APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2003, p. 2298–2305 Vol. 69, No. 4
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.4.2298–2305.2003

Characterization of TsaR, an Oxygen-Sensitive LysR-Type Regulator for the Degradation of *p*-Toluenesulfonate in *Comamonas testosteroni* T-2

Tewes Tralau,† Jörg Mampel,‡ Alasdair M. Cook, and Jürgen Ruff*

Department of Biology, The University of Konstanz, D-78457 Konstanz, Germany

Received 3 October 2002/Accepted 23 January 2003

TsaR is the putative LysR-type regulator of the *tsa* operon (*tsaMBCD*) which encodes the first steps in the degradation of *p*-toluenesulfonate (TSA) in *Comamonas testosteroni* T-2. Transposon mutagenesis was used to knock out *tsaR*. The resulting mutant lacked the ability to grow with TSA and *p*-toluenecarboxylate (TCA). Reintroduction of *tsaR* in *trans* on an expression vector reconstituted growth with TSA and TCA. The *tsaR* gene was cloned into *Escherichia coli* with a C-terminal His tag and overexpressed as $TsaR^{His}$. $TsaR^{His}$ was subject to reversible inactivation by oxygen, which markedly influenced the experimental approaches used. Gel filtration showed $TsaR^{His}$ to be a monomer in solution. Overexpressed $TsaR^{His}$ bound specifically to three regions within the promoter between the divergently transcribed *tsaR* and *tsaMBCD*. The dissociation constant (K_D) for the whole promoter region was about 0.9 μM, and the interaction was a function of the concentration of the ligand TSA. A regulatory model for this LysR-type regulator is proposed on the basis of these data.

p-Toluenesulfonate (TSA) is widely used in the metal and chemical industries and in laundry detergents in the home (2). TSA is degraded via *p*-sulfobenzoate (PSB) and protocatechuate (PCA) by the bacterium *Comamonas testosteroni* T-2 (Fig. 1) (9); this pathway is encoded in part on two plasmids, pTSA and pT2L, and in part on the chromosome (PCA). pTSA (12) is widespread (42).

The first three metabolic enzymes in the degradation of TSA by strain T-2 are encoded on pTSA in the operon *tsaMBCD* (Fig. 1); *tsaR* is located upstream of *tsaMBCD* in the reverse orientation (Fig. 1) (13). TsaR, a hypothetical protein of 298 amino acids (32.7 kDa) (13), was proposed to be a LysR-type regulator (35) of the *tsa* operon with amino acid sequence identities of 27.8% to Nac (nitrogen assimilation control regulator; 37) and 27.2% to ClcR (chlorocatechol operon regulator; 7). Most of the LysR-type regulatory proteins are known to bind specifically to their target DNA (for examples, see references 5, 8, and 35). The putative regulatory region between *tsaR* and *tsaMBCD* has all the elements characteristic of regulatory regions, including bacterial σ^{70} promoters, putative ribosomal binding sites, and transcriptional start, Pribnow box, and −35 regions (13).

We now confirm that TsaR, examined as the His-tagged $TsaR^{His}$, is the major regulator of expression of *tsaMBCD* in *C. testosteroni* T-2, and we describe some of its properties and interactions with the promoter region.

MATERIALS AND METHODS

Bacteria, mutants, and growth conditions. *C. testosteroni* T-2 (DSM 6577) was grown in minimal medium as described previously (36, 40). Mutants 4A10 and TT6 (Table 1) were grown with minimal medium supplemented with 50 to 70 μg of tetracycline/ml. The compounds used as growth substrates were pure (>99%). Growth was estimated as optical density at 580 nm and quantified according to levels of Lowry-type protein (15). Substrate utilization was determined after separation on reversed-phase high-performance liquid chromatography columns (17), and sulfate was quantified as a suspension of barium sulfate (39).

Escherichia coli S17-1λpir[pUT-Tc] was kindly provided by M. Kertesz and grown as described previously (10). *E. coli* DH5α[pJB866], as the donor of the broad-host-range vector pJB866 (GenBank accession no. U8200), was kindly provided by J. M. Blatny and grown as described previously (3). The *E. coli* host strain M15[pREP4] (Qiagen, Hilden, Germany), which contains an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression system, and the derived clone M10 (see below) were grown with Luria-Bertani (LB) medium (33) with 25 μg of kanamycin/ml and 100 μg of ampicillin/ml, respectively, according to the manufacturer's recommendations.

PCR, quantification of DNA, cycle sequencing, and Southern hybridization. PCR was done in a final volume of 20 to 50 μl. All reaction mixtures contained 10% (vol/vol) dimethyl sulfoxide. Genomic DNA (180 to 200 ng), prepared by cetyltrimethylammonium bromide precipitation (1), or a bacterial culture was used as the template. Templates (≤5 kb) were amplified with *Taq* polymerase (MBI Fermentas) in buffer system 2 of the Expand Long PCR system (Roche) or (>5 kb) with the Expand Long PCR System (Roche). The latter has a proofreading activity and was also used for the generation of PCR products designed for cloning. Reactions were done according to the manufacturers' recommendations. DNA was quantified fluorimetrically (DyNA Quant 200; Hoefer) according to the manufacturer's instructions. Primers used for PCR are listed in Table 2. The Tn*5* insertions were localized in strain 4A10 with primer pair TsaReg1-TsaReg2. The template for sequencing the Tn*5* insertion in strain 4A10 was amplified with primer pair TsaRu1-AntiRu1; the sequencing primers were TsaIG1, TsaReg4, and Tn55-Out.

Primers 866-MB1 and 866-21 or $tsaR^{His}$*Hin*dIII were used to detect by PCR those clones carrying pJB866 with the His-tagged *tsaR* gene; primer pair pQE70-1 and pQE70-2 was used for *E. coli* transformants carrying the His-tagged *tsaR* gene on pQE-70. Templates for sequencing the inserts of pJB866 and pQE70 were generated with primer pair 866-MB2 and 866-22 and primer pair pQE70-1 and pQE70-2, respectively.

The amplified template for cycle sequencing was purified with the Qiaquick PCR purification kit (Qiagen), and the fragment (85 ng of DNA/kb) was added to the sequencing reaction mixture (ABI Prism Big Dye terminator kit; Applied Biosystems) and subjected to the following PCR program: 70 s at 95°C and then 26 cycles of 20 s at 95°C, 30 s at 50°C, and 4 min at 60°C. The sequence was determined by capillary electrophoreses (GATC, Konstanz, Germany). Using standard software (Edit View [Perkin Elmer], a GCG program package, and DNA-STAR [Lasergene]) and the neural network analysis for prokaryotic promoters software of M. G. Reese (http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html) (11; M. G. Reese and F. H. Eeckman, abstract from the

* Corresponding author. Mailing address: Department of Biology, The University of Konstanz, Universitätsstr. 10, D-78457 Konstanz, Germany. Phone: 49 7531 88 2100. Fax: 49 7531 88 2966. E-mail: juergen.ruff@uni-konstanz.de.

† Present address: School of Biological Sciences, University of Manchester, Manchester, United Kingdom.

‡ Present address: Institute of Biotechnology, Swiss Federal Institute of Technology, Zürich, Switzerland.

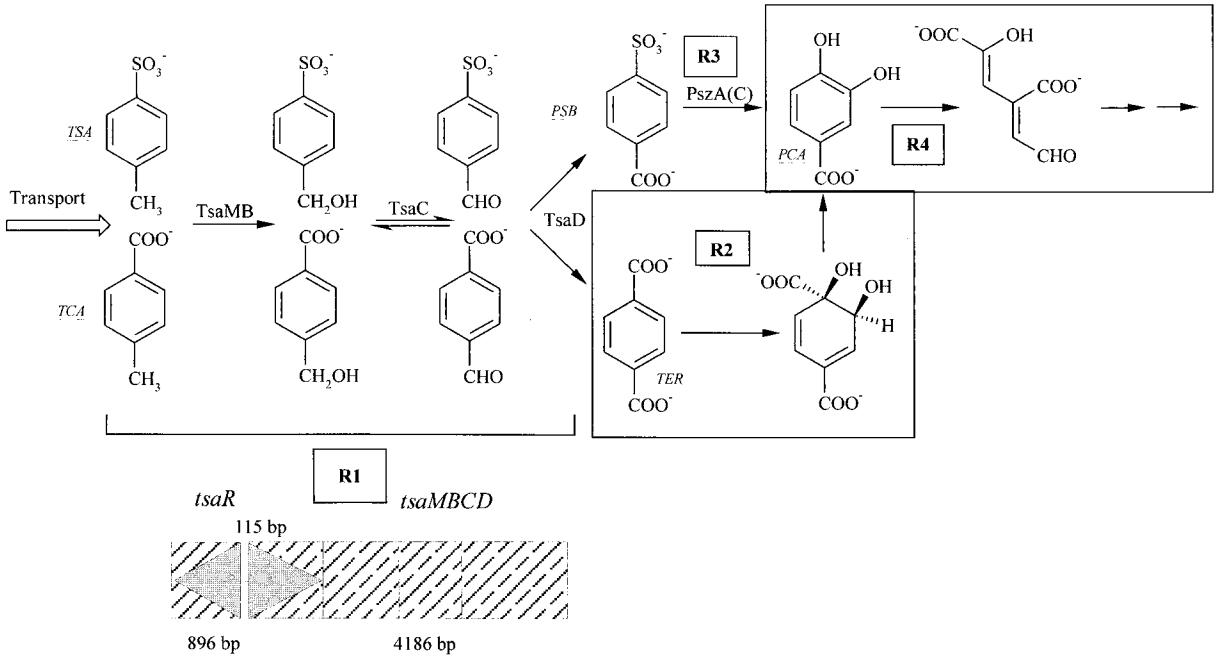

FIG. 1. Degradation of TSA and TCA to amphibolic intermediates by *C. testosteroni* T-2 and the four regulons involved (R1 to R4) (9, 29, 36). Reactions of gene products that are encoded by chromosomal genes are in insets. Regulatory units R1 (pTSA) and R3 (pT2L) are plasmid encoded (42; Ruff, unpublished). The *tsa* regulon is sketched schematically, with the directions of transcription indicated by shaded triangles. TsaMB, *p*-toluenesulfonate methylmonooxygenase (oxygenase M, reductase B); TsaC, *p*-sulfobenzylalcohol dehydrogenase; TsaD, *p*-sulfobenzaldehyde dehydrogenase; PszA(C), *p*-sulfobenzoate-3,4-dioxygenase.

Proceedings of the Seventh International Genome Sequencing and Analysis Conference, vol. 1, no. 1, p. 45, 1995, and M. G. Reese, N. L. Harris, and F. H. Eeckman, poster from the Proceedings of the 1996 Pacific Symposium, Biocomputing, 1996), we analyzed the sequence data on the basis of characterized *E. coli* promoters.

Southern blot hybridization was done as described elsewhere (24). Gene probes were generated according to the manufacturer's instructions by digestion with *Eco*RI (New England BioLabs) of pUT-Tc carrying Tn*5*. The 2.1-kb fragment was labeled with a DIG system (Roche) and used as a gene probe to confirm the presence of the Tn*5* insertion on pTSA.

The DNA markers used were the 0.1- and 1-kb ladders (MBI Fermentas), with ranges of 0.1 to 3 kb and 0.25 to 10 kb, respectively.

Pulsed-field gel electrophoresis. DNA was prepared from whole cells embedded in low-melting-point agarose as described elsewhere (38). Electrophoresis was done as described previously (42).

Transposon mutagenesis, competent cells, and cloning. The mini-Tn*5* transposon mutagenesis system (10) was used to knock out *tsaR* in *C. testosteroni* T-2. *E. coli* S17-1λpir[pUT-Tc] (6×10^9 cells; donor) was mated with *C. testosteroni* T-2 (2×10^9 cells; recipient) on LB agar at 30°C for 48 h. Prior to mating, cells were centrifuged separately, washed twice, resuspended in 0.9% (wt/vol) sterile NaCl solution, and mixed before a 50-μl volume was applied to the LB agar plate. Cells were then scraped off the agar and washed once with 0.9% (wt/vol) NaCl. Dilution series were plated on minimal agar with PCA or succinate as the carbon and energy source (antidonor selection) and 15 μg of tetracycline/ml (selection for the Tn*5* marker). A total of 7,500 mutants were characterized for growth with TSA, TCA, PSB, or terephthalate (TER) in microtiter plates. Mutants unable to grow on TSA or TCA were further analyzed, because this phenotype is expected from a knockout of *tsaR* (Fig. 1) (or of *tsaMBCD*). A total of 5‰ of the mutants corresponded to this phenotype, but only one *tsaR*-negative mutant was obtained.

Vectors with inserts were introduced into target cells either by electroporation or by chemical transformation. Cells of *C. testosteroni* were prepared for electroporation as described previously (23, 33, 43) and electroporated with 0.2 to 1 μg of DNA at 15 kV/cm in a Gene Pulser II (Bio-Rad) with 125 μF of capacity, 200 Ω of resistance, and a 4.8×10^{-3}-s pulse. Chemical transformation was done with *E. coli* M15[pREP4] according to the supplier's instructions (QIAexpress Kit Type ATG; Qiagen).

Three recombinant organisms were generated. First, *C. testosteroni* 4A10 (Table 1) was generated from *C. testosteroni* T-2 by Tn*5* transposon mutagenesis; the insertion was in *tsaR*. Second, *C. testosteroni* TT6 was generated from mutant 4A10 (Table 2); it contained wild-type *tsaR* in *trans*. The *tsaR* gene from strain T-2 was amplified with primers CT1 and CT2 (Table 2) and modified by nested PCR to encode a C-terminal, sixfold His tag (*tsaR*^{his}). For that purpose, the reverse primer, tsaR^{His}HindIII, encoded a C-terminal His tag upstream of a stop codon and a restriction site for *Hin*dIII. A restriction site for *Afl*III was generated in the forward primer, tsaRAflIII, by replacing the original start codon, GTG, with ATG plus a subsequent TTG (leucine) codon. The construct generated by PCR was cloned into the broad-host-range vector pJB866 under the control of a promoter inducible by 2 mM *m*-toluate. This plasmid was electroporated into mutant 4A10 to yield mutant TT6 (Table 1). Third, *E. coli* M10 was constructed from *E. coli* M15[pREP4] to express TsaR^{His}. The *tsaR* gene from strain T-2 was ligated into pQE-70, which encodes a His tag. The insert was generated from the PCR-amplified template (primers CT1 and CT2; see above) and modified by nested PCR. The primer pair was tsaRSphI, in which the original start codon was replaced with ATG and a subsequent CTC (leucine) codon to generate a restriction site for *Sph*I, and tsaRBglII, in which the original stop codon was replaced with TCT and AGA (serine and arginine) to fuse TsaR to the C-

TABLE 1. *C. testosteroni* T-2 and some mutants and the substrates they utilize

Organism	Property(ies)	Substrate utilized as carbon and energy source			
		TSA	TCA	PSB	TER
T-2	Parent strain	Yes	Yes	Yes	Yes
4A10	Lacking *tsaR*	No	No	Poor[a]	Yes
TT6	Lacking *tsaR*; *tsaR*^{his} in *trans*	Yes[b]	Yes[b]	Yes[b]	Yes

[a] Slow growth to about 30% of the yield expected from the parent strain.
[b] Utilization of these substrates requires induction of cloned *tsaR*^{his} with 2 mM *m*-toluate.

TABLE 2. PCR primers used in this study

Primer	Sequence[a]
For cloning, detection, and sequencing	
TsaReg1	5′-GCG GGG TAC AGC CAT TCT TG-3′
TsaReg2	5′-CGG TGA CGT CGG GGA ACT C-3′
TsaReg4	5′-GGC GCT GGG AGG GGC ACA TCA-3′
TsaRu1	5′-CTG CAG CAG GGC AAT GAC CAT-3′
AntiRu1	5′-TCG GGC ACC TGG CTC AAG ATT T-3′
TsaIG1	5′-ACT TCC TCA ATG CAG ATG AGT-3′
Tn55-Out	5′-ATC CCC GGG AAT TCG GCC TAG GT-3′
CT1	5′-CCA GCG CTC AAA GGT GTG GG-3′
CT2	5′-AGG TCT TGG GCG GAA TCT GTT CCT-3′
tsaRAfiII	5′-GGA CAA CA***T GTT G***AA GCT CCA GAC GCT GCA GGC ACT C-3′
tsaR^{His}HindIII	5′-GTA CAA GCT T**TC A***GT GAT GGT GAT GGT GAT G*GC CCG TCT GCA GGG CGT GGT GC-3′
866-MB1	5′-ACT TCT TCG CCC CCG TTT TCA CCA T-3′
866-21	5′-CTC ACA GCC AAA CTA TCA GGT CAA G-3′
866-MB2	5′-TCT TGC CCG CCT GAT GAA TGC-3′
866-22	5′-AGG CTG GCT TTT TCT TGT TAT CG-3′
tsaRSphI	5′-AAC CGG GAC A***GC ATG C***TC AAG CTC CAG ACG CTG CAG GCA C-3′
tsaRBgIII	5′-CCA GCC CAT G***AG ATC T***GC CCG TCT GCA GGG CGT GG-3′
pQE70-1	5′-GCT TTG TGA GCG GAT AAC AAT TAT-3′
pQE70-2	5′-GGC GGC AAC CGA GCG TTC TG-3′
For template amplification	
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′
Prom1	5′-CGA CAG GGC GGG TTG CGA GAG GTG-3′
Prom2	5′-CGA GCG GTG GCA GCA GCG GTT TTC-3′
Pro21	5′-TAA TCG GTT TTT CCA ATC ACT GAA CCG-3′
Pro12	5′-GGA AAA ACC GAT TAT ATC GCC G-3′
Pro23	5′-GTG AAG CTC CAG ACG CTG CAG G-3′
Pro13	5′-CTT CCT CAA TGC AGA TGA GTG-3′
Pro22	5′-GTC TCC TTG TTG GGG TTT TGG G-3′
Pro14	5′-GTT CAT CCG CAA TTG CTG GTA CG-3′

[a] Altered and inserted codons within the *tsaR* gene are in italics, start and stop codons are in bold letters, and restriction sites are underlined.

terminal His tag of pQE-70 and create a *Bgl*II restriction site. This plasmid was introduced into *E. coli* M15 by chemical transformation.

Preparation of TsaR^{His}, Western blotting, and determination of molecular mass under native and denaturing conditions. Suspensions of chilled *E. coli* M10 were disrupted by sonication (3 bursts for 1 s at 70 W, on ice), and TsaR was purified under native conditions as described elsewhere (QIAexpress kit type ATG; Qiagen) and stored in 20% glycerol at −20°C. Immunological detection of purified TsaR^{His} after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by Western blotting following standard protocols (32), which were modified as necessary according to the antibody's manufacturer. Monoclonal antibodies (QIAexpress detection kit and Tetra-His antibody) against a fourfold His epitope were provided by Qiagen; anti-mouse immunoglobulin coupled to alkaline phosphatase (Roche) was used as secondary antibody, and detection was done with a disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan)-4-yl)phenyl phosphate system (CSPD; Roche). The molecular mass of TsaR^{His} was estimated by gel filtration chromatography on a Superose 12 column calibrated with chymotrypsin, ovalbumin, bovine serum albumin, and aldolase (molecular mass values of 25, 43, 67, and 158 kDa, respectively). The mobile phase was 10 mM Tris, pH 7.0, containing 150 mM NaCl; the loading buffer was the elution buffer of the QIAexpress kit.

Denatured proteins were separated on SDS-PAGE (34) and stained with either standard (33) or colloidal (27) Coomassie brilliant blue. The protein markers used were a low-range marker (6.5, 14.4, 21.5, 31, 45, 66.2, and 97.4 kDa; Bio-Rad) and a 10-kDa protein ladder (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, and 200 kDa; Gibco). Soluble protein was quantified according to Bradford (4).

DNA-binding assays (20-μl volumes). Binding of TsaR^{His} to DNA was assayed by incubation of freshly prepared TsaR^{His} with 100 ng of PCR-amplified target DNA in binding buffer (5 mM Tris/HCl, 20 mM NaCl, 8 mM $MgCl_2$, 10% glycerol, 1.5 M fresh 2-mercaptoethanol, 30 μg of freshly added bovine serum albumin/ml, pH 8.0) for 40 min on ice. Binding assays of TsaR^{His} were done with 2 μg of protein, and assays to determine K_D, the dissociation constant, were done with 0.3 to 1.5 μg of protein. K_D was defined as the protein concentration at which 50% of the target DNA molecules were occupied, as assayed by band shift. The influence of TSA (0.01 to 6 mM) on the binding of TsaR^{His} to DNA was measured at constant protein concentration (30.6 μg/ml). Samples for analysis were supplemented (10% [vol/vol]) with a solution of 15% Ficoll containing 0.25% bromophenol blue and subjected to standard agarose gel electrophoresis (33) in 1.5% agarose at 14.7 V/cm. Staining with ethidium bromide and destaining (33) were done after electrophoresis to exclude intercalation into the DNA during separation. Stained gels were documented (Gel Doc 1000; Bio-Rad) and subject to densitometric analysis (Multi-Analyst software for Macintosh, version 1.02; Bio-Rad Laboratories, Hercules, Calif.).

Nucleotide sequence accession number. The putative promoter region sequence and partial gene sequence of *pszA* are available in the National Center for Biotechnology Information GenBank library under accession number AY044257.

RESULTS

Transposon mutagenesis and complementation confirm TsaR as a regulator. Junker et al. (13) suggested that TsaR is a LysR-type regulator of the *tsa* operon in *C. testosteroni* T-2 (Fig. 1). Transposon mutagenesis of *tsaR* (see below) yielded mutant 4A10 (Table 1), which was phenotypically *tsa* and *tca* negative but was genotypically *tsa* positive and tetracycline resistant (the resistance marker on the Tn*5* minitransposon). The transposon was located by PCR and cycle sequencing. It was inserted between two cytosines at positions 563 and 564 on the *tsa* locus (U32622) in reverse transcriptional orientation to the *tsaR* gene, thus truncating 33 C-terminal amino acids of TsaR. pTSA was separated by pulsed-field gel electrophoresis,

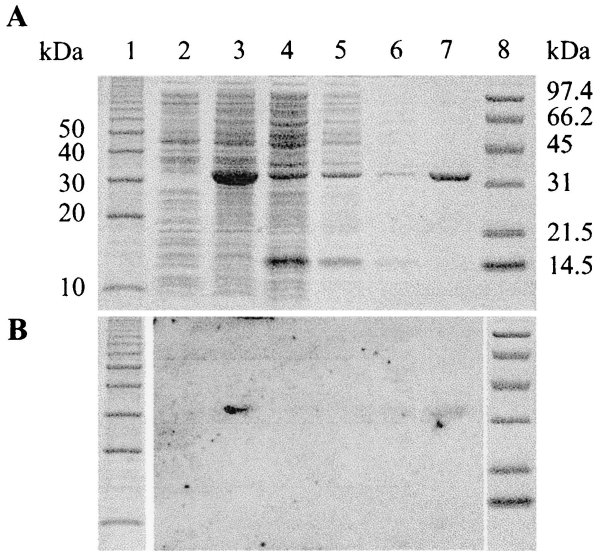

FIG. 2. Expression and purification of $TsaR^{His}$ from *E. coli* strain M10 monitored by SDS-PAGE (A) and Western blot analysis (B). *E. coli* M10 was grown in the absence and presence of IPTG, the inducer of expression of $tsaR^{his}$. Soluble proteins from disrupted cells were compared; $TsaR^{His}$ from induced cells was purified, and the His tag was identified immunologically. Lanes 1 and 8, calibration proteins with the molecular mass values given; lane 2, extract of noninduced cells; lane 3, extract of induced cells; lane 4, supernatant fluid after addition of nickel-agarose; lane 5, supernatant fluid from wash 1 of the purification protocol; lane 6, supernatant fluid from wash 2 of the purification protocol; lane 7, purified $TsaR^{His}$.

and Southern hybridization against the Tn*5* transposon confirmed the prediction that the latter was inserted in the plasmid (data not shown).

Mutant 4A10 grew with neither TSA nor TCA, and growth with PSB was poor (Table 1). In contrast, construct TT6 (Table 1) showed utilization of TSA, TCA, or PSB in the presence of 2 mM *m*-toluate to induce $TsaR^{His}$; substrate utilization was determined by high-performance liquid chromatography. Inducible expression of $TsaR^{His}$ in strain TT6 was confirmed by SDS-PAGE. Using antibodies against the His tag, separated proteins from crude extracts of cells grown in the presence or absence of *m*-toluate were subjected to Western blotting; only induced cells showed cross-reactivity, with a single band at about 33 kDa (Fig. 2B and data not shown). This proved that TsaR is a gene product which is essential for expression of *tsaMBCD* in *C. testosteroni* T-2. It also showed that the function of TsaR is not significantly affected by the C-terminal His tag.

Overexpression in and purification of $TsaR^{His}$ from *E. coli* M10 and specific binding of $TsaR^{His}$ to the *tsa* promoter region. No His-tagged protein was detected in noninduced cells of *E. coli* M10 (Fig. 2B, lane 2). Cells grown in the presence of 1 mM IPTG expressed a 33-kDa polypeptide (Fig. 2A, lane 3), which represented some 6% of soluble protein and carried a His tag (Fig. 2B, lane 3). This molecular mass value corresponded to that of the wild-type TsaR, 32.7 kDa (13), with a His tag of 0.7 kDa, as predicted by the DNA sequence encoding the recombinant protein. This protein was purified (Fig. 2, lane 7). The apparent molecular mass was 30 kDa (gel filtration), which implied that $TsaR^{His}$ is a monomer in solution.

Preliminary binding studies showed that $TsaR^{His}$ rapidly lost its ability to bind DNA within 5 h of cell disruption, unless it was stored under an atmosphere of nitrogen. Under the latter conditions, binding capacity was retained for at least 1 month. Samples with low levels of binding capacity were able to be restored to full capacity by incubation under nitrogen. Reproducible binding of $TsaR^{His}$ to its target DNA in vitro required the presence of high concentrations of 2-mercaptoethanol (1.5 M instead of the millimolar concentrations which are often used; for an example, see reference 25). Binding assays had to be done quickly, because even under optimal conditions, all activity was lost after 20 min. We presume that TsaR is sensitive to oxygen.

This rapid loss of binding capacity, and the requirement for 1.5 M mercaptoethanol, ruled out the use of standard footprint assays. We chose to avoid radiochemical techniques, so standard band shift assays of DNA binding (1) were not applicable and other approaches were developed.

DNA-binding assays with $TsaR^{His}$ and with specific fragments of DNA from promoter and coding regions were done in parallel, which compensated for the addition of competitor DNA normally used in radioactive DNA-binding assays to ensure sequence-specific binding of the protein. $TsaR^{His}$ did not bind significantly to a coding region of the *tsa* operon ($tsaM^{cod}$) (Table 3 and Fig. 3A); the mobility of the DNA fragments was unaltered by the presence of $TsaR^{His}$. The protein did cause a band shift when the putative promoter region was examined (tsa^{prom}) (Table 3 and Fig. 3), and the DNA fragment was shifted completely by addition of 1.5 μM $TsaR^{His}$ (tsa^{prom}; Fig. 3B). We were concerned that $TsaR^{His}$ might interact with a putative promoter region(s), so we explored the interaction with the appropriate region of the locus encoding PSB dioxygenase (Fig. 1) ($pszA^{prom}$; Table 3). $TsaR^{His}$ had no interaction with that fragment of DNA ($pszA^{prom}$; Fig. 3B). TsaR is thus a DNA-binding protein

TABLE 3. PCR products used for DNA-binding assays

Primer pair	Fragment (size in bp)	Location in U32622 (13) (if appropriate)
tsaM-C, tsaB-N	$tsaM^{cod}$ (386)	Coding region of *tsaMB* (13), position 2397–2782
Psz-Prom1, Psz-Prom2	$pszA^{prom}$ (320)	Promoter of *pszA*[a] (GenBank accession number AY044257)
Prom1, Prom2	tsa^{prom} (368)	Complete promoter region of *tsaR* and *tsaMBCD* and parts of *tsaR* and *tsaM*, position 1264–1632
Prom1, Pro21	Subfragment 1 (135)	First part of tsa^{prom}, position 1264–1399
Pro12, Prom2	Subfragment 2 (246)	Second part of tsa^{prom}, position 1386–1632
Prom1, Pro23	Subfragment 3 (98)	First 98 bp of *tsaR*, position 1264–1362
Pro13, Pro22	Subfragment 4 (151)	First 43 bp of *tsaR* and most of the *tsaR*/*tsaMBCD* promoter region, position 1320–1471
Pro14, Prom2	Subfragment 5 (153)	First 154 bp of *tsaM*, excluding A and T of the ATG start codon, position 1479–1632

[a] PSB dioxygenase (Fig. 1) (Ruff, unpublished).

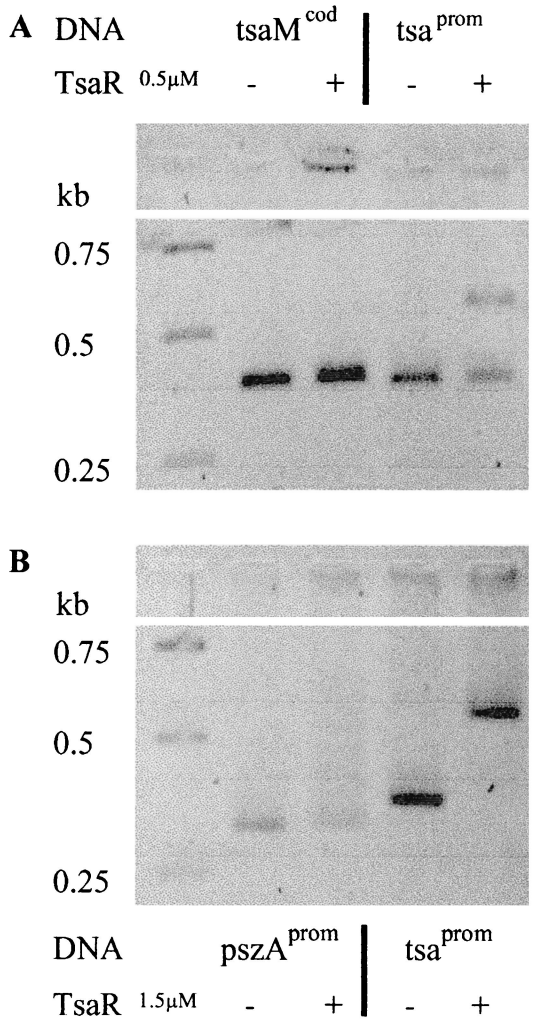

FIG. 3. Band shift assays with $TsaR^{His}$ and specific fragments of DNA from *C. testosteroni* T-2. (A) $tsaM^{cod}$, coding region of *tsaMB* (Table 3) used as a negative control for nonspecific DNA binding; (B) tsa^{prom}, promoter region between *tsaR* and *tsaM* (Table 3); $pszA^{prom}$, promoter of *pszA* (Table 3) used as a control for promoter specificity of DNA binding of $TsaR^{His}$.

specific for the *tsa* promoter region. The dissociation constant (K_D [Materials and Methods]) of $TsaR^{His}$ for the *tsa* promoter region (tsa^{prom}; Table 3) was found to be 0.9 μM.

Binding of TsaR to tsa^{prom} depends on the ligand (TSA) concentration. Changes in the affinity of binding (Fig. 4) of $TsaR^{His}$ to *tsa* promoter DNA (tsa^{prom}; Table 3) as a function of the substrate (TSA) concentration were observed and quantified as the amount of DNA subject to a band shift at various concentrations of TSA, the putative inducer of the *tsa* operon (13, 20). At low concentrations of TSA (0.01 mM), about 80% of the promoter DNA was shifted. When TSA concentrations were intermediate (0.1 to 1 mM), the amount of DNA shifted was lower (about 50%), which indicates a lower affinity of TsaR to its DNA-binding site(s). At higher concentrations of TSA (above 3 mM), more promoter DNA (about 80%) was shifted. Binding of TsaR to the *tsa* promoter region thus seems to depend on the substrate concentration, which suggests the presence of a regulatory system sensitive to levels of the growth substrate.

Location of several binding regions for $TsaR^{His}$ within the *tsa* promoter region. The putative *tsa* promoter region (positions 1363 to 1476 in U32622) is presumed to regulate expression of the divergently oriented *tsaR* and *tsaMBCD* genes (13). Newer software (30) allowed us to suggest transcriptional start sites for *tsaR* and *tsaMBCD* at positions 1389 and 1453, respectively (Fig. 5). We have now been able to explore the interaction of $TsaR^{His}$ with five defined, overlapping DNA subfragments in this region (tsa^{prom}) (Fig. 5 and Tables 3 and 4). In the absence of TSA, $TsaR^{His}$ bound strongly to only one fragment (subfragment 4; Table 4). In the presence of 6 mM TSA, about a quarter of subfragment 1 was shifted (Table 4), whereas two shifts were visible with subfragment 2. Subfragments 3 and 5 gave no significant shift, whereas subfragment 4 gave two strong shifts. We conclude from Table 4 that there are two experimentally confirmed, TSA-dependent binding sites for $TsaR^{His}$ (BS I on subfragment 1 and BS II on subfragment 2) (Fig. 5), whereas a third binding site (BS III, at the 5′ end of *tsaM*), which was derived from the consensus sequence (35) and may contribute to the double shift of subfragment 4, has negligible direct experimental support (subfragment 5; Table 4). In the absence of TSA, in contrast, there is only one binding site for $TsaR^{His}$ (BS IV; Fig. 5).

DISCUSSION

The hypothesis that TsaR regulates expression of the *tsa* operon (13) (Fig. 1) has been confirmed. Mutant 4A10 (Table 1) was generated by insertion of the Tn*5* minitransposon in the *tsaR* gene, and the effect of this specific knockout (Table 1) was complemented by providing an inducible copy of *tsaR* in *trans* (mutant TT6; Table 1). Further, $TsaR^{His}$ interacts specifically with the divergent *tsa* promoter region (Fig. 3) and its affinity is modulated by the putative inducer (Fig. 4) as required of a regulatory protein.

Knockout mutant 4A10 failed to grow with both TSA and TCA (Fig. 1), and utilization of both substrates was restored in mutant TT6 (Table 1). We thus confirmed the prediction that

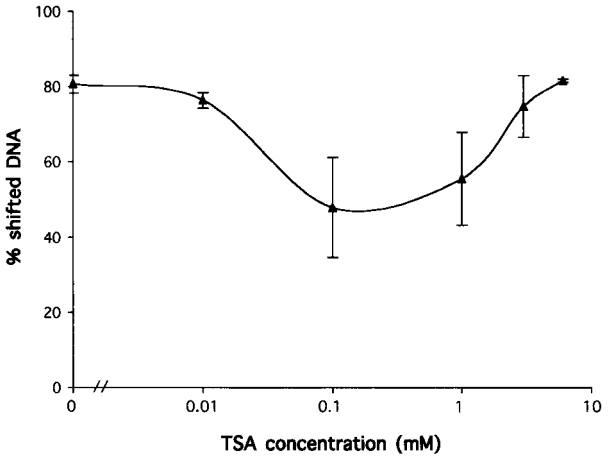

FIG. 4. The effect of TSA concentration on the band shift of tsa^{prom} caused by $TsaR^{His}$.

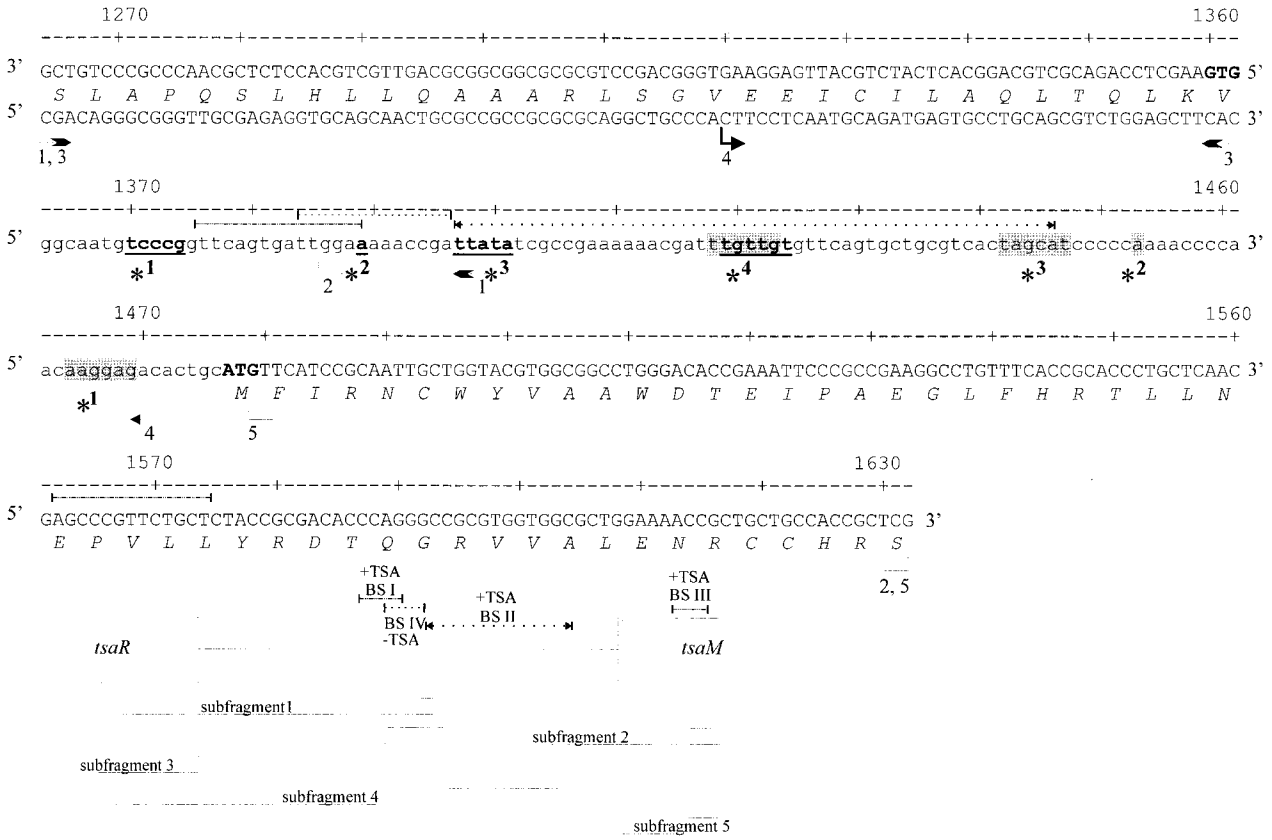

FIG. 5. Sequence and structure near the *tsa* promoter region. The nucleotide positions are those of U32622 (13). Data from genes *tsaR* (both strands) and *tsaM* are in uppercase characters, with the start codons in bold characters; amino acids are in uppercase italic characters. The putative ribosomal binding site ($*^1$), transcriptional start ($*^2$), Pribnow box ($*^3$), and −35 ($*^4$) regions are in bold characters and are underlined for *tsaR* and shaded grey for *tsaM*. Subfragments 1 to 5 (Table 3) are indicated with pairs of arrows. The dashed line indicates putative DNA-binding sites for $TsaR^{His}$ in the presence of TSA (I and III), and the dotted line indicates the putative binding site for $TsaR^{His}$ in absence of TSA (IV); the binding region II is also shown with a dotted line with terminal arrows. An overview depicting subfragments 1 to 5 with the binding sites and the requirement of TSA is sketched below the sequence.

TsaR regulates the degradation of both compounds (13, 36); the gene products from *tsaMBCD* are used in the initial attacks on each substrate (Fig. 1) (21). Tralau et al. (unpublished data) showed that mutants in TSA transport prevent neither growth with TCA, which has a different transport system (22), nor normal growth with PSB, for which TsaB is required to complement the missing PszC (9, 14; J. Ruff, unpublished data) (Fig. 1). We thus conclude that *tsaMBCD* is directly affected by the knocking out of *tsaR* and that failure to grow is not a result of, e.g., positional effects.

Mutant 4A10 grew poorly with PSB (Table 1), and the deleterious effect of the mutation was corrected on complementation of TsaR (mutant TT6; Table 1). The surprise is that mutant 4A10 grew at all with PSB, given that TsaB is required to complement the missing PszC. We presume that an unidentified reductase is able to complement PszC partially.

Gel filtration chromatography indicated that active TsaR is a monomer in solution, though le Maire et al. (18) warn of errors sometimes encountered with this method. TsaR as the active monomer differs from several LysR-type regulators. One example of a monomeric regulator exists, AmpR (31), whereas ClcR is a dimer (6) and CysB and OxyR are tetramers (16, 44).

TsaR is sensitive to oxygen. Thus, gel filtration chromatography could not be done with $TsaR^{His}$ bound to DNA. So it was impossible to determine whether the dissolved monomeric $TsaR^{His}$ binds to its target sequence in a multimeric form, as LysR-type regulators usually do. We examined band shifts in agarose gels instead of polyacrylamide gels for the same reason, because separation is faster, thus allowing us to assay the interaction with DNA before TsaR lost its binding capacity.

TABLE 4. Band shifts with $TsaR^{His}$ and subfragments of the *tsaR*/*tsaMBCD* regulon[a]

Conditions	Shifts(s) with DNA subfragment[b] no.:				
	1	2	3	4	5
No TSA	−	−	−	+ (97)[c]	−
6 mM TSA	+ (26)[d]	+ (31&11)[e]	−	+ (53&47)[f]	− (<5)[g]

[a] +, band shift on incubation of $TsaR^{His}$; −, no band shift. The values in parentheses are the percentages of shifted DNA in one or two bands; the first number in each number pair is for the smaller shift.
[b] See Table 3 and Fig. 5 for details of the subfragments examined.
[c] Binding site IV.
[d] Binding site I.
[e] Binding sites II and III.
[f] Binding sites I and II.
[g] Binding site III.

It is unclear what interaction causes this oxygen sensitivity. Redox sensitivity has been described previously for the DNA-binding regulator FurS (ferric iron uptake) in *Streptomyces reticuli*, for which the effect is attributed to the redox state of cysteine residues (28). There was no sequence similarity between TsaR and FurS, so we presume that a different interaction occurs between oxygen and TsaR. Furthermore, reactivation of TsaR under nitrogen occurs in the absence of a reducing agent; the biochemical mechanism of this oxygen sensitivity is unknown. Given that TsaR regulates an operon encoding an oxygenase (TsaMB; Fig. 1) (13, 19) and that it seems to be a negative autoregulator (Tralau et al., unpublished), this sensitivity to oxygen may be useful to the cell. TsaR is very stable in the cell (Tralau et al., unpublished). One possible explanation is that TsaR in its active conformation is stable under conditions in which oxygen levels were low. This would reduce the turnover of active TsaR, and the transcription of the *tsa* operon would be repressed efficiently, because all binding sites would be occupied. Alternatively, the stability of TsaR would decrease in the presence of oxygen. This would increase the turnover of TsaR and allow transcription of the *tsa* operon to start more easily. The mechanism might thus help to compensate for the relatively high K_D value (0.9 μM). This value is of the same magnitude as that for CatR, the regulator for catechol degradation at its internal binding site in *catB* in *Pseudomonas putida* (5), whereas the DNA-binding regulator ModE (26) shows higher affinity for its target promoter (24 nM).

The modulation by TSA of the interaction of $TsaR^{His}$ with the whole intergenic tsa^{prom} fragment (Fig. 4) involves regulatory sites for the expression of both *tsaR* and *tsaMBCD*, and we observed single shifts with this fragment only (Fig. 3). When we used shorter fragments of DNA, we obtained evidence for four binding regions and some double shifts, whose nature we do not understand (Table 4). Presumably the resolution of the agarose gels allowed double shifts to be detected with short fragments only. We realize that the multiple binding regions prevent a quantitative evaluation of the data presented in Fig. 4, but they imply that the system can react in a finely tuned manner. We also do not know how the ligands TSA and oxygen interact during the binding of TsaR to its target sites. However, regulatory complexity is already apparent at the physiological level, e.g., in defined mixed cultures utilizing multiple substrates (41).

Up to four binding sites for TsaR can be deduced in the sequence of tsa^{prom} (Table 3 and Fig. 5), three of which may be derived from the consensus sequences (T-N_{11}-A) presented by Schell for LysR-type regulators (35). BS I at positions 1376 to 1389 was proposed previously (13) and now has experimental support (subfragment I; Table 4). The location of BS II was not defined experimentally (Table 4 and Fig. 5), but of two possible binding motifs (35) at positions 1397 to 1409 and positions 1434 to 1446, the latter could bind $TsaR^{His}$ without blocking the putative ribosomal binding site, the transcriptional start, the Pribnow box, or the −35 region for gene *tsaR*. We tentatively located BS III at positions 1562 to 1574 near the 5′ end of *tsaM*; this site, derived from the consensus sequence, may contribute to the double shift with subfragment 2 (Table 4), but this could not be confirmed experimentally (subfragment 5; Table 4). BS IV, at positions 1384 to 1396, has clear experimental support in the absence of TSA (subfragment 4; Table 4).

We propose that in the absence of TSA, TsaR interacts at BS IV, which blocks the transcription of *tsaR*. In the presence of TSA, the regulator would shift to BS II (presumably position 1434 to 1446), thereby releasing the repression of transcription for *tsaR* and activating transcription of *tsaMBCD*. The existence of two additional binding sites is uncommon for LysR-type regulatory systems (35). The marginal activity of BS III resembles a situation seen with the CatR system in the *catBCA* operon of *P. putida*, which possibly represents a mechanism to save energy by reducing enzyme synthesis (5); induced TsaM represents 5% of soluble protein (19). The role of BS I may be involved with synthesis of TsaR, which reaches micromolar concentrations during growth (Tralau et al., unpublished).

ACKNOWLEDGMENTS

We are grateful to M. Kertesz and J. M. Blatny, who kindly made bacteria available to us.

T.T. and J.M. were funded by the Deutsche Forschungsgemeinschaft (through A.M.C. and J.R.), the EU project SUITE (to A.M.C.), the University of Konstanz, and the Fonds der Chemischen Industrie.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
2. **Behret, H., J. Ahlers, S. Ettel, E. Feicht, E. Futterer, I. Mangelsdorf, C. Pohlenz-Michel, H. Roβ, H. Sterzl-Eckert, D. Vogel, L. Weis, and K. Widmann.** 1991. *p*-Toluolsulfonsäure, Beratergremium für umweltrelevante Altstoffe (BUA)-Stoffberichte, vol. 63. Verlag Chemie, Weinheim, Germany.
3. **Blatny, J. M., T. Brautaset, H. C. Winther Larsen, P. Karunakaran, and S. Valla.** 1997. Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in gram-negative bacteria. Plasmid **38:**35–51.
4. **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.
5. **Chugani, S. A., M. R. Parsek, and A. M. Chakrabarty.** 1998. Transcriptional repression mediated by LysR-type regulator CatR bound at multiple binding sites. J. Bacteriol. **180:**2367–2372.
6. **Coco, W. M., M. R. Parsek, and A. M. Chakrabarty.** 1994. Purification of the LysR family regulator, ClcR, and its interaction with the *Pseudomonas putida clcABD* chlorocatechol operon promoter. J. Bacteriol. **176:**5530–5533.
7. **Coco, W. M., R. K. Rothmel, S. Henikoff, and A. M. Chakrabarty.** 1993. Nucleotide sequence and initial functional characterization of the *clcR* gene encoding a LysR family activator of the *clcABD* chlorocatechol operon in *Pseudomonas putida*. J. Bacteriol. **175:**417–427.
8. **Collier, L. S., G. L. Gaines III, and E. L. Neidle.** 1998. Regulation of benzoate degradation in *Acinetobacter* sp. strain ADP1 by BenM, a LysR-type transcriptional activator. J. Bacteriol. **180:**2493–2501.
9. **Cook, A. M., H. Laue, and F. Junker.** 1999. Microbial desulfonation. FEMS Microbiol. Rev. **22:**399–419.
10. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
11. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter sequences. Nucleic Acids Res. **15:**2343–2361.
12. **Junker, F., and A. M. Cook.** 1997. Conjugative plasmids and the degradation of arylsulfonates in *Comamonas testosteroni*. Appl. Environ. Microbiol. **63:**2403–2410.
13. **Junker, F., R. Kiewitz, and A. M. Cook.** 1997. Characterization of the *p*-toluenesulfonate operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2. J. Bacteriol. **179:**919–927.
14. **Junker, F., E. Saller, H. R. Schläfli Oppenberg, P. M. H. Kroneck, T. Leisinger, and A. M. Cook.** 1996. Degradative pathways for *p*-toluenecarboxylate and *p*-toluenesulfonate and their multicomponent oxygenases in *Comamonas testosteroni* strains PSB-4 and T-2. Microbiology (Reading) **142:**2419–2427.
15. **Kennedy, S. I. T., and C. A. Fewson.** 1968. Enzymes of the mandelate pathway in bacterium N.C.I.B. 8250. Biochem. J. **107:**497–506.
16. **Kullik, I., J. Stevens, M. B. Toledano, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J. Bacteriol. **177:**1285–1291.

17. **Laue, H., J. A. Field, and A. M. Cook.** 1996. Bacterial desulfonation of the ethanesulfonate metabolite of the chloroacetanilide herbicide metazachlor. Environ. Sci. Technol. **30:**1129–1132.
18. **le Maire, M., A. Ghasi, and J. V. Moller.** 1996. Gel chromatography as an analytical tool for the characterisation of size and molecular mass of proteins. ACS Symp. Ser. **635**.
19. **Locher, H. H., T. Leisinger, and A. M. Cook.** 1991. 4-Toluene sulfonate methyl-monooxygenase from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component. J. Bacteriol. **173:**3741–3748.
20. **Locher, H. H., T. Leisinger, and A. M. Cook.** 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.
21. **Locher, H. H., C. Malli, S. Hooper, T. Vorherr, T. Leisinger, and A. M. Cook.** 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.
22. **Locher, H. H., B. Poolman, A. M. Cook, and W. N. Konings.** 1993. Uptake of 4-toluene sulfonate by *Comamonas testosteroni* T-2. J. Bacteriol. **175:**1075–1080.
23. **Mampel, J.** 2000. Transport- und Regulationsphänomene beim Abbau von 4-Toluolsulfonat in *Comamonas testosteroni*. Ph.D. thesis. University of Konstanz, Konstanz, Germany.
24. **Mampel, J., J. Ruff, F. Junker, and A. M. Cook.** 1999. The oxygenase component of the 2-aminobenzenesulfonate dioxygenase system from *Alcaligenes* sp. strain O-1. Microbiology (Reading) **145:**3255–3264.
25. **Martínez-Costa, O. H., A. J. Martín-Triana, E. Martínez, M. A. Fernández-Moreno, and F. Malpartida.** 1999. An additional regulatory gene for actinorhodin production in *Streptomyces lividans* involves a LysR-type transcriptional regulator. J. Bacteriol. **181:**4353–4364.
26. **McNicholas, P. M., S. A. Rech, and R. P. Gunsalus.** 1997. Characterization of the ModE DNA-binding sites in the control regions of *modABCD* and *moaABCDE* of *Escherichia coli*. Mol. Microbiol. **23:**515–524.
27. **Neuhoff, V., N. Arold, D. Taube, and W. Ehrhardt.** 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.
28. **Ortiz de Orué Lucana, D., and H. Schrempf.** 2000. The DNA-binding characteristics of the *Streptomyces reticuli* regulator FurS depend on the redox state of its cysteine residues. Mol. Gen. Genet. **264:**341–353.
29. **Providenti, M. A., J. Mampel, S. MacSween, A. M. Cook, and C. R. Wyndham.** 2001. *Comamonas testosteroni* BR6020 possesses a single genetic locus for extradiol cleavage of protocatechuate. Microbiology (Reading) **147:**2157–2167.
30. **Reese, M. G.** 1998. NNPP-neural network promoter prediction. Baylor College of Medicine Human Genome Sequencing Center, Houston, Tex.
31. **Roh, I. K., I. J. Kim, J. H. Chung, and S. M. Byun.** 1996. Affinity purification and binding characteristics of *Citrobacter freundii* AmpR, the transcriptional regulator of the *ampC* β-lactamase gene. Biotechnol. Appl. Biochem. **23:** 149–154.
32. **Rosenberg, I. M.** 1996. Protein analysis and purification. Birkhäuser, Boston, Mass.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Schägger, H., and G. von Jagow.** 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.
35. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
36. **Schläfli Oppenberg, H. R., G. Chen, T. Leisinger, and A. M. Cook.** 1995. Regulation of the degradative pathways from 4-toluenesulphonate and 4-toluenecarboxylate to protocatechuate in *Comamonas testosteroni* T-2. Microbiology (Reading) **141:**1891–1899.
37. **Schwacha, A., and R. A. Bender.** 1993. The product of the *Klebsiella aerogenes nac* (nitrogen assimilation control) gene is sufficient for activation of the *hut* operons and repression of the *gdh* operon. J. Bacteriol. **175:**2116–2124.
38. **Smith, C. L., S. R. Klco, and C. R. Cantor.** 1988. Pulsed-field gel electrophoresis and the technology of large DNA molecules, p. 40–72. *In* K. E. Davies (ed.), Genome analysis: a practical approach. IRL Press, Oxford, United Kingdom.
39. **Tabatabai, M. A.** 1974. Determination of sulfate in water sample. Sulphur Inst. J. **10:**11–13.
40. **Thurnheer, T., T. Köhler, A. M. Cook, and T. Leisinger.** 1986. Orthanilic acid and analogues as carbon sources for bacteria: growth physiology and enzymic desulphonation. J. Gen. Microbiol. **132:**1215–1220.
41. **Tien, A. J.** 1996. The physiology of a defined four-membered mixed bacterial culture during continuous cultivation with mixtures of three pollutants in synthetic sewage. Ph.D. thesis. Swiss Federal Institute of Technology, Zürich, Switzerland.
42. **Tralau, T., A. M. Cook, and J. Ruff.** 2001. Map of the IncP1β plasmid pTSA encoding the genes (*tsa*) for *p*-toluenesulfonate degradation in *Comamonas testosteroni* T-2 and conservation of these genes in different genetic backgrounds. Appl. Environ. Microbiol. **67:**1508–1516.
43. **Trevors, J. T., and M. E. Starodub.** 1990. Electroporation of pKK1 silver-resistance plasmid from *Pseudomonas stutzeri* AG259 into *Pseudomonas putida* CYM318. Curr. Microbiol. **21:**103–107.
44. **Tyrrell, R., K. H. G. Verschueren, E. J. Dodson, G. N. Murshudov, C. Addy, and A. J. Wilkinson.** 1997. The structure of the cofactor-binding fragment of the LysR family member, CysB: a familiar fold with a surprising subunit arrangement. Structure **5:**1017–1032.