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TeiR, a LuxR-Type Transcription Factor Required for Testosterone Degradation in *Comamonas testosteroni*

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We have identified a new steroid-inducible gene (designated *teiR* [testosterone-inducible regulator]) in *Comamonas testosteroni* that is required for testosterone degradation. Nucleotide sequence analysis of *teiR* predicts a 391-amino-acid protein which shows homology between residues 327 and 380 (C-terminal domain) to the LuxR helix-turn-helix DNA binding domain and between residues 192 and 227 to the PAS sensor domain. This domain distribution resembles that described for TraR, a specific transcriptional regulator involved in quorum sensing in *Agrobacterium tumefaciens*. Analysis of the gene expression indicated that *teiR* is tightly controlled at the transcriptional level by the presence of testosterone in the culture medium. A *teiR*-disrupted mutant strain was completely unable to use testosterone as the sole carbon and energy source. In addition, the expression of several steroid-inducible genes was abolished in this mutant. Northern blot assays revealed that *teiR* is required for full expression of *sip48*–β-HSD gene mRNA (encoding a steroid-inducible protein of 48 kDa and 3β-17β-hydroxysteroid dehydrogenase) and also of other steroid degradation genes, including those encoding 3α-hydroxysteroid dehydrogenase, Δ^5–3-ketoisomerase, 3-oxo-steroid Δ^1-dehydrogenase, and 3-oxo-steroid Δ^4-(5α)-dehydrogenase enzymes. Moreover, when *teiR* was provided to the *teiR*-disrupted strain in *trans*, the transcription level of these genes was restored. These results indicate that TeiR positively regulates the transcription of genes involved in the initial enzymatic steps of steroid degradation in *C. testosteroni*.

Steroids, phenylalkanoic acids, resin acids, and different polycyclic aromatic hydrocarbons represent a group of molecules that are widespread in the environment as breakdown products of lignin or other plant-derived molecules (7, 22, 31). These compounds (known collectively as endocrine disruptors) interfere with the normal endocrine system physiology of vertebrates, particularly in the mechanisms governing reproductive development and function (10), and constitute an important group of bioactive environmental pollutants.

Comamonas testosteroni is a gram-negative bacterium able to use steroids (as well as many other aromatic compounds) as a sole carbon source; it is an attractive model for the study of the mechanisms involved in the mineralization of these bioactive compounds for their removal from the environment (3, 5, 6, 11, 19, 26, 30, 37, 38). *C. testosteroni* metabolizes certain steroids through a complex metabolic pathway involving many steps catalyzed by steroid-inducible enzymes (11, 26, 38, 42). Interestingly, recent works revealed that testosterone simultaneously induced both steroid- and PAH-metabolizing enzymes in this bacterium (29, 36). For this reason, the study of the mechanisms regulating the steroid-inducible gene transcription is concerned with understanding both catabolic pathways.

While the genes encoding some of the enzymes catalyzing the oxidoreduction at different positions of the steroid nucleus and the ring opening of the steroid molecule have been identified (1, 2, 8, 9, 12, 16, 18, 20, 21, 25, 29, 32, 36, 44, 45), only limited information is available about the mechanisms governing steroid-inducible gene expression. The following items have been reported to date. Horinouchi et al. (20) suggest that an intermediate compound produced in the course of testosterone degradation induces expression of *tesB*, encoding a *meta*-cleavage steroid-inducible enzyme. The induction of 3α-hydroxysteroid dehydrogenase–carbonyl reductase (α-HSD), in contrast, appears to represent a derepression in which the steroidal inducer prevents the binding of two repressor proteins (RepA and RepB) to the α-HSD gene promoter and mRNA, respectively (44, 45).

For this study we report the identification and characterization of the *teiR* gene, which is essential for testosterone degradation in *C. testosteroni*. *teiR* encodes a LuxR-type transcription factor required for the expression of several steroid-inducible genes, suggesting that a quorum-sensing mechanism is involved in its regulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium (34). *C. testosteroni* was grown at 30°C in LB medium or in M9 minimal medium (34) plus acetate (0.2% [wt/vol]) or testosterone (0.25 mg ml^{-1}) or both, as indicated in the text. Overnight cultures were diluted 1/100 in fresh medium and incubated for 2 h in LB medium or for 12 h in M9 medium, and cells were washed, diluted 1/50 in fresh medium, and incubated as indicated for each experiment.

Growth of *C. testosteroni* was monitored by measuring optical density at 600 nm (OD_{600}). Alternatively, growth in M9 medium plus testosterone was monitored by counting colonies that appeared on LB plates (on which appropriately diluted cultures have been spread) after incubation at 30°C. When needed, antibiotics were added at the following concentrations (in micrograms per mil-

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TABLE 1. Bacterial strains, plasmids, and cosmids used

Strains, plasmids, and cosmids	Relevant genotype	Source or reference
Strains		
Escherichia coli DH5α	F$^-$ *recA1 endA1 gyr96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ*lacZYA-argF U169* ϕ80*dlacZ*ΔM15; host strain for DNA manipulation	Stratagene
C. testosteroni		
Wild type		ATCC 11996
UT2.5	Cm^r; *C. testosteroni* bearing a chromosomal insertion of miniTn*5* Cm/*sip48*–β-HSD::*lacZ*	This study
UT2.5teiR-3′URΩ	Gm^r, Sp^r; mutant of *C. testosteroni* ATCC 11996 carrying *Sma*I 2-kb cassette from pHP45Ω inserted in *teiR* 3′ untranslated region	This study
UT2.5Mut50	Cm^r, Tc^r; *C. testosteroni* bearing chromosomal insertions of mini-Tn*5* Cm/*sip48*-β-HSD::*lacZ* and mini-*Tn*5 Tc	This study
Plasmids and cosmids		
pSL9	pGEM3 containing 3.2-kb *Hin*dIII fragment of β-HSD gene and contiguous genes; Ap^r	18
pRK2013	Km^r, Mob$^+$, Tra$^+$; donor of transfer functions	15
pBIISK	Ap^r; multipurpose cloning vector	
pGEMT easy	Ap^r; PCR fragment cloning vector	Promega
pUTmini-Tn5 Tc	Ap^r, Tc^r; *R6KoriV, RP4oriT,* mini-Tn*5* Tc transposon vector delivery plasmid	13
pBBR1MCS2	Km^r; broad-host-range plasmid	24
pVK102	Tc^r, Km^r, RP4[32] (IncP-1)	23
pHP45:Ω	Ap^r, Sm^r,[a] Sp^r	33
pVK102*teiR*	Tc^r, containing *Hin*dIII partially digested fragment DNA of *C. testosteroni*	This study
pAK1370	pUC19 containing-1,370-bp *Pst*I fragment encoding the C-terminal sequences of α-HSD and Δ^5-KSI genes	25
pTEK21	pUC19 containing 2.2 *Kpn*I fragment encoding Δ^1-DH and the N end of Δ^4-DH genes	32
pUT 2.5	Ap^r, Cm^r; *Hin*dIII-*Eco*V (from pSL9)-*lacZ* transcriptional fusion inserted as a *Not*I fragment in pUTminiTn5	This study
pG*teiR*	pGEMT easy containing 1.2-kb *teiR* DNA fragment from PCR	This study
pB*teiR*-3′URΩ	pBIISKMut50 containing Ω cassette insertion into the *Nde*I site located in *teiR* 3′ untranslated region	This study
pBIISKMut50	Ap^r; pBIISK containing 2.5-kb *Not*I fragment from genomic DNA of *C. testosteroni* UT2.5Mut50	This study
pBB*teiR*	Ap^r, Km^r; pBBR1MCS2 containing 1.2-kb *teiR* gene insertion into the *Eco*RI site	This study
pBB20H	Ap^r, Km^r; pBBR1MCS2 containing 20-kb *Hin*dIII fragment from pVK102 cosmid library of *C. testosteroni*	This study

[a] Sm^r, streptomycin resistance.

liliter): ampicillin (Ap), 100; chloramphenicol (Cm), 20; gentamicin (Gm), 10; kanamycin (Km), 20; spectinomycin (Sp), 600; and tetracycline (Tc), 10.

DNA manipulations and sequence determinations. Standard protocols or manufacturers' instructions were followed for DNA isolation and recombinant DNA procedures (34). DNA sequencing was performed on double-stranded templates derived (using the dideoxy chain termination method) (35) from pBIISKMut50 and pBB20H. For *Taq*DNA polymerase-initiated cycle sequencing reactions with fluorescently labeled dideoxynucleotide terminators (Applied Biosystems Inc.), standard protocols of the manufacturer were used. The sequencing reactions were analyzed using a model 377 automated DNA sequencer (Applied Biosystems Inc.). Blast software was used to screen DNA and protein databases for similar proteins (4). Multiple sequence alignments were made with ClustalW software (version 1.7) (40).

Construction of plasmids and allele replacement. Plasmid pBIISKMut50 was constructed by the ligation of *Not*I DNA fragments from *C. testosteroni* UT2.5Mut50 into pBIISK. After transformation into *E. coli* DH5α, Tc-resistant (Tc^r) strains were isolated.

For the construction of pBB20H, a pVK102 *C. testosteroni* cosmid library was screened using a 0.5-kb *Pst*I/*Not*I fragment from pBIISKMut50 as a probe (see Southern blot analysis results) and a cosmid containing a *teiR* gene was isolated (pVK102*teiR*). Finally, a 20-kb *Hin*dIII fragment from pVK102*teiR* (containing *teiR*) was subcloned into the *Hin*dIII site of pBBR1MCS2.

Plasmid pG*teiR* was constructed by PCR amplification of *teiR* coding sequence with the primers *teiR*-Fw (5′-ggaagcttgctagcATGTGCCCATATTTCGACAC-3′) and *teiR*-Rv (5′-cccggggctagcaagcttTCACTTGTTCCCCAGCCA-3′). The amplification product was ligated into the pGEMT easy vector.

Plasmid pB*teiR*-3′URΩ was constructed by insertion of a 2-kb *Sma*I fragment (obtained from pHP45::Ω) (33) into the *Nde*I site located in the *teiR* 3′ untranslated region of pBIISKMut50. The recombinant plasmid was transferred (using the mobilizing plasmid pRK2013) into *C. testosteroni* UT2.5 by triparental mating. Donor, helper, and recipient cells were grown overnight in LB medium. Cell suspensions (0.2 ml each) were mixed, filtered on a 0.4-μm-pore-size nitrocellulose membrane filter, and incubated at 30°C on an LB agar plate for 24 h. Cells were suspended in sterile 1% (wt/vol) NaCl and plated onto LB agar containing Gm (10 μg ml^{-1}) and Sp (600 μg ml^{-1}) to select transconjugants. Southern hybridization was performed to confirm the genomic structure of the mutant strain (*C. testosteroni* UT2.5*teiR*-3′URΩ).

β-Galactosidase assays. The standard procedures described by Miller (27) were used for quantitative measurements of β-galactosidase activity. Samples were collected after 12 h (LB medium) or 17 h (M9 medium) of incubation. The values given throughout this paper represent the averages of the results of three independent experiments, each of which was conducted with duplicate samples.

Southern blot analysis. Genomic DNAs were prepared essentially as described by Sambrook et al. (34). Southern blot analysis was performed as described previously (8). DNA fragments were transferred from agarose gels or from bacterial colonies to nylon membranes after alkali denaturation. A 650-bp *Eco*RV-*Hin*dIII fragment from pSL9 (complementary to the 3′ end of the gene encoding 3β-17β-hydroxysteroid dehydrogenase [β-HSD]) and a 540-bp *Pst*I-*Not*I fragment from pBIISKMut50 (complementary to the *teiR* 3′ untranslated region) were labeled with [α^{32}P]dATP (3,000 Ci mmol^{-1}) by a random priming method (14) and used as probes as indicated in the text.

Testosterone degradation. Testosterone degradation was performed as described previously (18). Briefly, bacterial cells (grown in LB medium plus testosterone during 12 h of culture) were harvested by centrifugation at 4°C. Aliquots of culture supernatants were extracted three times with 5 vol of ethyl ether and submitted (using benzene-ethanol [95:5 {vol/vol}] as a solvent system) to thin-layer chromatography on silica gel GF254 plates. The pattern of testosterone degradation was visualized using 254-nm UV light. Testosterone, 4-androstene-3,17-dione, and 1,4-androstadiene-3,17-dione were used as standards.

RNA isolation and Northern blot analysis. *C. testosteroni* was grown in LB medium or M9 medium plus acetate during the indicated periods of culture growth in the absence or presence of testosterone. Total RNA was extracted as

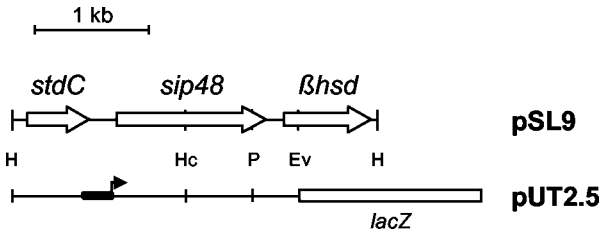

FIG. 1. Restriction map of the 3.2-kb *Hin*dIII fragment cloned into pSL9 plasmid. The regions carrying *stdC* and *sip48* and the β-HSD gene are indicated by boxes. H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; Ev, *Eco*RV. A schematic representation of pUT2.5 carrying the transcriptional fusion of the β-HSD gene upstream region (thick line) to a promoterless *lacZ* gene (open box) is shown. The black box and arrow indicate the *sip48*–β-HSD gene steroid-inducible promoter.

described previously (8). RNA samples (20 μg per lane) were electrophoresed on a 1.2% (wt/vol) agarose gel containing 18% (vol/vol) formaldehyde and transferred to nitrocellulose membranes (8). Equal levels of loading and transfer were assessed by methylene blue staining of membranes. Prehybridization and hybridization reactions were performed as described previously (8). A 600-bp *Hin*cII-*Pst*I restriction fragment from pSL9 (complementary to the *sip48* gene), a 650-bp *Eco*V-*Hin*dIII restriction fragment from pSL9 (complementary to the β-HSD gene), a 1,400-bp *Pst*I restriction fragment from pAK1370 (complementary to the α-HSD and Δ^5–KSI genes), a 2,200-bp *Kpn*I restriction fragment from pTEK21 (complementary to the 3-oxo-steroid Δ^1-dehydrogenase [Δ^1-DH] and 3-oxo-steroid Δ^4-(5α)-dehydrogenase [Δ^4-DH] genes), and a 1,200-bp *Eco*RI restriction fragment from pG*teiR* (complementary to *teiR*) were labeled with [α^{32}P]dATP (3,000 Ci mmol^{-1}) by a random priming method (14) and used as probes as indicated in the text.

Genetic complementation of *teiR* mutant. The complete coding sequence of *teiR* was obtained as a 1.2-kb *Eco*RI fragment from pG*teiR* and then subcloned into the *Eco*RI site of pBBR1MCS2 to generate pBB*teiR*. Plasmids pBBR1MCS2 (negative control) and pBB*teiR* were mobilized (using the mobilizing plasmid pRK2013) from *E. coli* to *C. testosteroni* UT2.5Mut50 by triparental mating. Donor, helper, and recipient cells were grown overnight in LB medium. Cell suspensions (0.2 ml each) were mixed, filtered on a 0.4-μm-pore-size nitrocellulose membrane filter, and incubated on an LB agar plate for 8 h at 30°C. Cells were suspended in sterile 1% NaCl, and transformants were selected on LB agar plates containing Gm (10 μg ml^{-1}) and Km (500 μg ml^{-1}).

Nucleotide sequence accession number. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession number AY363220. The 3.1-kb *Hin*dIII fragment of pSL9 bearing *stdC*, *sip48*, and the β-HSD gene has the following accession number: U41265.

RESULTS

Identification of a gene required for *sip48*–β-HSD gene steroid-inducible expression. Previously, we characterized two steroid-inducible genes encoding a protein of unknown function (Sip48) and β-HSD (which are transcribed as a polycistronic message). We localized the promoter activity responsible for *sip48*–β-HSD gene steroid-inducible transcription in the *sip48* 5′ untranslated region (unpublished data). A *lacZ* transcriptional fusion containing the promoter region inserted into the chromosome of *C. testosteroni* (*C. testosteroni* UT2.5) (Fig. 1) allowed us to measure promoter activity by quantifying the β-galactosidase activity produced by this strain in different experimental conditions. High levels of β-galactosidase activity (3,000 to 4,000 Miller units) were found when *C. testosteroni* UT2.5 was grown in presence of testosterone irrespective of whether LB medium or M9 medium was used (Fig. 2). In addition, this strain showed the same growth rate as wild-type *C. testosteroni* cells in LB medium and M9 minimal medium supplemented with acetate or testosterone (data not shown).

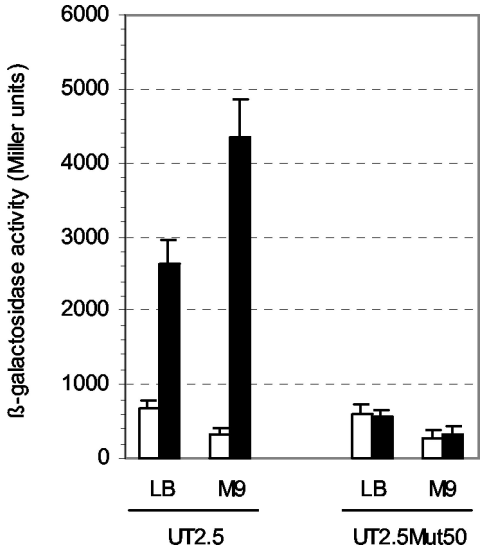

FIG. 2. Activity of *sip48*–β-HSD gene promoter in *C. testosteroni* UT2.5 and UT2.5Mut50 strains. The levels of β-galactosidase activity of a *sip48*–β-HSD gene promoter-*lacZ* transcriptional fusion in *C. testosteroni* UT2.5 and UT2.5Mut50 strains growing in LB medium and M9 minimal medium supplemented with acetate (M9) in the presence (black bars) and the absence (white bars) of testosterone are shown. β-Galactosidase activities were measured with permeabilized cells as described in Materials and Methods. Each value is the average of the results from three independent experiments (error bars indicate standard deviations).

To characterize genes involved in the regulation of *sip48*–β-HSD gene steroid-inducible expression, a mini-Tn*5* insertional mutagenesis procedure was carried out by the transference of a Tc minitransposon element (pUTminiTn5) into *C. testosteroni* UT2.5. A number of mutant strains that exhibited resistance to Tc were isolated. Screening of the resulting transconjugants revealed the presence of white-colored colonies grown on induction medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and testosterone. One of these colonies (designated UT2.5Mut50) was isolated and further characterized. The effect of the minitransposon insertion on *sip48*–β-HSD gene promoter activity in this mutant bacterium was confirmed by comparing the β-galactosidase activity produced by this strain to that produced by *C. testosteroni* UT2.5 when the strains were grown in LB medium and M9 minimal medium supplemented with acetate in the presence and the absence of testosterone (Fig. 2). The results clearly demonstrate that the testosterone-inducible expression of the reporter gene controlled by the *sip48*–β-HSD gene promoter is absent from the *C. testosteroni* UT2.5Mut50 mutant strain. To assess whether the β-galactosidase levels determined for the UT2.5Mut50 strain reflected the transcriptional state of the corresponding *sip48*–β-HSD gene transcript, their mRNA levels were analyzed by Northern blot analysis using *sip48* and β-HSD gene probes as described in Materials and Methods. Strong signals were revealed in the lanes corresponding to mRNAs from *C. testosteroni* wild-type and UT2.5 strains growing in the presence of testosterone. In contrast, no *sip48*–β-HSD gene transcript was observed with mRNAs from *C. testosteroni* UT2.5Mut50 grown under the same experimental

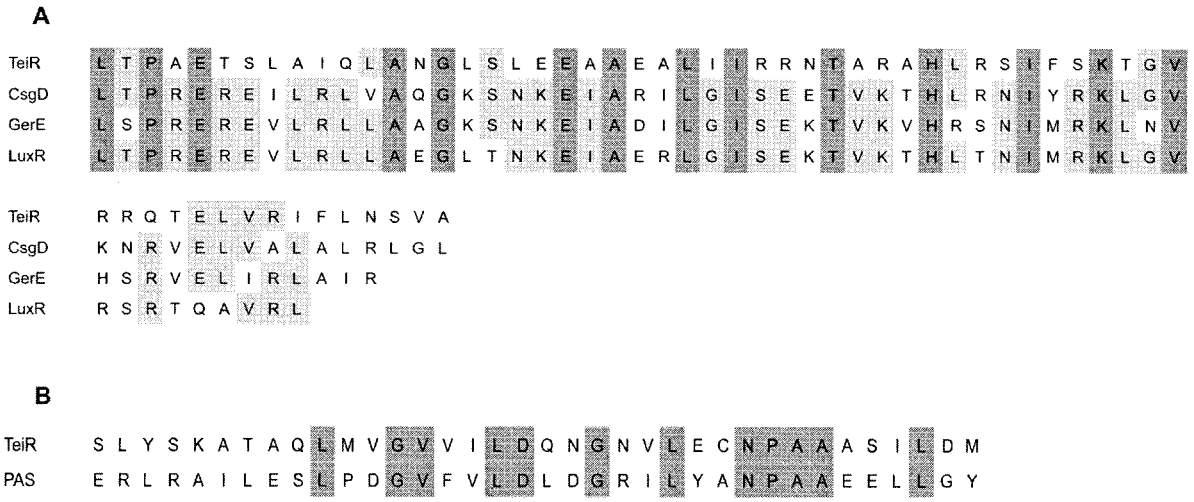

FIG. 3. (A) Alignment of the helix-turn-helix DNA binding domains of three transcriptional regulators (CsgD [COG2771], LuxR [smart00421], and GerE [pfam00196]) and amino acids 327 to 380 of the TeiR protein (accession number AY363220). (B) Alignment of the PAS sensor domain (smart00091) and amino acids 192 to 227 of the TeiR protein. For residues that were identical in all the aligned proteins, the characters representing the residues appear on a dark-gray background. For residues that were identical in 60% of the aligned proteins, the characters representing the residues appear on a light-gray background.

conditions (data not shown), thus confirming the results obtained with the β-galactosidase reporter fusion.

Characterization of the identified gene. Chromosomal DNA of the *C. testosteroni* UT2.5Mut50 strain was isolated and digested with a *Not*I enzyme, which does not cut within the minitransposon. The digested mixture was ligated into the *Not*I site of pBIISK plasmid, and transformants able to grow on Tc were selected. The resulting plasmid (pBIISKMut50) was found to contain a 2.5-kb insert (including the 1.8-kb Tc^r fragment). Complete analysis of the 0.7-kb fragment sequence located downstream of the Tc^r gene revealed an incomplete open reading frame (3′ end). To obtain the 5′ coding sequence of the interrupted gene, a DNA pVK102 *C. testosteroni* cosmid library was screened with a probe complementary to the 3′ end of the disrupted gene. A positive-testing clone was isolated and characterized by Southern blot analysis. A 20-kb *Hin*dIII fragment was subcloned into pBBR1MCS2 (pBB20H) and partially sequenced, and the complete sequence of the disrupted open reading frame was obtained. The deduced amino acid sequence of this gene predicts a 391-amino-acid protein with a molecular mass of 43 kDa. Computer analysis showed that this amino acid sequence shows high-level similarity (93.1%) between residues 327 and 380 (C-terminal domain) to that of the LuxR helix-turn-helix DNA binding domain (smart00421) (Fig. 3A). In addition, the sequence between residues 192 and 227 shows mild similarity (53.7%) to that of the PAS sensor domain (smart00091) (Fig. 3B). This novel gene (called *teiR* [testosterone-inducible regulator]) encodes a protein with 28% identity (44% similarity) over an aligned length of 384 amino acid residues with a putative protein of *Novosphingobium aromaticivorans* (accession number ZP 00092325.1). Other related proteins are putative transcription regulator proteins of *Mesorhizobium loti* (accession number NP 103400.1), *Bradyrhizobium japonicum* (accession number NP 770508.1), *Ralstonia solanacearum* (accession number NP 523029.1), and a DNA-binding protein of *Vibrio vulnificus* (accession number NP 761512.1). A 60-amino-acid region in the C-terminal domain (where a potential helix-turn-helix DNA binding motif is located along with several highly conserved amino acids such as those found in CsgD [COG2771], GerE [pfam00196], and LuxR [smart00421] regulatory proteins) is typically present in these proteins.

teiR gene expression was investigated in *C. testosteroni* UT2.5 and UT2.5Mut50 strains growing on LB medium with or without testosterone. Total RNA was extracted at different culture times under each set of experimental conditions, and Northern blot assays were performed. The *teiR* probe recognized strong signals corresponding to a 1,150-nucleotide transcript when *C. testosteroni* UT2.5 bacteria were grown in the presence of testosterone, indicating that this is a steroid-inducible gene. In contrast, no *teiR* expression was found when the UT2.5Mut50 RNA was probed, thus indicating the absence of *teiR* expression in the UT2.5Mut50 strain (Fig. 4).

Expression analysis of other steroid-inducible genes in *C. testosteroni* UT2.5Mut50. To establish whether the *teiR* gene is required for the transcription of other steroid-inducible genes, Northern blot assays were performed. Total RNAs from *C. testosteroni* UT 2.5 and UT2.5Mut50 strains grown on LB medium with or without testosterone were isolated. DNA fragment probes complementary to the sequences of the steroid-inducible genes encoding α-HSD–KSI and Δ^1-DH–Δ^4-DH recognized the corresponding strong signals in the RNA samples obtained from C. *testosteroni* UT 2.5 (the control strain) grown in the presence of testosterone. In contrast, no steroid-inducible gene expression was observed in *C. testosteroni* UT2.5Mut50 strain (Fig. 5). These results demonstrate that in addition to *sip48*–β-HSD gene transcription, the expression of other testosterone-inducible genes such as the α-HSD–KSI and Δ^1-DH–Δ^4-DH genes is impaired by *teiR* gene disruption.

Altered phenotypes in *C. testosteroni* UT2.5Mut50. Having obtained evidence that *teiR* disruption abolishes not only *sip48*–β-HSD gene testosterone-inducible expression but also

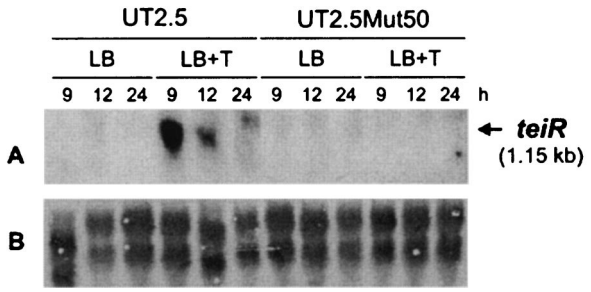

FIG. 4. Expression of *teiR* in *C. testosteroni* UT2.5 and UT2.5Mut50 strains in response to the presence of testosterone. Total RNA samples (20 μg per lane) were prepared from bacteria grown on LB medium in the absence (LB) or presence (LB+T) of testosterone during 9, 12, and 24 h of culture growth. (A) The membrane was hybridized with a DNA fragment complementary to the *teiR* gene. (B) The samples were analyzed by electrophoresis on formaldehyde-agarose gels, transferred to a nylon membrane, and stained with methylene blue.

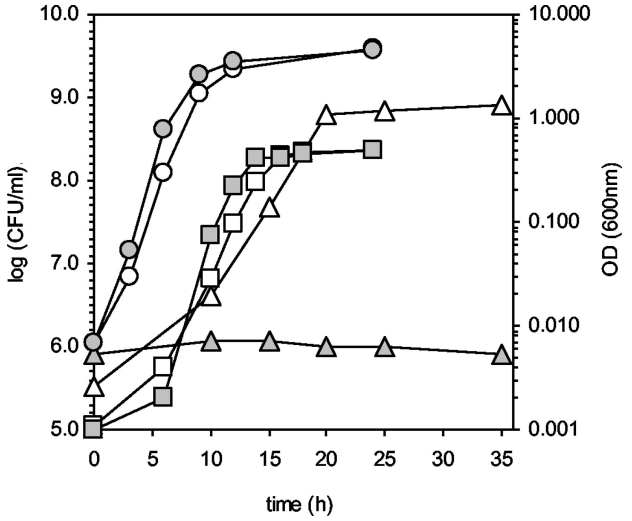

FIG. 6. Growth of *C. testosteroni* UT2.5 (white shading) and UT2.5Mut50 (grey shading) strains in LB medium (circle), M9 medium plus acetate (square), and M9 medium plus testosterone (triangle). Growth of bacteria in LB medium or M9 plus acetate was monitored by measuring OD_{600}. Growth of bacteria in M9 medium plus testosterone was monitored by counting colonies (in CFU per milliliter) that appeared on LB plates (on which appropriately diluted cultures have been spread) after incubation at 30°C.

the transcription of other steroid-inducible genes, we analyzed the growth of *C. testosteroni* UT2.5Mut50 in M9 minimal medium supplemented with testosterone as the sole carbon and energy source. The results (shown in Fig. 6) indicate that the mutant strain is completely unable to use testosterone as the sole carbon source. Moreover, measurement of testosterone degradation in *C. testosteroni* UT2.5Mut50 indicates that this strain cannot transform testosterone into androstenedione (data not shown). Nevertheless, the mutant and wild-type strains showed identical duplication times (70 min) when they were grown in LB medium or M9 minimal medium supplemented with acetate (Fig. 6). These results demonstrate that *teiR* disruption abolishes testosterone metabolism and (as a consequence) *C. testosteroni* growth in medium containing this steroid as the sole carbon source.

Insertional transcription inactivation downstream of the *teiR* coding sequence. To investigate whether the phenotype observed in the *C. testosteroni* UT2.5Mut50 strain was due to a polar mutation caused by *teiR* disruption, we inserted a Sp cassette after the coding sequence of this gene. A new *C. testosteroni* UT2.5 mutant strain was constructed by insertion of the Sp interposon into the *Nde*I restriction site located 50 bp downstream of a *teiR* stop codon. Southern blot analysis of the selected mutant strain (*C. testosteroni* UT2.5teiR-3′URΩ) allowed us to confirm that the wild-type sequence was completely replaced by the interposon-disrupted construct (data not shown). The UT2.5teiR-3′URΩ mutant strain grew on LB medium or M9 minimal medium plus acetate at the same growth rate (70 min) as *C. testosteroni* UT2.5. Moreover, the *C. testosteroni* UT2.5teiR-3′URΩ strain was able to grow on M9 minimal medium supplemented with testosterone as the sole carbon source (Fig. 7A), indicating that no genes required for growth on testosterone are 3′ of *teiR* and cotranscribed with *teiR*. In addition, the level of *sip48*–β-HSD gene promoter activity (measured as the level of β-galactosidase activity in *C. testosteroni* UT2.5teiR-3′URΩ grown in testosterone-containing medium) was equal to that of the control strain (UT2.5) (Fig. 7B). Thus, no genes required for testosterone-inducible gene expression are located downstream of *teiR* coding sequence and cotranscribed with it.

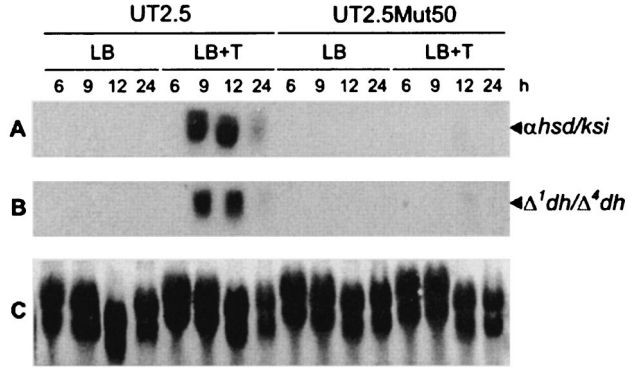

FIG. 5. Expression of steroid-inducible genes in *C. testosteroni* UT2.5 and UT2.5Mut50 strains. Total RNA samples (20 μg per lane) were prepared from bacteria grown on LB medium in the presence or absence of testosterone during 6, 9, 12, and 24 h of culture growth. (A and B) The membrane was hybridized with a DNA fragment complementary to α-HSD and Δ^5–KSI (*αhsd/ksi*) (A) and to Δ^1-DH and Δ^4-DH (Δ^1*dh*/Δ^4-*dh*) (B) steroid-inducible genes. (C) The samples were analyzed by electrophoresis on formaldehyde-agarose gels, transferred to a nylon membrane, and stained with methylene blue.

Genetic complementation of *teiR* mutant. To demonstrate that *teiR* encodes a protein involved in the testosterone-inducible expression of *sip48*–β-HSD genes and to confirm that this gene is essential for growth on testosterone as the sole carbon source, we performed a complementation assay. The aim of this experiment was to analyze the expression of the reporter *lacZ* fusion in *C. testosteroni* UT2.5Mut50 after *trans*-complementation with *teiR* and to ascertain whether the complemented strain regains the growing phenotype of the *C. testosteroni* UT2.5 parental strain. The complementation procedure

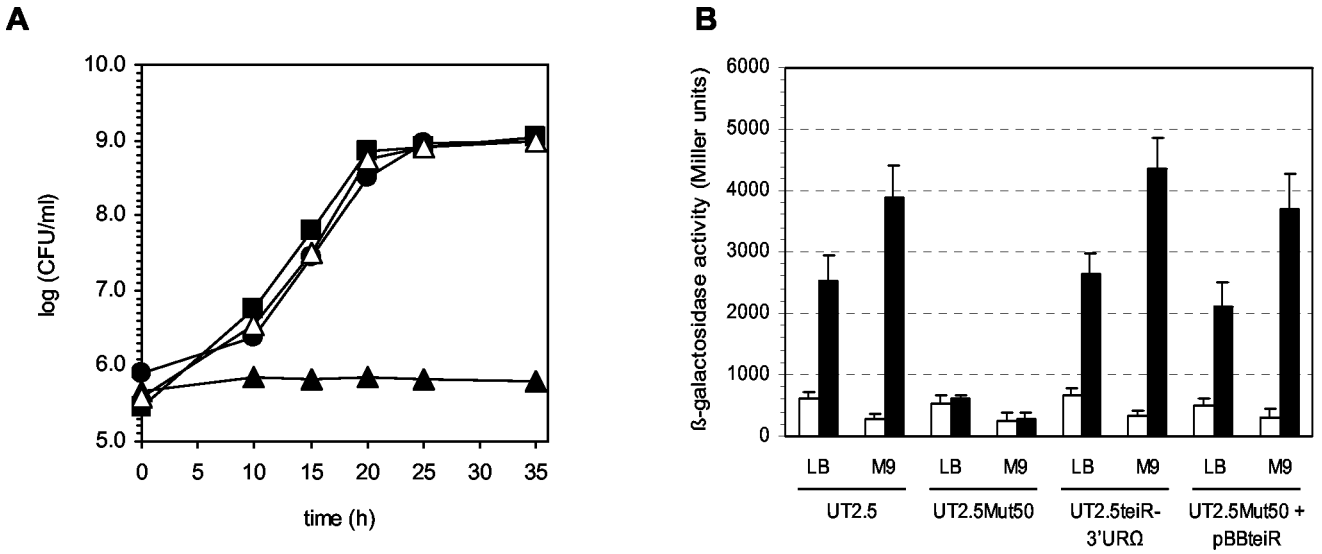

FIG. 7. Growth and *sip48*–β-HSD gene promoter activity in *C. testosteroni* UT2.5, UT2.5Mut50, and UT2.5*teiR*-3′URΩ strains and in the UT2.5Mut50 strain complemented with *teiR*. (A) Growth of *C. testosteroni* UT2.5 (black circle), UT2.5*teiR*-3′URΩ (black square), and UT2.5Mut50 (black triangle) strains and of the UT2.5Mut50 strain complemented with pBB*teiR* (white triangle). Growth of bacteria in LB medium or M9 plus acetate was monitored by measuring OD_{600}. Growth of bacteria in M9 minimal medium plus testosterone was monitored by counting colonies (in CFU per milliliter) that appeared on LB plates (on which appropriately diluted cultures have been spread) after incubation at 30°C. (B) β-Galactosidase activity levels of *sip48*–β-HSD gene promoter-*lacZ* transcriptional fusion in *C. testosteroni* strains UT2.5, UT2.5Mut50, UT2.5*teiR*-3′URΩ, and UT2.5Mut50 complemented with pBB*teiR* (UT2.5Mut50 + pBBteiR) growing in LB medium and in M9 minimal medium supplemented with acetate (M9) in the presence (black bars) and the absence (white bars) of testosterone. β-Galactosidase activities were measured with permeabilized cells as described in Materials and Methods. Each value is the average of the results from three independent experiments (error bars indicate standard deviations).

was performed with pBB*teiR* plasmid containing the *teiR* gene. As expected, the testosterone-inducible *sip48*–β-HSD gene promoter activity was restored in the complemented UT2.5Mut50 strain. The β-galactosidase levels determined when the cells were growing in the presence of testosterone were similar to those of the isogenic UT2.5 strain (Fig. 7B). In addition, the complemented bacterium was able to grow when testosterone was used as the sole carbon source (Fig. 7A). In both experiments, the behavior of a complemented strain carrying pBBR1MSC2 without an insert was indistinguishable from that of *C. testosteroni* UT2.5Mut50 (data not shown). Northern blot assays performed with the complemented strain also demonstrated steroid-inducible expression of the *sip48*–β-HSD gene, the α-HSD–KSI gene, and the Δ^1-DH–Δ^4-DH genes (data not shown). Altogether, these data confirm that *teiR* is essential for the steroid-inducible transcription of different steroid-inducible genes and for steroid metabolism in *C. testosteroni*.

DISCUSSION

In this work, we report the identification and characterization of *teiR*, a novel steroid-inducible gene that is necessary for testosterone degradation in *C. testosteroni* strains. The C-terminal domain of TeiR has a high level of similarity to the LuxR DNA binding domain (belonging to the family of LuxR-type transcription regulators). It has been established by genetic analyses that LuxR is composed of two functional modules or domains: an amino-terminal domain with an autoinductor binding region and a carboxy-terminal transcription regulatory domain (28). LuxR-like proteins bind autoinducers that have achieved a critical threshold concentration, after which the LuxR-autoinducer complexes generally activate gene transcription. Two interesting features among LuxR-type transcriptional regulators are the high level of homology in the DNA binding domain and the variability in the sensor domain (17). In this regard, the TeiR C-terminal domain shows high-level homology to the helix-turn-helix DNA binding motif present in LuxR and other related transcriptional regulators. In addition to the LuxR helix-turn-helix DNA binding domain (residues 327 to 380), a PAS sensor domain located between amino acids 192 and 227 was observed. This domain distribution resembles that described for different LuxR-type proteins; it is particularly similar to that of TraR, a specific transcriptional regulator involved in quorum sensing in *Agrobacterium tumefaciens* (whose three-dimensional structure has been recently described) (41).

It has been suggested that quorum sensing is an integral component of gene regulatory networks in gram-negative bacteria (43). Particularly, *C. testosteroni teiR*-disrupted strains are unable to induce the expression of several steroid-inducible genes, such as those encoding a steroid-inducible protein of 48 kDa, β-HSD, α-HSD, Δ^5–KSI, Δ^1-DH, and Δ^4-DH enzymes. The absence of these proteins probably determines the complete impairment of the *C. testosteroni teiR* mutant strain with respect to the use of different steroid compounds as a sole carbon and energy source. The results of the complementation assay and the transcriptional interruption downstream of the *teiR* coding sequence clearly demonstrate the importance of this gene for the observed phenotypes. Taken together, these results suggest that TeiR is a global regulator of the steroid catabolic pathway in *C. testosteroni*.

One or more regulatory proteins often control the expression of bacterial catabolic pathways for aromatic compounds, and the effectors of these regulatory proteins are usually either the initial substrates or catabolic intermediates of the pathways (39). It has been reported in particular that the regulation of α-HSD gene expression appears to be a derepression mechanism in which the steroidal inducer (testosterone) prevents the binding of two repressor proteins (RepA and RepB) to the α-HSD gene promoter and mRNA, respectively (45). The complete lack of activation of α-HSD gene steroid-inducible transcription when the *teiR*-disrupted mutant was grown in the presence of testosterone indicates that this steroid is not the true inducer of this pathway, suggesting that the mechanisms regulating α-HSD gene expression might be more complex than previously reported. In agreement with our results, the data obtained by Horinouchi et al. (20) suggest that expression of *tesB* (encoding a *meta*-cleavage steroid-inducible enzyme) in *C. testosteroni* TA441 is induced by an intermediate compound produced in the course of testosterone degradation.

In conclusion, mutation of *teiR* is sufficient to block the ability of *C. testosteroni* to use testosterone as a sole carbon source, indicating that TeiR is an integral part of the degradation portion of the steroid catabolic pathway. Furthermore, *teiR* encoding a LuxR-type transcription factor is required for the expression of several steroid-inducible genes, suggesting that a quorum-sensing mechanism might be involved in its regulation.

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