 3 gave only a single signal. The same was observed with the PCR primer probe against tsaB. Lanes: 1, DNA size marker; 2, PstI digest of T-2 total DNA; 3, PCR of T-2 total DNA; 4, PstI digest of PSB-4 total DNA; 5, PCR of PSB-4 total DNA; 6, Pstl digest of TER-1 total DNA; 7, PCR of TER-1 total DNA; 8, λ-HindIII DNA marker.

PsbAC<sub>PSB-4</sub>

The specific activity of p-sulfobenzoate- and NADH-dependent oxygen uptake in crude extract of strain PSB-4 was directly proportional to the protein concentration (not shown), which was preliminary evidence for a multicomponent enzyme system (cf. Locher et al., 1991a), presumably analogous to oxygenase system PsbAC<sub>T-2</sub>. When proteins in this extract were separated by anion-exchange chromatography, no single fraction catalysed the reaction, but the combination of two sets of fractions
2.5
2
1.5
1
0.5
10
20
30
40
50
60
Fraction no.

Fig. 3. Anion-exchange chromatography of extracts of \( p \)-sulfobenzoate-grown \( C. \) testosteroni PSB-4 and the elution of components of dioxygenase system \( \text{PsbAC}_{\text{PSB-4}} \). Protein was monitored as \( A_{280} \) (---). Gradient elution with \( \text{Na}_2\text{SO}_4 \) (---) was used. Reductase \( \text{PsbC} \) activity (○) was determined as NADH-dependent reduction of cytochrome \( c \) (fraction C). Oxygenase \( \text{PsbA} \) activity (●) was determined in the presence of fraction 24 as sulfobenzoate- and NADH-dependent oxygen uptake (fraction A).

(C and A, Fig. 3) was active and this activity was increased by the addition of Fe\(^{2+} \). The organic product from sulfobenzoate was protocatechuate, which was identified by co-chromatography with authentic material and by its UV spectrum (cf. Locher \textit{et al.}, 1989). We recovered 1 mol protocatechuate (mol sulfobenzoate)\(^{-1} \) in a reaction which consumed 1 mol O\(_2\) and 1 mol NADH. The reaction catalysed is thus:

\[
p\text{-sulfobenzoate} + \text{NADH} + \text{H}^+ + \text{O}_2 \rightarrow \text{protocatechuate} + \text{NAD}^+ + \text{HSO}_4^- \]

and we presumed we were working with the \( \text{PsbAC} \) system from strain PSB-4, which we termed \( \text{PsbAC}_{\text{PSB-4}} \), to distinguish it from the corresponding enzyme system in strain T-2, \( \text{PsbAC}_{\text{T-2}} \).

We concluded that fractions C (Fig. 3) represented the reductase component (termed reductase \( \text{PsbC}_{\text{PSB-4}} \)), because these fractions contained a NADH-cytochrome \( c \) reductase [and a NADH-2,6-DCPIP reductase] as observed with reductase \( \text{PsbC}_{\text{T-2}} \) (Locher \textit{et al.}, 1991a). The reductase(s) for cytochrome \( c \) displayed a specific activity.
that was independent of the protein concentration. Reductase PsbC in strain T-2 is not expressed in more recent studies where TsaB is the effective reductase in vivo (Schläfli Oppenberg et al., 1995).

We concluded that the red-coloured fractions A (Fig. 3) represented the oxygenase (termed oxygenase PbA_{PSB-4}). Proteins from fractions A were further separated by hydrophobic interaction chromatography, to yield an active protein of at least 90% purity (Fig. 4a, lane 3). The identical Mr values for the oxygenase components PbA_{PSB-4} and PbA_{T-2} (Fig. 4a, compare lanes 1 and 4 (from strain T-2) with lanes 2 and 3 (from strain PSB-4)), led us to examine the immunological cross-reactivity of the two proteins (Fig. 4b). The two proteins were indistinguishable on the basis of the nine characteristics we tested (Table 2, interactions 2-1494). This was explored immunologically with strain T-2 (Fig. 5a, lane 5) under conditions which caused expression of oxygenase TerZ@R,-, at low levels (Table 4, line 1) shows the major bands from oxygenase PsbA, oxygenase TsaM and reductase TsaB and a minor band for oxygenase TerZ@R, especially in cells grown with terephthalate-

Table 2. Common properties of PbAC or TerZ@R systems from different strains of C. testosteroni

<table>
<thead>
<tr>
<th>Components</th>
<th>PbAC in strains T-2 and PSB-4</th>
<th>TerZ@R in strains T-2, PSB-4 and TER-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reductase</strong></td>
<td>PbC (TsaB*)</td>
<td>PsbA</td>
</tr>
<tr>
<td><strong>Dioxygenase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M_r, denaturing conditions</td>
<td>50 kDa</td>
<td>46 kDa (x) + 18 kDa (β)</td>
</tr>
<tr>
<td>Antigenicity (anti-PsbA_T-2)</td>
<td>Positive</td>
<td>None</td>
</tr>
<tr>
<td>Antigenicity (anti-TerZ@R)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>N-terminal amino acid sequence</td>
<td>MLTSENNQILTRVGPTAM</td>
<td>MNEIQIAAF (Za)</td>
</tr>
<tr>
<td>Substrate range</td>
<td>p-Sulfobenzoate</td>
<td>Terephthalate, naphthalene 1,4-dicarboxylic acid, pyridine 2,5-dicarboxylic acid</td>
</tr>
<tr>
<td>Elution from Mono Q†</td>
<td>50 ± 10</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>Elution from Phenyl-Sepharose‡</td>
<td>50 ± 10</td>
<td>Not used</td>
</tr>
<tr>
<td><strong>Reductase</strong></td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Antigenicity (anti-TsaB_T-2)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Elution from Mono Q†</td>
<td>40 ± 10</td>
<td>30 ± 10</td>
</tr>
</tbody>
</table>

* Reductase PsbC in strain T-2 is not expressed in more recent studies where TsaB is the effective reductase in vivo (Schläfli Oppenberg et al., 1995).
† Position in the gradient (mM Na_2SO_4) where the enzyme eluted.
‡ Position in the gradient [mM (NH_4)_2SO_4] where the enzyme eluted.

so we knew that each organism expressed a dioxygenase reaction analogous to that in strain T-2 (Schläfli et al., 1994). This was explored immunologically with strain PSB-4 (Fig. 5). A control experiment with extract from strain T-2 (Fig. 5a, lane 5) under conditions which caused expression of oxygenase TerZ@R at low levels (Table 1, line 1) shows the major bands from oxygenase PsbA, oxygenase TsaM and reductase TsaB and a minor band for oxygenase TerZ@R close to an unrelated protein. The corresponding Western blot (Fig. 5b) shows this single relatively weak band of TerZ@R, a strong protein band of the same Mr, especially in cells grown with terephthalate, is seen in extracts from strain PSB-4 (Fig. 5a, lanes 3 and 4) and the cross-reactivity with the specific antibody is seen in Fig. 5b. Oxygenase component TerZ@R is absent from succinate-grown cells (Fig. 5b, lane 1) but partially induced in sulfobenzoate-grown cells (Fig. 5b, lane 2). Similar experiments with strain TER-1 showed the presence of inducible TerZ@R, no evidence for the occurrence of oxygenase system TsaMB or of oxygenase PsbA (cf. Fig. 4).
Proteins from each organism were then separated by anion exchange chromatography, which is a one-step purification of the oxygenase component, TerZaβ_{TER-2} (Schlafli et al., 1994). The characteristics of oxygenase component TerZaβ from strain PSB-4 and mutant TER-1 were identical with those from strain T-2 (Table 2). We were unable to purify reductase component TerR (as with strain T-2), but the preliminary data (Table 2) indicate close similarity, and each reductase was active with each oxygenase TerZaβ.

**DISCUSSION**

We knew of the taxonomic similarity of our strains of C. testosteroni T-2 and PSB-4 (Busse et al., 1992) and of the similarity in their degradative characteristics (Thurnheer et al., 1986). Indeed, the latter similarity has been shown to be identity (e.g. Table 2), where tested. We were previously unable to define the difference in metabolism between the strains, represented by the enzymes of regulatory unit R1 (Fig. 1). These enzymes, mono-oxygenase system TsaMB and two dehydrogenases TsaCD, could not be detected by assays of activity in strain PSB-4, and immunological studies (Fig. 4b, c) showed that neither oxygenase component (TsaMB) was present. We could explain this absence thoroughly, however, only with a genetic method (Fig. 2) which could confirm the absence of the appropriate structural genes, and eliminate the case for solely regulatory effects.

We have found *tsaMBC* to be contiguous (F. Junker & R. Kiewitz, unpublished) so we presume that the genes in regulatory unit R1 (Fig. 1) are part of an operon. We presume that the whole regulatory unit R1 is absent from strain PSB-4, because neither dehydrogenase (TsaCD) is detected (Table 1). We also conclude that mutant TER-1, derived from strain T-2, was generated by a spontaneous deletion of regulatory unit R1 (Fig. 2), and not by a mutation in regulation. It is less easy to establish the fate of the genes encoding oxygenase system PsbAC (regulatory unit R3) during the generation of mutant TER-1 because we have no satisfactory gene probe. Our data (analogous to Figs 4 and 5) show that no putative Psb_{TER-1} is expressed in our experiments. We postulate deletion of regulatory unit R3 concomitant with the deletion of operon R1, but we cannot exclude the possibility of altered regulation resulting in a cryptic *psb-A*. Regulatory units R2 (terephthalate to protocatechuate) and R4 (ring cleavage), in contrast, appear to be unaffected by the mutation (Table 1), as judged by the identity of oxygenase system *TerZaβ* in parent and mutant (Table 2) and by the similarities in induction patterns and enzyme levels in parent and mutant (Table 1).

Regulatory unit R2 in strains T-2 and PSB-4 encodes identical proteins (oxygenase system *TerZaβ*<sup>TER-1</sup>, Table 2), within the limits of our data, and shows the same pattern of induction (Table 1). Regulatory unit R3, in contrast, while representing one enzyme activity (oxygenase system PsbAC) in strains T-2 and PSB-4, is subject to different regulation in these organisms. Whereas oxygenase PsbA<sub>TER-2</sub> is usually expressed constitutively (Saller et al., 1995; Schlafli Oppenberg et al., 1995) and operon R1 must be expressed at a low level to enable oxygenase PsbA<sub>TER-2</sub> to function as an oxygenase system by supplying reductase TsaB (Schlafli Oppenberg et al., 1995), oxygenase PsbA<sub>PSB-4</sub> is strictly inducible (Fig. 5a, lanes 1 and 2) as is reductase PsbC<sub>PSB-4</sub>. Were oxygenase system PsbAC<sub>PSB-4</sub> incomplete, it could not be complemented by reductase TsaB, as *tsaB* is absent from this organism (see above).

Our work on the enzymology of the degradation of toluenesulfonate and toluene-carboxylate in C. testosteroni T-2 has twice failed to yield proteins, reductases PsbC and TerR from oxygenase systems PsbAC and TerZaβ<sup>R</sup>, respectively. We have now found an alternative source for these proteins, C. testosteroni PSB-4, which obviously synthesizes appropriate isofunctional (and possibly ident-
ical) proteins (Table 2), but new approaches are clearly needed to purify these proteins for characterization.

Reductase TerR of oxygenase system TerZaPR transfers electrons to oxygenase component TerZaA, independent of the source of TerR or TerZaB. Reductase TerR, however, does not transfer electrons to oxygenases PsbA or TsaM, perhaps because TerZaPR belongs to a different subclass of the mononuclear iron oxygenases (cf. Schläfli et al., 1994). Reductase PsbC_{PsbA}, transfers electrons to oxygenase PsbA, independent of the source of PsbA, but there is no transfer from reductase PsbC to oxygenase TsaM or to oxygenase TerZaB. Solely reductase TsaB can transfer electrons to a heterologous oxygenase (albeit in the same subclass, IA), oxygenase PsbA. Functional hybrid dioxygenases are known in the literature (Furukawa et al., 1993; Harayama et al., 1992). The class IIIB reductase components BphA4 and TodA (involved in the dioxygenation of biphenyl (bph operon) and toluene (tod operon) form active enzymes with the additional components of the other oxygenase (Tod and Bph, respectively) (Furukawa et al., 1993; Hirose et al., 1994). The reductase components (BphA4 and TodA) have 60% sequence identity. The sequenced reductases in subclass IA have about 40% identity (Nakatsu et al., 1995; F. Junker & R. Kiewitz, unpublished), so we wonder whether hybrid enzymes can be formed and whether reductases TsaB and PsbC have a higher similarity than 40%. More experiments are needed to explore these effects, which involve the sites of interaction and electron transfer between proteins.

The utilization of terephthalate is widespread in C. testosteroni (Willems et al., 1991) and we have concluded that terephthalate is a natural product (Schlafli et al., 1994), so presumably it is not unusual that the strains T-2 and PSB-4 have a common oxygenase system, TerZaPR. We used to presume that the degradation of sulfobenzoate was more recent, given that only one naturally occurring aromatic sulfonate of low molecular mass was known (Bentley & Holliman, 1970). Now it is clear that a natural sulfonated polymer (humic acid) is widespread (van Loon et al., 1993) and likely, based on its low pK value, to contain sulfonated benzene rings, so it is possible that the degradation of sulfobenzoate is also ancient. The identity of the oxygenase PsbA proteins in strains T-2 and PSB-4 makes it unlikely that they evolved independently. Some tentative hypotheses can thus be made. (i) Strain PSB-4 is a deletion mutant of strain T-2 (lacking a functional side-chain oxidation); two-dimensional protein gels might answer this suggestion. (ii) Strain T-2 evolved from strain PSB-4 by the recruitment of side-chain oxidation, the high G+C content (70%) (F. Junker & R. Kiewitz, unpublished) of tsaMB (encoding TsaMB) compared with the G+C content (62%) of C. testosteroni chromosomal DNA (Busse et al., 1992) would support this hypothesis. (iii) Pre-strain T-2 (toluene carboxylate”, toluenesulfonate”) acquired oxygenase system PsbAC from strain PSB-4; this would be difficult to distinguish from (i). (iv) Both strains acquired oxygenase system PsbAC independently from a third strain.

The regulatory units postulated to be involved in the degradation of toluenesulfonate and toluene carboxylate (Schlafli Oppenberg et al., 1995) are found in three different combinations in the strains or mutant we have studied here. This surely implies high genetic flexibility in these organisms, and many subgroup IA oxygenases are appropriately plasmid-encoded (e.g. Nakatsu et al., 1995). We are now exploring this possibility in strain T-2.

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