Degradative pathways for p-toluene carboxylate and p-toluene sulfonate 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-toluene carboxylate 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-toluene carboxylate via terephthalate and protocatechuate, and has the unusual property of requiring the reductase (TsaE) of the toluenesulfonate methyl monooxygenase system (TsaMB) in an incompletely expressed sulfobenzoate dioxygenase system (PsbAC) [Schläfli Oppenberg, H. R., Chen, G., Leisinger, T. & Cook, A. M. (1995). Microbiology 141, 1891-1899]. The 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 monooxygenase 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 (PsbA<sub>PSB-4</sub>), turned out to be indistinguishable from the corresponding component from strain T-2 (PsbA<sub>T-2</sub>). Reductase PsbC<sub>PSB-4</sub>, which we could separate but not purify, was active with oxygenase PsbA<sub>PSB-4</sub> and PsbA<sub>T-2</sub>. Oxygenase PsbA<sub>PSB-4</sub> 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
INTRODUCTION

The degradation of p-toluenesulfonate 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; Schläfi et al., 1994), the physiology of their regulation examined (Schläfi Oppenberg et al., 1995) and the genes encoding the toluenesulfonate monooxygenase 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 (Schläfi 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-toluenesulfonate methyl monooxygenase (TsaMB) and p-sulfobenzoate dioxygenase (PsbAC)] (cf. Batie et al., 1992), whereby the loss of a reductase (PsbC) over time is suspected (Schläfi 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 (Schläfi 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 (Schläfi 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. Polyvalent antibodies from rabbit serum were prepared previously (Schläfi et al., 1994; Schläfi Oppenberg et al., 1995). Chemicals were of the highest purity available commercially (Locher et al., 1989, 1991a; Saller et al., 1995; Schläfi et al., 1994, 1995; Schläfi Oppenberg et al., 1995).

Materials and apparatus for the purification and characterization of proteins are described elsewhere (Locher et al., 1991a; Schläfi 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; Schläfi 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 1⁻¹), 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; Schläfli et al., 1994).

Analytical methods. Oxygenase system PsbAC or oxygenase system TerZaPR was assayed as substrate-dependent oxygen uptake in a Clark-type oxygen electrode (Locher et al., 1991a; Schläfli et al., 1994). NADH reductase activity was assayed photo-electrometrically as NADH-dependent reduction of dichloro-phenolindophenol (DCPIP) or cytochrome c (Locher et al., 1991a). TsaC was assayed photo-electrometrically as NADH-dependent reduction of carboxybenzaldehyde, and sulfobenzoic acid dehydrogenase (TsaD) was assayed as the NADH-dependent oxidation of carboxybenzaldehyde, and sulfobenzoic acid dehydrogenase (TsaD) was assayed as described elsewhere (Schlafli et al., 1996). Products of enzyme reactions were identified (co-chromatography) and quantified by reversed-phase HPLC at room temperature (Locher et al., 1991a, c; Schlafli et al., 1994). 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 (Schläfli Oppenberg et al., 1995). Affinity-purified antisera raised against oxygenase component PsbA₂ of oxygenase system PsbAC₂, against component TsaB of oxygenase system TsaMB, and against oxygenase subunit TerZa of oxygenase system TerZaPR was used to detect cross-reacting proteins from C. testosteroni PSB-4 in blotted extracts and in fractions of purified enzymes (Schläfli 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 OD₅₅₀ (an OD₄₅₀ of 1.0 represented 200 mg protein 1⁻¹). The N-terminal amino acid sequences were determined as described elsewhere (Schläfli 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 TsaB (F. Junker & R. Kiewitz, unpublished). Total DNA from 100 ml batch cultures (200 mg protein 1⁻¹) 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' TTGAGCTTTTCCTGTAATC 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 14% agarose gel. The DNA was blotted on to a Hybond-N nylon membrane (Amersham International) and hybridized with 5' end-labelled γ[³²P]dATP oligonucleotide probes (primers for tsaM and tsaB used for PCR reaction) (Ausubel et al., 1987).

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 terephthalate but not toluenesulfonate was utilized. In mutant TER-1, terephthalate 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 terephthalate oxygenating enzymes, whereas a basal level of the protocatechuate 4,5-dioxygenase (PcpA) was observed in strains PSB-4 and T-2 (Table 1). Inducible oxygenation of sulfobenzoate and of protocatechuate or of terephthalate 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, PsbAC_TER₁, but may reflect solely the lack of reductase component TsaB, which is needed for activity of oxygenase system PsbAC in the parent strain T-2 (see Introduction). However, the largely constitutive synthesis of oxygenase component PsbA_ter₁, which was readily visualized on SDS-PAGE gels (Schläfli Oppenberg et al., 1995; visible in Fig. 4a, lane 1 and Fig. 5a, lane 5), was not matched by a band of putative PsbA_TER₁ 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>TsaMB</td>
<td>TsAC</td>
</tr>
<tr>
<td>T-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluenesulfonate</td>
<td>4.0</td>
<td>152</td>
</tr>
<tr>
<td>Toluene-carboxylate</td>
<td>1.5</td>
<td>94</td>
</tr>
<tr>
<td>Sulfo-benzoate</td>
<td>0.5</td>
<td>48.2</td>
</tr>
<tr>
<td>Terephthalate</td>
<td>ND</td>
<td>5.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PSB-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluenesulfonate</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Toluene-carboxylate</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sulfo-benzoate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Terephthalate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Succinate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TER-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluenesulfonate</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Toluene-carboxylate</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sulfo-benzoate</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Terephthalate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Succinate</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not detected; +, not quantified; —, no growth.
†Data from Locher et al. (1983).
‡Data from Schläfli 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 tsaA. 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, PstI digest of TER-1 total DNA; 7, PCR of TER-1 total DNA; 8, λ-HindIII DNA marker.

**PsbAC**

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_T2. 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...
p-Sulfobenzoate and terephthalate dioxygenases

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

Fig. 4. Immunological characterization of oxygenase component PsbA and reductase component TsaB. (a) Separation of extracts and protein fractions on SDS-PAGE with visualization by Coomassie staining. (b) The same samples after reaction with antiserum specific for oxygenase PsbA$_{T-2}$. (c) The same samples after reaction with antiserum specific for reductase TsaB. Lanes: M, prestained marker proteins; 1, crude extract of C. testosteroni T-2, grown on sulfobenzoate (10 µg protein); 2, crude extract of C. testosteroni PSB-4 grown with sulfobenzoate (10 µg protein); 3, partially purified oxygenase PsbA$_{PSB-4}$ (2 µg protein); 4, partially purified reductase TsaB of sulfobenzoate-grown T-2 (2 µg protein). The oxygenase component gave a single band on Western blots only if the experiment was done immediately after purification; on storage, minor bands of lower $M_r$ than the parent were detected. The contrast of lanes M was digitally enhanced.

(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 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:

\[
p$-$sulfobenzoate + NADH + H^+ + O_2 \rightarrow protocatechuate + NAD^+ + HSO_4^-\]

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

We concluded that fractions C (Fig. 3) represented the reductase component (termed reductase PsbC$_{PSB-4}$), because these fractions contained a NADH-cytochrome $c$ reductase [and a NADH-2,6-DCPIP reductase] as observed with reductase PsbC$_{T-2}$ (Locher et al., 1991a). The reductase(s) for cytochrome $c$ displayed a specific activity
Table 2. Common properties of PsbAC or TerZ/βR systems from different strains of C. testosteroni

<table>
<thead>
<tr>
<th>Components</th>
<th>PsbAC in strains T-2 and PSB-4</th>
<th>TerZ/βR in strains T-2, PSB-4 and TER-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reductase</td>
<td>PsbC (TsaB*)</td>
</tr>
<tr>
<td></td>
<td>Dioxigenase</td>
<td>PsbA</td>
</tr>
<tr>
<td>Dioxygenase</td>
<td>M₆, denaturing conditions</td>
<td>50 kDa</td>
</tr>
<tr>
<td></td>
<td>Antigenicity (anti-PsbA₆)</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Antigenicity (anti-TerZα₆-₉)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>N-terminal amino acid sequence</td>
<td>MLTSENQILTRVGGPTAM</td>
</tr>
<tr>
<td></td>
<td>Substrate range</td>
<td>p-Sulfobenzoate</td>
</tr>
<tr>
<td></td>
<td>Elution from Mono Qt†</td>
<td>50 ± 10</td>
</tr>
<tr>
<td></td>
<td>Elution from Phenyl Sepharose‡</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>Reductase</td>
<td>Antigenicity (anti-TsaB₆)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Elution from Mono Qt†</td>
<td>40 ± 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 Na₂SO₄) where the enzyme eluted.
‡ Position in the gradient [mM (NH₄)₂SO₄] where the enzyme eluted.

that was independent of the protein concentration. Reductase PsbCₖ₅ reduced the oxygenases PsbAₖ₅ (see below) and PsbA₄, but not oxygenase TsaM. We did not examine the protein further because its activity was lost on hydrophobic interaction chromatography. Reductase PsbC had no immunological cross-reaction with the alternative electron donor to oxygenase PsbA, reductase TsaB (Fig. 4a, c, lanes 1 and 2 with control lane 4).

We concluded that the red-coloured fractions A (Fig. 3) represented the oxygenase (termed oxygenase PsbA₄). 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 M₆ values for the oxygenase components PsbA₄ and PsbA₄ [Fig. 4a, compare lanes 1 and 4 (from strain T-2) with lanes 2 and 3], 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 with reductase TsaB and with reductase PsbCₖ₅ and UV-visible spectrum; cf. Locher et al., 1991a).

The UV-visible spectrum of oxygenase PsbA₄ was assumed to represent a Rieske [2Fe-2S] centre (Locher et al., 1991a). We have confirmed this conclusion with oxygenase components TerZαB and TsaM (Schläfli et al., 1994; F. Junker & R. Kiewitz, unpublished). The EPR spectrum of oxygenase PsbA₄ shows the typical g values (gₓ = 2.025; gᵧ = 1.921; gₘ = 1.745) for a Rieske [2Fe-2S] centre (Mason & Cammack, 1992) and we thus confirm that the oxygenase component PsbA contains a Rieske [2Fe-2S] centre.

TerZαβRₖ₅ and TsaB were expressed in T-2, while TsaM and TerR were expressed in strain PSB-4. 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 TerZαβRₖ₅ 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 TerZα close to an unrelated protein. The corresponding Western blot (Fig. 5b) shows this single relatively weak band of TerZαₕ. 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 TerZαPsb₄ 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 TerZαPsb₄; no evidence for the occurrence of oxygenase system TsaMB or of oxygenase PsbA (cf. Fig. 4) was detected.
Proteins from each organism were then separated by anion exchange chromatography, which is a one-step purification of the oxygenase component, TerZa$_{PB-4}$ (Schlafli et al., 1994). The characteristics of oxygenase component TerZa$_{PB-4}$ 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 TerZa$_{PB-4}$.

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 PsbATER-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 TerZa$_{PB-4}$ 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 TerZa$_{PB-4}$, 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$_{T-2}$ is usually expressed constitutively (Saller et al., 1995; Schlafli Oppenberg et al., 1995), oxygenase PsbA$_{PSB-4}$ is strictly inducible (Fig. 5a, lanes 1 and 2), and reductase PsbC$_{PSB-4}$ is expressed at a low level to enable oxygenase PsbA$_{T-2}$ to function as an oxygenase system by supplying reductase TsaB (Schlafli Oppenberg et al., 1995). Oxygenase PsbA$_{PSB-4}$ is strictly inducible (Fig. 5a, lanes 1 and 2), as is reductase PsbC$_{PSB-4}$. Were oxygenase system PsbAC$_{PSB-4}$ 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-2-carboxylate in C. testosteroni T-2 has twice failed to yield proteins, reductases PsbC, and TerR from oxygenase systems PsbAC and TerZa$_{PB-4}$, respectively. We have now found an alternative source for these proteins, C. testosteroni PSB-4, which obviously synthesizes appropriate isofunctional (and possibly ident-

![Fig. 5. Immunological detection of the dioxygenase component TerZa of TerZa/Psb-A. (a) Separation by SDS-PAGE of extracts (5 µg protein per lane) of C. testosteroni PSB-4 grown with succinate (lane 1), sulfobenzene (lane 2), terephthalate (lane 3) and terephthalate plus toluenesulfonate (lane 4) or of C. testosteroni T-2 grown with toluenesulfonate (lane 5). Proteins were stained with Coomassie blue and oxygenase components TerZa, PsbA, TsaM and reductase component TsaB from strain T-2 are indicated by arrows. (b) The samples were blotted onto nitrocellulose membranes and probed for reaction with antiserum specific for oxygenase TerZa$_{PB-4}$ from C. testosteroni T-2.](image-url)
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 TerZaβ, independent of the source of TerR or TerZaβ. Reductase TerR, however, does not transfer electrons to oxygenases PsbA or TsaM, perhaps because TerZaβR belongs to a different subclass of the mononuclear iron oxygenases (cf. Schläfli et al., 1994). Reductase PsbCPSB-4 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 TerZaβ. 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 TadA (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 TadA) 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 (Schläfli et al., 1994), so presumably it is not unusual that the strains T-2 and PSB-4 have a common oxygenase system, TerZaβR. 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 testable 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 (Schläfli 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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