Identification and Characterization of the LysR-type Transcriptional Regulator HsdR for Steroid-inducible Expression of the 3α-Hydroxysteroid Dehydrogenase/Carbonyl Reductase Gene in Comamonas testosteroni

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Running Title: HsdR positively regulates the 3α-HSD/CR expression.
Abstract

3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) from Comamonas testosteroni (C. testosteroni) is a key enzyme in steroid degradation in soil and water. 3α-HSD/CR gene (hsdA) expression can be induced by steroids like testosterone and progesterone. Previously, we have shown that induction of hsdA expression by steroids is a derepression where steroidal inducers bind to two repressors, RepA and RepB, thereby preventing blocking of hsdA transcription and translation, respectively. In the present study, a new LysR-type transcriptional factor HsdR for 3α-HSD/CR expression in C. testosteroni has been identified. The hsdR gene locates 2.58 kb downstream from hsdA on the C. testosteroni ATCC 11996 chromosome with an orientation opposite to hsdA. The hsdR gene was cloned and recombinant HsdR protein was produced, as well as anti-HsdR polyclonal antibodies. While heterologous transformation systems revealed that HsdR activates the expression of hsdA gene, electrophoresis mobility shift assays (EMSA) showed that HsdR specifically binds to the hsdA promoter region. Interestingly, the activity of HsdR is dependent on decreased repression by RepA. Furthermore, in vitro binding assays indicated that HsdR can contact with RNA polymerase. As expected, an hsdR knock-out mutant expressed low levels of 3α-HSD/CR compared to wild type C. testosteroni after testosterone induction. In conclusion, HsdR is a positive transcription factor for the hsdA gene and promote induction of 3α-HSD/CR expression in C. testosteroni.
Introduction

*Comamonas testosteroni* (C. *testosteroni*) is a Gram-negative bacterium that belongs to the beta group of Proteobacteria (34). These strictly aerobic, nonfermentative, chemoorganotrophic bacteria rarely attack sugars, but grow well on organic acids and amino acids (2). Moreover, *C. testosteroni* strains are able to use steroids as the sole carbon source and may be an attractive means for the removal of these stable compounds from the environment. Interestingly, the catabolic enzymes for steroid degradation are usually not constitutively expressed, but are induced by their respective steroid substrates (18, 20, 24). Hence, steroids play a particularly important role in certain prokaryotes, as they may simultaneously serve both as signal molecules and carbon source.

Since the pioneering work of Talalay and co-workers (18, 33), it is well known that 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) is one of the first enzymes of the steroid-catabolic pathway and therefore plays an important role in steroid metabolism. In previous investigations, 3α-HSD/CR has been identified to catalyze the oxidoreduction at position 3 of the steroid nucleus of a variety of C19-27 steroids (24). Surprisingly, this enzyme was also capable of catalyzing the carbonyl reduction of a variety of nonsteroidal xenobiotic aldehydes and ketones (24). It has been demonstrated that this substrate pluripotency not only enhances the metabolic capacity of insecticide degradation but also increases the resistance of *C. testosteroni* towards the steroid antibiotic fusidic acid (23).

3α-HSD/CR from *C. testosteroni* is one of the enzymes, whose expression is induced by steroids such as testosterone and progesterone (18, 20, 23, 24), and this is why we were
interested in the mode of the molecular regulation of its gene (hsdA). In previous investigations, we identified two genes, repA and repB, involved in hsdA regulation and reported a “two repressor model” to control hsdA gene expression. RepA was identified to specifically bind to both operators Op1 and Op2 and to force the DNA between them to form a loop structure. The two palindromic 10-bp motifs Op1 (TCAAAGCCCA) and Op2 (TGGGCTTTGA), working as cis-acting operator elements for hsdA regulation, were localized at 0.935 kb and 2.568 kb upstream of hsdA, respectively. While Op2 overlaps the -10 binding site (TTTGAT) of the σ70 RNA polymerase by 5 bp. Thus, the resulting DNA-loop-RepA complex strongly blocks transcription of the hsdA gene. In the presence of appropriate steroids, however, they bind to RepA, thereby reducing its ability to bind to the operator region (38). Upon dissociation of RepA from the operators, RNA polymerase may bind to the promoter and transcription of 3α-HSD/CR mRNA is initiated. RepB was demonstrated to bind to the mRNA of 3α-HSD/CR and to interfere with 3α-HSD/CR translation (39). Later, the teiR gene encoding a positive regulator of steroid-degrading enzymes, including 3α-HSD/CR, was identified to mediate steroid sensing and signaling in C. testosteroni ATCC 11996 via a kinase mechanism (7).

In the present study, a novel regulator “3α-Hydroxysteroid dehydrogenase/carbonyl reductase Regulator” (HsdR) for 3α-HSD/CR expression in C. testosteroni has been identified, which was recognized as a member of the LysR-type transcriptional regulator family. The LysR-type transcriptional regulator (LTTR) family, formally documented by Henikoff et al. (9), is a well-characterized group of transcriptional regulators. LTTRs are dual function regulators acting as both auto-repressors and activators of target promoters, frequently of genes co-located with the LTTRs in the chromosome (9, 29, 32). The common features of this family comprise sequence
lengths of around 300 residues, high sequence similarity at the N-terminal winged-helix-turn-helix (wHTH) motif for DNA binding, less conserved C-terminal inducer binding domain and, with few known exceptions (29), LTTRs act as homotetramers (32). LTTRs regulate the expression of a wide variety of genes, including operons involved in amino acid metabolism, oxidative stress, bacterial virulence and degradation of aromatic compounds (29, 32).

Here, the hsdR gene was found to be a positive transcriptional regulator for hsdA expression and locates 2.58 kb downstream of the hsdA gene on the C. testosteroni chromosome with an orientation opposite to hsdA. Studies with wild type and hsdR knock-out mutant strains confirmed that HsdR is necessary for the induced expression of the hsdA gene. Electrophoretic mobility shift assays showed that HsdR and RepA can simultaneously bind to different sites of the hsdA promoter region. In addition, HsdR interacts with RNA polymerase as revealed by HsdR-RNA polymerase binding. The expression of HsdR itself in C. testosteroni is not induced by testosterone. From these results we conclude that HsdR is a positive transcription factor for induced hsdA expression.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Host strains E. coli HB101 (Promega) and C. testosteroni ATCC 11996 (Deutsche Sammlung für Mikroorganismen) were used for cloning and gene expression. Subcloning of fragments was carried out in plasmids pUC18 (containing the ampicillin resistance gene and obtained from Invitrogen) and plasmid pK18 (containing the kanamycin resistance gene; a gift from Ciba Pharmaceuticals, Inc., Department of Biotechnology, Basel, Switzerland). The plasmid copy
numbers determined were 80 copies of pK18 and pUC18 per cell in *E. coli*. For overexpression and purification of HsdR, *E. coli* strain BL21(DE3)pLysS together with plasmid pET15b from Novagen was used. The tac promoter (274 bp) was obtained by BamHI digestion from plasmid pHA10, which was a gift from H. Arai (1). Plasmid pCR2.1-TOPO (Invitrogen) served for PCR cloning of *hsdR* fragments and sequencing. Bacterial cells were grown in a shaker (180 rpm) in Standard I Nutrient broth medium (SIN) (Merck) or LB medium at 37°C (*E. coli*) or 27°C (*C. testosteroni*). Growth media contained 100 µg/ml ampicillin and/or 30 µg/ml kanamycin when required.

**DNA manipulations, sequencing and reagents**

Recombinant DNA work was carried out following standard techniques according to Sambrook and Russel (30). All of the primers were prepared by MWG (Ebersberg, Germany). Before further cloning, fragments prepared by PCR were cloned into pCR2.1-TOPO and then checked for correct sequence by MWG. Restriction enzymes, T4 ligase and shrimp alkaline phosphatase were obtained from Roche Applied Science, New England Biolabs, MBI, Promega, and Amersham Biosciences, and used according to the manufacturers’ instructions. Sodium lauroyl sarcosinate was from Fluka. The steroid compounds were supplied by Sigma. Ampicillin and kanamycin were from AppliChem and Calbiochem, respectively. Incomplete Freund’s adjuvant was from MP Biomedicals.

**Plasmid construction**

The *hsdR* gene was cloned from *C. testosteroni* ATCC 11996 chromosomal DNA by using the following pair of primers: forward primer containing an NdeI site (5'-
CATATGGATTCTAATGCGC-3’) and reverse primer containing a BamHI site (5’-
GGATCCAAGAGCGTATGC-3’). The full hsdR gene was then cloned into pCR2.1-TOPO to
yield plasmid pTOPOHsdR, which after sequence confirmation (MWG), was used as template
for further PCR reactions (Fig. 1). To generate hsdR gene constructs that are controlled by the lac
or the tac promoter, respectively, plasmid pTOPOHsdR was digested with KpnI and XbaI and
the resulting KpnI-XbaI fragment was ligated into either pK18 downstream from the lac
promoter to yield pKHsdR3 or downstream from the tac promoter to yield pKtaHsdR1. In
addition, pTOPOHsdR was digested by BamHI and NdeI and subcloned into pET15b
downstream from the N-terminal His-tag coding sequence to yield pETHsdR2, which was used
for recombinant HsdR protein production.

To elucidate if HsdR regulates transcription of the hsdA gene, a series of plasmids constructed
in our lab were used. As described previously, a 5.257 kb EcoRI fragment of C. testosteroni
chromosomal DNA was cloned into pUC18 to yield p6 (38). Plasmid p6 contains the 3α-
HSD/CR gene, hsdA, together with its regulatory region and the two repressor genes repA and
repB (38, 39). AvrII and XbaI were used for double digestion of p6 and ligation to yield pAX1
(38). With pAX1 as template, a shorter derivative, pDel13n, was constructed, in which 13
upstream bases critical for HsdR action had been deleted (Fig. 2). To vary the distance between
Op1 and Op2, restriction enzymes AvrII and MluI together with PCR reactions were used to
generate mutant plasmids p67, p67-3, p67-5 and p67-7 from p6 which comprised a spacing of 67,
64, 62 and 60 bp between Op1 and Op2 (40), respectively (Fig. 3). The expression of hsdA
served as a detection system for HsdR transcriptional regulation in co-transformation
experiments with the plasmids described above.
Construction of an \textit{hsdR} disrupted mutant of \textit{C. testosteroni}

An \textit{hsdR} disrupted mutant of \textit{C. testosteroni} was prepared by homologous integration. A DNA fragment ranging from 251 bp to 650 bp of the \textit{hsdR} gene was generated by PCR using forward primer 5’-CTGCCGCCAGCGGGC-3’ and reverse primer 5’-CAGTGCATGGGCTGC-3’ and plasmid pTOPOHsdR as template. The 400 bp fragment was cloned into pCR2.1-TOPO containing the kanamycin resistance gene to yield pTOPOHsdR3-1. Since plasmid pTOPOHsdR3-1 cannot replicate in \textit{C. testosteroni}, and because of the sensitivity of wild type \textit{C. testosteroni} to kanamycin, only mutants of \textit{C. testosteroni} harbouring the kanamycin resistance gene of plasmid pTOPOHsdR3-1 integrated within the chromosomal DNA can grow in medium containing kanamycin. Accordingly, \textit{C. testosteroni} was transformed with 10 \(\mu\)g of pTOPOHsdR3-1 by electroporation (1.8 kV, 1-cm cuvette, BIO-RAD), which contains \textit{hsdR} sequences homologous to \textit{C. testosteroni} chromosomal DNA. The cells were spread on 30 \(\mu\)g/ml kanamycin SIN agar plates and cultured in a 27°C incubator overnight. The colonies were proven by PCR for homologous integration.

Preparation and purification of recombinant HsdR protein

Overexpression of HsdR was performed in \textit{E. coli} strain BL21(DE3)pLysS with plasmid pETHsdR2, and the recombinant protein was purified by its His-tag sequence. In brief, cells transformed with plasmid pETHsdR2 (Fig. 1) were grown in SIN medium with 100 \(\mu\)g/ml ampicillin at 37°C in a shaker (180 rpm). One hundred microliter of the overnight culture was used to inoculate 3 ml of fresh medium. When the bacteria had grown to an optical density of 0.4-0.6 at 595 nm, target protein expression was induced by the addition of isopropyl-\(\beta\)-D
thiogalactoside to a final concentration of 1 mM. After induction for 4 h at 37°C or overnight at room temperature, cells were harvested by centrifugation. The cell pellet was either stored at -80°C for further usage or directly suspended in 200 µl of lysis buffer (50 mM sodium dihydrogenphosphate, 300 mM sodium chloride, 10 mM imidazole, pH 8.0) (Qiagen) containing different concentrations of sodium lauroyl sarcosinate (Fluka Chemie AG, Buchs, Switzerland). Cells were lysed by freezing (-20°C, 30 min) and thawing (room temperature, 30 min) 3 times, and the resulting mixture was centrifuged at 10,000 x g for 20 min. The supernatant was applied to a mini nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography column (Qiagen). After washing 2 times with 600 µl of washing buffer (50 mM sodium dihydrogenphosphate, 300 mM sodium chloride, 75 mM imidazole, and different concentrations of sodium lauroyl sarcosinate, pH 8.0) (Qiagen), HsdR was eluted from the column by applying 100 µl of elution buffer for 4 times (50 mM sodium dihydrogenphosphate, 300 mM sodium chloride, 250 mM imidazole and different concentrations of sodium lauroyl sarcosinate, pH 8.0). Samples containing pure and soluble HsdR protein were assessed by SDS-polyacrylamide gel electrophoresis. Purification of RepA protein was performed as described previously (10). The concentration of purified protein was determined by the method of Bradford with Roti-Quant solution (Roth) using bovine serum albumin as standard (3). Protein analysis by SDS polyacrylamide gel electrophoresis was carried out according to Laemmli (12).

**Immunization and preparation of antisera against HsdR**

On the first day, rabbits were injected subcutaneously with an emulsion of 0.5 ml water and 0.5 ml incomplete Freund’s adjuvant. For immunization, one microgram of purified HsdR protein was dissolved in an emulsion of 0.5 ml water and 0.5 ml incomplete Freund’s adjuvant, and
rabbits were immunized on the following days: 7 d, 37 d, 67 d. The antiserum was collected at day 74 and antibody titer determination in the rabbit serum was performed by ELISA.

ELISA of 3α-HSD/CR and HsdR

Proteins for 3α-HSD/CR or HsdR ELISA detection were prepared from 3 ml of bacterial cell culture and subsequent centrifugation at 10,000 x g for 10 s. The pellet was washed 3 times with 1 ml of phosphate buffered saline (PBS) and resuspended in 200 µl of PBS with 100 µg/ml lysozyme. The suspension was frozen at -20°C and thawed at room temperature 3 times. Finally, the samples were centrifuged again at 10,000 x g for 20 min. The supernatant was diluted into 1 mg/ml protein and used for 3α-HSD/CR or HsdR ELISA detections.

To quantify 3α-HSD/CR protein expression, an ELISA was established, and respective antibodies directed against 3α-HSD/CR from C. testosteroni were prepared in rabbits (19). For HsdR detection, antibodies against HsdR were prepared in rabbits as described above. ELISA plates were coated with protein containing 3α-HSD/CR or HsdR diluted in coating buffer. After washing, antibodies against 3α-HSD/CR or HsdR were added in a 1:1000 dilution. As the secondary antibodies, peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO, Denmark) were used in a 1:1000 dilution. The further procedure corresponded to that of the chloramphenicol acetyl transferase ELISA kit from Roth.

HsdR-RNA polymerase interaction

300 ng RNA polymerase (a generous gift from Prof. Ruth Schmitz-Streit, Kiel University) was used to coat each well (coating buffer: Na₂CO₃ 1.59 g, NaHCO₃ 2.93 g and NaN₃ 0.2 g, pH 9.6
in 1 liter). The plate was incubated at 37°C for 30 min. After washing 3 times (washing buffer: NaCl 8 g, KH$_2$PO$_4$ 0.2 g, Na$_2$HPO$_4$ 1.5 g, KCl 0.2 g and Tween 20 0.5 ml, pH 7.4 in 1 liter), different amounts of purified HsdR diluted with washing buffer were added. Following an incubation at 37°C for 30 min, rabbit antibodies against HsdR diluted with washing buffer (1:1000) were added into the wells. After incubation for another 30 min at 37°C and washing, washing buffer diluted (1:1000) peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO, Denmark) was added. Following a further incubation for 30 min at 37°C and washing, 100 µl ABTS solution (2'-'azino-di-(3-ethylbenzthiazoline-6-sulfonate) with H$_2$O$_2$ in glycin/citric acid buffer; Roche, Mannheim, Germany) was added into each well and incubated at 37°C for 30 min. Finally, the samples were assayed in an ELISA reader at 415 nm with reference at 490 nm (BIO-RAD, Hercules, California).

**Electrophoretic mobility shift assays (EMSA)**

For gel mobility shift assays, digoxigenin-11-dUTP-labeled 203 bp DNA fragments containing the hsdA promoter region were generated by PCR with forward primer p7-1: 5'-'AGGGGAATTACATCGTCCGTTTGCATGTAGCC-3', reverse primer pCla: 5'-CCGCATCGGTATATCG-3' and plasmid p6 as template. Incubation mixtures (20 µl) contained 10 ng of labeled DNA fragment, 100 ng of herring sperm DNA and different concentrations of purified HsdR and RepA protein in binding buffer consisting of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 12.5 mM MgCl$_2$, 1 mM EDTA, 5% glycerol, 100 μg/ml bovine serum albumin and 1 mM DTT.

After incubation at 27°C for 30 min, samples were separated on 5% polyacrylamide nondenaturing gels in 0.05 M Tris borate/EDTA buffer (pH 8.3) for 2 h at 10 V/cm and blotted onto nitrocellulose membranes. Labeled DNA was visualized by chemiluminescence using the
digoxigenin luminescence detection kit (Roth).

Results

Cloning and sequence analysis of HsdR from *C. testosteroni*

A new transcriptional factor, HsdR, named from “3α-Hydroxysteroid dehydrogenase/carbonyl reductase Regulator” was identified in *C. testosteroni*. The *hsdR* gene locates 2.58 kb downstream from *hsdA* on the *C. testosteroni* ATCC 11996 chromosome with an orientation opposite to *hsdA* (Fig. 1). The open reading frame of *hsdR* consists of 912 bp and translates into a protein of 303 amino acids. BLAST analysis showed that HsdR is a member of the LysR-type transcriptional regulator family (LTTRs). The nucleotide sequence of the *hsdR* gene reported in this study has been submitted to the GenBank database with the accession no. JF747025.

Activation of *hsdA* expression by HsdR

For the identification of HsdR as an activator of *hsdA* expression, plasmid pKHsdR3 containing *hsdR* gene was co-transformed into *E. coli* HB101 with plasmids p6, pAX1 or pDel13n, respectively. After co-transformation with pKHsdR3, the expression level of 3α-HSD/CR with pAX1 increased compared to the control vector pK18, but not with p6 (Fig. 2).

According to our previous findings, there are two operators (Op1 and Op2) present in plasmid p6 which form a DNA-loop structure upon binding of both with repressor RepA (38). In addition, Op2 overlaps the -10 region of the *hsdA* promoter region (38). Both the loop structure and the occupation of the *hsdA* promoter by RepA lead to a strong repression of *hsdA* gene expression (38). Since in pAX1 there is only operator Op2 present, the DNA-loop structure cannot be
formed, such that hsdA expression already increases with empty control vector pK18. However, after co-transformation with plasmid pKHsdR3, an increased expression of hsdA could be observed. This indicates that HsdR drives hsdA gene expression as a transcriptional activator (Fig. 2).

Co-transformation of pDel13n, in which 13 bases were deleted upstream of the AvrII-EcoRI fragment, with pK18 also lead to a slight increase in 3α-HSD/CR expression compared to p6 (Fig. 2). Also here, the DNA-loop structure could not be formed due to the lack of bases important for RepA binding (38). Interestingly, co-transformation of the hsdR gene (pKHsdR3) did not significantly enhance 3α-HSD/CR expression with pDel13n, a fact which lead us conclude that the deleted nucleotides may be necessary for HsdR action (Fig. 2).

In order to demonstrate that the ability of HsdR to activate hsdA expression is affected by RepA binding, a series of plasmids p67, p67-3, p67-5 and p67-7, in which a various number of bases were present between the two operators Op1 and Op2, were employed. With these plasmids, we have previously shown that a critical distance between Op1 and Op2 together with additional -3, -5 and -7 nucleotides deletions result in DNA rotations that lead to altered orientations of both operators to each other (40). As a consequence, RepA binding and subsequent hsdA repression was influenced (40).

Here, E. coli strain HB101 was transformed with these plasmids and plasmid pKtacHsdR1 (Fig. 3). 3α-HSD/CR expression was lowest with plasmid p67-3, probably because the 64 bp distance and relative orientation between Op1 and Op2 is suitable for a strong RepA binding and hsdA
repression (38, 40). In this conformation, RepA is able to prevent HsdR from activating the \textit{hsdA} promoter. With plasmid p67-7, which harbours a 60 bp spacing between Op1 and Op2, co-
transformation of HsdR lead to a significant increase in 3\alpha-HSD/CR expression when compared
to the empty control vector pK18. Here, the altered DNA conformation, in which repression by
RepA is not as strong as that in p67-3, allows HsdR to perform its action on \textit{hsdA} expression.
This effect becomes most clear with plasmids p67 and p67-5, in which the positioning between
Op1 and Op2 was turned by at least 72º along the DNA axis compared to p67-3 and p67-7, such
that RepA-Op1 and RepA-Op2 binding became sterically unlikely (40). In the absence of the
Op1-RepA-Op2, HsdR could bind to the \textit{hsdA} promoter domain and activate 3\alpha-HSD/CR
expression (Fig. 3). This result clearly shows that HsdR activity is dependent on decreased
repression by RepA.

\textbf{Overexpression and purification of HsdR}

To produce purified HsdR protein, \textit{E. coli} BL21(DE3)pLysS cells were transformed into
plasmid pETHsdR2 and induced by IPTG at 37°C. However, the recombinant HsdR protein was
present in form of inclusion bodies, even after attempting to dissolve it with different detergents
such as sodium lauroyl sarcosinate (SLS). To solve this problem, the protein was then induced at
room temperature overnight and dissolved with SLS. Surprisingly, the solubility of HsdR
induced at room temperature highly increased compared to that induced at 37°C, especially in
the presence of SLS. Probably, the low temperature slowed down the speed of protein production
and gave the protein enough time to fold properly (37). Then the dissolved HsdR protein was
purified with a Ni-NTA chromatography column under native conditions. The molecular mass of
the recombinant protein (33.4 kDa) plus the His-tag sequence (2.2 kDa) as seen on the SDS-
polycrylamide gel (35.6 kDa) was identical to that predicted from the amino acid sequence. The purified protein was used for binding assays to DNA and RNA polymerase, as well as for the preparation of polyclonal antibodies.

**Binding of HsdR to the promoter region of the hsdA Gene**

The specific interaction between HsdR and the promoter of the hsdA gene was demonstrated by gel mobility shift assays. A DNA fragment from -65 to +137 bp relative to the transcription start site was labeled with digoxigenin and incubated with purified HsdR protein. After electrophoresis, formation of the HsdR-DNA complex was seen as shifted bands in Fig. 4A (lanes 2 and 3). Two shifted bands were observed when 100 pM of HsdR protein was present. It seems that there are several binding sites at the hsdA promoter, including high affinity binding sites and low affinity binding sites, the former being occupied after addition of small amounts of HsdR protein which resulted in the formation of the fast-migrating band. When large amounts of HsdR protein are present, both high affinity and low affinity binding sites are bound and the slower migrating band is formed. The extent of the shifted bands became weaker upon reducing the amount of HsdR protein as shown in Fig. 4A (lane 3). This result indicates that HsdR can bind to the hsdA promoter.

To figure out if RepA competes with HsdR to bind to the hsdA promoter, the same DNA fragment used above was also used for competition binding of RepA with HsdR. This fragment contained one high affinity determinant (Op2) of RepA (9). The shifted bands formed by RepA (Fig. 4B, lane 1) and HsdR (Fig. 4B, lane 4), respectively, indicated that HsdR and RepA can independently bind to the same DNA probe. It is proposed that if they compete for the same
binding determinant, formation of the HsdR-DNA complex will be inhibited by RepA. However, it turned out that two shifted bands occurred after addition of 6 pM of RepA to the HsdR reaction mixture (Fig. 4B, lane 3): one shifted band compares to that formed by HsdR, and the other one, with slower electrophoretic mobility, obviously represents an HsdR-DNA-RepA complex. Furthermore, the shifted HsdR-DNA-RepA complex became very strong after addition of more RepA protein (12 pM) to the HsdR reaction mixture (Fig. 4B, lane 2). Taken together, HsdR and RepA can simultaneously bind to different sites of the \textit{hsdA} promoter.

**Interaction between HsdR and RNA polymerase**

To determine HsdR as an activator on the transcription level for the target gene, \textit{in vitro} binding activity of HsdR and RNA polymerase was measured (Fig. 5). In this ELISA experiment, two distinct negative controls were set up, one with no RNA polymerase coated to the wells and no HsdR protein added, and the other with only HsdR protein (20 ng) added. As shown in Fig. 5, the optical density value with 20 ng HsdR and 300 ng RNA polymerase was approximately 3-fold compared to that with 0.625 ng HsdR. At the same time the value gradually decreased with decreasing amounts of HsdR protein shown in Fig. 5. This indicates that HsdR binds to RNA polymerase, thereby potentially increasing the concentration of RNA polymerase in the promoter domain of the target gene and enhancing its expression.

**HsdR is essential for the induced expression of \textit{hsdA} gene**

To demonstrate if HsdR is involved in the induction of \textit{hsdA} expression, an \textit{hsdR} gene knock-out mutant of \textit{C. testosteroni} (CT-HsdR-Ko) was prepared by homologous integration. Wild type \textit{C. testosteroni} and the \textit{hsdR} knock-out mutant strain CT-HsdR-Ko were induced overnight with
0.5 mM testosterone, and ELISA was used to measure hsdA expression. As shown in Fig. 6, the expression level of 3α-HSD/CR in wild type C. testosteroni highly increased after incubation with testosterone, which was not the case in the hsdR knock-out mutant. In the absence of the inducer testosterone, 3α-HSD/CR expression in both the wild type strain and the hsdR knock-out mutant was at the same basal level. It seems that testosterone binding to RepA decreases its affinity to the operators Op1 and Op2 such that the loop unfolds (38). As a consequence, the hsdA promoter becomes accessible for the transcription factor HsdR which now can perform its function to enhance 3α-HSD/CR expression. According to this result, HsdR is a critical factor for hsdA gene regulation.

**HsdR expression is not induced by testosterone**

To determine whether the expression of HsdR itself is sensitive to testosterone induction, the amount of HsdR in C. testosteroni was measured by ELISA with respective primary antibodies. After addition of testosterone, 3α-HSD/CR expression increased, whereas HsdR expression did not change (Fig. 7). This reveals that HsdR expression is not induced by testosterone in C. testosteroni.

**Discussion**

Microorganisms capable of utilizing various naturally occurring steroids as carbon and energy sources are relatively widespread in nature (16, 17, 31, 41). Complete assimilation of these substrates is achieved through an adaptive complex metabolic pathway involving many enzymatic steps of oxidation responsible for the breakdown of the steroid nucleus (8, 13). Catabolic enzymes for steroid degradation are not constitutively expressed, but are induced by
their respective steroid substrates (18, 20, 24). There is considerable interest in the mechanism of
the induction of steroid-catabolic enzymes expression and the basis of their transcriptional
regulation. In previous investigations, 3α-HSD/CR from *C. testosteroni* ATCC 11996 was
identified to be induced by steroids such as testosterone and the expression of this enzyme was
controlled by several regulators including repressors, RepA and RepB, and positive regulator
TeiR (7, 38, 39).

In the present investigation, the novel transcription factor HsdR was found to serve as an
activator of *hsdA* expression. To search for further cis- or trans-acting elements for 3α-HSD/CR
expression which might locate upstream or downstream from the *hsdA* gene, a 5.257 kb EcoRI
genomic fragment containing *hsdA* was extended leftward or rightward. Sequence analysis
revealed that a LysR-type transcriptional regulator (LTTR) gene (which we later named “hsdR”)
is located 2.58 kb downstream from the *hsdA* gene. Extensive studies revealed that LTTRs have a
highly conserved N-terminal DNA binding domain and a less conserved C-terminal coinducer
recognition domain, and they often act as homotetramers (29, 32). The HELIXTURNHELIX
program (http://www.pasteur.fr) predicted that HsdR contained a winged helix-turn-helix DNA-
binding motif in the N-terminal region. Moreover, at the C-terminus a LysR-type substrate
binding domain was identified, as revealed by BLAST search. In the course of our further
investigation, HsdR was shown to bind to the promoter region of the *hsdA* gene. In general,
LysR-type regulators act as tetramers or dimers to perform their function and to recruit RNA
polymerase for subsequent gene transcription. Recruitment of RNA polymerase was also shown
in this study for HsdR induced *hsdA* expression.
LTTRs constitute the largest family of prokaryotic regulatory proteins identified so far (15). The genes regulated by LTTRs have diverse functions including the degradation of organic compounds. In this study, HsdR has been identified to regulate the expression of 3α-HSD/CR, which is one of the first enzymes in the steroid-catabolic pathway and also catalyzes the carbonyl reduction of nonsteroidal aldehydes and ketones (24). Apart from this enzyme, many LTTRs are associated with degradation pathways of aromatic compounds. A large group of LTTRs regulates a single target operon only, such as CatR controlling catBCA expression for catechol metabolism in Pseudomonas putida (28). Operons such as the clcABDE operon of plasmid pAC27 (4), the tcbCDEF operon in plasmid pP51 (36) and the cbnABDE operon from Ralstonia eutropha (21) involved in chlorocatechol metabolism were controlled by ClcR, TcbR and CbnR (4, 21). Two paralogous LTTRs, BenM and CatM from Acinetobacter sp. strain ADP1 controlling the expression of several operons involved in benzoate degradation, were extensively studied (5, 27).

In general, LTTRs have been described as transcriptional activators of a single divergently transcribed gene, which exhibited negative autoregulation (14, 25, 32). Extensive research has now led to them being regarded as global transcriptional regulators, acting as either activators or repressors of single or operonic genes; they are often divergently transcribed, but can be located elsewhere on the bacterial chromosome (10, 11). In this study, hsdR was located 2.58 kb downstream from its target gene hsdA on the C. testosteroni chromosome. In addition, a further short-chain dehydrogenase/reductase gene, SDRx, was found to be divergently transcribed from the hsdR gene. SDRx is seemingly related to 7α-HSDs as revealed by phylogenetic analysis. 7α-HSD is an NADP(H)-dependent oxidoreductase belonging also to the short-chain dehydrogenase/reductase (SDR) superfamily (34). 7α-HSDs are widespread among bacteroides.
and clostridia and occur as well as in *E. coli* and *Ruminococcus* species. They catalyze the dehydrogenation of a hydroxyl group at position 7 of the steroid skeleton of bile acids (26). Interestingly, degradation velocities of the steroids cholic acid, testosterone and estradiol decreased in *SDRx* knock-out mutants of *C. testosteroni* (6).

Based on the results obtained in the present study, a model for regulation of *hsdA* expression by HsdR is proposed. In the absence of “inducing” steroids, the transcriptional repressor RepA binds to Op1 and Op2 to form a loop structure which contains the *hsdA* promoter domain. Due to the resulting DNA configuration, other transcriptional regulators such as HsdR cannot perform their function at the *hsdA* promoter even if already bound to the promoter (Fig. 8A). Therefore, 3α-HSD/CR expression is only at basal levels in both the wild type strain and the *hsdR* knock-out mutant (cf. Fig. 6). In the presence of an inducer such as testosterone, RepA is released from the operators and the loop structure is disrupted, such that HsdR can activate the *hsdA* promoter and increase the concentration of RNA polymerase at the promoter domain (Fig. 8B). Finally, *hsdA* expression increases. In conclusion, HsdR is a positive transcription factor for the *hsdA* gene and promotes induction of 3α-HSD/CR expression in *C. testosteroni*. Furthermore, HsdR activity is dependent on decreased repression by RepA.

**Acknowledgements**

We would like to give our thanks to the “State-Sponsored Scholarship Program for Graduate Students” funded by the China Scholarship Council for financial support. This work was supported by grants from the Deutsche Forschungsgemeinschaft (MA 1704/4-1; MA 1704/4-2).
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Figure Legends

Fig. 1. Genetic organization of the *hsdA* gene and its regulatory elements together with the schematic illustration of the various constructs of the *hsdR* gene. A, two negative regulator genes, *repA* and *repB*, are located in the vicinity of the *hsdA* gene (38, 39), and a positive regulator gene, *teiR* (7), upstream of the *hsdA* gene. A novel transcriptional regulator, HsdR, encoding by *hsdR*, of *hsdA* expression was identified 2.58 kb downstream of the *hsdA* gene. Open reading frames are indicated as arrows and restriction sites are abbreviated as one-letter code (A, AvrII; B, BamHI; Bg, BglII; Bs, BssHI; C, ClaI; E, EcoRI; H, HindIII; K, KpnI; M, MluI; N, NdeI; No, NotI; P, PstI; X, XamI). Op: operator; *ksi*: gene encoding ketosteroid isomerase. B, the *hsdR* gene and fragments thereof are drawn as horizontal lines and were subcloned into vectors, pK18 (pK), pET15b (pET), or pCR2.1-TOPO (pTOPO). The first four plasmids were used for *E. coli* and *C. testosteroni* transformation, and the plasmid in the bottom line for the preparation of an *hsdR* knock-out mutant in *C. testosteroni*.

Fig. 2. HsdR activates expression of 3α-HSD/CR. 3α-HSD/CR (µg/mg protein) was assayed by ELISA after co-transformation of *E. coli* with pKHsdR3 and plasmids containing *hsdA*. Plasmid p6 contained the entire 5.257 kb EcoRI fragment of *C. testosteroni* chromosomal DNA including *hsdA* and its regulatory elements (38). In plasmid pAX1 operator Op1 was deleted. Finally, in plasmid pDel13n additional 13 bases upstream of *hsdA* promoter had been deleted. Induced expression of 3α-HSD/CR is observed with plasmid pAX1, but not with p6 (control) nor with pDel13n. Bars represent the average and standard deviation of at least three independent
measurements (*, p < 0.05, t test; ***, p < 0.0001, t test).

Fig. 3. HsdR activity is associated with the function of RepA. Under normal conditions, active Repressor A (RepA) binds to two operators Op1 and Op2 upstream of the \textit{hsdA} gene and forces the DNA between these two palindromic sequences to form a loop structure (38, 40). Plasmids p67, p67-3, p67-5 and p67-7 with varying distances between Op1 and Op2 (67, 64, 62 and 60 bp, respectively) upstream of the \textit{hsdA} gene, were co-transformed into \textit{E. coli} HB101 with plasmids pK18 (control) or pKtacHsdR1 (containing \textit{hsdR}). \(3\alpha\)-HSD/CR expression in \textit{E. coli}, as determined by ELISA, was sensitive to the DNA-loop topology (for details see text). Bars represent the average and standard deviation of at least three independent measurements (***, p < 0.0001, t test).

Fig. 4. HsdR binds to the \textit{hsdA} promoter region. A DNA fragment of 203 bp containing the promoter domain of the \textit{hsdA} gene was labeled with DIG-11-dUTP upon amplification by PCR. The labeled DNA fragment was mixed with different concentrations of HsdR and/or RepA, respectively, and herring sperm DNA was used as the competitor DNA. The reaction mixtures were subjected to 5% native PAGE. A, 10 ng of DNA probe was incubated with different amounts of HsdR protein. Compared to free DNA (lane 1), HsdR-DNA binding leads to a shift of the corresponding bands (lanes 2 and 3). B, different concentrations of purified RepA were added to the HsdR-DNA reaction mixture. Compared to the control (lane 5), RepA and HsdR were shown to bind to different sites of the \textit{hsdA} promoter (lanes 1 to 4) (for details see text).

Fig. 5. HsdR interacts with RNA polymerase. RNA polymerase (RNAP) was first coated onto
the ELISA plates followed by addition of various concentrations of HsdR protein. After incubation with primary antibodies against HsdR, peroxidase-conjugated swine anti-rabbit immunoglobulin (anti-rabbit) was added as secondary antibodies. Finally, the samples were assayed in an ELISA reader (BIO-RAD). A clear and concentration dependent binding of RNAP to HsdR is seen. Bars represent the average and standard deviation of at least three independent measurements.

Fig. 6. HsdR is necessary for the induced expression of 3α-HSD/CR. Wild type cells (C.T.) and hsdR knock-out mutants of C. testosteroni (CT-HsdR-Ko) were cultured overnight in SIN medium in the presence or absence of 0.5 mM testosterone. ELISA revealed that after testosterone induction 3α-HSD/CR expression increased considerably in wild type C. testosteroni, compared to that observed in hsdR knock-out mutants. In the absence of the steroidal inducer testosterone, 3α-HSD/CR expression in the hsdR knock-out mutants and in wild type cells occurred at the same basal level. Bars represent the average and standard deviation of at least three independent measurements (***, p < 0.0001, t test).

Fig. 7. 3α-HSD/CR but not HsdR expression is induced by testosterone. C. testosteroni was cultured overnight at 27°C in the presence or absence of 0.5 mM testosterone. Total protein was extracted and adjusted to 1 mg/ml. 3α-HSD/CR and HsdR expression was determined by ELISA. Whereas 3α-HSD/CR expression was induced by testosterone (A), HsdR expression did not change upon testosterone induction (B). Therefore, HsdR itself is not induced by testosterone but necessary for steroid signaling in C. testosteroni. Bars represent the average and standard deviation of at least three independent measurements (***, p < 0.0001, t test).
Fig. 8. Model for the regulation of \textit{hsdA} expression by HsdR in \textit{C. testosteroni}. A, in the absence of the inducing steroid testosterone, the RepA protein binds to operators Op1 and Op2 and blocks \textit{hsdA} transcription. The loop structure formed by RepA binding does also affect other transcription regulators such as HsdR to activate the \textit{hsdA} promoter. B, in the presence of testosterone, RepA is released from the operators and the loop structure is disrupted. Thus, HsdR can now activate the \textit{hsdA} promoter and recruit RNA polymerase (RNAP) to activate \textit{hsdA} transcription.
FIGURE 1

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Op1 (TCAAGCCCA)  0.935

Op2 (TGGGCTTTGA)  2.568

hsdA  (2.615 - 3.388) (3.544 - 3.921)

ksi  (2.065 - 3.328)

repA  (4.084 - 4.320)

repB  (4.084 - 4.320)

hsdR  (5.975 - 6.887)

SDRx  (6.994 - 7.762)

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teIR  (-55.135 - -53.961)

B

pTOPOHsdR  912 bp

pKhsdR3  912 bp  plac

pKtaHsdR1  912 bp  ptac

pETHsdR2  912 bp  6xHis

pTOPOHsdR3-1  650 bp  251 bp

31
FIGURE 3

![Graph showing 3H-ISDCH µg/mg total protein levels for p67, p67-3, p67-5, and p67-7.]

- p67
  - 67 bp hsdA
  - 64 bp repA

- p67-3
  - 64 bp hsdA
  - 62 bp repA

- p67-5
  - 62 bp hsdA
  - 60 bp repA

- p67-7
  - 60 bp hsdA

Legend:
- pK18
- pK tac HsdR1

Statistical significance:
- **p < 0.001
FIGURE 4

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1 2 3 4 5
FIGURE 5

- OD of HsdR binding
- RNAP (ng): 0, 300, 300, 300, 300, 300, 300, 300, 0, 0
- HsdR (ng): 20, 20, 10, 5, 2.5, 1.25, 0.625, 0, 0, 0
- Anti-HsdR (1:1,000): +, +, +, +, +, +, +, +, -
- Anti-rabbit (1:1,000): +, +, +, +, +, +, +, +, -
FIGURE 6

![Bar graph showing the comparison of Se-HSD/CR µg/mg with and without testosterone between C.T. and CT-HsdR-Ko samples.]

- Without testosterone
- With testosterone

C.T. CT-Hs dR-Ko

0 20 40 60 80 100
Se-HSD/CR µg/mg soluble protein

Legend:
- Square: without testosterone
- Solid square: with testosterone

Statistical significance: ***
FIGURE 7

A

![Graph A]

B

![Graph B]

3α-HSD/CR μg/mg total protein

Testosterone

0 20 40 60 80

0 10 20 30 40 50 60 70 80 90 100

Testosterone

0 5 10

0 37
FIGURE 8

A: in the absence of inducing steroids

B: in the presence of appropriate steroids