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Cloning, Sequencing and Expression of *Pseudomonas testosteroni* Gene Encoding 3α-Hydroxysteroid Dehydrogenase

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We describe the cloning, sequencing and expression of the 3α-hydroxysteroid dehydrogenase (3α-HSD) gene of *Pseudomonas testosteroni*. A genomic library of *P. testosteroni* total DNA constructed from SauIIIA digests ligated to an λgt11 vector was probed with a polyclonal antibody raised against purified enzyme. Subclones derived from a recombinant phage containing a 1746 bp insert were sequenced and found to contain an open reading frame of 696 bp that corresponds to a protein of 231 amino acid residues. A search for homologous proteins was performed. No similarity was observed when comparing 3α-HSD with known members of the short-chain dehydrogenase family. However a small proteic fragment (80 amino acids) shows homology with the N-terminal sequence of bacterial L7/L12 ribosomal proteins.

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INTRODUCTION

3α-hydroxysteroid dehydrogenase [3α-HSD; EC 1.1.1.50] catalyzes the reversible oxidation of 3α-hydroxyl groups of the steroid nucleus [1]. This enzymatic reaction was described, the enzyme characterized and purified in *Pseudomonas testosteroni* [2, 3], a gram-negative bacteria that can grow on steroids as its only source of carbon [2-4]. Based on the molecular weight and the substrates specificities, *Pseudomonas* 3α-HSD seems to belong to the short-chain alcohol dehydrogenase family [5].

Recently, the short-chain alcohol dehydrogenase family has attracted renewed attention by discovering that procaryotic as well as eukaryotic proteins belong to this family. Although related, all these enzymes are highly dissimilar, with residue identity only at the 15–35% level, compatible with distant relationships [6, 7].

In a previous work, we have cloned and sequenced the structural gene encoding the (3-17)β-HSD of *P. testosteroni*; a member of this enzyme family [8]. To obtain more information on the short-chain dehydrogenase family, we have cloned and sequenced the gene of the 3α-HSD.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

The wild type, *P. testosteroni* (ATCC 11996) used in this study was originally obtained from the Pasteur Institute (Paris). *E. coli* Y1090 was used for construction of a bacterial genomic library in vector λgt11, HB101 for identification; JM101 for sequencing and K38 harbouring pGP1-2 [9] for gene expression studies. *E. coli* strains (HB101, and Y1090) were grown at 37°C in LB broth; ampicillin was added, when needed, at the following concentration 50 μg/ml, K38 strain was grown at 30°C in the presence of kanamycin at 40 μg/ml.

Pseudomonas testosteroni was routinely grown at 30°C in a liquid medium according to Schultz *et al.* [10]. Induction of 3α-HSD was obtained by adding testosterone (250 μg/ml) in an overnight culture, and growth was continued for 24 h.

3α-HSD antiserum

Polyclonal antiserum against purified bacterial 3α-HSD was raised in rabbits. Antiserum was absorbed with *E. coli* strain Y1090 lysate to remove antibodies that recognize *E. coli* antigens [11].

Preparation of P. testosteroni DNA

Pseudomonas testosteroni DNA was isolated following Marmur's method [12] and partially digested by SauIIIA under conditions rendering partially digested DNA fragments with an average fragment size of 2–5 kb. Digested DNA ends were filled in and then transformed into EcoRI cohesive ends by ligation of EcoRI linkers. Digested DNA was ligated into the EcoRI site of λgt11 and packaged into particles following the conditions recommended by the supplier (Amersham Corp. Arlington, U.S.A.).

Antibody screening of λgt11 library

About 10^6 plaque-forming units (pfu) of recombinant phages from a bacterial genomic library were screened with an 3α-HSD antiserum, diluted 1:2000 in Tris-buffered saline containing 3% bovine serum albumin. Immunopositive plaques were further purified by three cycles of screening at low plaque density with antiserum until all phages produced positive signals. Twelve positive phage clones were amplified and the recombinant DNA was isolated as described [13]. The inserts were excised by digestion with EcoRI, purified after electrophoresis using the Geneclean kit (Bio 101, La Jolla, CA, U.S.A.); and subcloned into pBR322, pT7-3 and pT7-4 vectors.

DNA sequencing

The DNA fragments to be sequenced were ligated into M13mp18 and M13mp19 and transformed into *E. coli* JM101. Nucleotide sequence determination was carried out by the chain termination of Sanger [14] using a commercial sequencing kit from Amersham. Open reading frame region was determined by using the DNASIS software from Pharmacia.

3α-HSD activity assay

Assay for 3α-HSD was performed in 5 ml mixture containing: 100 mM Tris–HCl, pH 8.0, 2.7 nmol 3α-androstanediol + 200,000 dpm [^{3}H]3α-androstanediol, 1 mM NAD and appropriate amounts of bacterial extracts. Dehydrogenation was assayed at 25°C for 10 min. The reactions were terminated by addition of 5 ml cold ethyl acetate. All radioactive metabolites were extracted four times with 5 ml of ethyl acetate. The extracts were pooled and evaporated under nitrogen. Recovery of steroid was monitored by counting the radioactivity from an aliquot and was always close to 100%. The extracts were dissolved in 200 μl of ethyl acetate containing 20 μg of nonradioactive steroids (3α-androstanediol, testosterone, 5α-dihydrotestosterone, Δ4-androstenedione, isoandrosterone and 5α-androstanedione) and applied on silicagel TLC plates (60F 254, size 20 × 20 cm, thickness 0.25 mm; Merck, Darmstadt, Germany). The plates were developed twice in a solvent system of toluene-ethanol (9:1, v/v). The regions of the chromatograms corresponding to the standard steroids were scrapped. Radioactivity present in fractions was measured using a Packard Liquid scintillation Spectrophotometer Tricarb 3000. Specific activity was expressed as nmol 5α-dihydrotestosterone formed/mg protein/min.

Protein determination

Protein was measured by the method of Lowry *et al.* [15], using bovine serum albumin as the reference standard.

Gene expression in the bacteriophage T7 promoter—RNA polymerase system

The inserts were cloned downstream of the strong T7φ10 promoter, in the two possible orientations with respect to φ10, depending in the use of pT7-3 or pT7-4. The hybrid plasmids were introduced into *E. coli* K38, which contained the compatible plasmid pGP 1-2, encoding T7 RNA polymerase under the control of the inducible promoter and the gene encoding the heat sensitive λ repressor cI 857.

Labelling of the products of the genes cloned downstream of φ10, was performed as described by Tabor and Richardson [9], slightly modified by Trautwetter and Blanco [16].

Electrophoresis and immunoblotting

Pure 3α-HSD and crude bacterial extracts were electrophoresed on a denaturing 10% polyacrylamide (29-1) gel according to Laemmli [17]. After electrotransfer to nitrocellulose membrane, the unspecific membrane protein binding sites were blocked with 5% bovine serum albumin, and the membrane incubated with a 1/5000 dilution of 3α-HSD antiserum. The immunocomplex was detected with anti-rabbit IgG-alkaline phosphatase complex.

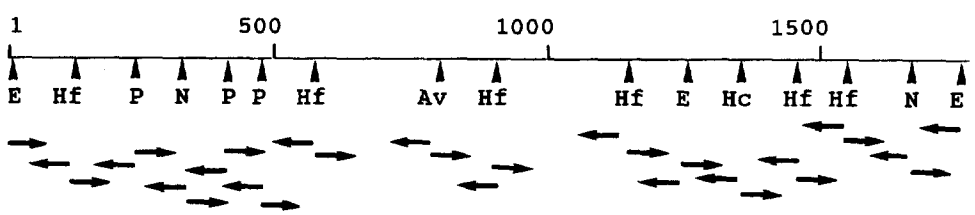

Fig. 1. Restriction map and sequencing strategy of the cloned DNA. The 696 bp region encoding the HSD is indicatied by the box. The horizontal arrows below the restriction map indicate the start points and the direction of the DNA-primed synthesis used for sequence analysis of subclones by the dideoxynucleotide method [14]. E, EcoRI; P, PstI; Hc, HincII; Hf, HinfI; N, NcoI; Av, AvaII.

```
5'                                                                    80
GAATTCCGATCCTGGGCCCCCGCGGCTGATGCCCAACCCCAAGGTTGGCACCGTGACTCCTGACGTCGCTACGGCTGTGA
                                                                     160
AGAACGCCAAGGCTGGTCAAGTGCAGTTCCGCGTGGACAAGGCTGGTATCGTGCACGGCACCGATCGGTCGCCGTTCGTT
                                                                     240
CGATGCCGACAAGCTGCAGGGCAACCTGGCTGCTCTGATCGACGCACTGAACAAGGCCAAGCCTGCTTCCAGCAAGGGTC
                                                                     320
TGTACCTGCGCAAGGTGGCTGTTTCGTCGACCATGGGTGTGGGCGTTCGCGTCGATACACAATCCATCTCGGCGTAATTG
                                                                     400
CTAAAAATCGTCAGATGAGCTTTGCTCATCTGGTGTGGTGGGCTGCGGGAGTTGTTGCTCCTGCAGGTCATCCAAGACCG
                                                                     480
TTGGTGTGATTTGATCACTTAATCACGAAGGCCAACGCAGATGGCGATCCCGCTGCAGATGGAATTTCTAAACAGTTGGT
                                                                     560
CGCTGCAACAAGAGCGTGTATGCAGGGCTTGCCTTGTATATGCATTTGAAGGAGTAGACCTTGAGTCTGAATCGCAGTGA
                                                                     640
GAAAGAAGCGGTCATTTCCGAAGTGACCAGCCTCGCCGCTAAAGCTCAAACGCTTGTGATCGCGGAATACCGTGGCATCA
                                                                     720
CGGTCGCCGACATGACCAAACTGCGTGCCGATGCTCGCAGCAAGGGTGTGAGCCTGAGCGTGTTGAAGAACACCTGGCAC
                                                                     794
CGCCGTGCTGTTGCCGGTAGCGCGTTTGAGAGGAGGCTGACCAGATGACTGGTCCCCTGATCTATGGCTTCTCT
                                                  MetThrGlyProLeuIleTyrGlyPheSer
                                                                     860
GAAGACGCAGTGCGTGCCGCTAAGGTGGTGGCCGACTTCGCGAAGACCAACGACAAGTTGGTGATT
GluAspAlaValArgAlaAlaLysValValAlaAspPheAlaLysThrAsnAspLysLeuValIle
                                                                     926
CGCGGTGGCGCGTTCGGAGGCAAGGCCCTGGACATCGACGGCGTTAAGCAACTGGCAAACATCCCC
ArgGlyGlyAlaPheGlyGlyLysAlaLeuAspIleAspGlyValLysGlnLeuAlaAsnIlePro
                                                                     992
TCCAAGGAAGTACTGCTCGCACAAGTGTGCGGCTTGCTCATGTCGCCTATCTCGCGTACAGCCGTT
SerLysGluValLeuLeuAlaGlnValCysGlyLeuLeuMetSerProIleSerArgThrAlaVal
                                                                    1058
GTGCTGGGCGCTCTGCGCAGAGGCGAAGCTCTGCGAAGCGGCAAACAGAAACAGCCGCAGCCTGAG
ValLeuGlyAlaLeuArgArgGlyGluAlaLeuArgSerGlyLysGlnLysGlnProGlnProGlu
                                                                    1124
TGCTCGGCAAACCAACCAAATTTTAGGAAAATCAAAATGGCATTCGATAAAGACGCATTCCTGACC
CysSerAlaAsnGlnProAsnPheArgLysIleLysMetAlaPheAspLysAspAlaPheLeuThr
                                                                    1190
GCCCTGGACAGCATGACTGTTCTGGAACTGAACGACCTGGTCAAGGCTATTGAAGAGAAGTTTGGT
AlaLeuAspSerMetThrValLeuGluLeuAsnAspLeuValLysAlaIleGluGluLysPheGly
                                                                    1256
GTCCGCCGCTGCTATGGCTGCTCCGCTGCTGGCGGCGCTGCTGGTGGCGCTGCTGCTGTTGAAGAA
ValArgArgCysTyrGlyCysSerAlaAlaGlyGlyAlaAlaGlyGlyAlaAlaAlaValGluGlu
                                                                    1322
AAGACCGAATTCAACGTGGTGCTGACCGACGCTGGCGCCAACAAGGTGTCCGTGATTAAGGCTGTG
LysThrGluPheAsnValValLeuThrAspAlaGlyAlaAsnLysValSerValIleLysAlaVal
                                                                    1388
CGCGAAATCACCGGCCTGGGTCTGAAGGAAGCCAAGGATCTGGTCGACGGCGCTCCCAAGACCGTG
ArgGluIleThrGlyLeuGLyLeuLysGluAlaLysAspLeuValAspGlyAlaProLysThrVal
                                                                    1454
AAGGAAGCCGCTCCCAAGGCTGACGCTGAAGCCGCTGTGAAGAAGCTGGTTGAAGCCGTGCCACTG
LysGluAlaAlaProlysAlaAspAlaGluAlaAlaValLysLysLeuValGluAlaValProLeu
                                                                    1532
AACTGACTGAAGTAATTCAGTGAAAATCAAGGCTGAGACTCGTAAAAGGGTCTCCGACCTTTGGCGCTTGTGAAGTGC
Asn***
                                                                    1611
CTTAAAGAACACACCAGAAAGCAGGTTTCCTAGGGAGTCTGCTTTTTAGTGTCTTCTGACCAATCCGACAGCAGAAGAT
                                                                    1690
GCCTTGGTTCGGGCGATGTGCAACGCATCGCCGTCAGCCATGGTTGGTAGTGGCCAACCGCCAAGCCCGCAGGAGAACC
                                                                    1746
TGTGGGGCAGTCGTCGCAGACCCAGGACTCATGTCTTTGCCCGGAGATCGGAATTC 3'
```

Fig. 2. Nucleotide and deduced amino acid sequence of the open reading frame of the 3α-HSD gene. The underlining indicates the Shine–Dalgarno (ribosome binding sequence); a putative promoter has an overbar.

RESULTS

Isolation of the 3α-HSD gene

We screened 1,000,000 recombinant clones carrying *P. testosteroni* chromosomal fragments of 2–5 kb pairs for the presence of 3α-HSD with a specific 3α-HSD antiserum.

One positive clone, reacting with antiserum was selected and further purified by immunoscreening. From the last screening 12 clones were selected. λgt11 recombinant DNA from these clones were isolated, digested with EcoRI, and the fragments were separated by agarose-gel electrophoresis. The size of the insert was estimated to be 1.7 kb pairs with an internal EcoRI restriction site. A restriction map of the insert in shown in Fig. 1.

DNA sequencing of the 3α-HSD gene

The nucleotide sequence (1746 bp) of the cloned DNA was determined by the strategy shown in Fig. 1. The nucleotide sequence of the strands (Fig. 2) contained a single open reading frame of 695 bp, which includes the initiation codon at ATG and the termination codon TGA ending at position 1460, of sufficient length to accommodate 3α-HSD.

The molecular weight of the processed protein with amino acid residues is calculated from the predicted amino acid sequence, to be 24,195 Da, which is in good agreement with that of the purified 3α-HSD of 23,000 Da determined by SDS–polyacrylamide gel electrophoresis. The G + C% content of the coding region is 58.9 mol%, which is close to that of other *Pseudomonas* genes and suggest its origin from the genus *Pseudomonas* [18, 19].

There is a purine-rich region, 5-AGAGGA which could be regarded as the ribosome-binding sequence (S.D. sequence [20]) for the 3α-HSD gene. Upstream of the Shine–Dalgarno sequence there was a sequence (TGGC-11 bases-TGGC) homologous with those of the promoters of several other *Pseudomonas* genes [21] located 30–15 bases upstream of the SD sequence.

Table 1. 3α-HSD activity of P. testosteroni and E. coli containing recombinant plasmids

Strain	3α-HSD (nmol/mg Prot./min)
HB101	—
HB101 + Testosterone	—
HB101-pBR322	—
HB101-pBR322 + Testosterone	—
HB101-PT3A	53
HB101-pPT3A + Testosterone	52
Pseudomonas	44
Pseudomonas + Testosterone	6140

(—) not detectable.

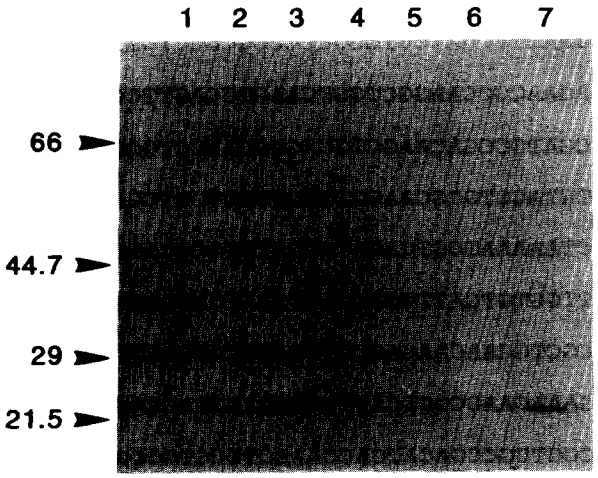

Fig. 3. Immunoblot analysis of purified 3α-HSD and of bacterial crude extracts with 3α-HSD antiserum. Lanes 1, *E. coli*; 2, *E. coli* + testosterone; 3, *P. testosteroni* induced by testosterone; 4, *P. testosteroni* uninduced; 5, *E. coli* + pPt3A uninduced; 6, *E. coli* + pPT3A induced by testosterone; 7, purified 3α-HSD. Molecular mass size standards: bovine serum albumin (66 kDa); ovalbumin (44.7 kDa); carbonic anhydrase (29 kDa); trypsine inhibitor (21.5 kDa).

Expression of 3α-HSD in E. coli

The cloned DNA with its internal EcoRI site, obtained by partial digestion of λgt11 recombinant DNA, was isolated from agarose gel and subcloned into pBR 322. The recombinant plasmid, designated pT3A was then introduced into *E. coli* strain HB101.

P. testosteroni grown on a medium containing no added testosterone has a very low 3α-HSD specific activity. Addition of testosterone to the growth medium enhanced the specific activity 140 times (Table 1).

The parental *E. coli* strain bearing the pBR322 vector has no detectable enzymatic activity. On the contrary, the recombinant strain containing pT3A expressed 3α-HSD at a low level close to that observed in

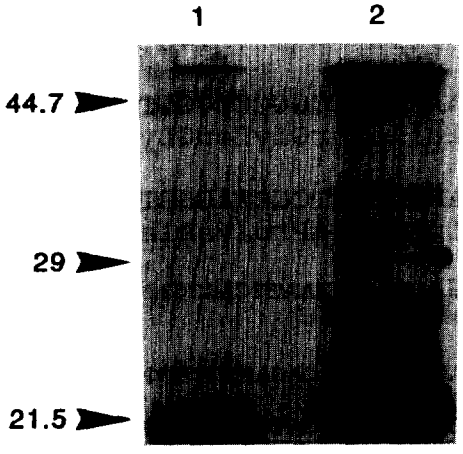

Fig. 4. Synthesis of 3α-HSD protein in the T7 expression system of Tabor and Richardson [9]. Autoradiograph of SDS–PAGE gels showing [^{35}S]methionine-labelled polypeptides encoded by the cloned sequences. Lanes: 1, pT7-4; 2, pT7-4 + Insert.

uninduced *P. testosteroni*. Testosterone did not induce any increase in specific activity in recombinant *E. coli* (Table 1).

Immunoblotting of crude extracts from *E. coli* containing the plasmid pT3A and from *P. testosteroni* showed that the 3α-HSD antiserum detects a single protein only in extracts from *P. testosteroni* grown in the presence of testosterone. Extracts from uninduced *P. testosteroni* and *E. coli* strains bearing pT3A contain 3α-HSD protein at a concentration too low to be detected by immunoblotting (Fig. 3).

To ascertain the presence of 3α-HSD gene in the cloned DNA, the insert was subcloned into pT7-4 and introduced into *E. coli* strain K38, the recombinant plasmid expressed a polypeptide of 23 kDa (Fig. 4).

DISCUSSION

We have identified the genomic region coding for 3α-hydroxysteroid dehydrogenase from *P. testosteroni* using a specific antibody, and cloned this gene on a 1.7 kb DNA fragment.

The base composition of the 1.7 kb DNA fragment was found to be 59% G + C, which is similar to that reported previously for other species of *Pseudomonas* [22]. This high G + C value is due in large part to the codon usage preference found in the coding region for the 3α-HSD gene, where there is a strong bias (68%) toward guanine—or cytosine—terminated codons, this preferential codon usage appears to be common in *Pseudomonas* [23].

Analysis of the region upstream of the translational start of 3α-HSD gene revealed a sequence of 30 nucleotides which shows strong homology to a set of positively controlled promoters proposed to be transcribed by a σ^{54}-like RNA polymerase holoenzyme [24]. This class of promoters is recognized on the basis of a minimal canonical sequence of GG-10 bp-GC at position −24 to −12 from the mRNA start site [21]. There was no DNA sequence preceding the RBS that showed homology to *E. coli* promoters [25, 26]. 3α-HSD was expressed at a very low level in recombinant *E. coli* strain HB101, close to that observed in uninduced *P. testosteroni*. Furthermore, testosterone (when added to the culture medium) did not induce any increase in 3α-HSD specific activity in *E. coli* recombinant strains. It is known that genes derived from *Pseudomonas* species are usually expressed at low levels in *E. coli* [27] owing to differences in promoter structure. Therefore, it is likely that the 3α-HSD gene in pPT3A was transcribed from its own promoter resulting in the low gene expression. In a previous work we have found, in the 5′ flanking region of the (3-17)β-HSD gene from *P. testosteroni*, several sequences presenting high homology with positively controlled promoters from other *Pseudomonas* genes [8]. (3-17)β-HSD was expressed at a high level in recombinant *E. coli* strains. The sequences could represent a very strong gene promoter, activatable in heterologous bacterial strains. On the contrary, in the 5′ flanking region of the 3α-HSD gene we could only find a single sequence representing *Pseudomonas* gene promoter, as a result, 3α-HSD was expressed at a low level, the promoter was not strong enough to promote expression in the heterologous strain.

The nucleotide sequence of the 3α-HSD gene was compared with all known sequences [28, 29]. The nucleotide sequence showed no significant overall homology with any other sequence in the Genbank or EMBL data base.

The amino acid sequences of several dehydrogenases involved in the metabolism of steroids, members of the short-strain alcohol dehydrogenase family, did not exhibit any sequence similarity to 3α-HSD. The deduced amino acid sequence of 3α-HSD was compared with the sequences in the NBRF protein data base. Surprisingly, a conserved region between residues 120–200 of 3α-HSD showed significant similarity (50 %) to N-terminal sequences from bacterial L7/L12 ribosomal proteins. These ribosomal proteins are involved in binding elongation factors during protein synthesis [30]. This elongation factor-binding site is located at the C-terminal part of L7/L12 protein, a sequence which does not share any similarity with 3α-HSD. At the moment we have no reasonable explanation for this surprising similarity.

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