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Cloning, expression and characterization of a putative 7alpha-hydroxysteroid dehydrogenase in *Comamonas testosteroni*

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ABSTRACT

The short-chain dehydrogenase/reductase (SDR) superfamily is a large and diverse group of genes with members found in all forms of life. *Comamonas testosteroni* (*C. testosterone*) ATCC11996 is a Gram-negative bacterium which can use steroids as carbon and energy source. In the present investigation, we found a novel SDR gene 7alpha-hydroxysteroid dehydrogenase (*7α-HSD*) which is located 11.9 kb upstream from *hsdA* with the same transcription orientation in the *C. testosteroni* genome. The open reading frame of this putative 7alpha-hydroxysteroid dehydrogenase gene consists of 771 bp and translates into a protein of 256 amino acids. Two consensus sequences of the SDR superfamily were found, an N-terminal Gly-X-X-X-Gly-X-Gly cofactor-binding motif and a Tyr-X-X-X-Lys segment (residues 161–165 in the *7α-HSD* sequence) essential for catalytic activity of SDR proteins. To produce purified 7α-HSD protein, the *7α-HSD* gene was cloned into plasmid $p^{ET\text{-}15b}$ and the over expressed protein was purified by His-tag sequence on metal chelate chromatography. To prove that 7α-HSD is involved in the metabolic pathway of steroid compounds, we constructed a *7α-HSD* knock-out mutant of *C. testosteroni*. Compared to the wild type *C. testosteroni*, degradation of testosterone, estradiol and cholesterol were decreased in the *7α-HSD* knock-out mutant.

Furthermore, growth in the medium with testosterone, estradiol and cholesterol was impaired in *7α-HSD* knock-out mutant. The results showed that 7α-HSD is involved in steroid degradation.

1. Introduction

Short-chain dehydrogenase/reductases (SDRs) constitute one of the largest enzyme superfamilies of NAD (P) (H)-dependent oxidoreductases which are distinct from the medium-chain dehydrogenase/reductases (MDR) and aldo-ketoreductase (AKR) superfamilies (Hyndman et al. 2003; Joernvall et al. 1995; Nordling et al. 2002). Found in all forms of life (archaea, bacteria and eukaryotes), SDRs metabolize a range of substrates including aliphatic aldehydes and ketones, monosaccharides, steroids, prostaglandins, flavonoids, polycyclic aromatic hydrocarbons, and retinoids. Several of these SDR substrates are known to serve as important intra or intercellular signal molecules in pro and eukaryotes, which is especially true for steroids, retinoids, and flavonoids (Duester 1996; Persson et al. 2005).

Comamonas testosteroni ATCC11996 is a Gram-negative bacterium that belongs to the beta group of the Proteobacteria. These strictly aerobic, non-fermentative, chemoorgano-trophic bacteria rarely attack sugars, but grow well on organic acids and amino acids (Willems et al. 1992). Moreover, this bacterium is able to grow on steroids or aromatic hydrocarbons as sole carbon and energy source and thus may represent an important means in the mineralization of these stable compounds. Genome Sequence of *C. testosteroni* ATCC 11996 has been reported. In earlier investigations that 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) from *C. testosteroni* is a member of the SDR superfamily and catalyzes the reversible interconversion of hydroxyl and oxo groups at position 3 of the steroid nucleus of a great variety of C19–27 steroids (Möbus et al. 1997; Möbus and Maser 1998). In addition, 3α-HSD/CR mediates the carbonyl reduction of non-steroidal aldehydes and ketones. Interestingly, the 3α-HSD/CR gene (*hsdA*) expression is induced by steroids such as testosterone and progesterone (Möbus et al. 1997; Oppermann et al. 1993; Oppermann and Maser 2004). 3α-HSD/CR from *C. testosteroni* is a member of the SDR superfamily and catalyzes the reversible interconversion of hydroxyl and oxo groups at position 3 of the steroid nucleus of a great variety of C19–27 steroids. The 7-dehydroxylation of bile acids is considered to be the most important of these reactions in a

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qualitative sense, because of the potential toxicity of the secondary bile acid products. Although the reaction products (deoxycholic acid and lithocholic acid) may constitute a significant proportion of the circulating bile acid pool, the ability to carry out the reaction is apparently restricted to a few species, mainly in the genera of Clostridium, Eubacterium, and possibly Bacteroides. On the other hand, the presence of bile acid and steroid dehydrogenases appears to be widespread among intestinal as well as soil microflora (Coleman et al. 1994). 7α-Hydroxysteroid dehydrogenase (7α-HSD) (EC 1.1.1.159), catalyzing the dehydrogenation of a hydroxyl group at position 7 of the steroid skeleton of bile acids, was found in numerous bacteria and mammalian liver (Ueda et al. 2004). It was shown recently, that a novel short-chain dehydrogenase/reductase gene (*SDRx*) in *C. testosteroni* ATCC11996 was found and shown to be located 3.6 kb downstream of the *hsdA* gene (Gong et al. 2012a,b). Phylogenetic analyses revealed that SDRx is a 7α-hydroxysteroid dehydrogenase (7α-HSD) that is involved in steroid metabolism.

In the present work, another novel short-chain dehydrogenase/reductase gene in *C. testosteroni* was found and locates 11.9 kb upstream of the *hsdA* gene. As a member of the SDR superfamily, this novel SDR also contains the common sequence motifs of SDRs that define the cofactor binding site Gly-X-X-X-Gly-X-Gly in the N-terminal region and the catalytic residue motif Tyr-X-X-X-Lys. Phylogenetic analyses revealed that the SDR is also a 7α-hydroxysteroid dehydrogenase (7α-HSD) and might be involved in steroid metabolism. In this work *7α-HSD* knock-out mutant was prepared. The result showed that 7α-HSD is important to steroid degradation and bacterial growth in *C. testosterone*.

2. Materials and methods

2.1. Bacterial strains and plasmids

Host strains *Escherichia coli* HB101 (Promega) and *C. testosteroni* (ATCC 11996) were used for cloning and gene expression. Cloning of PCR fragments was carried out in $p^{CR2.1\text{-}TOPO}$ (Invitrogen). For overexpression and purification of 7α-HSD, *E. coli* strain BL21 (DE3) pLysS was transformed with plasmid $p^{ET\text{-}15b}$ from Novagen.

2.2. Growth media and growth conditions

Bacterial cells were grown in a shaker (180 rpm) in LB medium at 37 °C (*E. coli*) or 27 °C (*C. testosteroni*). Growth media contained 100 μg/ml ampicillin or/and 30 μg/ml kanamycin.

2.3. Restriction enzymes and other reagents

Restriction enzymes were obtained from TaKaRa, TOYOBO, MBI, and used according to the manufacturers' instructions. Ampicillin and kanamycin were from Sangon Shanghai. Recombinant DNA work was carried out following standard techniques according to Sambrook and Russel (2001).

2.4. Cloning of the 7α-HSD gene from C. testosteroni and subcloning of 7α-HSD gene fragments

Chromosomal DNA sequence of *C. testosteroni* ATCC11996 was published in 2012 (Gong et al. 2012a,b). According to the sequence a novel 7α-HSD gene which is located 11.9 kb upstream from *hsdA* with the same transcription orientation was found. The *7α-HSD* gene in *C. testosteroni* was amplified by PCR. The corresponding primers for PCR were prepared. The forward primer contains an *Nde*I site: 5′-GCCATATGAACGAGATTTTTCG-3′ and the reverse primer contains a *Bam*HI site: 5′-CCGGATCCTCAGAAGTTGGGAGC-3′. The full 7α-HSD gene was then cloned into $p^{CR2.1\text{-}TOPO}$ to yield plasmid $p^{TOPO7\alpha\text{-}HSD}$. After sequence confirmation (Sangon), the *7α-HSD* gene fragment was subcloned into the *Nde*I/*Bam*HI restriction sites of $p^{ET\text{-}15b}$, which additionally codes for an N-terminal His tag sequence with an integrated thrombin cleavage site. The plasmid $p^{ET7\alpha\text{-}HSD}$ was used to prepare purified 7α-HSD protein.

2.5. Overexpression and purification of the 7α-HSD protein

Overexpression of *7α-HSD* was performed in *E. coli* strain BL21 (DE3) pLysS (Novagen) with plasmid $p^{ET7\alpha\text{-}HSD}$, and the recombinant protein was purified by its His-tag sequence. Cells transformed with plasmid $p^{ET7\alpha\text{-}HSD}$ were grown at 37 °C in a shaker (180 rpm), and maintenance of plasmids was ensured by adding 100 μg/ml ampicillin to the culture medium. An amount of 100 μl of the overnight culture was used to inoculate 3 ml of fresh medium. At an OD_{600} of 0.6, expression was induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After 4 h at 37 °C, cells were sedimented by centrifugation. The cell pellet was either stored at −80 °C for further usage or directly suspended in 200 μl of lysis buffer (8 M urea, pH 8.0) (Qiagen). Cells were lysed by freezing (−20 °C, 30 min) and thawing (room temperature, 30 min) 3 times, and the resulting mixture was centrifuged (20 min 13,000 rpm 4 °C). The supernatant was applied to a mini nickel agarose affinity column (Qiagen). After 2 times washing with 600 μl of washing buffer (8 M urea, pH 4.5) (Qiagen), the 7α-HSD protein was eluted from the column by applying 100 μl of elution buffer (8 M urea, pH 3.5) for 4 times.

2.6. ELISA of 7α-HSD

To quantify 7α-HSD protein expression, an ELISA was established and antibodies were generated. Rabbit antibodies against 7α-HSD from *C. testosteroni* were prepared according to standard methods (Xiong and Lutz 1992). ELISA plates were coated with supernatant of the cell were lysed by freezing (−20 °C, 30 min) and thawing (room temperature, 30 min) 3 times diluted with coating buffer. The protein concentration of the supernatant samples was adjusted to 1 mg/ml, and 200 μl was added to each well. After washing, antibodies against 7α-HSD were diluted in 1:8000 and 200 μl was added into each wells. The further procedure corresponded to that of the CAT ELISA kit from Jet Biofil (literature).

2.7. Generation of a 7α-HSD knock-out mutant of C. testosteroni

A *7α-HSD* knock-out mutant of *C. testosteroni* was prepared by homologous integration. A central part of the *7α-HSD* gene from 204 bp to 569 bp was generated by PCR using forward primer 5′-GCGATGTGAACAAAGAGGAAGA-3′ and reverse primer 5′-GCTCGATGGCGTTGATACG-3′ with plasmid template $p^{TOPO7\alpha\text{-}HSD}$. The PCR fragment was cloned into $p^{CR2.1\text{-}TOPO}$ to yield $p^{TOPO7\alpha\text{-}HSD366}$. Because of its sensitivity to kanamycin, only the mutant *C. testosteroni* in which $p^{TOPO7\alpha\text{-}HSD366}$ was integrated into the chromosomal DNA could grow in kanamycin medium, while $p^{TOPO7\alpha\text{-}HSD366}$ could not replicate as a plasmid in *C. testosteroni*. Accordingly, 10 μg of $p^{TOPO7\alpha\text{-}HSD366}$ was transformed into *C. testosteroni* by electroporation (1.8 kV, 1-mm cuvette, Bio-Rad), and the *7α-HSD* gene was disrupted upon integration of the plasmid into the chromosomal DNA. The cells were cultured and spread on 30 μg/ml kanamycin SIN agar plates, and then cultured in a 27 °C incubator overnight. The colonies on the plates were cultured in 1 ml kanamycin SIN medium and PCR (forward primer 5′-GCCATATGAACGAGATTTTTCG-3′ and reverse primer 5′-TCAGAAGAACTCGTCAAGAAGGCG-3′) was used to prove the right integration. The PCR sequence was confirmed by DNA sequencing (Shanghai Sangon Bioengineering Co., Ltd.).