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ENVIRONMENTAL BIOTECHNOLOGY

Characterization of 17β-hydroxysteroid dehydrogenase and regulators involved in estrogen degradation in *Pseudomonas putida* SJTE-1

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Abstract

In bacteria, the enzyme catalyzing the transformation of 17β-estradiol is considered the key enzyme for its metabolism, whose enzymatic activity and regulatory network influence the biodegradation efficiency of this typical estrogen. In this work, a novel 17β-hydroxysteroid dehydrogenase (17β-HSD) was characterized from the estrogen-degrading strain *Pseudomonas putida* SJTE-1, and two regulators were identified. This 17β-HSD, a member of the short-chain dehydrogenase/reductase (SDR) superfamily, could be induced by 17β-estradiol and catalyzed the oxidization reaction at the C_{17} site of 17β-estradiol efficiently. Its K_m value was 0.068 mM, and its V_{max} value was 56.26 μmol/min/mg; over 98% of 17β-estradiol was oxidized into estrone in 5 min, indicating higher efficiency than other reported bacterial 17β-HSDs. Furthermore, two genes (*crgA* and *oxyR*) adjacent to *17β-hsd* were studied which encoded the potential CrgA and OxyR regulators. Overexpression of *crgA* could enhance the transcription of *17β-hsd*, while that of *oxyR* resulted in the opposite effect. They could bind to the specific and different sites in the promoter region of *17β-hsd* gene directly, and binding of OxyR could be released by 17β-estradiol. OxyR repressed the expression of *17β-hsd* by its specific binding to the conserved motif of GATA-N_9-TATC, while CrgA activated the expression of this gene through its binding to the motif of T-N_{11}-A. Therefore, this 17β-HSD transformed 17β-estradiol efficiently and the two regulators regulated its expression directly. This work could promote the study of the enzymatic mechanism and regulatory network of the estrogen biodegradation pathway in bacteria.

Keywords 17β-hydroxysteroid dehydrogenase · 17β-estradiol · Estrone · OxyR · CrgA · Biodegradation

Introduction

Environmental estrogens (EEs) are important contaminants that can cause significant hormone disruptions in plants, animals, and humans (Katori et al. 2002; Yin et al. 2002). Typical natural estrogens contain 17β-estradiol (E2), estrone (E1), and estriol (E3), with 17β-estradiol having the highest estrogenic activity (Luine 2014). Biodegradation using microorganisms is considered a promising strategy to decompose environmental estrogens, owing to its high efficiency, low cost, and lack of secondary pollution (Yu et al. 2013; Khanal et al. 2006; Combalbert and Hernandez-Raquet 2010). Microorganisms can utilize estrogenic chemicals as their carbon or energy sources and transform them into non-estrogenic chemicals (Yu et al. 2013). Strains of bacteria, fungi, and algae with estrogen degradation capabilities have been found, such as *Sphingomonas* spp., *Rhodococcus zopfii*, *Rhodococcus equi*, *Nitrosomonas europaea*, and *Pseudomonas* spp. (Hom-Diaz et al. 2015; Jianghong et al. 2004; Liang et al. 2012; Yang et al. 2017; Takeshi et al. 2004; Tamagawa et al. 2006; Yu et al. 2007). Their degradation efficiencies, degradation periods, and substrate spectrums are varied. For example, *Rhodococcus* sp. and *Sphingomonas* sp. could degrade 50% of E2 (0.8 mg) in 24 h and 90% of E2 in 120 h (Futoshi et al. 2010). *N. europaea* can degrade E1 and E2 efficiently but cannot utilize the synthetic estrogen 17α-ethynylestradiol (EE2) (Jianghong et al. 2004); while *Sphingobacterium* sp. can degrade typical natural estrogens and the synthetic estrogen EE2 (Haiyan et al. 2007).

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Although several estrogen-degrading strains have been isolated, the mechanisms underlying the degradation of estrogenic chemicals remain unclear. In most bacteria, the transformation from 17β-estradiol to estrone is considered the first step in its metabolism. Therefore, the enzyme catalyzing this step is the key enzyme in this pathway. In humans, hydroxysteroid dehydrogenases (HSDs), belonging to the short-chain dehydrogenase (SDR) family, have been demonstrated to be responsible for the mutual transformation of active to inactive forms of estrogens by the NAD(P)H-linked oxidization-reduction at the 17-position of steroid molecules (Chang et al. 2010). In bacteria, HSDs also widely exist and were found to participate in the oxidation/reduction process of steroids. For example, 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD) catalyzed the oxidoreduction at the 3-position of testosterone in *Comamonas testosteroni* (Maser et al. 2001). 17β-hydroxysteroid dehydrogenase (17β-HSD) was also found to oxidize the 17β-hydroxyl steroids such as 17β-estradiol (Yuanhua et al. 2015; Ye et al. 2017). In addition, several regulators, including Rep1/Rep2, HsdR, PhaR, TetR, LuxR, and BRP protein, were found to regulate the expression of *3α-hsd* or *3,17β-hsd* in *C. testosteroni* and are involved in the degradation of testosterone (Gong et al. 2012a, b; Ji et al. 2017; Li et al. 2013; Pan et al. 2015; Wu et al. 2015). However, because of the unclear genetic backgrounds and various metabolism networks in different estrogen-degrading bacteria, only a few enzymes catalyzing the metabolism of 17β-estradiol were identified; their characteristics and their regulators were still unclear.

Pseudomonas spp. strains are known for their remarkable tolerance to environmental stresses and efficient degradation capability, and a number of *Pseudomonas* strains have been utilized in wastewater treatment and environmental bioremediation (Matsumura et al. 2009; Zeng et al. 2009). Previously, we have isolated *P. putida* SJTE-1 (CGMCC NO. 6585) from sludge and confirmed that it could utilize 17β-estradiol as a sole carbon source and could transform it into non-estrogenic chemicals efficiently. 17β-estradiol of 1 mg/L or 10 mg/L could be degraded completely in 24 h or 48 h, respectively; it was first converted into estrone, and the accumulated estrone was then degraded into non-estrogenic chemicals. The whole genome sequence of this strain has been obtained, and its comparative proteomics in different culture environments have also been analyzed (Liang et al. 2012; Xu et al. 2017). Significant changes were observed in the expression of proteins involved in carbon catabolism, transportation, and transcription regulation when exposed to 17β-estradiol (Xu et al. 2017). Therefore, characterization of the key enzyme for 17β-estradiol transformation and identification of the related regulator could elucidate the estrogen degradation pathway. In this work, the key 17β-HSD responsible for the metabolism of 17β-estradiol in *P. putida* SJTE-1 was characterized, and two novel transcriptional regulators (OxyR and CrgA) were identified to be involved in this pathway.

Materials and methods

Strains, chemicals, and cultures

All strains and plasmids used in this study are listed in Table 1. *P. putida* SJTE-1 (CGMCC No. 6585) was cultured at 30 °C. Luria-Bertani (LB) medium (tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 10 g/L) 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; and

Table 1 Strains and plasmids used in this study

Name	Description	Source/reference
Strains		
P. putida SJTE-1	Strain with the estrogen-degradation capability, wild type (CGMCC NO. 6585)	(Liang et al. 2012)
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	*E. coli* expression plasmid, Km^r	Novagen
pET28a-hsd	Plasmid pET28a inserted with *hsd* gene at *Eco*R I/*Nde* I sites, Km^r	This study
pET28a-oxyR	Plasmid pET28a inserted with *oxyR* gene at *EcoR* I/*Nde* I sites, Km^r	This study
pET28a-crgA	Plasmid pET28a inserted with *crgA* gene at *Bam* HI/*Nde* I sites, Km^r	This study
pBSPPc-Gm	*oriT*$^+$gene replacement vector derived from pBR322, Ap^r, Gm^r	(Xu et al. 2013)
pBSPPc-Gm-eGFP	*egfp* gene inserted into plasmid pBSPPc-Gm without promoter, Ap^r, Gm^r	This study
pBSPPc-P_{hsd}-eGFP	*egfp* gene inserted into plasmid pBSPPc-Gm with promoter of *17β-hsd* gene, Ap^r, Gm^r	This study
pBSPPc-crgA	*crgA* gene inserted into plasmid pBSPPc-Gm with promoter of lac gene, Ap^r, Gm^r	This study
pBSPPc-oxyR	*oxyR* gene inserted into plasmid pBSPPc-Gm with promoter of lac gene, Ap^r, Gm^r	This study

$MgCl_2$, 0.02 g/L) were used. 17β-estradiol, estrone, estriol, and testosterone (of HPLC grade, > 99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical reagent grade. As DMSO could not be utilized by *P. putida* SJTE-1, all the estrogenic chemicals were dissolved in DMSO (Fig. S1). All steroid chemicals were dissolved in dimethyl sulfoxide (DMSO) to form 10 mg/mL solutions, and the final concentration in solution ranged from 10 to 20 mg/L.

Standard DNA manipulation

Oligonucleotides, used for PCR amplification, reverse transcription (RT), quantitative PCR (q-PCR), homologous recombination, plasmid construction, and electrophoretic mobility shift assay (EMSA), were synthesized at Invitrogen Ltd. (Shanghai, China) and listed in Table 2. Restriction enzymes and DNA-modifying enzymes were purchased from TaKaRa Biocompany (Dalian, China). The PCR protocol used was first denaturation at 95 °C for 5 min, then denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1.5 min. This process was repeated 30 times, and then elongation was performed at 72 °C for another 5 min. The plasmids were constructed by ligating the restriction enzyme-treated PCR fragments and plasmid bones with T4 DNA ligase, and chemical transformation was performed with *E. coli* DH5α strain to obtain the target plasmids. The PCR products were separated by agarose gel electrophoresis and recovered with the QIAquick gel extraction kit (Qiagen, Shanghai, China). Plasmid DNAs were isolated using the QIAprep Mini-spin kit (Qiagen, Shanghai, China), and genome DNA was obtained using the QIAamp DNA minikit (Qiagen, Shanghai, China). Other general techniques for restriction enzyme manipulation, molecular cloning, and agarose gel electrophoresis were carried out with standard protocols. All the constructs were confirmed by DNA sequencing (Invitrogen, Shanghai, China).

Multiple sequence alignment

Multiple sequence alignment of 17β-HSD/OxyR/CrgA proteins from *P. putida* SJTE-1 and other organisms was performed with ClustalW software. The phylogenetic tree was constructed by MEGA 6.0 on the basis of amino acid sequences using the neighbor-joining method. The DNA sequence conservation analysis was performed with the Weblogo online software (http://weblogo.berkeley.edu/).

Expression and purification of the recombinant proteins

The recombinant 17β-HSD/OxyR/CrgA proteins were expressed and purified as described previously (Lu et al. 2012). Briefly, plasmid pET28a-hsd/pET28a-oxyR/pET28a-crgA was transformed into competent *E. coli* BL21 (DE3) cells. A single colony was inoculated in 10 mL LB media containing 50 μg/mL kanamycin and cultured overnight at 37 °C. The cultures were transferred into 1 L fresh LB containing 50 μg/mL kanamycin and cultured at 37 °C until OD_{600} = 0.5–0.6. Next, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the culture and then

Table 2 Oligonucleotides used in this study

Primers	Sequences (5′-3′)	Usage
eGFP-F	GGGGGGAAGCTTATGGTGAGCAAGGGCGAGGAG	Primers used for *egfp* gene amplification
eGFP-R	GGGGGGGGATCCTTAGTACAGCTCGTCCATGCCGAGA	
Hsd-P-F	GGGGGGCTCGAGAATTAGCAACCAAAGGTTTACTCTG	Primers used to amplify the promoter of *hsd* gene
Hsd-P-R	GGGGAAGCTTGCTGTTCTCCTGCTGGAAG	
Hsd-F	GGGGGGCATATGTCCAAGCAACTTACACTCGAAGG	Primers used for *hsd* gene amplification
Hsd-R	GGGGGGGAATTCTCAGGCTGCAAACCCACCAT	
OxyR-F	GGGGCATATGATGAACCTCAAGCAGCTCGAATAT	Primers used for *oxyR* gene amplification
OxyR-R	GGGGGAATTCCTAGCCTTTCTGTGCCCCCA	
CrgA-F	GGGGCATATGATGGCCATGGAATCATTCAGCG	Primers used for *crgA* gene amplification
CrgA-R	GGGGGATCCCTAAAGGGCCTGCAGGGCAT	
pBS-T-F	TCGACTAACCCAGATGCCG	Primers used for plasmid detection
pBS-T-R	AGGTAGCGAACCCGTCTC	
Hsd-P-F2 (5′-FAM)	AATTAGCAACCAAAGGTTTACTCTG	Primers used to amplify the promoter region of *hsd* gene for EMSA detection
Hsd-P-R2	GCTGTTCTCCTGCTGGAAG	
Hsd-Q-F	GGCTGAAGAACTGGTTCGAG	Primers for the q-PCR amplification of *hsd* gene
Hsd-Q-R	CCAGGTCGAACTCTGTCACC	
OxyR-Q-F	CATGTTCGTCTGGTGGAGAA	Primers for the q-PCR amplification of *oxyR* gene
OxyR-Q-R	TGTTCCATCACGTCCAGAAA	
CrgA-Q-F	TAGTACCCGCAAGCTGACCT	Primers for the q-PCR amplification of *crgA* gene
CrgA-Q-R	CAACGGCAACACGTACAAAC	

induced for 3 h at 37 °C. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C and resuspended with 25 mL ice-cold lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol (β-ME), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10% glycerol, pH 7.9). The cell solution was sonicated on ice, and then the supernatant was collected by centrifugation at 4 °C, 12,000 rpm for 30 min. The cell supernatants were loaded for affinity chromatography of Ni-NTA resin (Bio-Rad, CA, USA) at 4 °C and washed with 100 mL of washing buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM β-ME, 10% glycerol, 1 mM PMSF, and 50 mM imidazole, pH 7.9). Finally, the recombinant protein was eluted from the column using 5 mL of elution buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM β-ME, 10% glycerol, 1 mM PMSF, 200 mM imidazole, pH 7.9). All eluted solutions were analyzed by 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO). The eluted recombinant proteins were dialyzed and stored in the storage buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM 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).

Enzymatic assay of 17β-HSDs

The enzymatic activity of recombinant 17β-HSD protein was measured by detecting the yield of NADH at 355 nm (excitation)/460 nm (emission) as described previously (Pan et al. 2015). The reaction system contained 1 × reaction buffer (20 mM Tris-HCl, 25 mM NaCl), 200 μM NAD^+, and different concentrations of 17β-HSD protein and 17β-estradiol. A reaction mixture without 17β-HSD or 17β-estradiol was used as a blank. The parameters of 17β-HSD (K_m, V_{max}, optimal pH, and optimal temperature) were detected. Effect of different metal ions (Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, and Ni^{2+}) on the enzymatic activity was determined. All the experiments were repeated five times, and the results were the average values with standard errors.

HPLC detection

The solutions of enzymatic reaction were extracted with ethyl acetate, dried in the nitrogen blowing apparatus, dissolved with acetonitrile, and detected with HPLC (high-performance liquid chromatography, Agilent 1260 Infinity LC, USA). The Agilent Eclipse Plus C18 column (3.5 μm, 4.6 × 150 mm, Agilent, USA) and diode array detector (DAD) were used with the mobile phase of acetonitrile and water (1:1, *v*/*v*) at a flow rate of 1 mL/min at 30 °C, and the UV wavelengths were 280 nm (Ribeiro et al. 2010). Five independent experiments were performed, and results were calculated as average values with standard errors. The quantity of steroids was 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. The presented data are the averages of five independent measurements.

RT-qPCR

The transcription profiles of target genes in *P. putida* SJTE-1 cultured with different carbon sources were detected with RT-qPCR. The strain was cultured in minimal medium with 0.2% glucose or 10 mg/L 17β-estradiol to the mid-exponential phase, and the total RNA was extracted using the total RNA extraction reagents (Vazyme, Nanjing, China). 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). Quantitative PCR was performed using the Premix Ex Taq and gene-specific primers (Table 2) in the IQTM 5 Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA). The cycling conditions were 95 °C for 3 min, then 40 cycles of 95 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s. A final melting analysis was obtained by slow heating in increments of 0.5 °C for 10 s from 57 to 95 °C. The threshold cycle (Cq) value of each sample was determined during the exponential phase of amplification, and the relative fold change in mRNA quantity was calculated using the DDCt method (Livak and Schmittgen 2001). Five independent experiments were conducted for each RNA sample, and the average values with the standard errors were calculated.

Green fluorescent protein fluorescence assay

Gene *egfp* was cloned into vector pBSPPc-Gm to generate plasmid pBSPPc-eGFP (Table 1). The promoter fragment of *17β-hsd* was inserted into the upstream of *egfp* forming plasmid pBSPPc-P_{hsd}-eGFP (Table 1), which was electrotransformed into the *E. coli* BL21(DE3) strain. The recombinant strain was cultured in MM with 0.2% glucose or 10 mg/L of 17β-estradiol. The cells were collected at different time points, and the green fluorescent protein (GFP) fluorescence was measured at the fluorescence microplate reader 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.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed to detect the interaction of recombinant protein OxyR or CrgA with DNA fragments. The upstream DNA regions of *17β-hsd* gene were selected and amplified using primers labeled with

FAM at 5′-site (Table 2). The OxyR or CrgA protein and the DNA fragments were mixed in different molecular ratios and incubated in binding buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, pH 7.5) at 37 °C for 30 min. Then the mixture was analyzed with 8% native polyacrylamide gel, and the gel was displayed and photographed in the Bio-Rad Imaging System (Bio-Rad, CA, USA).

Detection of 17β-estradiol effect on protein-DNA interaction

17β-estradiol was supplied to detect its effect on the binding of OxyR or CrgA to the upstream region of the *17β-hsd* gene. The control used was 0.5% DMSO. The binding of OxyR or CrgA to the upstream fragments of *17β-hsd* gene was performed as described above. Different concentrations of 17β-estradiol were supplied and incubated for 15 min at 37 °C. Then the mixture was analyzed using an 8% native polyacrylamide gel and displayed in the Bio-Rad Imaging System (Bio-Rad, CA, USA).

Results

Sequence alignments showed that the predicted HSD is a member of 17β-HSD

According to the genome analysis of *P. putida* SJTE-1, one gene was predicted as a *hsd* gene, encoding an HSD protein (ANI04816.1). Multiple sequence alignment showed that the amino acid sequence and the secondary structure of this protein were consistent with those of the 17β-HSDs from *Rhodococcus* sp. P14, *E. coli* MG1655, *Homo sapiens*, *C. testosteroni* ATCC 11966, *Plasmodium falciparum*, and *Arabidopsis thaliana* (Fig. 1). All these proteins contain two conserved sequence motifs identical to those of the SDR family members: the N-terminal Gly-X-X-X-Gly-X-Gly motif for cofactor binding and the Tyr-X-X-X-Lys motif for proton acceptance. The secondary structure of this HSD of *P. putida* SJTE-1 is also composed of βαβ units such as a parallel β-sheet sandwiched between two α-helixes, which exists in many SDR proteins (Kavanagh et al. 2008). The conserved triad motif of HSD, Ser-Tyr-Lys (residues 141, 155, 159), was also observed in this HSD protein of *P. putida* SJTE-1. The serine residue probably functions as the active site, and the highly conserved tyrosine 155 may act as a proton acceptor (Beck et al. 2017). Therefore, this HSD is 17β-HSD, a member of SDR family, and its gene was named *17β-hsd*.

This 17β-HSD was induced by 17β-estradiol and efficiently transformed it into estrone

The transcription and expression levels of *17β-hsd*, *oxyR*, and *crgA* genes in *P. putida* SJTE-1 cultured with 17β-estradiol as a sole carbon source were measured. Results showed that when 17β-estradiol was used, the transcription of *17β-hsd* increased approximately 2.6-fold, while there were minimal changes to the transcription levels of *oxyR* and *crgA* (Fig. 2a). The expression of GFP protein under the promoter of *17β-hsd* gene was also observed with 2.4-fold enhancement after 3 h by the induction of 17β-estradiol, and this enhancement was sustained for several hours (Fig. 2b). These demonstrated that this 17β-HSD in *P. putida* SJTE-1 could be induced by strongly 17β-estradiol and may play a role in the metabolism of this chemical.

Next, the enzymatic characteristics of the 17β-HSD were studied. This *17β-hsd* gene was cloned and expressed in *E. coli* BL21 (DE3), and the purified 17β-HSD protein of 27.5 KD was obtained (Fig. 3a). The enzymatic assay showed that this 17β-HSD protein used NAD^+ rather than $NADP^+$ as its reaction cofactor. Detecting the fluorescence of NADH at 460 nm could quantify the oxidization efficiency of this enzyme. Only 17β-estradiol and testosterone could be oxidized by this 17β-HSD protein *in vitro* when different estrogenic substrates used, and the transformation of 17β-estradiol was more efficient than that of testosterone, indicating a substrate preference (Table 3). Enzymatic analysis showed that the optimal reaction temperature of this 17β-HSD was 37 °C, and the optimal reaction pH was pH 9.0, similar to other HSDs (Fig. 3b, c) (Mythen et al. 2018). Mg^{2+} could significantly enhance the reaction efficiency while Ca^{2+} and Cu^{2+} repressed the enzyme activity greatly (Fig. 3d). The K_m value of the 17β-HSD was 0.068 mM, and its V_{max} value was 52.26 μmol/min/mg (Table 4). Further, HPLC detection showed that estrone was the product of the oxidation reaction with 17β-estradiol as a substrate, and the transformation efficiency was above 98% within 5 min (Fig. 4). In summary, this 17β-HSD was induced by 17β-estradiol and oxidized it into estrone efficiently, confirming its involvement in the estrogen metabolism of *P. putida* SJTE-1.

OxyR and CrgA could bind to the specific and conserved sites in the promoter of *17β-hsd* gene and regulate the expression of 17β-HSD

Genome analysis of *P. putida* SJTE-1 showed that adjacent to the *17β-hsd* gene, there were two genes, *oxyR* and *crgA*, encoding two potential regulators, OxyR (ANI04815.1) and CrgA (ANI04817.1) (Fig. 5a). Multiple sequence alignment showed the OxyR and CrgA proteins of *P. putida* SJTE-1 both contained a typical helix-turn-helix (HTH) structure, including six α-helixes at the N-terminal for sequence-specific DNA

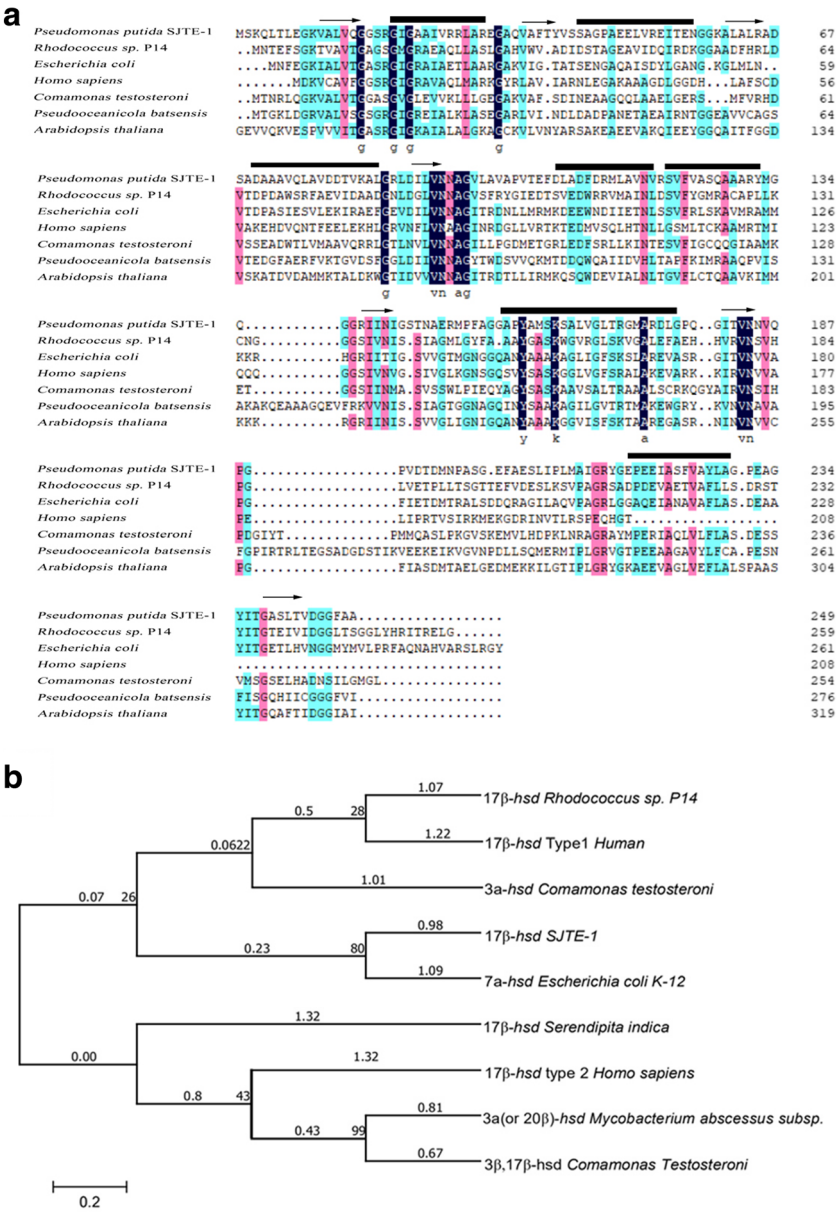

Fig. 1 Multiple sequence alignment (MSA) of 17β-HSDs. **a** The 17β-HSD from *P. putida* SJTE-1 (WP_014754112.1), *Rhodococcus sp*. P14 (WP_010595922.1), *E. coli* MG1665 (WP_001499617.1), Homo sapiens (XP_005263372.1), *C. testosteroni* ATCC11966 (WP_003080542.1), *Plasmodium falciparum* (WP_009803484.1), and *Arabidopsis thaliana* (NP_564216.1) was aligned with ClustalW. Conserved amino acids were indicated in blue bars, identical amino acids were marked in pink bars, and the similar amino acids were showed in lighter blue bars. The secondary structure of seven α-helixes and seven β-strands was shown in black rectangles and arrows. **b** The phylogenetic tree of this 17β-HSD and other related enzymes was constructed by MEGA 6.0 on the basis of amino acid sequences using neighbor-joining method. The bar represents a genetic distance of 0.20

binding and several β-strands in the C-terminal for substrate binding. The highly conserved motifs LXXXXA, FXRAA, QXXL, and TXXG of HTH-type regulators used for specific DNA binding were found in the two regulators (Fig. 5b, c). Secondary structures showed similarities to those of other OxyR regulators and CrgA regulators, respectively, which belong to the LysR family of regulators (Fig. 5b, c). Therefore, the two proteins encoded by *oxyR* and *crgA* near *17β-hsd* were considered to be the OxyR regulator and the CrgA regulators, respectively.

Since the transcription of OxyR and CrgA proteins could be induced by 17β-estradiol, they may regulate the degradation of this chemical (Fig. 2a). RT-qPCR results showed that overexpression of CrgA protein could enhance the transcription of *17β-hsd* approximately 2.1-fold, while overexpression of OxyR protein could decrease the transcription of *17β-hsd* significantly (Fig. 5d, e). This indicates that the two regulators may participate in the transformation of 17β-estradiol by regulating the transcription of *17β-hsd*.

To determine whether the OxyR and CrgA proteins were involved in the regulation of 17β-HSD directly, the two proteins were obtained through heterogeneous expression and affinity purification, and their molecular weights were 32.4 KD (CrgA) and 34.4 KD (OxyR) (Fig. 5f). EMSA results showed that the two proteins could both bind to the 118 bp upstream fragment of *17β-hsd* at a low protein/DNA ratio (Fig. 6a, b). OxyR protein bound to this DNA fragment at a lower ratio than did CrgA protein, indicating the stronger binding capability of OxyR from the promoter region of *17β-hsd* (Fig. 6a, b). Interestingly, addition of 17β-estradiol

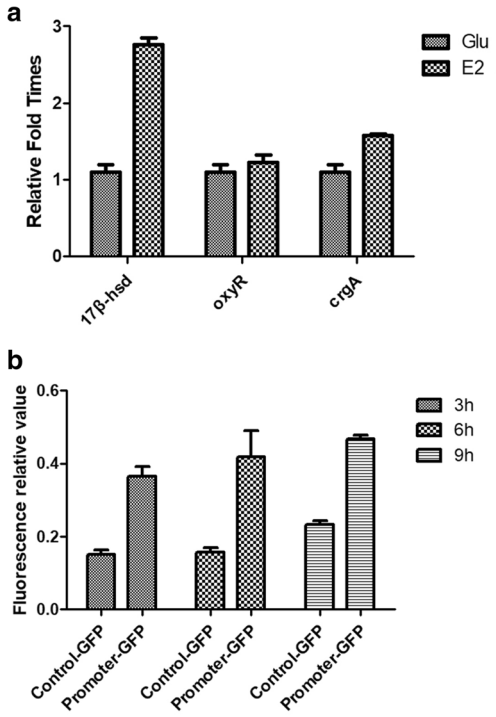

Fig. 2 The transcription and expression analysis of *17β-hsd* gene. **a** The transcription levels of 17*β-hsd*, *oxyR*, and *crgA* genes in *P. putida* SJTE-1 cultured with glucose or 17β-estradiol for different hours were detected. **b** The fluorescence values of *E. coli* BL21 (DE3) containing plasmid pBSPPc-P_{hsd}-eGFP were detected by inducing with 17β-estradiol of 20 mg/L for different hours

into the reaction mixture could release the binding of OxyR protein to the DNA fragment (Fig. 6c). However, CrgA bound to this DNA fragment could not be released by 17β-estradiol, and, in fact, seemed to strengthen the binding (Fig. 6d). These results demonstrated that the OxyR and CrgA regulator could regulate the expression of *17β-hsd* directly by binding to its promoter region, and 17β-estradiol could influence their binding abilities.

To find whether the conserved sites for OxyR and CrgA binding exist in the promoter of *17β-hsd* gene, sequence alignment and conservation analysis of the upstream region of *17β-hsd* gene were performed. Results showed that there existed two conserved recognition and binding regions of OxyR regulators, GATA-N_9-TATC, similar to that in the promoter region of *pdh* gene (Fig. 7a) (Zhang et al. 2015). Two possible binding regions of the CrgA regulator, T-N_{11}-A, were also found in the *crgA-17β-hsd* intergenic region (Fig. 7a) (Morelle et al. 2003). In addition, several sites were found to be conserved in the promoter of *crgA-17β-hsd* genes, likely used as binding sites (Fig. 7b). In summary, the two OxyR and CrgA regulators encoded by genes (*oxyR* and *crgA*) could directly bind to the specific sites in the promoter region of *17β-hsd* gene and regulate the expression of 17β-HSD in different manners.

Discussion

Environmental estrogen pollution is considered one of the most significant environmental problems, and

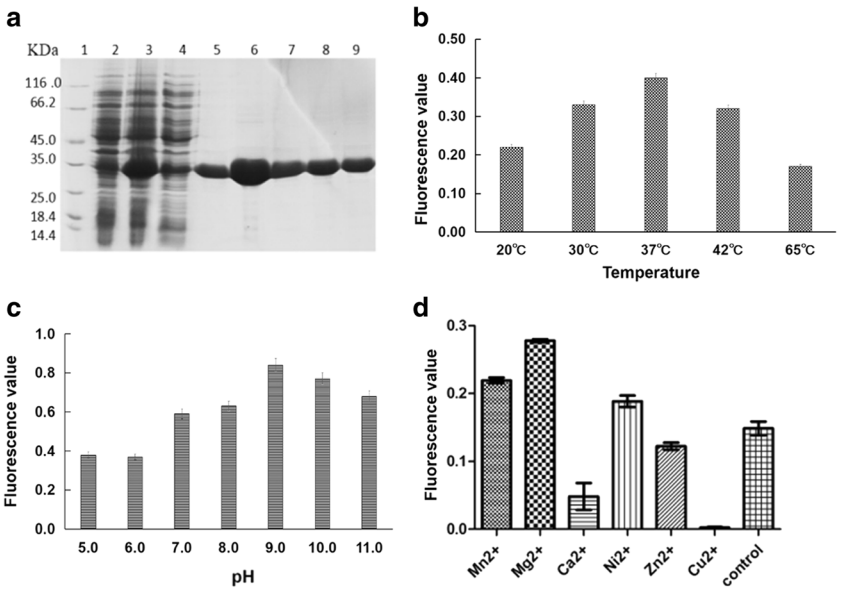

Fig. 3 Purification and characterization of 17β-HSD. **a** SDS-PAGE detection of recombinant 17β-HSD protein. Lane 1, protein marker; Lane 2, the pre-induction cell lysates; Lane3, the post-induction cell lysates; Lane 4, the solution after loading on the column; Lanes 5–9, the eluted protein solution. **b** The fluorescence value of NADH detected from the enzyme reaction performed at different temperatures (20–65 °C). **c** The fluorescence value of NADH detected from the enzyme reaction performed under different pHs (pH 5.0–11.0). **d** The fluorescence value of NADH detected from the enzyme reaction suppled with different metal ions of 1 mM (Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, and Ni^{2+}). The average values of three repeats were calculated and the standard errors were shown

Table 3 The substrates spectrum of the recombinant 17β-HSD protein

Substrates	17β-estradiol	Testosterone	Estrone	Estriol	Ethanol
Fluorescence values of NADH at 460 nm	0.937	0.790	− 0.165	− 0.006	− 0.145

The reactions were performed in 1 × reaction buffer (20 mM Tris-HCl, 25 mM NaCl), 200 μM NAD^+, 100 μM steroids, and 150 μM 17β-HSD protein. The reactions were performed at 37 °C for 5 min. Mixture without 17β-HSD or 17β-estradiol was used as blank

microorganisms with estrogen degradation capabilities could be used for efficient environmental remediation. In the steroid-degradation bacteria, the HSDs belonging to the SDR family were considered the first restriction enzymes to start the transformation of steroid chemicals (Chang et al. 2010; Maser et al. 2001; Yuanhua et al. 2015; Ye et al. 2017). In humans, HSDs have been well studied and confirmed to oxidize/reduce the active and inactive forms of estrogens at the C17 site with NAD(P)H as the cofactor (Chang et al. 2010). Previous reports have shown that bacterial HSDs could also catalyze the oxidation of testosterone and 17β-estradiol (Edmund et al. 2001; Yuanhua et al. 2015; Ye et al. 2017). In this work, one 17β-HSD in *P. putida* SJTE-1 was identified and confirmed to catalyze the transformation of 17β-estradiol into estrone efficiently, and its enzymatic activity was similar to or higher than those of reported HSDs (Yuanhua et al. 2015; Ye et al. 2017). The K_m and V_{max} values of the 17β-HSD in *Rhodococcus* sp. P14 were 18.9 μM and 12.5 μmol/min/mg; however, the V_{max} value of this 17β-HSD in *P. putida* SJTE-1 was about 4.2 times higher (52.26 μmol/min/mg). Although its K_m value (0.068 mM) was much larger, over 98% transformation from 17β-estradiol to estrone could be observed after 5 min, indicating high catalytic activity and interaction with substrates of this 17β-HSD protein. Since it could be induced by 17β-estradiol, this 17β-HSD participated in the transformation of 17β-hydroxyl steroids in *P. putida* SJTE-1, and its oxidization efficiency probably restricted the biodegradation of 17β-estradiol.

The SDR family consists of over 47,000 members, including isomerases, dehydrogenases, and decarboxylases found in prokaryotes, eukaryotes, and archaea (Kallberg et al. 2010). Although the identity of these proteins was only about 20%, the members share some conserved structures (Kallberg et al. 2010). Besides the classic motifs GXXXGXG and YXXXK, the classical SDRs also contain an efficient catalytic center formed by four highly conserved residues (Asn-Ser-Tyr-Lys), and NAD(P) or NAD(P)H always acted as their reaction co-factors (Kavanagh et al. 2008). HSD, a member of SDR family, also contained typical and conserved structures including seven parallel β-sheets at the center and three α-helices on each side. This core structure is the binding site of the cofactor NAD(P)H and the catalytic center with the conserved amino acid sequence Tyr-$(Xaa)_3$-Lys (Kallberg et al. 2010; Kavanagh et al. 2008). For example, 3α-HSD catalyzed the oxidoreduction at carbon 3 of steroid hormones in *C. testosteroni*, and three residues (Ser114, Tyr155, and Lys159) in 3α-HSD form a triad essential for the catalysis (Kavanagh et al. 2008). As for the 17β-HSD of *P. putida* SJTE-1 studied in this work, the conserved motifs, the βαβ units of SDR family, and the conserved Ser-Tyr-Lys-triad (residues 141, 155, 159) of HSDs were all observed in its sequence and structure; therefore, this enzyme was a typical HSD.

Although several 3,17β-HSDs have been found to participate in the metabolism of steroids, especially the oxidization at 17-position of these chemicals, the enzyme catalyzing degradation of 17β-estradiol was not yet clear. This was due to the fact that although the transformation of 17β-estradiol could be catalyzed by these enzymes *in vitro*, the reported 3,17β-HSDs could not be induced by 17β-estradiol (Gong et al. 2012a, b; Yuanhua et al. 2015). Thus, there must exist multiple isoenzymes for efficient transformation of 17β-estradiol *in vivo*. Previous work showed that *P. putida* SJTE-1 could utilize 17β-estradiol efficiently as a sole carbon source; its proteomics analysis indicated the expression of several hydroxysteroid dehydrogenases changed in the culture with 17β-estradiol (Liang et al. 2012; Xu et al. 2017). The 17β-HSD of this work is one of the upregulated

Table 4 The enzymatic characteristics of the recombinant 17β-HSD protein

Enzyme	K_m (mM)	V_{max} (μmol/min/mg)	*Kcat* (/s)	*Kcat*/K_m (/s mM)
17β-HSD	0.068	56.26	2.073	30.485

The reactions were performed in 1 × reaction buffer (20 mM Tris-HCl, 25 mM NaCl), 200 μM NAD^+, and 17β-estradiol and 17β-HSD protein of different concentrations. The reactions were performed at 37 °C for 5 min. Mixture without 17β-HSD or 17β-estradiol was used as blank

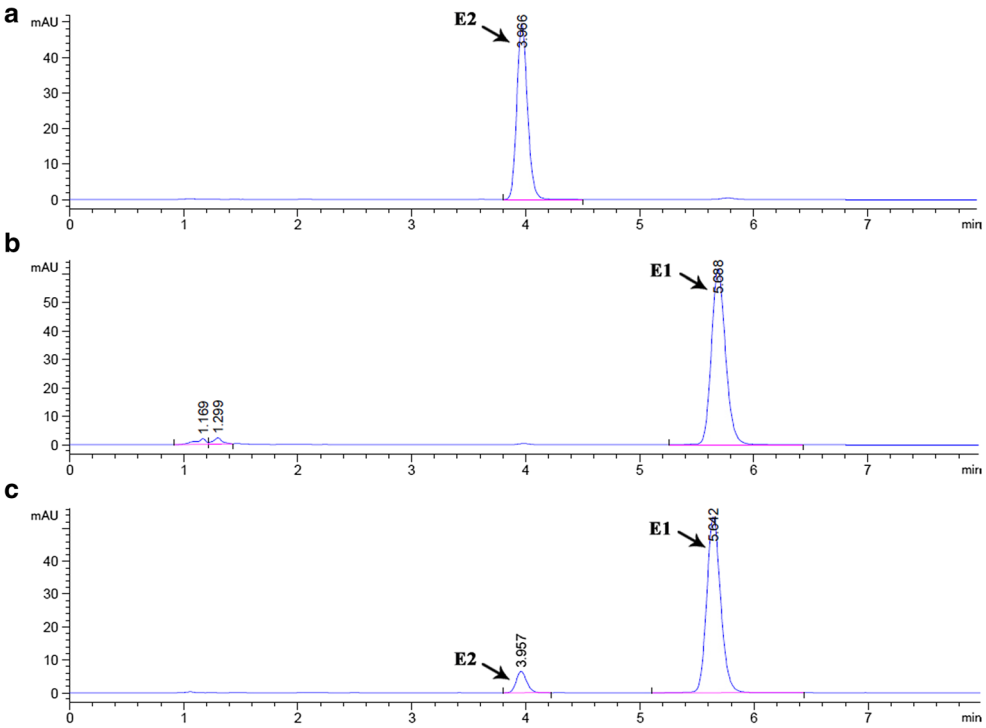

Fig. 4 HPLC detection of the reaction product of 17β-HSD. **a**The HPLC map of 0.2 μM 17β-estradiol. **b** The HPLC result of 0.2 μM estrone. **c** The HPLC map of the 17β-HSD oxidation reaction with 17β-estradiol as a substrate. The reaction mixture contained 0.2 μM of 17β-estradiol, 0.4 μM NAD^+, and 0.3 μM 17β-HSD in 1 × reaction buffer (20 mM Tris-HCl, 25 mM NaCl) and the reaction was performed at 37 °C for 2.5 min

proteins, and its induction by 17β-estradiol was also confirmed in this work. These results demonstrated this 17β-HSD was probably the key enzyme for estrogen transformation in *P. putida* SJTE-1. Considering its high enzymatic activity, this transformation could be completed efficiently, achieving the quick utilization of this chemical and fast energy transfer into the cell growth.

In addition to the uncertainty regarding major enzymes for 17β-estradiol transformation, the regulation networks and potential regulators involved in the biodegradation of 17β-estradiol were also unclear. Several regulators have been identified to participate in the regulation of the reported HSDs (Gong et al. 2012a, b; Ji et al. 2017; Li et al. 2013; Pan et al. 2015; Wu et al. 2015). Two repressors, Rep1 and Rep2, could inhibit the expression of 3α-HSD genes in the absence of substrates; when there is a substrate, the substrate can prevent the binding of the transcription suppressors to the promoter region upstream of the 3α-*hsd* gene (Gong et al. 2012a). HsdR belonging to the LysR-type transcriptional regulator (LTTR) family activates the expression of the *hsdA* gene in *C. testosteroni* (Gong et al. 2012b). As to the regulation of the *3,17β-hsd* gene, PhaR, TetR, LuxR, and BRP proteins were found to regulate the expression of this enzyme negatively and influence the degradation of testosterone in *C. testosteroni* (Ji et al. 2017; Li et al. 2013; Pan et al. 2015; Wu et al. 2015). However, this regulation was not obvious when 17β-estradiol used, not only because the utilization of 17β-estradiol in *C. testosteroni* was not as good as that of testosterone but also because the expression of *3,17β-hsd* gene could not be induced by 17β-estradiol in this microorganism (Hwang et al. 2005; Yuanhua et al. 2015). In addition, the binding characteristics and regulation mode of these regulators were still not analyzed clearly. As shown in this work, the *17β-hsd* gene could be strongly induced by 17β-estradiol, and the transcription levels of the two genes (*oxyR* and *crgA*) also increased with the addition of 17β-estradiol; overexpression of *oxyR* or *crgA* could generate adverse effects on the transcription of 17β-*hsd*. *In vitro* EMSA detection demonstrated that the OxyR and CrgA proteins could bind to the promoter region of the *17β-hsd* gene from *P. putida* SJTE-1 efficiently, and the specific binding of OxyR to this DNA fragment could be released by 17β-estradiol. This meant the OxyR protein probably acts as a repressor of 17β-*hsd*, and its transcriptional repression of 17β-HSD could be relieved by its substrate. In contrast, the CrgA protein is more likely to work as an activator of this *17β-hsd* gene, and the addition of 17β-estradiol may strengthen its activation.

Although the OxyR and CrgA proteins were members of the LysR-type transcription regulator (LTTR) family, their binding characteristics were specific and varied from each other. The common features of the LTTR family include sequence lengths of around 300 residues, high

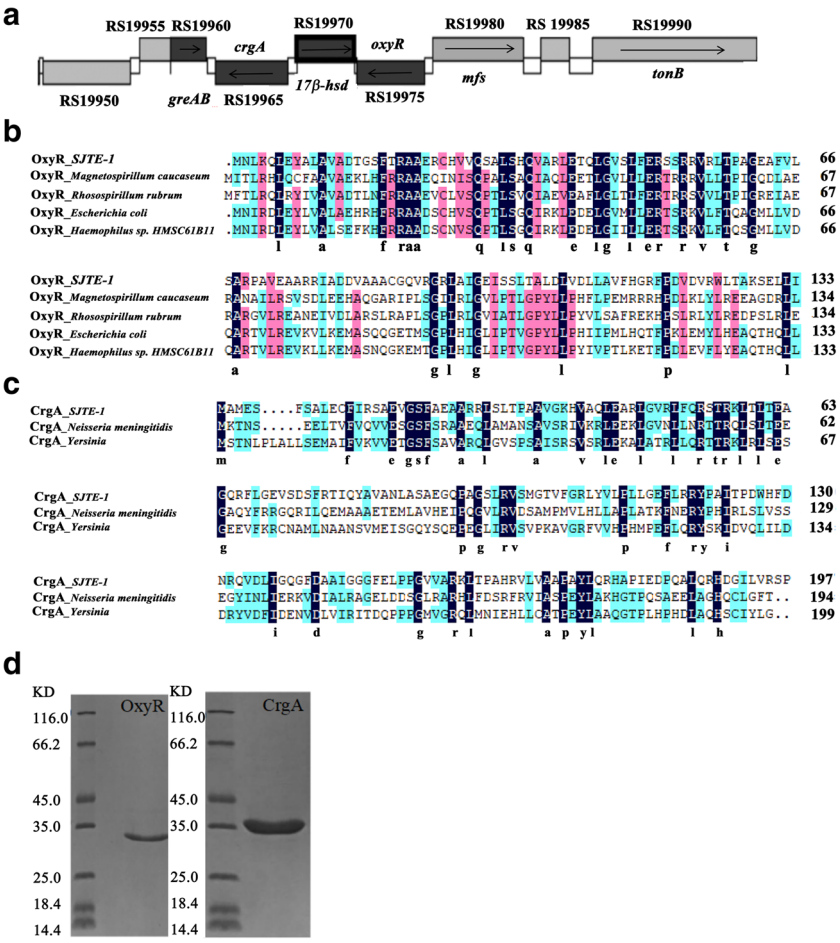

Fig. 5 Genes map, sequences alignment, and proteins purification of OxyR and CrgA. **a** The genes map of *oxyR*, *crgA*, and *17β-hsd* in the genome of *P. putida* SJTE-1. **b** The OxyR protein from *P. putida* SJTE-1 (ANI04815), *Magnetospirillum caucaseum* (EME68759.1), *Rhodospirillum rubrum ATCC 11170* (YP_427866.1), *Escherichia coli str. K-12 substr. MG1655* (NP_418396.1), and *Haemophilus* sp. *HMSC61B1* (OHR67871.1) was aligned with ClustalW. **c** The CrgA protein from *P. putida* SJTE-1 (ANI04817.1), *Neisseria meningitides* (AAF37819.1), and *Yersinia pestis Pestoides A* (EEO91441.1) was aligned with ClustalW. Conserved amino acids were indicated in blue bars, identical amino acids were marked in pink bars, and the similar amino acids were showed in lighter blue bars. The secondary structure of seven α-helixes and seven β-strands was shown in black rectangles and arrows. **d** The transcription levels of *17β-hsd* and *oxyR* genes in *P. putida* SJTE-1 containing plasmid pBSPPc-crgA were detected, which was cultured with 17β-estradiol of 20 mg/L and induced with 0.5 mM IPTG for 3 h. **e** The transcription levels of *17β-hsd* and *crgA* genes in *P. putida* SJTE-1 containing plasmid pBSPPc-oxyR were detected, which was cultured with 17β-estradiol of 20 mg/L and induced with 0.5 mM IPTG for 3 h. **f** The SDS-PAGE profiles of purified OxyR and CrgA proteins

sequence similarity at the N-terminal HTH motif for DNA binding, an inducer binding C-terminal domain, and a homotetrameric quaternary structure of the active species. Proteins belonging to the LTTR family normally are dual function regulators acting as both auto-repressors and activators of target promoters (Xiong and Maser 2015). It has been demonstrated that there is a conserved repression binding site (RBS, −80 to −50) and an activation binding site (ABS, −50 to −20) in the target promoters controlled by LTTRs (Schell 1993). The sequences of conserved recognition and binding region of the OxyR regulators were -GATA-N_9-TATC-, and that of the CrgA regulators was -T-N_{11}-A- (Morelle et al. 2003; Zhang et al. 2015). In this study, two potential conserved binding motifs of the OxyR regulator and two possible binding regions of the CrgA regulator were found in the *crgA-17β-hsd* intergenic region. OxyR, acting as a global regulator of oxidative stress response in *E. coli*, regulates the expression of genes required for protection against oxidation toxicity, heat stress, and phagocyte-mediated killing (Wei et al. 2012; Henikoff et al. 1988). It can also facilitate the efficient utilization

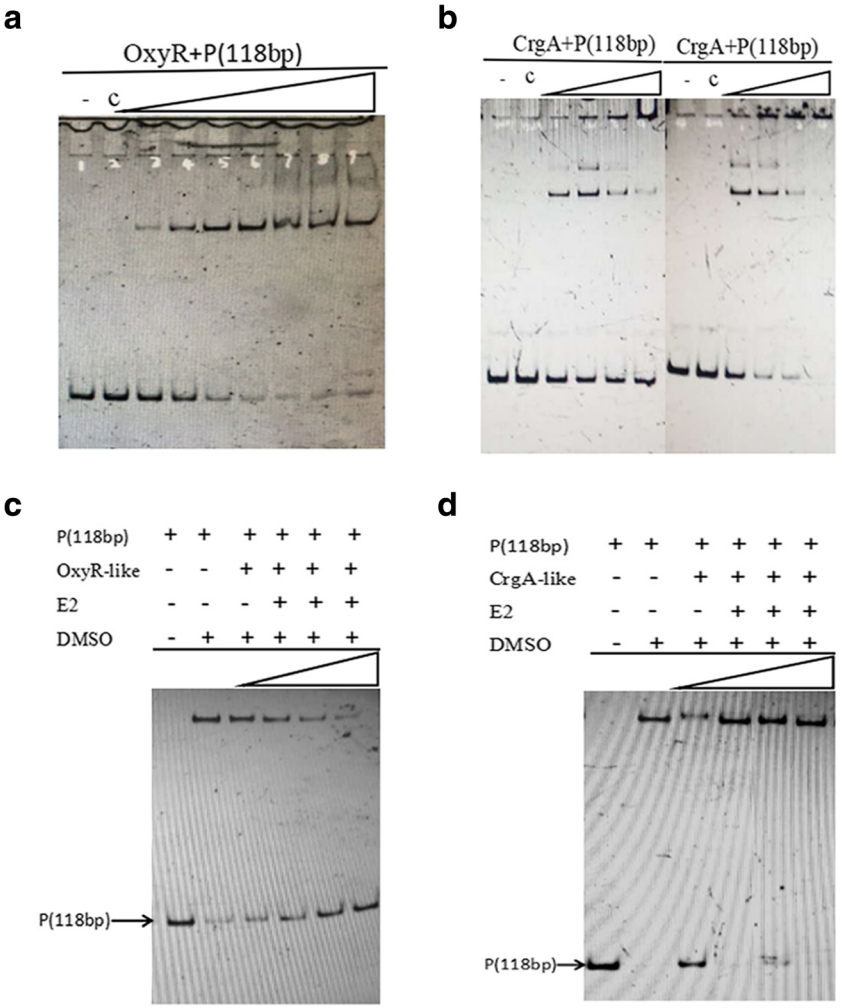

Fig. 6 EMSA detection of the binding of OxyR and CrgA to the promoter region of *17β-hsd* and the effect of 17β-estradiol. **a** EMSA results of OxyR protein to the 5′-FAM labeled 118 bp fragment of *17β-hsd* gene. Two micromolar labeled DNA was used for each reaction. The first lane (−) was the free-labeled DNA and the second lane (c) was added with 10 μM BSA. Other lanes were supplied with the increased amounts of OxyR protein in the protein/DNA ratio from 0.1:1, 0.2:1, 0.4:1, 0.6:1, 0.8:1, 1.2:1 to 1.5:1 (lane 3–lane 9). **b** EMSA results of CrgA protein to the 5′-FAM labeled 118 bp fragment of *17β-hsd* gene. Two micromolar labeled DNA was used for each reaction. The first lane (−) was the free-labeled DNA and the second lane (c) was added with 10 μM BSA. Other lanes were supplied with the increased amounts of CrgA-like protein in the protein/DNA ratio from 0.2:1, 0.45:1, 1:1 to 1.5:1 (lane 3–lane 6, left) and 3:1, 4.5:1, 6:1 to 7.5:1 (lane 3′–lane 6′, right). **c** The effect of 17β-estradiol on the binding of OxyR to DNA fragment. Two micromolar labeled DNA was used for each reaction. The first lane was the free-labeled DNA, and lane 2 was added 1 μL DMSO. Lane 3 was added with OxyR protein (the protein/DNA ratio was 3:1) and 1 μL DMSO. Lanes 4–6 was added 5 μM of E2, 10 μM of E2, and 15 μM of E2, all solved in 1 μL DMSO. **d** The effect of 17β-estradiol on the binding of CrgA to DNA fragment. The first lane was the free-labeled DNA, and lane 2 was added 1 μL DMSO. Lane 3 was added with CrgA protein (the protein/DNA ratio was 7.5:1) and 1 μL DMSO. Lanes 4–6 was added 5 μM of E2, 10 μM of E2, and 15 μM of E2, all solved in 1 μL DMSO

of limited resources during the oxidative stress condition by activating crucial cellular mechanisms (Staudinger et al. 2002). Thus, this OxyR protein of *P. putida* SJTE-1 could probably function as a global regulator to resist the oxidative stress generated by 17β-estradiol and enhance the transformation of this chemical. On the other hand, CrgA was initially identified in *Neisseria meningitidis* involved in the regulation of cell infection to human epithelial cells and appears to be a part of a group of genes that are coordinately upregulated during initial adhesion (Deghmane et al. 2000; Morelle et al. 2003). The HsdR activating the expression of the *hsdA* gene in *C. testosteroni* was also found to be a CrgA-type regulator (Gong et al. 2012a). These implied that the CrgA protein of *P. putida* SJTE-1 may help the strain's fast adaption to the 17β-estradiol environment and facilitate the efficient utilization of this chemical. Therefore, the OxyR regulator and CrgA regulator can both directly recognize and bind to the specific motifs in the promoter of *17β-hsd* genes, regulate the expression of *17β-hsd* in

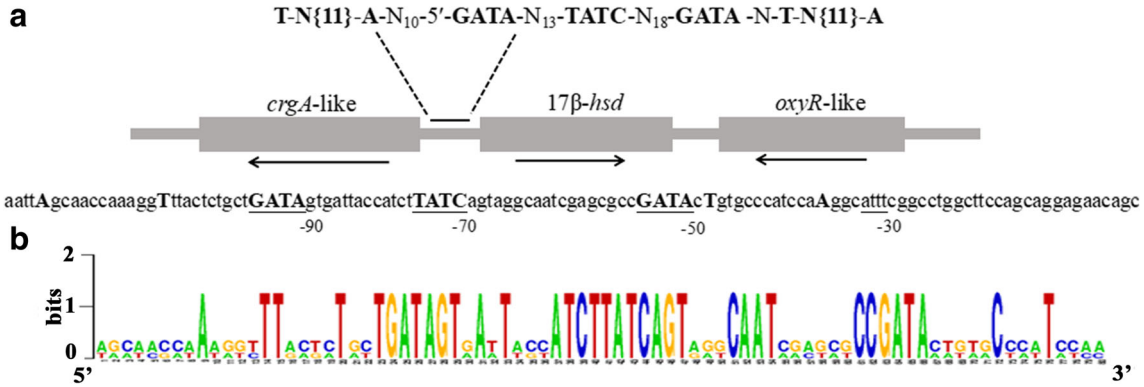

Fig. 7 Conserved nucleic acid analysis of the promoter region of *17β-hsd* gene. **a** The sequences and the potential conserved motifs in the promoter region of *17β-hsd* gene. Conserved motif T-N11-A was binding site of protein CrgA, and that of protein OxyR was motif GATA-N_X-TATC. The big and bold words meant the potential conserved motifs. **b** The conserved nucleic acid analysis results of the promoter region of *17β-hsd* gene with MEME software. The big words meant the high conserved nucleic acids

different patterns, and play important roles in the 17β-estradiol metabolism in *P. putida* SJTE-1. In summary, the degradation of 17β-estradiol in *P. putida* SJTE-1 was complex with multiple regulators participating in the process; however, further work is needed.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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