微生物学报
Acta Microbiologica Sinica
2020, 60(2): 306–319
http://journals.im.ac.cn/actamicrocn
DOI: 10.13343/j.cnki.wsxb.20190150

Research Article 研究报告

Characterization of 17β-hydroxysteroid dehydrogenase 2 and the AraC regulator involved in 17β-estradiol oxidization in *Pseudomonas putida* SJTE-1

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Abstract: [Objective] *Pseudomonas putida* SJTE-1 can degrade17β-estradiol (E2) efficiently, but its degradation mechanism is still unclear. Here we characterized a 17β-hydroxysteroid dehydrogenase 2 (17β-HSD2) and one AraC regulator responsible for oxidization and regulation of E2 biodegradation. **[Methods]** We detected the transcription of *17β-hsd2* and *araC* by reverse transcription and quantitative PCR. We overexpressed 17β-HSD2 and AraC in *Escherichia coli* BL21(DE3) strain and purified them with metal-ion affinity chromatography. We characterized the enzymatic properties of 17β-HSD2 *in vitro* and detected the product with High Performance Liquid Chromatography. We determined the binding capability and binding sites of AraC by electrophoretic mobility shift assay and DNase I footprinting assay. **[Results]** Results showed the transcription of 17β-HSD2 and AraC were induced by E2. Multiple sequences alignment showed 17β-HSD2 contained the conserved structure and residues of short-chain dehydrogenase/reductase and β-hydroxysteroid dehydrogenase. 17β-HSD2 oxidized E2 at C_{17} site using NAD^+ as cofactor, with 0.0802 mmol/L K_m value and 56.26±0.02 μmol/(min·mg) V_{max} value; over 97.4% of E2 was transformed into estrone in five minutes. AraC protein could directly bind to the specific sites in the promoter region of *17β-hsd2*, which could be released by E2 or estrone. Overexpression of AraC repressed the transcription of *17β-hsd2* significantly. **[Conclusion]** 17β-HSD2 catalyzed the transformation of 17β-estraiol efficiently and was regulated by AraC in *P. putida* SJTE-1. This work promoted the enzymatic mechanism and the regulatory network studies about bacterial estrogen biodegradation.

Keywords: 17β-hydroxysteroid dehydrogenase 2, 17β-estradiol, AraC regulator, estrone, biodegradation

Environmental estrogens (EEs) are one of the most important environmental contaminants with great disorder effect and sex influence, and can cause serious environment problems[1–2]. Typical natural estrogens include 17β-estradiol (E2), estrone (E1), and estriol (E3); 17β-estradiol exhibits the highest estrogenic activities[3]. Bioremediation using microorganisms are considered as the efficient way

Supported by the National Natural Science Foundation of China (31570099, 31370152)
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Received: 4 April 2019; Revised: 29 April 2019; Published online: 19 January 2020

to eliminate the EEs pollution because of its high efficiency, low cost and little secondary pollution[4–5]. Microorganisms can utilize estrogens as carbon source and degrade them into non-estrogenic compounds[5]. Series of estrogen-degrading strains have been isolated from activated sludge and farm composting, such as *Sphingomonas* spp., *Rhodococcus zopfii*, *Rhodococcus equi*, *Nitrosomonas europaea* and *Pseudomonas* spp.[6–8]. Some fungi and algae were also found with the estrogen-degrading capabilities[9–11].

In bacteria, 17β-estradiol oxidization was considered as the first step for E2 biodegradation; therefore, enzyme catalyzing this reaction is the key enzyme for E2 transformation. In human, hydroxysteroid dehydrogenases (HSDs) belonging to the short-chain dehydrogenase/reductase (SDR) family, catalyze the conversion of inactive/active forms of estrogens by NAD(P)H-linked oxidization/ reduction of steroid molecules[12]. 3,17β-HSD and 17β-HSD in *Comamonas testosterone* have been proved to catalyze the oxidoreduction at the C_3 site of testosteroneand at C_{17} site of 17β-estradiol[8,13–14]. Several regulators (Rep1 and Rep2, HsdR, PhaR, TetR, LuxR, and BRP protein) have been found to participate in the degradation of testosterone in *C. testosteroni* by regulating the expression of 3α-*hsd* or 3,17β-*hsd* genes[15–19]. However, up to now, only a few enzymes involved in the bacterial degradation pathway of 17β-estradiol were identified; the enzymatic characteristics and their regulation factors were not very clear.

Pseudomonas putida SJTE-1 (CGMCC No. 6585) has been confirmed with efficient estrogen-degrading capability; its whole genome sequence and comparative proteomics have been obtained and analyzed[6,20]. In 17β-estradiol environment, significant expression changes of many proteins involved in carbon catabolism, cellular transportation and transcriptional regulation have been observed[20]. In this work, a novel 17β-HSD (17β-HSD 2) was characterized responsible for E2 oxidization in *P. putida* SJTE-1, and one AraC regulator was identified to participate in the regulation of E2 degradation in this strain.

1 Materials and methods

1.1 Strains, chemicals and cultures

All strains and plasmids used in this work were listed in Table 1. *E. coli* strains were cultured at

Table 1. Strains and plasmids used in this study

Strains/Plasmids	Descriptions	Sources/References
Strains		
P. putida SJTE-1	Estrogen-degradation strain, wild type	[6]
E. coli DH5α	*F'/ endA1 hsdR17* (*rK-mK-*) *glnV44 thi-1 recA1 gyrA* (*NalR*) *relA1 Δ* (*lacIZYA-argF*)*U169 deoR* (*Φ80dlacΔ* (*lacZ*)*M15*)	Invitrogen
E. coli BL21(DE3)	Protein allogeneic expression strains	Novagen
Plasmids		
pET28a	Expression plasmid in *E. coli* , Km^r	Novagen
pET28a-hsd2	Plasmid pET28a inserted with *17β-hsd2* gene at *Eco*R I/*Nde* I sites, Km^r	This study
pET28a-araC	Plasmid pET28a inserted with *araC* gene at *EcoR* I/*Nde* I sites, Km^r	This study
pBSPPc-Gm	Plasmid derived from pBR322 for gene replacement, Ap^r, Gm^r	[21]
pBS-araC	*araC* gene inserted into plasmid pBSPPc-Gm under the *lac* promoter, Ap^r, Gm^r	This study
pBS-eGFP	*egfp* gene inserted into plasmid pBSPPc-Gm without promoter, Ap^r, Gm^r	This study
pBS-P_{hsd2}-eGFP	*egfp* gene inserted into plasmid pBSPPc-Gm under the promoter of *17β-hsd2* gene, Ap^r, Gm^r	This study
pBS-P_{hsd2}-hsd2	*17β-hsd2* gene and its promoter inserted into plasmid pBSPPc-Gm, Ap^r, Gm^r	This study

37 °C and *P. putida* SJTE-1 was cultured at 30 °C. Luria-Bertani (LB) medium (tryptone 10.0 g, yeast extract 5.0 g, NaCl 10.0 g/L, pH 7.0) and minimal medium (MM) (K_2HPO_4 3.815 g, KH_2PO_4 0.5 g, $(NH_4)_2HPO_4$ 0.825 g, KNO_3 1.2625 g, Na_2SO_4 0.2 g, $CaCl_2$ 0.02 g, $FeCl_3$ 0.002 g, $MgCl_2$ 0.02 g/L, pH 7.0) were used for strains culture. 17β-Estradiol, estrone, estriol, 17α-ethinyl estradiol, testosterone, phenanthrene (of HPLC grade, >98.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical reagent grade. Estrogenic compounds were dissolved in dimethyl sulfoxide (DMSO) to form 10 mg/mL solution as DMSO was not utilized by strains.

1.2 Standard DNA manipulation

Oligonucleotides used for PCR amplification, reverse transcription (RT), quantitative PCR (q-PCR), homologous recombination, plasmid construction, electrophoretic mobility shift assay (EMSA) and DNase I footprinting were synthesized at Invitrogen Ltd. (Shanghai, China) and listed in Table 2. Restriction endonucleases and DNA-modifying enzymes were purchased from TaKaRa Biocompany (Dalian, China). The PCR products were recovered with the QIAquick gel extraction kit (Qiagen, Shanghai, China). Plasmid DNAs were isolated using the QIAprep Mini-spin kit (Qiagen, Shanghai, China), and genomic DNA was obtained using the QIAamp DNA minikit (Qiagen, Shanghai, China). All the constructed plasmids were confirmed by DNA sequencing (Invitrogen, Shanghai, China). Other general techniques for restriction endonuclease manipulation, agarose gel electrophoresis, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE were carried out with standard protocols.

1.3 Multiple sequences alignment (MSA) and homology modelling

Multiple sequences alignment of 17β-HSD proteins and AraC proteins from *P. putida* SJTE-1 and other organisms were performed with Clustal W software. The structure conformation of 17β-HSD2 protein was constructed by homology-modelling method achieved in SWISS-MODEL, with the short-chain dehydrogenase/reductase (5jla.1.A) from *Homo sapiens* as template.

1.4 Reverse transcription and quantitative PCR (RT-qPCR)

The transcription profiles of target genes (*17β-hsd2* and *araC*) in *P. putida* SJTE-1 cultured with different carbon sources were detected with

Table 2. Oligonucleotides used in this study

Primers	Sequences (5′→ 3′)	Usage
eGFP-F	GGGGGGAAGCTTATGGTGAGCAAGGGCGAGGAG	Primers for *egfp* gene amplification
eGFP-R	GGGGGGGGATCCTTAGTACAGCTCGTCCATGCCGAGA	
U176-F	FAM-GGTGTTGTAATTGTTCTGTTCAGT	Primers used to amplify the promoter fragment of *17β-hsd2* gene
U176-R	GCTGCTCTCCAGAGCGTT	
Hsd2-F	GGGGGGCATATGCACAACAACAAGATCCTTTCTC	Primers used to amplify 17β-*hsd2* gene
Hsd2-R	GGGGGGGAATTCTCATTGGCCTGCCCCCTC	
AraC-F	GGGGCATATGATGAACAAGATCCCCAATTACA	Primers for *araC* gene amplification
AraC-R	GGGGGAATTCTTACTGGCCCTCCCGAAATC	
Hsd-Q-F	GGCTGAAGAACTGGTTCGAG	Primers for the q-PCR detection of *17β-hsd2* gene
Hsd-Q-R	CCAGGTCGAACTCTGTCACC	
AraC-Q-F	TAGTACCCGCAAGCTGACCT	Primers for the q-PCR detection of *araC* gene
AraC-Q-R	CAACGGCAACACGTACAAAC	

RT-qPCR. Strain SJTE-1 was cultured in the minimal medium with 0.2% glucose, 10 mg/L or 20 mg/L different steroid compounds (17β-estradiol, estrone, estriol, 17α-ethinyl estradiol, testosterone) as sole carbon source to the mid-exponential phase; and the total RNA was extracted using the Total RNA Extraction Reagents (Vazyme, Nanjing, China) according to the protocol. The yield of RNA was estimated using a Nanodrop UV spectrometer (Thermo Scientific, DE, USA). Reverse transcription was achieved with 1 μg RNA and 20 ng random primers using the PrimeScript Reverse Transcriptase Kit (TaKaRa, Dalian, China). The quantitative PCR was performed using the Premix Ex Taq and the gene-specific primers (Table 2) in the IQTM 5 Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA). At the meanwhile, the transcription profiles of target genes (17*β-hsd2* and *araC*) in *P. putida* SJTE-1 transformed with plasmids pBS-araC cultured with different carbon sources were also detected. The strain was cultured in MM meidium with 0.2% glucose or 20 mg/L E2 to the mid-exponential phase, and 0.1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplied. The RNA extraction and RT-qPCR detection were performed as above. The relative fold change in mRNA quantity was calculated using the DDCt method. Five independent experiments were performed for each RNA sample and the average values with the standard errors were calculated.

1.5 Heterogeneous expression and affinity purification of recombinant proteins

The recombinant 17β-HSD2/AraC proteins were expressed and purified as described before[22]. Plasmids pET28a-hsd2/pET28a-araC were transformed into the competent *E. coli* BL21 (DE3) cells. Single colony was inoculated in LB media with 50 μg/mL kanamycin and cultured at 37 °C overnight. The culture were transferred into fresh LB with 50 μg/mL kanamycin and cultured to OD_{600}=0.5. 0.1 mmol/L IPTG was added and cells were induced at 37 °C for 3 h. Cells were harvested and resuspended with ice-cold lysis buffer [20 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L imidazole, 5 mmol/L β-mercaptoethanol (β-ME), 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 10% glycerol, pH 7.9]. After sonication (300 W, 10 s/ 10 s, 20 min), the supernatant was obtained and loaded into affinity chromatography of Ni-NTA resin (Bio-Rad, CA, USA) at 4 °C. After washed with washing buffer (20 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L β-ME, 10% glycerol, 1 mmol/L PMSF, and 50 mmol/L imidazole pH 7.9), the recombinant protein was eluted from the column using elution buffer (20 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L β-ME, 10 % glycerol, 1 mmol/L PMSF, 200 mmol/L imidazole, pH 7.9). All eluted solutions were analyzed by SDS-PAGE. The eluted recombinant proteins were dialyzed and stored in the storage buffer (20 mmol/L Tris-HCl, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 50% glycerol, pH 8.0) or 1× PBS buffer (Na_2HPO_4 1.42 g/L, KH_2PO_4 0.27 g/L KCl 0.2 g/L, NaCl 8.0 g/L, pH 7.4) at –80 °C. The concentration of proteins was determined using a BCA protein assay kit (TaKaRa, Dalian, China).

1.6 Enzymatic assay of 17β-HSD2

The enzymatic activity of recombinant 17β-HSD2 protein was measured by detecting the yield of NADH at 355 nm (excitation) /460 nm (emission)[17]. The reaction system (20 μL) contained 20 μmol/L NAD^+, and different concentrations of 17β-HSD2 protein and steroids (0–0.5 μmol/L of 17β-HSD2, 0-0.5 μmol/L of 17β-estradiol, estrone, estriol, 17α-ethinyl estradiol, testosterone, phenanthrene) in reaction buffer (20 mmol/L Tris-HCl, 25 mmol/L NaCl). The samples without 17β-HSD2 or steroids were used as blank controls. The enzymatic parameters of 17β-HSD2 (K_m, V_{max}, optimal pH and temperature) were determined. Effect of metal ions (Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, and Ni^{2+}) was analyzed. One unit

of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of E2 per min under the above-indicated conditions at 25 ℃. All the experiments were repeated five times, and results were the average values with standard errors.

1.7 High Performance Liquid Chromatography (HPLC) detection of enzymatic products

The solutions of enzymatic reaction were extracted with ethyl acetate, dried in the nitrogen blowing apparatus, dissolved with acetonitrile, and detected with HPLC system (Agilent 1260 Infinity LC, US). The Agilent exclipse plus C18 column (3.5 μm, 4.6 mm×150 mm, Agilent, US) and Diode Array Detector (DAD) detector were used with the mobile phase of acetonitrile and water (1:1 *V*/*V*) at a flow rate of 1 mL/min at 30 ℃, and the UV wavelengths of 280 nm and 315 nm. The quantities of estrogens were calculated from their respective peak areas by using a standard curve of individual standards; the R^2 values for the standard curves were >0.99. Five independent experiments were performed and results were calculated as average values with standard errors.

1.8 Green fluorescent protein (GFP) fluorescence assay

Plasmid pBS-P_{hsd2}-eGFP (Table 1) was transformed into *E. coli* BL21(DE3) strain and the recombinant strain was cultured in the minimal medium with 0.2% glucose or 10 mg/L 17β-estradiol. The cells were collected at different time points, and the fluorescence values of eGFP were measured with excitation at 485 nm and emission at 527 nm. Five independent experiments were performed, and the average values with the standard errors were calculated.

1.9 Electrophoretic mobility shift assay (EMSA)

The DNA fragments were amplified from the upstream region of *17β-hsd2* gene with 5′-FAM labeled primers (Table 2). The recombinant AraC protein and DNA fragments were mixed in different molecular ratios, and incubated in the binding buffer (20 mmol/L Tris-HCl, 50 mmol/L NaCl, 1 mmol/L DTT, 0.1 mmol/L EDTA, pH 7.5) at 37 ℃ for 30 min. All the input DNA amounts were 4 pmol in 20 μL binding system. The reaction mixture was analyzed with native PAGE, and the gel was photographed in the Bio-Rad Imaging System (Bio-Rad, CA, USA).

1.10 Detection of estrogens effect on protein-DNA interaction

The binding of AraC protein to the upstream fragments of *17β-hsd2* gene was performed as above. 17β-estradiol and estrone (50 pmol or 250 pmol) were supplied into the binding system and DMSO was used as negative control. After 15 min incubation at 37 ℃, the mixture was analyzed with native PAGE and the gel was photographed in BioRad Imaging System (Bio-Rad, CA, US).

1.11 DNase I foot-printing assay

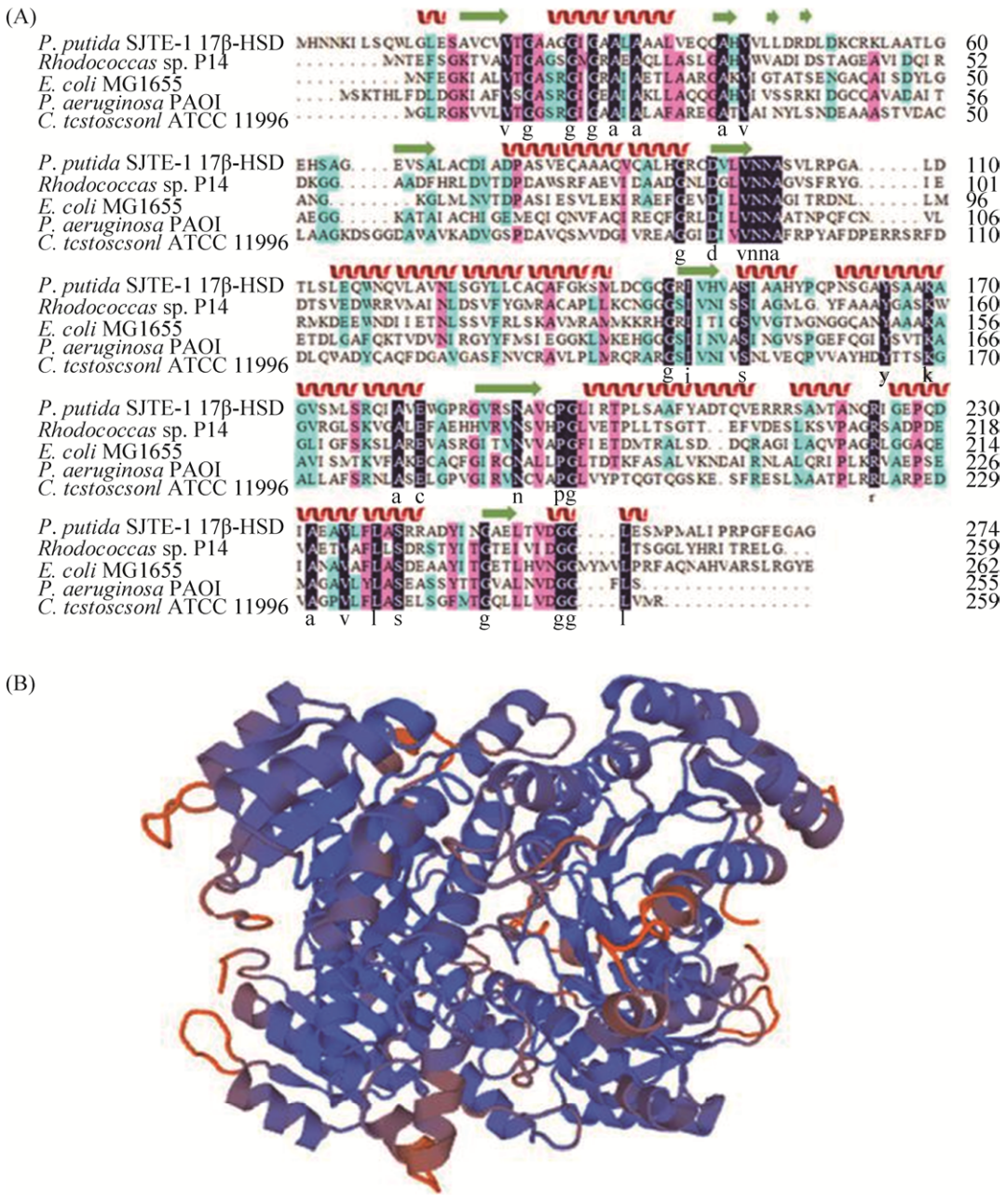
The 1.87 μg of DNA (the upstream fragments of *17β-hsd2* gene) was bound with AraC protein at 25 ℃ for 30 min performed as above. Then 1.33 U of DNase I was added into the binding mixture and incubated at 25 ℃ for 1 min. The DNA fragment without AraC protein addition was used as control. The reaction products were extracted with phenol to remove proteins and ethanol was added to precipitate DNA. The mixture were analyzed with native PAGE and displayed in the BioRad Imaging System (Bio-Rad, CA, US).

2 Results

2.1 Sequence alignments and structure conformation showed that 17β-HSD2 contained the conserved structure of SDR

Based on the analysis of *P. putida* SJTE-1 genome sequence (CP015876.1), one gene (A210_13000, 2861306..2862133) was predicted as *hsd* gene and its encoding protein was probably an HSD protein (ANI03512.1). Multiple sequences alignment showed that its secondary structure

contained the conserved Rossmann-fold of SDRs, a central twisted parallel β-sheets consisting of 6–7 β-strands flanked by 3–4 α-helices from each side[23]. The amino acid sequences and the secondary structure of this protein were similar to those of 17β-HSDs from *P. aeruginosa* PAO1, *Rhodococcus* sp. P14, *C. testosteroni* ATCC 11966, and *E. coli* MG1655 (Figure 1-A). They all contained the two



Figures 1. Multiple sequences alignment of 17β-HSDs. A: the 17β-HSD2 from *P. putida* SJTE-1 (ANI03512.1), *P. aeruginosa* PAO1 (NP_250519.1), *C. testosteroni* ATCC 11996 (WP_003080542.1), *Rhodococcus* sp. P14 (WP_010595922.1), and *E. coli* MG1655 (WP_001499617.1) were aligned with Clustal W. Similar and conserved amino acids were marked in colour. The secondary structure of α-helices and β-strands were indicated. B: structure conformation result of 17β-HSD2 based on homology modelling was shown, with SDR (5jla.1.A) as the template.

conserved motifs of SDR members, the N-terminal Rossmann-fold Gly-X-X-X-Gly-X-Gly motif for cofactor binding and the Tyr-X-X-X-Lys motif for proton acceptance. The conserved triad motif of HSD, Ser-Tyr-Lys (residues 152, 165, 169), was also found in this protein. Serine at residue 152 probably functioned as the active site, and the highly conserved tyrosine (residue 165) may act as a proton acceptor[24]. Structure modelling showed that this protein exhibited 39.43% sequence similiarity to the template, the SDR from *Homo sapiens* (5jla.1.A), and its coverage was 89%. Their structures were quite similar as the local quality estimate of this HSD protein to the template was about 0.8 (Figure 1-B). Therefore, this protein was a 17β-HSD.

2.2 Protein ANI03512.1 could be induced by 17β-estradiol and was a 17β-HSD

The transcription levels of 17β-*hsd2* gene in *P. putida* SJTE-1 cultured with different steroid compounds as sole carbon sources were measured. Results showed when 10 μg/mL or 20 μg/mL 17β-estradiol were used, the transcription of *17β-hsd2* increased about 3.6 or 4.8 folds compared to that in the culture with glucose. Tetosterone could also enhance the transcription of 17β-*hsd2* gene about 2.5 folds, while estrone and estriol only had a little induction (Figure 2-A). 17α-ethinyl estradiol and phenanthrene also exhibited a little inductive activity (data not shown). The eGFP fluorescence results showed that the expression of *egfp* under the 17β-*hsd2* promoter showed an increase of about 1.7 folds after 3 h induction with E2, and an increase of about 2.8 folds after 9 h induction (Figure 2-B). This demonstrated that protein ANI03512.1 could be induced by steroidic compounds, especially by 17β-estradiol.

Then the enzymatic properties of protein ANI03512.1 were studied. Its encoding gene was cloned and expressed in *E. coli* BL21 (DE3), and the purified protein exhibited 29.0 kDa with over 96% purity (Figure 3-A). The yield of recombinant protein was over 11 mg/L and the specific activity of the purified protein for E2 transformation was 25.28 U/mg. Enzymatic assay showed that the recombinant protein used NAD^+ but not $NADP^+$ as its reaction cofactor; 17β-Estradiol, testosterone and estradiol could be oxidized effciently *in vitro*. Obvious substrate preference for protein ANI03512.1 to 17β-estradiol was observed with efficient transformation efficiency (Table 3). The optimal reaction temperature was 37 °C and the optimal reaction pH was 9.0, similar to other HSDs (Figure 3-B, 3-C)[25]. Mg^{2+} and Mn^{2+} could enhance the reaction efficiency, while Zn^{2+} and Cu^{2+} repressed the enzyme activity greatly (Figure 3-D). The K_m value of protein ANI03512.1 for E2 was 0.0802±0.004 mmol/L and its V_{max} value was 56.26±0.02 μmol/min·mg; its specificity constant (k_{cat}/K_m) for E2 was 28.267/s·mmol·L^{-1}

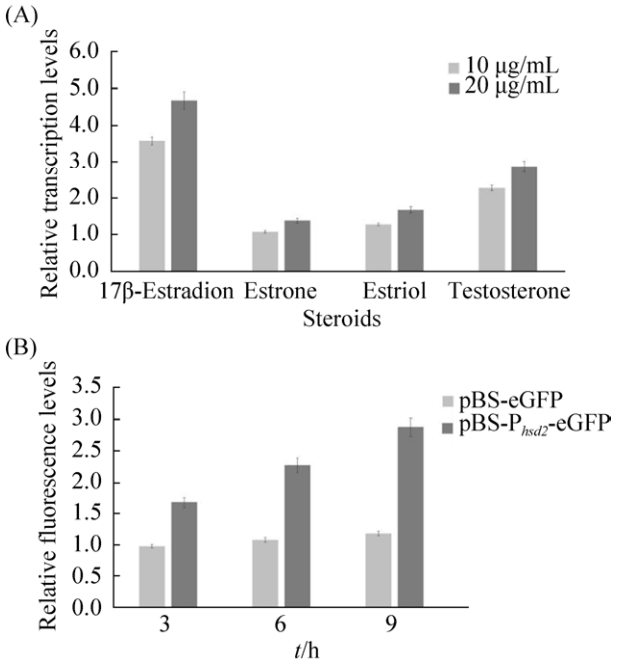


Figure 2. The transcription and expression analysis of 17*β-hsd2* gene under different conditions. A: The transcription levels of 17*β-hsd2* gene in *P. putida* SJTE-1 cultured with glucose or different steroid compounds of 10 μg/mL for six hours were determined. B: The fluorescence values of *E. coli* BL21 (DE3) containing plasmid pBSPPc-P_{hsd2}-eGFP were determined by inducing with 20 μg/mL 17β-estradiol for different hours.

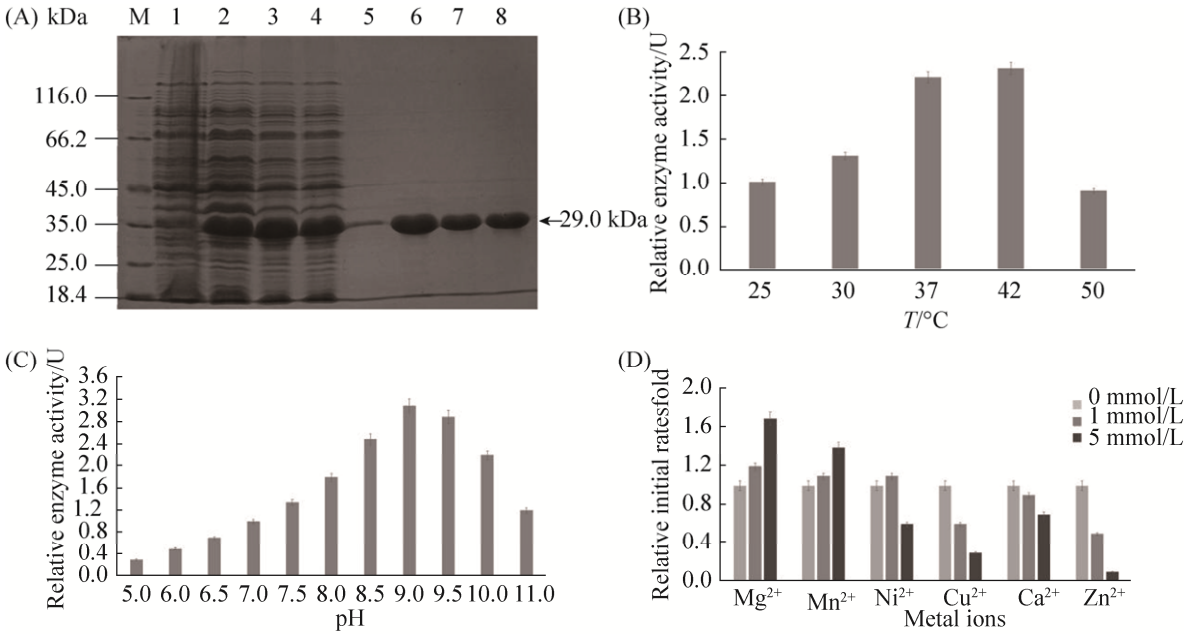


Figure 3. The purification and characterization of 17β-HSD2. A: SDS-PAGE detection of the recombinant 17β-HSD2 protein. M: protein marker; Lane 1: cell lysis supernant before induction; Lane 2: cell lysis supernant after induction; Lane 3: the supernant after sonication; Lane 4: the solution after column loading; Lane 5–8: the eluted protein solutions. B: The relative enzyme activities of 17β-HSD2 calculated with the NADH fluorescence values produced by enzyme reaction at different temperatures. The NADH fluorescence values of enzyme reaction at 25 °C was set 1.0. C: The relative enzyme activities of 17β-HSD2 calculated with the NADH fluorescence values produced by enzyme reaction under different pH. The NADH fluorescence values produced by enzyme reaction at pH 7.0 and 37 °C was set 1.0. D: The relative enzyme activities of 17β-HSD2 calculated with the NADH fluorescence values produced by enzyme reaction supplied with different metal ions. The NADH fluorescence values produced by enzyme reaction without divalent metal ion at pH 7.0 and 37 °C was set 1.0. All the reaction mixture contained 0.2 μmol/L of 17β-estradiol, 20 μmol/L NAD^+, and 0.3 μmol/L 17β-HSD2 in 1× reaction buffer (20 mmol/L Tris-HCl, 25 mmol/L NaCl); the reaction volume was 20 μL and the reaction time was 5 min.

Table 3. The substrates spectrum of 17β-HSD2

Substrates	17β-estradiol	Estrone	Estriol	17α-ethinyl estradiol	Testosterone	phenanthrene
Fluorescence values of NADH	0.713	–0.08	0.127	–0.10	0.480	0.043

The reaction mixture (20 μL) contained 0.2 μmol/L of steroid compounds, 20 μmol/L NAD^+, and 0.3 μmol/L 17β-HSD2 in 1× reaction buffer (20 mmol/L Tris-HCl, 25 mmol/L NaCl) and the reaction was performed at 37 °C for 5 min.

(Table 4). Further HPLC analysis showed that estrone was the oxidation product of 17β-estradiol catalyzed by protein ANI03512.1 and the transformation efficiency was above 97.4% in five minutes (Figure 4). Therefore, protein ANI03512.1 could be induced by 17β-estradiol and could oxidize 17β-estradiol into estrone *in vitro* efficiently. It was named as 17β-HSD2.

2.3 AraC could bind to the speicific sites in the promoter region of 17β-*hsd2* gene and repress the expression of 17β-HSD2

Genome analysis of *P. putida* SJTE-1 showed that one *araC* gene (A210_12995, 2860257. 2861129) encoding a potential regulator AraC (ANI06277.1) located in the upstream of 17β-*hsd2*

gene. RT-qPCR detection showed that this *araC* gene could also be induced by 17β-estradiol, dependent on the concentration of E2 (Figure 5-A). To analyze the potential role of AraC to 17β-HSD2, we detected the transcription levels of *hsd2* gene in *P. putida* SJTE-1 with plasmids pBS-araC. IPTG induced the overexpression of AraC greatly, and then the transcription of *hsd2* gene was repressed significantly. When E2 was used, the transcription of *hsd2* gene was induced and increased; while with IPTG added, overexpressed AraC caused transcriptional repression of *hsd2* gene (Figure 5-B). The repression extent of AraC to the transcription of *hsd2* gene decreased significantly in E2 environment compared to that in glucose environment, even the expression of AraC induced greatly with IPTG; this implied that E2 could release the binding of AraC and enhance the transcription of *hsd2* gene. Multiple

Table 4. The enzymatic characteristics of 17β-HSD2

Enzyme	K_m/(mmol/L)	V_{max}/(μmol/(min·mg))	k_{cat}/(/s)	k_{cat}/K_m/[/s/(mmol/L)]
17β-HSD2	0.0802±0.004	56.26±0.02	2.267±0.07	28.267

The reaction mixture (20 μL) contained 0–0.5 μmol/L of 17β-estradiol, 20 μmol/L NAD^+, and 0–0.5 μmol/L 17β-HSD2 in 1× reaction buffer (20 mmol/L Tris-HCl, 25 mmol/L NaCl) and the reaction was performed at 37 °C for 5 min.

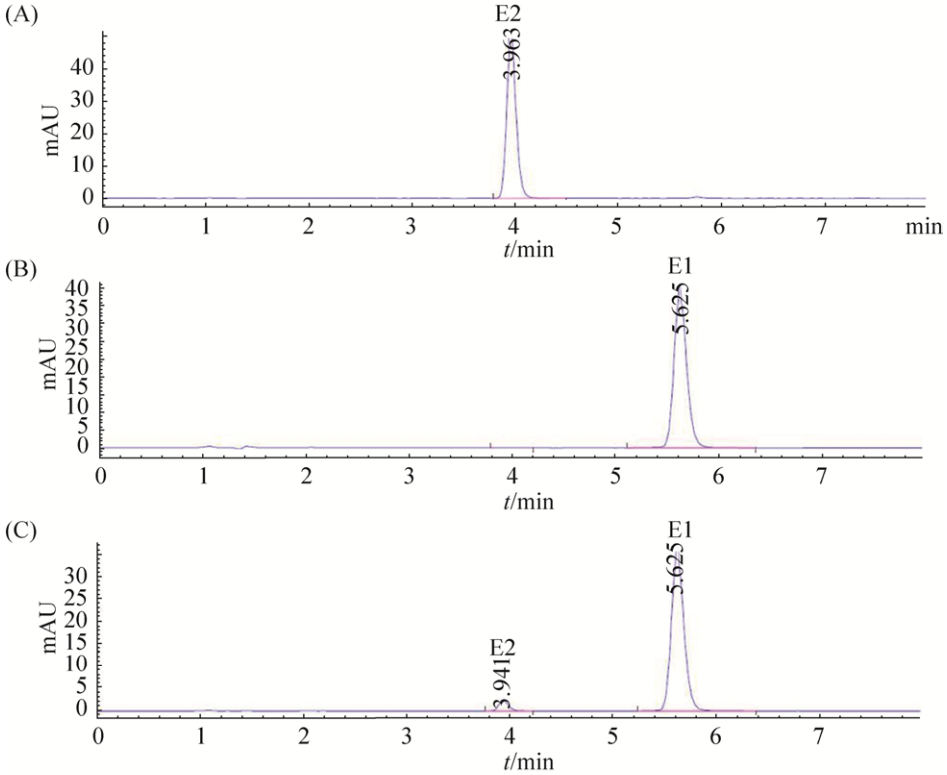


Figure 4. HPLC profiles of the reaction products catalyzed by 17β-HSD2. A: HPLC profile of 0.2 μmol/L 17β-estradiol. B: HPLC profile of 0.2 μmol/L estrone. C: HPLC profile of the products in 17β-HSD2 oxidation reaction with 0.2 μmol/L of 17β-estradiol. The reaction mixture contained 0.2 μmol/L of 17β-estradiol, 20 μmol/L NAD^+, and 0.3 μmol/L 17β-HSD2 in 1× reaction buffer (20 mmol/L Tris-HCl, 25 mmol/L NaCl) and the reaction was performed at 37 °C for 5 min.

sequences alignment showed that the secondary structure of AraC protein in *P. putida* SJTE-1 was similar to that of other AraC regulators, with the conserved N-terminal HTH (helix-turn-helix) structure and the variable C-terminal (Figure 5-C). These indicated this AraC protein could be induced by 17β-estradiol and may participate in the 17β-estradiol degradation via regulating the transcription of *17β-hsd2*.

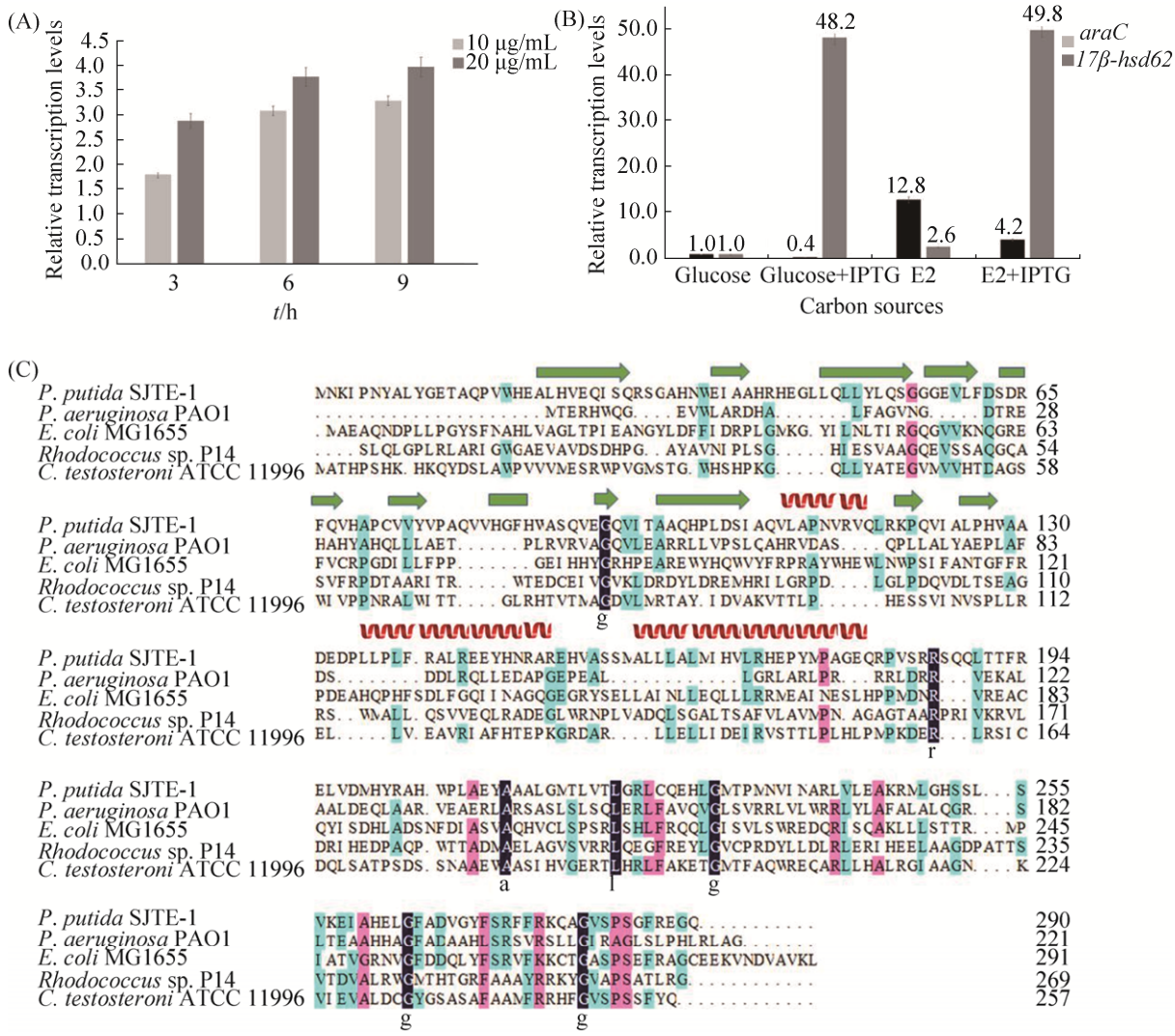


Figure 5. Affinity purification and multiple sequences alignment of AraC protein. A: The transcription levels of *araC* gene in *P. putida* SJTE-1 cultured with 17β-estradiol of 10 μg/mL or 20 μg/mL for different hours (3 h, 6 h, 9 h) were detected. The transcription levels of *araC* gene cultured with 0.2% glucose was set 1.0. B: The transcription levels of 17*β-hsd2* gene and *araC* gene in *P. putida* SJTE-1 transformed with plamids pBS-araC cultured with different carbon sources. 0.2% glucose or 20 μg/mL 17β-estradiol were supplied as carbon sources; 0.1 mmol/L IPTG was used for inducer. The transcription levels of 17*β-hsd2* gene and *araC* gene cultured with 0.2% glucose were set 1.0. C: Multiple sequences alignment of AraC proteins from *P. putida* SJTE-1 (ANI06277.1), *P. aeruginosa* PAO1 (YP_008719734), *E. coli* MG1655 (NP_414606.1), *Rhodococcus* sp. P14 (WP_010595765.1) and *C. testosteroni* ATCC 11996 (EHN63024.1) were performed with Clustal W. Similar or conserved amino acids were marked with colour.

The binding ability of AraC protein to the upstream fragment of 17β-*hsd2* gene was determined. The recombinant AraC protein (30.5 kDa) was obtained by hetergenous expression and affinity purification (Figure 6-A). EMSA detection showed this AraC protein could directly bind to the 176 bp upstream fragment of 17β-*hsd2* gene at low protein/DNA ratio, and the binding was in a concentration dependent mode (Figure 6-B). Addition of high-concentration 17β-estradiol or

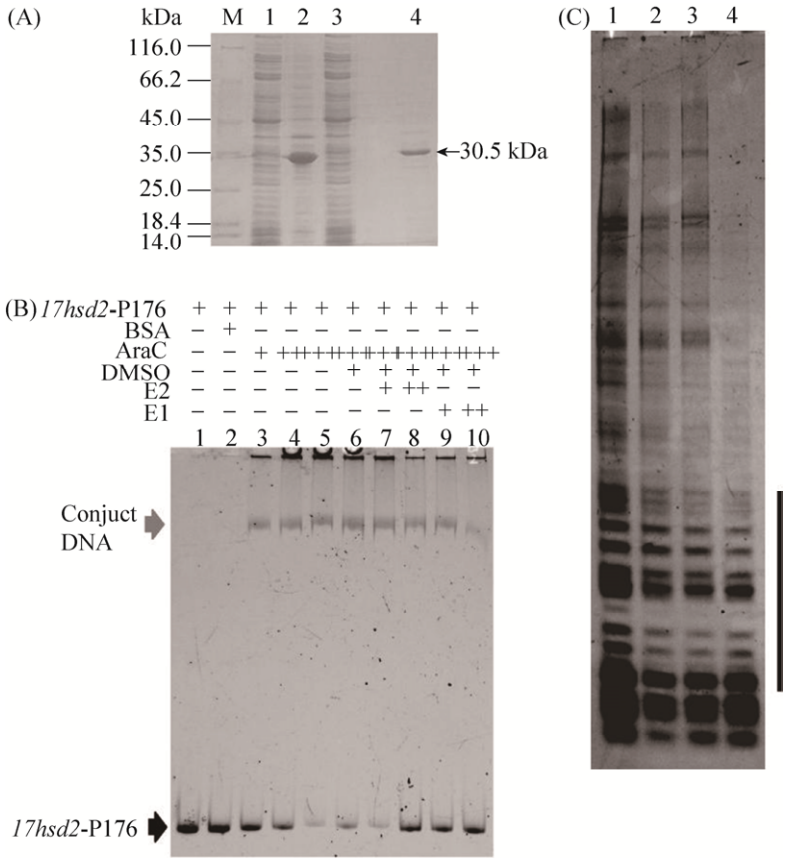


Figure 6. EMSA assay and DNase I-footprinting detection of AraC protein binding to the promoter region of *17β-hsd2*. A: SDS-PAGE profiles of recombinant AraC protein. M: protein marker; lane 1: cell lysis supernant before induction; lane 2: cell lysis supernant after induction; lane 3: the solution after column loading; lane 4: the eluted protein solution. B: EMSA results of AraC protein to the 5′-FAM-labeled 176 bp fragment of *17β-hsd2* gene (17*hsd2*-P176). The input of 17*hsd2*-P176 fragments in each sample was 4 pmol. The band of free DNA fragments were marked with black arrow and the conjunct DNA were marked with grey arrow. lane 1: the free labeled DNA; lane 2: the labeled DNA mixed with BSA in the protein/DNA ratio of 10:1. lanes 3–5: the labeled DNA mixed with AraC protein in the protein/DNA ratio from 9:1, 18:1, and 36:1. lane 6: 5% DMSO was added into the mixture of AraC protein and labeled DNA in the protein/DNA ratio of 36:1. lane 7–8: the mixture of AraC protein and labeled DNA (36:1) supplied with 1 μL E2 solution (dissoved in DMSO) with the final amount of 50 pmol or 250 pmol. lane 9–10: the mixture of AraC protein and labeled DNA (36:1) supplied with 1 μL E1 solution (dissoved in DMSO) with the final amount of 50 pmol or 250 pmol. C: The DNase I footprinting profile of AraC protein with the fragment 17*hsd2*-P176. 1.33U of DNase I and 1.87 μg of labeled DNA were added into each lane. lane 1: the free labeled DNA fragment treated with DNase I. lane 2–4: the mixutre of the labeled DNA and AraC protein in ratio of 0.5:1, 1.5:1 and 2.5:1 treated with DNase I. The potential binding regions was marked with black lines.

estrone could release the binding of AraC protein from the upstream fragment of 17β-*hsd2* gene to some extent; 17β-estradiol of low-concentration had little influence on the AraC binding to DNA (Figure 6-B). Further DNase I foot printing assay showed when AraC protein added, the regions close to its transcription start site in the promoter of *17β-hsd2* gene were covered, probably acting as the specific sites for AraC protein binding (Figure 6-C). This region was around 40 bp upstream of *17β-hsd2* with the sequence ATATGCXXGTTXCCCTGAXX, similar to the conserved binding sites of AraC regulator[26]. These results demonstrated that AraC protein could directly bind to the specific sites in the promoter region of *17β-hsd2* gene and repress its expression; the substrate (E2) and the product (E1) of 17β-HSD2 could release the binding of AraC and facilitate the transcription of *17β-hsd2* gene.

Biodegradation with microorganisms is considered as efficient strategy to remediate the pollution of environmental estrogens. HSDs were considered as the key enzyme for 17β-estradiol transformation in bacteria[12–14,27]. Although several 3,17β-HSDs were found to participate in the oxidization at C_{17}-site of steroid compounds, the key catalyzing enzyme of 17β-estradiol was not really clear. The reason was that the reported 3,17β-HSDs could not be induced by 17β-estradiol, even the oxidization of 17β-estradiol could be achieved *in vitro*[13,15,28]. Previous work showed that *P. putida* SJTE-1 could degrade 17β-estradiol efficiently and several hydroxysteroid dehydrogenases were induced in the culture with 17β-estradiol as carbon source[6,20]. In this work, 17β-HSD2 in *P. putida* SJTE-1 could oxidize 17β-estradiol into estrone efficiently, whose enzymatic activity was higher than or similar to those of the reported HSDs[13,27]. The 17β-HSD2 could be sigificantly induced by 17β-estradiol and testosterone, and catalyzed the transformation of two compounds. It implied 17β-HSD2 was probably the key enzyme for estrogen transformation in *P. putida* SJTE-1 and important for the estradiol degradation of this strain.

At the meanwhile, the regulators involved in the biodegradation of 17β-estradiol in bacteria and their regulation mechnisms were also unclear. PhaR, TetR, LuxR, and BRP protein have been found to regulate the 3,17β-HSD and influence testosterone utilization in *C. testosteroni*[16–19]. However, these regulation was not obvious when 17β-estradiol used; not only because 17β-estradiol was not utilized in *C. testosteroni* as good as testosterone, but also because this 3, 17β-*hsd* gene could not be efficiently induced by 17β-estradiol[13,29]. In this work, AraC regulator was proved to able to bind to the specific sites in the promoter region of *17β-hsd2* gene directly and repress the transcription of *17β-hsd2* gene. Estrogens could induce the expression of AraC, and overexpressed AraC could repress the transcription of *17β-hsd2* gene. The binding of AraC could be released by high concentration of E2 and its repression effect would be reduced to generate more 17β-HSD2 and facilitate the E2 biodegradation. The potential binding region was found in the *araC*-17*β-hsd2* intergenic region, close to the transcription start sites of 17*β-hsd2*. The sequences of the binding site were ATATGCXXGTTXCCCTG AXX, similar to the conserved binding sites (ATATG CXXGAAAXXXTCCTTA) of reported AraC regulator[26]. Therefore, AraC protein regulated the expression of *17β-hsd2* gene in a negative mode in *P. putida* SJTE-1. In a word, this work could promote the estrogen degradation mechanism study and the regulatory network analysis in bacteria.

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恶臭假单胞菌 SJTE-1 中氧化 17β-雌二醇的 17β-羟甾类脱氢酶 2 及其转录调控因子 AraC 的鉴定

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摘要：【目的】假单胞菌 SJTE-1 可高效转化 17β-雌二醇，但其代谢机制尚不清楚。本文鉴定和表征了该菌株中参与雌二醇降解与调控过程的 17β-羟甾类脱氢酶 2（17β-HSD2）和转录调控因子 AraC。【方法】我们通过荧光定量 PCR 分析了 *17β-hsd2* 和 *ara*C 的转录水平；我们在大肠杆菌 BL21(DE3)菌株中异源表达了 17β-HSD2 和 AraC 基因，并利用金属离子亲和层析法纯化获得了重组蛋白；我们体外表征了 17β-HSD2 的酶学性质，利用高效液相色谱鉴定了其产物；通过电泳迁移转移法和 DNase 酶 I 足迹试验，我们鉴定了重组蛋白 AraC 的结合能力与结合位点。【结果】17β-HSD2 和 AraC 可被 17β-雌二醇诱导表达；蛋白序列比对结果表明 17β-HSD2 含有短链脱氢酶/还原酶(SDR)和 β-羟甾类脱氢酶的保守结构与残基。该酶以 NAD^+为辅助因子，在 C_{17} 位点氧化 17β-雌二醇，其 K_m 值为 0.082 mmol/L，V_{max} 值为 56.26±0.02 μmol/(min·mg)；5 min 内可转化 97.4%以上的雌二醇。转录调控因子 AraC 可直接结合 *17β-hsd2* 基因启动子区的特异位点；雌二醇与雌酮可解除这一结合，启动 *17β-hsd2* 基因转录；过表达 AraC 蛋白可抑制 *17β-hsd2* 的转录。【结论】假单胞菌 SJTE-1 的 17β-羟甾类脱氢酶 2 可高效催化 17β-雌二醇转化，并受到转录因子 AraC 的直接调控。本工作可推进细菌的雌激素降解酶学机制与调控网络研究。

关键词：17β-羟甾类脱氢酶 2，17β-雌二醇，转录因子 AraC，雌酮，生物降解

(本文责编：张晓丽)

基金项目：国家自然科学基金(31570099，31370152)

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收稿日期：2019-04-04；**修回日期：**2019-04-29；**网络出版日期：**2020-01-19