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ORIGINAL PAPER

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An additional regulator, TsaQ, is involved with TsaR in regulation of transport during the degradation of *p*-toluenesulfonate in *Comamonas testosteroni* T-2

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Abstract The degradation of *p*-toluenesulfonate (TSA) by *Comamonas testosteroni* T-2 is initiated by a transport system (TsaST) and enzymes (TsaMBCD) encoded on the *tsa* transposon, Tn*tsa*, on the TSA plasmid (pTSA). Tn*tsa* comprises an insert of 15 kb between two IS*1071* elements. The left-hand 6 kb and the right-hand 6 kb are nearly mirror images. The regulator of the $tsaMBCD_1$ genes (right-hand side) is the centrally located LysR-type TsaR, which is encoded upstream of $tsaMBCD_1$ on the reverse strand. The other centrally located genes are *tsaS* and *tsaT*, encoded downstream of *tsaR* and on the same strand as both *tsaR* and $tsaMBCD_2$. The latter four genes are not expressed. Downstream of $tsaD_1$ ($tsaD_2$) is $tsaQ_1$ ($tsaQ_2$) and another open reading frame of unknown function. The *tsaQ* genes have identical sequences. Sequence analysis indicated that TsaQ could be an IclR-type regulator, whose expression during degradation of TSA was proven by data from RT-PCR. Both copies of *tsaQ* could be knocked-out by homologous recombination. Double mutants failed to grow with TSA but grew with *p*-toluenecarboxylate (TCA), which is also degraded via TsaMBCD. This showed TsaQ to be essential for the degradation of TSA but not TCA. We attributed this to regulation of the transport of TSA, especially to regulation of the expression of *tsaT*, which was expressed solely during growth with TSA. Seven independently isolated bacteria containing the *tsa* operon were available. Those six which contained *tsaT* on Tn*tsa* also contained *tsaQ*. The promoter region of *tsaT* was found to be a target of the regulator TsaR. Band-shift data indicate that TsaR is required for the expression of *tsaT*, which suggests that *tsaR* and $tsaQ_{1,2}$, together with $tsaMBCD_1$, belong to a common regulatory unit.

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Introduction

p-Toluenesulfonate (TSA) is a biodegradable xenobiotic compound which is used in formulating household detergents and in industrial settings, so it enters many sewage works (e.g. Riediker et al. 2000). Three catabolic pathways for TSA are recognized in bacteria (Cook et al. 1999), but only that for sidechain oxygenation, which seems to be ubiquitious and has been characterized in *Comamonas testosteroni* T-2, is understood in any detail (Fig. 1; Locher et al. 1989; Cook et al. 1999; Tralau et al. 2001, 2003).

The degradation of TSA and its analogue *p*-toluenecarboxylate (TCA) requires enzymes encoded in four regulatory units (R1–R4; Fig. 1), two of which are plasmid-encoded (R1 on pTSA, R3 on pT2L; Junker and Cook 1997; Tralau et al. 2001) and two are chromosomally encoded (R2, R4; Junker and Cook 1997; Providenti et al. 2001), while regulon R3 seems to involve an IclR-type regulator (Ruff, unpublished data). The known portion of regulatory unit R1 is encoded as part of the insert in transposon Tn*tsa* (Tralau et al. 2001, 2003; Fig. 1). The initial enzymatic reactions in the degradation of TSA (Fig. 1, R1) are identical with those in the initial steps of degradation of TCA (Locher et al. 1991), but physiological data show that the transport systems for these two growth substrates are different (Locher et al. 1993). The degradative reactions (TsaMBCD) are encoded by $tsaMBCD_1$ (Fig. 1); and the nearly identical $tsaMBCD_2$ genes are silent, as shown by RT-PCR (Tralau et al. 2001) and Tn5 knock-out of $tsaM_1$, which was not complemented by $tsaMBCD_2$ (Mampel 2000). The transport of TSA involves *tsaT* and *tsaS* (Fig. 1; Mampel 2000), but the location of transport genes for TCA is unknown.

The expression of $tsaMBCD_1$ is under the control of the LysR-type regulator, TsaR, which binds to perhaps four different sites in the divergent promoter region between *tsaR* and $tsaMBCD_1$ (Tralau et al. 2003). Downstream of

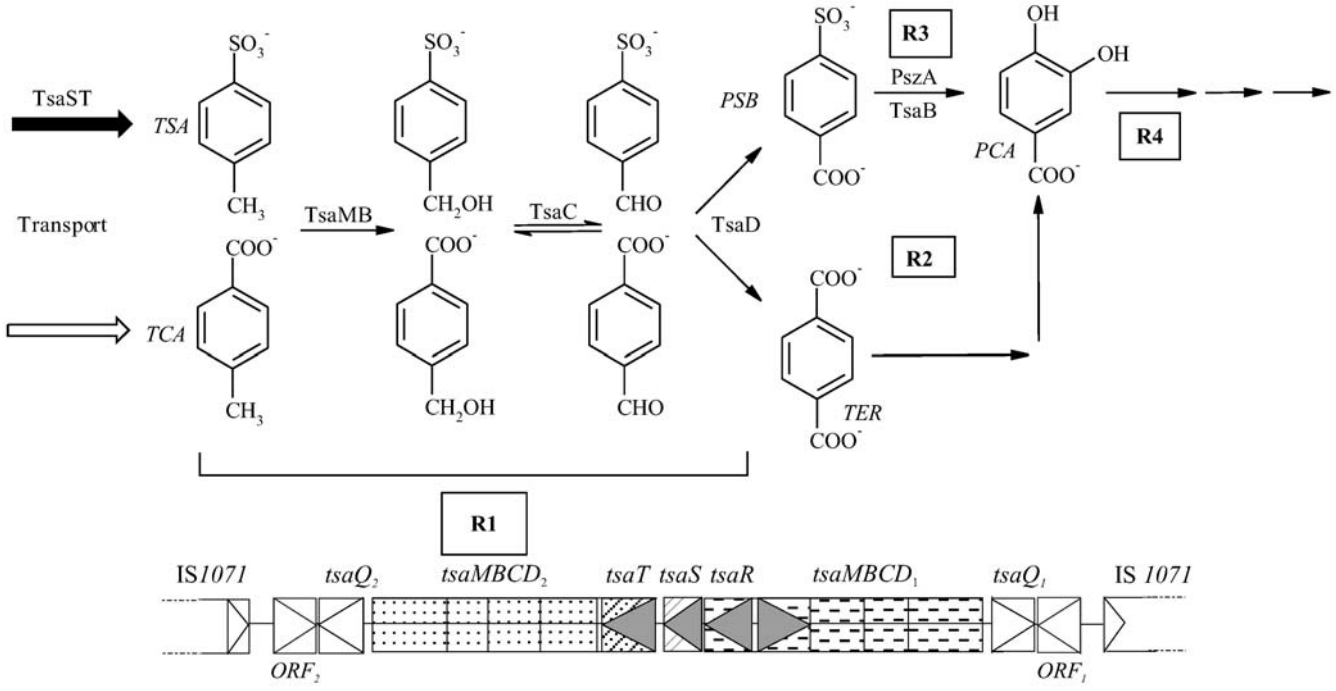


Fig. 1 The degradative pathway for *p*-toluenesulfonate (TSA) and *p*-toluenecarboxylate (TCA) in *Comamonas testosteroni* T-2 and the structure of Tn*tsa* on plasmid pTSA. The pathway is encoded in four regulons (R1–R4, see Introduction). *Triangles* indicate the direction of transcription of relevant genes in Tn*tsa* (Tralau et al. 2001). PCA Protocatechuate, PSB p-sulfobenzoate, PszA p-sulfobenzoate-3,4-dioxygenase, TER terephthalate, TsaMB p-toluenesulfonate methylmonooxygenase, TsaC p-sulfobenzylalcohol dehydrogenase, TsaD p-sulfobenzaldehyde dehydrogenase, TsaR (*tsaR*) LysR type regulator, TsaST (TsaS, TsaT; *tsaS*, *tsaT*) components of transport of TSA (Mampel 2000), TsaQ IclR-type regulator, ORF_1 and ORF_2 encode similar putative proteins of unknown function

$tsaMBCD_1$ and $tsaMBCD_2$, but within the transposon (Tn*tsa*), are two identical open reading frames (ORFs) which were designated $tsaQ_1$ and $tsaQ_2$ (Tralau et al. 2001) and which could encode an IclR-type regulator.

Molecular and physiological data are now presented which show that TsaR also binds to the promoter region of *tsaT* and that both copies of *tsaQ* function as additional regulator(s) for the *tsa* locus, specifically for *tsaT*. Thus, the whole *tsa* system, including $tsaMBCD_1$, *tsaT*, *tsaR* and $tsaQ_{1,2}$, can be considered as a common functional regulatory unit, R1 (Adhya 1999), which is controlled by at least two regulators.

Materials and methods

Bacteria, growth conditions, mutants and clones

Comamonas testosteroni T-2 (DSM 6577), *C. testosteroni* TER-1 and three metabolically different groups of TSA-degrading bacteria (Fig. 2; Schläfli Oppenberg et al. 1995; Tralau et al. 2001) were grown in minimal medium as described by Thurnheer et al. (1986). The *tsaQ*-mutants TE_7, TE_{11} and TE_{7C} (Table 2) were grown in 6 mM terephthalate (TER)-minimal salts medium with 30 µg tetracycline/ml (TE_7, TE_{11}) or 30 µg tetracycline/ml and 110 µg chloramphenicol/ml (TE_{7C}). Growth was estimated by turbidity measurements (optical density at 580 nm) and converted to protein using a correlation curve (Tralau et al. 2001).

Escherichia coli M10, generated to over-express TsaR with a C-terminal, 6-fold His-tag ($TsaR^{His}$), was grown in Luria-Bertani medium (Tralau et al. 2003). *E. coli* DH5α[pJB866], as donor for the broad-host-range vector pJB866 (GenBank accession number U8200), was kindly provided by J.M. Blatny and grown according to Blatny et al. (1997). *E. coli* JM109, as a donor of pMP141.1 (Cm^r, chloramphenicol resistance cassette), was a gift from M. Providenti (Providenti et al. 2001).

PCR, RT-PCR, quantification of DNA and cycle-sequencing

PCR was done as described by Tralau et al. (2003). Cells for the preparation of RNA were pre-grown in TSA-salts medium before transfer to the required selective medium and were then harvested before the mid-log phase (≤180 µg protein/ml). Total RNA from these cells ($1–4\times10^9$ cells/preparation) was prepared with the RNeasy mini kit and the RNase-free DNase set (Qiagen, Hilden, Germany), following the protocol of the manufacturer. The First strand cDNA synthesis kit (MBI Fermentas) was used for reverse transcription of RNA (0.5 µg). RNA was quantified photometrically (absorbance at 260 nm) and DNA was quantified fluorimetrically (DyNA Quant 200; Hoefer), according to the manufacturer's instructions.

The following primers were used for RT-PCR (Table 1). TsaOp 11 and TsaReg 4 were used for the reverse transcription of mRNA for *tsaR* and for amplification of cDNA, TsaOp 12 and TsaPrim 1 were used for the reverse transcription of mRNA for *tsaMB* and for amplification of cDNA, PsTs-11 and TsaTrf were used for the reverse transcription of mRNA for *tsaQ* and for amplification of cDNA and TT-orf-1 and TT-orf-2 were used for the reverse transcription of mRNA for *tsaT* and for amplification of cDNA. Primers for PCR detection of *tsaQ* in enrichment cultures (Fig. 2) were TsaQ-a and TsaQ-b. Fragments used for band-shift assays within the *tsaT* promoter region were amplified with the following primer pairs (Table 1): tsaTC1 and tsaTC2 (fragment C), tsaTD1 and tsaTC2 (fragment D), tsaTE1 and tsaTC1 (fragment E) and tsaTF1 and tsaTC2 (fragment F). Fragments A and B (Table 3) were amplified as described by Tralau et al. (2003).

DNA was sequenced as described by Tralau et al. (2003). Sequence data were analyzed using standard software (Edit View from

Table 1 PCR primers, their sequence and function. Relevant restriction sites are in italics

Primer name	Sequence
RT-PCR	
TsaOp 11	5′-CCG GCG CAG CAC GTA AAT GGT-3′
TsaReg 4	5′-GGC GCT GGG AGG GGC ACA TCA-3′
TsaOp 12	5′-TGG GCA GGG CGA GGT CAA TGT-3′
TsaPrim 1	5′-CGT GGT GGC GCT GGA AAA C-3′
PsTs-11	5′-GCG CGT GCC CAT GGT CAC GGT CAG-3′
TsaTrf	5′-GGG GCT CGA AAT CCT GTC TTG TT-3′
TT-orf-1	5′-GGC CCG GGG CGG CCG CAC ACT AGT GGC TTA GCG GTT GGC GGC GGC C-3′
TT-orf-2	5′-GGA GAC AAA CAT GGA TTT CCG CCG CCG CC-3′
PCR and cloning	
TsaQ-a	5′-GCG CGT GTT TTC ACG GGG CGC TGG TGT CGC-3′
TsaQ-b	5′-CAG CCC AAG CAA CTG GTG CTG CTG CCG GGC GA-3′
pJB866-Tet1EcoRI	5′-CAT CGC G*GA ATT C*GA GCA GCG CCT GCC TGA A-3′
pJB866-Tet2HindIII	5′-GGG CCG AGC GCA G*AA GCT T*TC CTG CAA CTT TAT CC-3′
TsaQ-12HindIII	5′-GTT CCT GGT GGG *AAG CTT* CGA TGC GGT TGC GCG-3′
TsaQ-21EcoRI	5′-CGC GCA ACC GC*G AAT TC*C GTG CCC ACC AGG AAC-3′
CmapMP141.1r	5′-CGG GCC TCT TCG CTA TTA C-3′
CmapMP141.11	5′-GGC ACC CCA GGC TTT ACA CTT TAT-3′
TsaQ-12EcoRI	5′-GTT CCT GGT GGG *GAA TTC* CGA TGC GGT TGC GCG-3′
IS772u	5′-TTG GCT ATG GGC TGC ACC GGC GAA ACA CTC A-3′
IS7721	5′-TGA GTG TTT CGC CGG TGC AGC CCA TAG CCA A-3′
TnTet-1	5′-CGC CTG TTT CGG GTT CGG GAT GGT-3′
PsTs-9	5′-CGC GCC GCT CAG AAC TCT TTG G-3′
Cm 3	5′-AAG ATC CGA AGG TCA TTG AGC AG-3′
Generation of fragments used in band-shift assays	
tsaTC1	5′-GCC CTG CCC ATC GCC TTC A-3′
tsaTC2	5′-ATC TTG GTC ATG GGG TTC TGC-3′
tsaTD1	5′-CGA TAA CAC TGG AGA CAA ACA TG-3′
tsaTE1	5′-GTG TTA TCG AGG CCG CTG GGC ATG-3′
tsaTF1	5′-CCT TTG CAC CGC CGC CCT GAT CG-3′
tsaM-C	5′-AAA AAT CTT GAG CCA GGT-3′
tsaB-N	5′-TTG AGC TTT TCG TGA ATC-3′
Psz-Prom1	5′-GGT CGG GGC AGA GCG GAT GTC-3′
Psz-Prom2	5′-CGG TTG CCA AAA GTG TCG GAA GAG-3′

Perkin Elmer, with the GCG program package, and the DNAstar package from Lasergene) and the Internet-based Neural network promoter prediction tool (NNPP, http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html; Reese et al. 1996).

The DNA size markers used were λ-DNA cut with *Eco*RI and *Hin*dIII (NewEngland BioLabs) and a 1-kb ladder (MBI Fermentas) with a range from 0.25 kb to 10 kb.

Knock-out mutagenesis

Knock-out of *tsaQ* was done by homologous recombination of the original gene locus with a linear PCR product of *tsaQ* with either an inserted tetracycline-resistance gene (Tet^r) or a Cm^r cassette. The appropriate inserts were constructed as follows. Tet^r from pJB866 was amplified with primers pJB866-Tet1EcoRI and pJB866-Tet2HindIII, thus introducing primer-encoded restriction sites for both *Eco*RI and *Hin*dIII. The first two-thirds of gene *tsaQ* (positions 39–738 in GenBank sequence AY227144) were amplified with primers TsaQ-a and TsaQ12HindIII and the last third (positions 742–1,146 in sequence AY227144) with primers TsaQ-b and TsaQ-21EcoRI. This introduced a restriction site for either *Hin*dIII or *Eco*RI. The resulting PCR products were digested with *Eco*RI and *Hin*dIII and ligated with the amplified Tet^r, which had been pre-digested with the same restriction enzymes. The ligation product was amplified with TsaQ-a and TsaQ-b. This resulted in a 2,811-bp Tet^r-*tsaQ* knock-out construct, where Tet^r was inserted in reverse orientation between bases 572 and 573 of *tsaQ*. A knock-out construct of *tsaQ* with an inserted Cm^r gene was similarly generated. Primers CmapMP141.1r and CmapMP141.11 were used to amplify the Cm^r gene from pMP141.1 (Providenti et al. 2001). With the two primer pairs, TsaQ-a+TsaQ-12EcoRI and TsaQ-21EcoRI+TsaQ-b, *tsaQ* was again amplified in two segments. The three PCR products were digested with *Eco*RI, ligated and the resulting Cm^r-*tsaQ*-knock-out construct (4,247 bp, with Cm^r in reverse orientation to *tsaQ* between bp 572 and 573, as confirmed by sequencing) was amplified with TsaQ-a and TsaQ-b. Religation of *tsaQ* without insert was avoided by dephosphorylation of the *tsaQ*-fragments prior to ligation. In both knock-out constructs, palindromic stem-loop-stem structures at the end of the resistance cassette (tet^rgene) or within the inserted sequence (cm^r cassette), in addition to termination sequences 14 bp 3′ to the *tsaD* genes (Junker et al. 1997) should prevent transcription of anti-sense mRNA from the *tsaMBCD* operons.

PCR-amplified knock-out constructs were introduced into target cells by electroporation as described by Tralau et al. (2003). The knock-out mutants of *C. testosteroni* T-2, which were generated during this work, are listed in Table 2. The knock-out was tested by PCR with primer pairs TsaQ-a and IS772u for $tsaQ_2$ and primer pairs TsaQ-a and IS772l for $tsaQ_1$; and the lengths of the

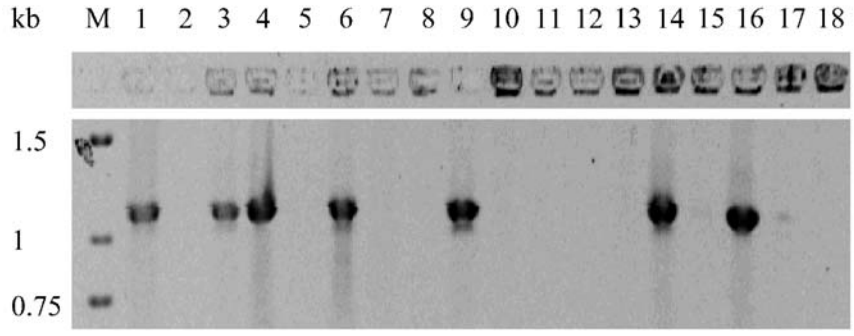


Fig. 2 Detection of *tsaQ* by PCR. Our collection of 17 cultures able to utilize TSA and a derivative of strain T-2 lacking the pTSA plasmid were examined by PCR, using primers TsaQ-a and TsaQ-b and an expected PCR product length of 1,108 bp. Cultures containing the *tsa* operon are *indicated in bold*. *Lane 1 C. testosteroni***T-2** (positive control), *lane 2 C. testosteroni* TER-1 (negative control), *lane 3 Delftia acidovorans***Mu4**, *lane 4 Stenotrophomonas maltophilia***MuF**, *lane 5* mixed culture KNP1, *lane 6 Delftia acidovorans***MoP1**, *lane 7 Hydrogenophaga teniospiralis* EWM1, *lane 8 C. testosteroni* EW13, *lane 9 Pseudomonas pseudoalcaligenes***EWL2**, *lane 10 Acidovorax delafieldii* TKA, *lane 11 S. maltophilia* VLB, *lane 12* mixed culture VLB1, *lane 13 S. maltophilia* RLB, *lane 14 Burkholderia pickettii***TKR**, *lane 15* unidentified isolate **TA12**, *lane 16 Pseudomonas* sp. **ISP2**, *lane 17* mixed culture SCM, *lane 18 C. testosteroni* OrL1, *lane M* 1-kb marker

predicted PCR products were 4.8 kb (Tet^r, 3.1 kb for wild type) and 8.1 kb (Cm^r, 4.7 kb for wild type), respectively. PCRs with primers TnTet-1 and PsTs-9 were done to localize Tet^r and with primers Cm3 and PsTs-9 to localize Cm^r in *tsaQ*. The insertion sites were confirmed by sequencing.

Protein purification and quantification, gel electrophoresis, preparation of His-tagged protein and band-shift assays

His-tagged regulator protein (TsaR^{His}) was expressed heterologously in *E. coli* and isolated as described by Tralau et al. (2003). Soluble protein was quantified colorimetrically (Bradford 1976) and the purity of protein fractions was examined by SDS-PAGE (Schägger and von Jagow 1987). Protein was stained with either soluble (Sambrook et al. 1989) or colloidal (Neuhoff et al. 1988) Coomassie brilliant blue and compared with protein standards given by Tralau et al. (2003). Gel retardation assays to quantify the binding of TsaR^{His} to DNA (using 2 µg of protein for band-shifts) or to determine the values of the dissociation constant (K_D; using 0.3–1.5 µg of protein) were as described by Tralau et al. (2003).

Nucleotide sequence accession numbers

The putative promoter region with the partial gene sequence of *tsaT* is available in the NCBI GenBank library under accession number AY044256; and the Tn*tsa* sections covering $tsaQ_1$ and $tsaQ_2$ are available under accession numbers AY227144 and AY227145, respectively.

Results and discussion

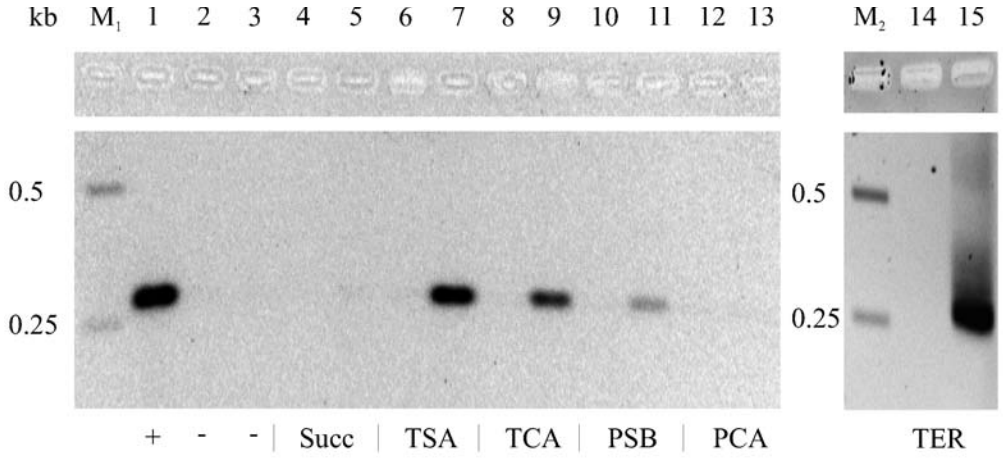
tsaQ is part of the *tsa* transposon

The *tsa* operon is widespread (Tralau et al. 2001), but the general composition of Tn*tsa* (Fig. 1) has not been established. We have now examined our TSA-utilizing cultures (Fig. 2) to discover which ones contain the *tsaQ* gene(s). The clear positive and negative controls using the PCR primer pair TsaQ-a and TsaQ-b (Fig. 2, lanes 1, 2) allow us to state that those cultures which lacked the *tsa* operon also lacked the *tsaQ* gene (Fig. 2, lanes 5, 7, 8, 10–13, 17, 18). Of the seven *tsa*$^+$ cultures (apart from strain T-2), six contained *tsaQ* (Fig. 3, lanes 3, 4, 6, 9, 14, 16) and one, strain TA12, did not (Fig. 3, lane 15). This observation coincides with the distribution of the complete *tsa* transposon, which lacks *tsaT* in TA12 (Mampel 2000; Tralau et al. 2001). The *tsaQ* gene is thus, presumably, a common component of *tsa* transposons. Strain TA12 obviously has a different overall strategy, especially concerning regulation and transport, to degrade TSA via TsaMBCD$_1$.

The transcription of *tsaQ* in *C. testosteroni* T-2 under a range of growth conditions was examined by RT-PCR (Fig. 3). The positive and negative controls allowed us to conclude that at least one of the two *tsaQ* genes was transcribed when TSA, TCA, *p*-sulfobenzoate (PSB) or TER was utilized, but not when the ring-cleavage substrate protocatechuate (PCA) was the sole carbon source. This induction pattern differs from the results obtained for the transcription of *tsaR* and *tsaMBCD* (Table at the foot of Fig. 3). The latter genes were transcribed in the presence of TSA, TCA and PSB but not with TER. The transcription of *tsaQ* during the growth of strain T-2 with TER is presumably largely irrelevant to the present work, because strain TER-1 grows normally with TER. Strain TER-1 is a mutant of strain T-2 which has lost pTSA. Further, the complete or partial loss of TsaQ in strain T-2 was without effect on growth with TER (see below). However, the data do show that the regulation of *tsaQ* is different from that of *tsaR* and *tsaMBCD*.

The sequence of *tsaQ* and another ORF

The *tsaQ* gene comprised 837 bp, and the deduced amino acid sequence (278 amino acids) was analyzed (NCBI BLAST; Altschul et al. 1997). No enzymic function could be attributed to the sequence. The most similar protein (NC_00431.11, 33% identity) was found in the genome of *Brucella suis* 1330. It encodes a protein (284 amino acids) which is annotated as a transcriptional regulator of the IclR family. PcaR (AAC38247) from *Rhodococcus opacus*, a proven IclR-type regulator (Eulberg and Schloemann 1998;



Gene	Growth substrate					
	succinate	TSA	TCA	PSB	PCA	TER
tsaR	-	+	+	+	-	-
tsaMBCD	-	+	+	+	-	-
tsaQ	-	+	+	+	-	+

Fig. 3 Detection by RT-PCR of transcripts of *tsaQ* in cells of *C. testosteroni* T-2 grown with different substrates, with tabulation of detection of transcripts of *tsaQ*, *tsaR* and *tsaMBCD*. *Lanes* M_1, M_2 DNA markers with sizes indicated in kilobases, *lane 1* PCR with total DNA from *C. testosteroni* T-2 (positive control), *lane 2* PCR with water as template (negative control), *lane 3* RT-PCR with water as template (negative control), *lane 4* PCR with total RNA from succinate-grown cells, *lane 5* RT-PCR with total RNA from succinate-grown cells, *lane 6* PCR with total RNA from TSA-grown cells, *lane 7* RT-PCR with total RNA from TSA-grown cells, *lane 8* PCR with total RNA from TCA-grown cells, *lane 9* RT-PCR with total RNA from TCA-grown cells, *lane 10* PCR with total RNA from PSB-grown cells, lane 11 RT-PCR with total RNA from PSB-grown cells, *lane 12* PCR with total RNA from PCA-grown cells, *lane 13* RT-PCR with total RNA from PCA-grown cells, *lane 14* PCR with total RNA from TER-grown cells, *lane 15* RT-PCR with total RNA from TER-grown cells. Symbols in Table: + transcription, – no transcription

Eulberg et al. 1998), shares 25% identity with TsaQ. A helix-turn-helix motif for DNA-binding could be located over amino acids 19–107, based on a search for conserved domains (Altschul et al. 1997). The amino acid sequence over positions 77–260 showed similarities to the consensus sequence for IclR-type regulators (Altschul et al. 1997), so we hypothesize that TsaQ has a regulatory function.

The left and right ends of Tn*tsa* were believed to contain no ORFs (Tralau et al. 2001). Renewed analysis revealed the presence of ORFs of identical length (807 bp) and high sequence identity (98.5%; Fig. 1), in the opposite orientation to the *tsaQ* genes. Their function is unknown, but some similarity to esterases allows the hypothesis that a TCA ester might be the substrate, if a gene is expressed.

The phenotype of *tsaQ* mutants

Two mutants with a single disruption in a *tsaQ* gene and a double mutant were generated. Growth of *C. testosteroni* T-2 with succinate, PCA or TER as substrate was not affected by elimination of either a single copy of the *tsaQ* gene, or both *tsaQ* genes (Table 2). When only $tsaQ_1$ was inactivated, growth of mutant TE_7 with TSA was prevented, whereas full growth with TCA or PSB was attained, but very slowly. Loss of $tsaQ_2$ (mutant TE_{11}) was less severe in its effect, full growth with TSA or TCA was very slow and growth with PSB was somewhat slower than strain T-2 (Table 2). When both copies of *tsaQ* were inactivated, the mutant (TE_{7C}) was unable to grow with TSA but showed normal growth with TCA, while growth with

Table 2 Growth of *Comamonas testosteroni* T-2 and of $tsaQ_1$- and $tsaQ_2$-mutants with different substrates (see Materials and methods). Growth media were inoculated with a 1:50 dilution of exponentially growing cells. *NG* No growth

Organism		Substrate (days to full growth)					
Strain	Genotype	Succinate	TSA	TCA	PSB	TER	PCA
T-2	$tsaQ_1^+ tsaQ_2^+$	1.5	1.5	1.5	1.5	1.5	1.5
TE_7	$tsaQ_1$, tet^r	1.5	NG	12.0	12.0	1.5	1.5
TE_{11}	$tsaQ_2$, tet^r	1.5	12.0	12.0	3.0	1.5	1.5
TE_{7C}	$tsaQ_1 tsaQ_2$, tet^r, cm^r	1.5	NG	1.5	1.0	1.5	1.5

PSB was slightly faster than growth of the parent organism (Table 2). Both copies of the *tsaQ* gene are identical, so we have no explanation for the different growth patterns of strains TE_7 and TE_{11}.

Normal growth of the double mutant with TCA indicates that the regulator TsaR and the degradative enzymes ($TsaMBCD_1$) are fully functional. The differences between the two single mutants (Table 2) imply that more $TsaQ_1$ was expressed than $TsaQ_2$. The faster growth with PSB (Table 2) possibly indicates some regulatory interaction of TsaQ with the IclR-type binding site for PSB regulation: cross-binding of distinct but evolutionarily related regulators to each others′ promoters has been observed with some LysR-type regulators (van der Meer et al. 1991; von Lintig et al. 1994; McFall et al. 1997).

The effects in the double mutant, in particular growth with TCA, argue against a direct interaction of TsaQ with *tsaR* or $tsaMBCD_1$ at the common promoter region between the *tsaR* and *tsaM* genes (Fig. 1; see Tralau et al. 2003). We thus looked for gene(s) independent of the metabolism of TCA, but essential for the degradation of TSA, that could be a target for regulatory TsaQ(s). The only serious candidate is a gene attributed to the transport of TSA (Mampel 2000), *tsaT*, which lies downstream of *tsaR* (Fig. 1; Tralau et al. 2001). Another transport component, TsaS (Fig. 1), is expressed constitutively (Mampel and Cook, unpublished).

Transcription of *tsaT* in *C. testosteroni* T-2 with different growth substrates was examined by RT-PCR (data not shown). TSA-grown cells of strain T-2 showed transcription of *tsaT*, but no mRNA for *tsaT* could be detected in cells grown with succinate, PCA, PSB, TER or TCA. These results link *tsaT* exclusively with the degradation of TSA. Similarly, *tsaQ* regulates a function exclusive to the degradation of TSA. So we presumed that TsaQ plays a regulatory role in the expression, or function, of *tsaT*. Whether this is a direct interaction between TsaQ and the transporter TsaT, as was found for MalT binding to the ABC transporter subunit MalY (Böhm et al. 2002; Schlegel et al. 2002), or an interference with the binding of the other regulator (TsaR; see next section) to the promoter of *tsaT* will be the subject of future studies.

The regulator of the *tsa* operon (TsaR) binds to the promoter region of *tsaT*

The putative promoter region of *tsaT*, 400 bp between *tsaS* and extending into *tsaT* (GenBank accession number AY044256; Fig. 5) was examined for interactions with TsaR in band-shift assays. Control experiments with $TsaR^{His}$ and a coding region in the *tsa* transposon (Table 3, fragment A; Fig. 4) and with a promoter region independent of Tn*tsa* (Table 3, fragment B; data not shown) showed no interaction. In contrast, DNA from the promoter region of *tsaT* migrated a shorter distance in the presence of $TsaR^{His}$; and larger amounts of $TsaR^{His}$ caused reduced mobility for larger amounts of DNA, independent of the presence of TSA (data not shown) or its absence (Fig. 4). There is, thus,

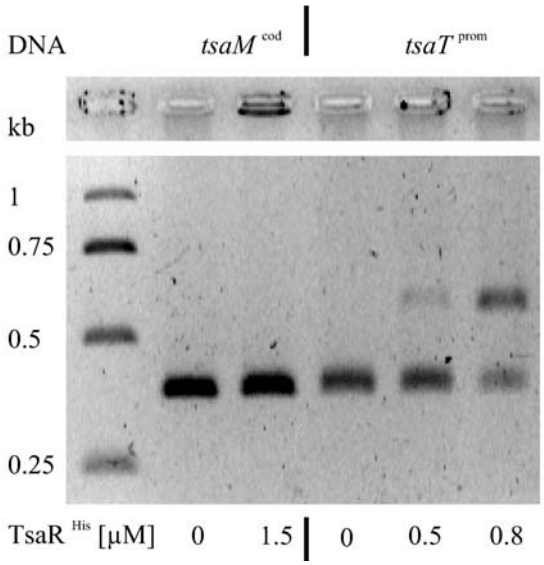


Fig. 4 Effect of $TsaR^{His}$ on the mobility of different fragments of DNA from the *tsa* transposon in the absence of TSA. *Lane 1* 1-kb marker, *lane 2* coding region of *tsaMB* in the absence of $TsaR^{His}$ (negative control), *lane 3* coding region of *tsaMB* in the presence of 1.5 μM $TsaR^{His}$ (negative control), *lane 4* promoter region of *tsaT* in the absence of $TsaR^{His}$, *lane 5* promoter region of *tsaT* in the presence of 0.5 μM $TsaR^{His}$, *lane 6* promoter region of *tsaT* in the presence of 0.8 μM $TsaR^{His}$

a specific interaction of $TsaR^{His}$ with the promoter region of *tsaT*, so transcription of the latter is presumably under the control of both TsaR and TsaQ.

The K_D of $TsaR^{His}$ for the whole promoter region of *tsaT* (Fig. 5, fragment C) was determined in the absence of TSA (K_D=0.9 μM) and in its presence (K_D=1.3 μM at 0.1 mM TSA, 1.2 μM at 1 mM TSA, 0.8 μM at 6 mM TSA). These values are very similar to those observed for the promoter region between *tsaR* and *tsaM* (Tralau et al. 2003).

The DNA sequence upstream of *tsaT* was analyzed in order to find consensus sequences for σ^{70}-dependent promoters and possible binding sites for LysR-type regulatory proteins (Schell 1993). A putative Pribnow box was found at positions 238–245. The corresponding putative recognition site for the RNA polymerase was located at positions 214–219. The transcriptional start-site could be predicted by NNPP at position 249 and a consensus for a Shine-Dalgarno sequence could be found at positions 266–270 (Fig. 5). However, a consensus motif ($T\text{-}N_{11}\text{-}A$; see Schell 1993) for the binding of LysR-type regulators was not detected.

There are several binding sites for TsaR in the promoter region between *tsaR* and *tsaM* (Tralau et al. 2003), so we probed for multiple binding sites in the complete promoter region of *tsaT*. The whole promoter, fragment C (Fig. 5; Table 3), gave a single band-shift with $TsaR^{His}$ both in the presence and absence of TSA. Band-shift assays were then done with overlapping subfragments of the promoter region. This showed that there was no binding site in the coding region of fragment C (Table 3, fragment F;

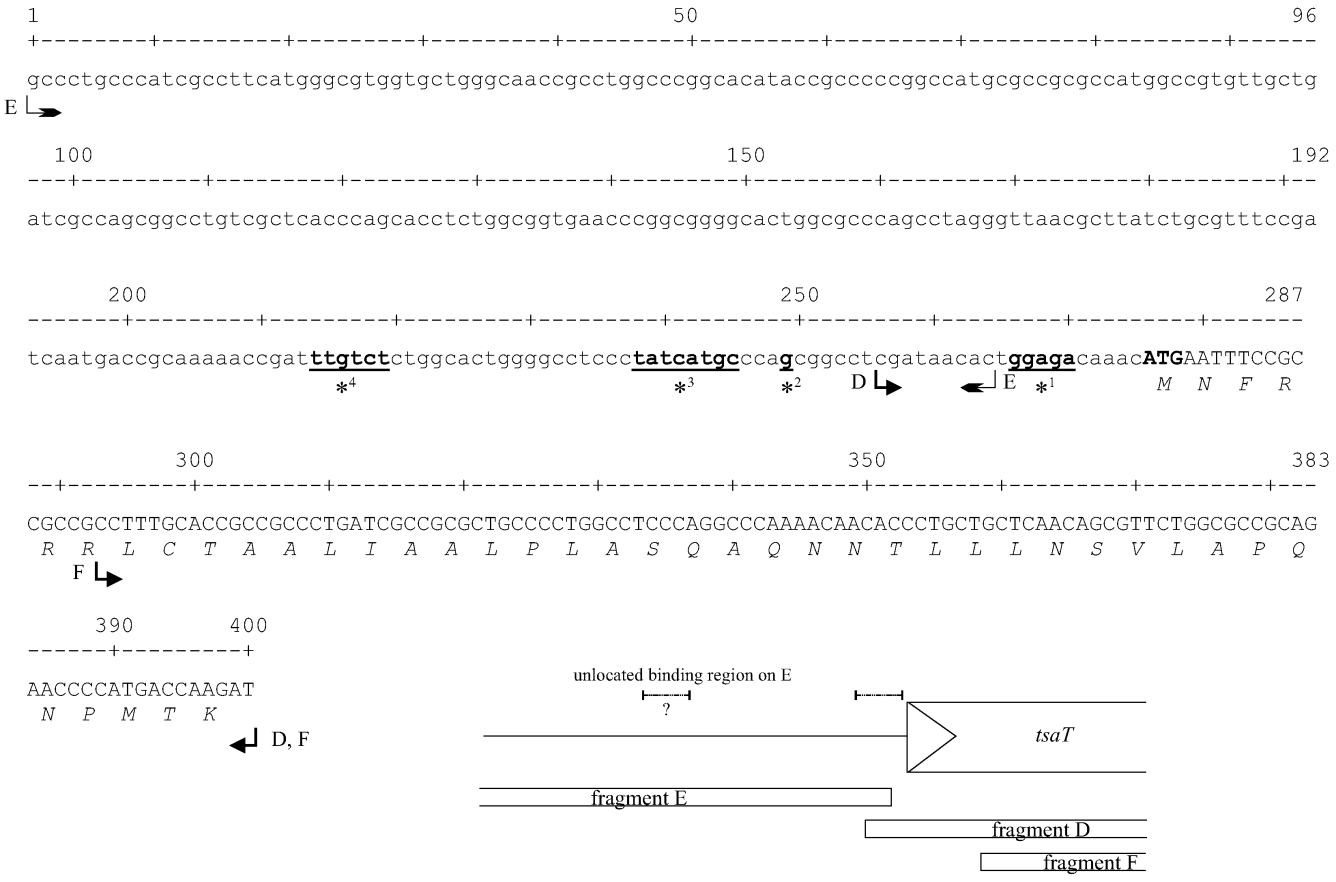


Fig. 5 Sequence of PCR-fragment C (GenBank accession number AY044256) and structure of the promoter of *tsaT*. Fragment C (Table 3) extends over the putative promoter region of *tsaT* into the coding region of the gene. Gene *tsaT* is *shown in uppercase*, the start codon is *shown in bold*. The putative ribosomal binding site (*^{1}), transcriptional start (*^{2}), Pribnow box (*^{3}) and –35 region (*^{4}) are *shown in bold and underlined*. Primers for fragment D are *marked in bold, with long arrowheads*; and those for fragment E and F are *marked in bold, with short arrowheads* (see also Table 3). Putative binding regions for TsaR^{His} are *indicated in the sketch* of fragments D, E and F (*below main sequence*)

Fig. 5) and one binding region could be detected upstream of the coding region, indicated by a weak shift (17%) of fragment D (Table 3; Fig. 5). Fragment E showed two shifted bands (Table 3). This indicates that at least two binding sites are located on this fragment, one in the region overlapping fragment D (nucleotides 250–267) that probably mediates the weak shift of fragment D and another which could not be specified (Table 3; Fig. 5). The binding of TsaR^{His} to these sites was independent of the presence of TSA. This allows the conclusion that TsaR cannot be the only regulator for *tsaT*, because the inducing substrate TSA does not mediate any change in the occupational state of the binding regions for TsaR^{His}. This contrasts with our finding for the promoter of *tsaMBCD*$_1$, where TSA-dependent and TSA-independent binding was observed (Tralau et al. 2003). We presume that the second regulator, TsaQ, mediates a regulatory separation of the enzymes required for the transformation of TSA to PSB (*tsaMBCD*; Junker et al. 1997) and the transport for TSA (probably *tsaT*; Locher et al. 1993; Mampel 2000). This enables *C. testosteroni* T-2 to use the same set of enzymes

Table 3 Results for the DNA-binding assays with TsaR^{His} and PCR products of the promoter region of *tsaT* (cf. Fig. 5). Values in parentheses are the percentages of band-shifted DNA; and, if there are two bands, the first value is for the smaller shift. A coding region of *tsaMB* (fragment A) and the promotor region of *pszA* (fragment B) were used as negative controls

Fragment	Location of fragment	Band shift(s)	
		no TSA	6 mM TSA
A	2,397–2,782 in U32622; coding region in *tsaMB*	None	None
B	Promoter region of *pszA*, AY044257	None	None
C	1–400; promoter region of *tsaT* and 5′ region of *tsaT*, AY044256	1 (100%)	1 (100%)
D	256–400, AY044256	1 (17%)	1 (17%)
E	1–264, AY044256	2 (69%, 31%)	2 (67%, 33%)
F	293–400, AY044256	None	None

very efficiently for the degradation of several substrates as, e.g. TCA. The location of *tsaR* and *tsaQ* within Tn*tsa* ensures that the transfer of this part of the degradative pathway (Fig. 1; Junker and Cook 1997; Tralau et al. 2001) is accompanied by the transfer of the complete regulatory system.

We conclude that not only *tsaR* and *tsaMBCD* but also *tsaQ* and *tsaT* are part of one common regulatory unit, regulon R1, leaving open the question why *tsaQ* is transcribed when TCA or TER are growth substrates, although growth with these substrates is not prevented when *tsaQ* is knocked-out.

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References

Adhya S (1999) Regulation of gene expression: operons and regulons. In: Lengeler JW, Drews G, Schlegel HG (eds) Biology of the prokaryotes. Thieme, Stuttgart, pp 437–468

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Blatny JM, Brautaset T, Winther Larsen HC, Karunakaran P, Valla S (1997) Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in gram-negative bacteria. Plasmid 38:35–51

Böhm A, Diez J, Diederichs K, Welte W, Boos W (2002) Structural model of MalK, the ABC subunit of the maltose transporter of *Escherichia coli*: implications for mal *gene* regulation, inducer exclusion, and subunit assembly. J Biol Chem 277:3708–3717

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Cook AM, Laue H, Junker F (1999) Microbial desulfonation. FEMS Microbiol Rev 22:399–419

Eulberg D, Schloemann M (1998) The putative regulator of catechol catabolism in *Rhodococcus opacus* 1CP – an IclR-type, not a LysR-type transcriptional regulator. Antonie Van Leeuwenhoek 74:71–82

Eulberg D, Lakner S, Golovleva LA, Schlömann M (1998) Characterization of a protocatechuate catabolic gene cluster from *Rhodococcus opacus* 1CP: evidence for a merged enzyme with 4-carboxymuconolactone-decarboxylating and 3-oxoadipate enol-lactone-hydrolyzing activity. J Bacteriol 180:1072–1081

Junker F, Cook AM (1997) Conjugative plasmids and the degradation of arylsulfonates in *Comamonas testosteroni*. Appl Environ Microbiol 63:2403–2410

Junker F, Kiewitz R, Cook AM (1997) Characterization of the *p*-toluenesulfonate operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2. J Bacteriol 179:919–927

Lintig J von, Kreusch D, Schroder J (1994) Opine-regulated promoters and LysR-type regulators in the nopaline (*noc*) and octopine (*occ*) catabolic regions of Ti plasmids of *Agrobacterium tumefaciens*. J Bacteriol 176:495–503

Locher HH, Leisinger T, Cook AM (1989) Degradation of *p*-toluenesulphonic acid via sidechain oxidation, desulphonation and *meta* ring cleavage in *Pseudomonas* (*Comamonas*) *testosteroni* T-2. J Gen Microbiol 135:1969–1978

Locher HH, Malli C, Hooper S, Vorherr T, Leisinger T, Cook AM (1991) Degradation of *p*-toluic acid (*p*-toluenecarboxylic acid) and *p*-toluene sulphonic acid via oxygenation of the methyl sidechain is initiated by the same set of enzymes in *Comamonas testosteroni* T-2. J Gen Microbiol 137:2201–2208

Locher HH, Poolman B, Cook AM, Konings WN (1993) Uptake of 4-toluenesulfonate by *Comamonas testosteroni* T-2. J Bacteriol 175:1075–1080

Mampel J (2000) Transport- und Regulationsphänomene beim Abbau von 4-Toluolsulfonat in *Comamonas testosteroni*. PhD thesis, University of Konstanz, Konstanz

McFall SM, Parsek MR, Chakrabarty AM (1997) 2-Chloromuconate and ClcR-mediated activation of the *clcABD* operon: in vitro transcriptional and DNase I footprint analyses. J Bacteriol 179:3655–3663

Meer JR van der, Frijters AC, Leveau JH, Eggen RI, Zehnder AJ, Vos WM de (1991) Characterization of the *Pseudomonas* sp. strain P51 gene *tcbR*, a LysR-type transcriptional activator of the *tcbCDEF* chlorocatechol oxidative operon, and analysis of the regulatory region. J Bacteriol 173:3700–3708

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis 9:255–262

Providenti MA, Mampel J, MacSween S, Cook AM, Wyndham RC (2001) *Comamonas testosteroni* BR6020 possesses a single genetic locus for extradiol cleavage of protocatechuate. Microbiology 147:2157–2167

Reese MG, Harris NL, Eeckman FH (1996) Large scale sequencing specific neural networks for promoter and splice site recognition. In: Hunter L, Klein TE (eds) Biocomputing: proceedings of the 1996 Pacific symposium. World Scientific Publishing, Singapore

Riediker S, Ruckstuhl S, Suter MJ-F, Cook AM, Giger W (2000) *p*-Toluenesulfonate in landfill leachates: leachability from foundry sands and aerobic biodegradation. Environ Sci Technol 34:2156–2161

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schägger H, Jagow G von (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Schell MA (1993) Molecular biology of the LysR family of transcriptional regulators. Annu Rev Microbiol 47:597–626

Schläfli Oppenberg HR, Chen G, Leisinger T, Cook AM (1995) Regulation of the degradative pathways from 4-toluenesulphonate and 4-toluenecarboxylate to protocatechuate in *Comamonas testosteroni* T-2. Microbiology 141:1891–1899

Schlegel A, Böhm A, Lee SJ, Peist R, Decker K, Boos W (2002) Network regulation of the *Escherichia coli* maltose system. J Mol Microbiol Biotechnol 4:301–307

Thurnheer T, Köhler T, Cook AM, Leisinger T (1986) Orthanilic acid and analogues as carbon sources for bacteria: growth physiology and enzymic desulphonation. J Gen Microbiol 132: 1215–1220

Tralau T, Cook AM, Ruff J (2001) Map of the IncP1β plasmid pTSA encoding the widespread genes (*tsa*) for *p*-toluenesulfonate degradation in *Comamonas testosteroni* T-2. Appl Environ Microbiol 67:1508–1516

Tralau T, Mampel J, Cook AM, Ruff J (2003) Characterization of TsaR, an oxygen-sensitive LysR-type regulator for the degradation of *p*-toluenesulfonate in *Comamonas testosteroni* T-2. Appl Environ Microbiol 69:2298–2305