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Research Paper

Identification of a 17β-estradiol-degrading *Microbacterium hominis* SJTG1 with high adaptability and characterization of the genes for estrogen degradation

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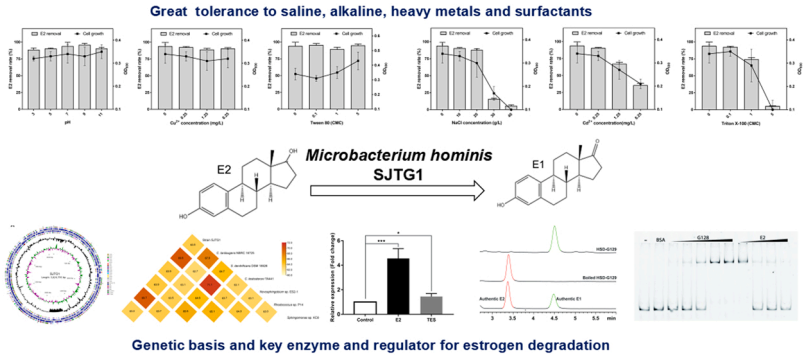
HIGHLIGHTS

- *Microbacterium hominis* SJTG1 grew normally and degraded E2 efficiently under various harsh conditions.
- *M. hominis* SJTG1 removed E2 in the simulated E2-polluted soil environment effectively and stably.
- Comparative genome analysis predicted the potential genes involved in steroid degradation in strain SJTG1.
- The 3β/17β-hydroxysteroid dehydrogenase HSD-G129 catalyzed the 3β-/17β-dehydrogenation of different steroids.
- Regulator LysR-G128 repressed the transcription of *hsd-G*129 and regulated the steroid degradation in strain SJTG1.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental estrogen contamination poses severe threat to wildlife and human. Biodegradation is an efficient strategy to remove the wide-spread natural estrogen, while strains suitable for hostile environments and fit for practical application are rare. In this work, *Microbacterium hominis* SJTG1 was isolated and identified with high degrading efficiency for 17β-estradiol (E2) and great environment fitness. It could degrade nearly 100% of 10 mg/L E2 in minimal medium in 6 days, and remove 93% of 1 mg/L E2 and 74% of 10 mg/L E2 in the simulated E2-polluted solid soil in 10 days. It maintained stable E2-degrading efficiency in various harsh conditions like non-neutral pH, high salinity, stress of heavy metals and surfactants. Genome mining and comparative genome analysis revealed that there are multiple genes potentially associated with steroid degradation in strain SJTG1. One 3β/17β-hydroxysteroid dehydrogenase HSD-G129 induced by E2 catalyzed the 3β/17β-dehydrogenation of E2 and other steroids efficiently. The transcription of *hsd-G*129 gene was negatively regulated by the adjacent LysR-type transcriptional regulator LysR-G128, through specific binding to the conserved site. E2 can release this binding and initiate the degradation process. This work provides an efficient and adaptive E2-degrading strain and promotes the biodegrading mechanism study and actual remediation application.

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1. Introduction

Environmental estrogens (EEs) are distributed ubiquitously in many environments, such as lake, soil, wastewater, sludge, and livestock manure. Due to the stable chemical structure and low functional concentration, estrogenic chemicals can persist in environments for quite a long period and threaten the health of wildlife and human. EEs pollution has been becoming a severe and emerging environmental issue (Zhong et al., 2021). 17β-estradiol (E2), a typical and prevalent member of natural estrogens excreted by animals and human beings, can disrupt reproductive development and increase the risk of breast cancer at trace concentration (ng/L). The chronic exposure under the E2-contaminated environments can affect reproduction, growth, and behavior of organisms. When reaching certain concentration, EEs cause malformation, infertility, and reduced population size (Adeel et al., 2017; Zhao et al., 2019). The industrial farming and human activities generated more estrogenic discharge and the continual accumulation of EEs in the environment exacerbates the harmful effects, which have drawn worldwide attention.

Biodegradation using microorganisms has become the principle and efficient strategy to remove environmental estrogenic pollutants, as its higher efficiency, lower costs and more environmentally friendly compared with physico-chemical methods (Zhao et al., 2019). Bacteria are the main species of E2-degraders. A series of estrogen-degrading bacteria have been isolated from different environmental habitats, such as *Pseudomonas*, *Novosphingobium*, *Stenotrophomonas*, *Rhodococcus*, *Acinetobacter* (Liu et al., 2018; Li et al., 2018, 2017; Wang et al., 2019; Xiong et al., 2020a, 2020b; Ye et al., 2019; Qiu et al., 2019). The degradation of E2 in bacteria can be achieved through the 9, 10-*seco* pathway, the 4, 5-*seco* pathway, and the 2, 3-*seco* pathway (Holert et al., 2018; Chen et al., 2017, 2018; Li et al., 2020; Ibero et al., 2020; Wu et al., 2019; Hsiao et al., 2022; Wang et al., 2020). In these estrogen-degrading bacteria, the hydroxysteroid dehydrogenases (HSDs), the member of short chain dehydrogenase/reductase (SDR), catalyzed the initial and restricted step in the biodegradation pathway of typical steroids (Wang et al., 2019; Xiong et al., 2020a, 2020b; Chen et al., 2017, 2018; Li et al., 2020; Wu et al., 2019). It is worth noting that although strains can remove estrogens effectively in laboratory conditions, the exogenously inoculated strains into wild environments confront the adverse and execrable conditions and the furious competition with the inherent microorganisms, leading to the apparent lag in cell growth and the desperate degeneration in degradation capacity (Li et al., 2018). Besdides, only several degrading strains have been sequenced and a few enzymes involved in the E2 degradation have been found. It is still indistinct about the metabolic and regulatory mechanism of estrogen degradation in bacteria. It also limits the practical use of estrogen-degrading strains in actual environments. Therefore, it is in extreme necessity to isolate and identify effective bacterial strains with high degrading efficiency, great environmental suitability and clear genetic background, used for practical bioremediation of estrogens.

In this work, an estrogen degrading strain, *Microbacterium hominis* SJTG1 was isolated with efficient steroid-degrading capacity and great environmental suitability. Based on its whole genome sequence and the comparative genome analysis, a series of potential genes involved in E2-biodegrading process of this strain was aligned. One novel 3β/17β-HSD responsible for the first key step in E2 degradation was identified; its regulator and the regulatory mechanism was also discovered. These findings enrich the degrading strain pool, promote the estrogen-degrading mechanism study in bacteria and facilitate practical bioremediation for estrogen removal in environment.

2. Materials and methods

2.1. Chemicals, cultures and standard DNA operation

17β-estradiol (E2, >99%), estrone (E1, >99%), testosterone (TES, >99%), and dehydroepiandrosterone (DHEA, >99%) were the products of Sigma-Aldrich Company (Allentown, PA, USA); 4-androstenedione (4-AD, >99%), acetonitrile (HPLC grade, >99.5%) and ethyl acetate (HPLC grade, >98%) were the products of Aladdin Biochemical Technology Company (Shanghai, China); all other chemicals were analytical grade. All the steroids were dissolved in DMSO to the concentration of 10 mg/mL and stored at − 20 °C. Luria-Bertani medium (LB) and basic salt medium (BSM) was used for strain culture (Xiong et al., 2020a). The solid plated were made with the addition of 15 g/L agar into the liquid medium. All the strains, plasmids and oligonucleotides used in this work were listed in Table S1. The genome DNA was obtained using TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) and the 16 S rDNA gene fragments were amplified by Bacterial 16 S rDNA Kit (TaKaRa Biotechnology, Dalian, China). The total RNA was extracted by the RNAiso Plus reagent (TaKaRa Biotechnology, Dalian, China). The synthesis of the oligonucleotides and sequencing of the PCR-amplified fragments and the constructed plasmids were performed by Invitrogen Inc. (Shanghai, China).

2.2. Isolation and identification of the E2-degrading strain

The wastewater collected from a wastewater treatment plant in Shanghai, China (no specific permissions were required), was mixed with BSM medium with 25 mg/L of E2 in 1:10 ratio and cultured for 15 days at 30 °C. After six-round enrichment, the culture was spread on the BSM plates with E2 and cultured for 3 days at 30 °C. The colonies with fast grow rate on the E2-supplied plate were picked and named as SJTG1. It was deposited in the China General Microbiological Culture Collection Center (CGMCC) with accession NO. 15437 and its physiological properties were determined (Breed et al., 1944). The 16 S rDNA gene of strain SJTG1 was amplified and sequenced, with the sequence deposited in GenBank (MH180761.1). Based on the alignment of 16 S rDNA gene sequences, the nearest relatives of strain SJTG1 were identified and the phylogenic tree was constructed by MEGA-X using neighbor-joining method with 1000 replications (Kumar et al., 2018).

2.3. Whole genome sequencing and the average nucleotide identity (ANI) analysis

The genome DNA of strain SJTG1 was extracted for construction of a 10-kb insert SMRT-bell library and a 400-bp insert library. The first library was sequenced by PacBio RS II platform of the Pacific Biosciences (PacBio) RS II sequencer (Pacific Biosciences, CA, USA) and the data assembled using the Celera® Assembler and PBjelly software. The latter library was sequenced by Illumina Miseq platform and the data were corrected with Kmer and assembled with Newbler (Glenn, 2011; Denisov et al., 2008; English et al., 2012; Fletez-Brant et al., 2013; Margulies et al., 2005). The gaps were closed by specific PCR and Sanger sequencing. The genome sequence was annotated using RAST database and submitted into GenBank database (NZ_CP025299.1) (Aziz et al., 2008). The coding sequences (CDSs) were predicted with Glimmer 3.0 and genes were annotated in NCBI-NR, eggNOG, Swiss-Prot, and KEGG databases (Delcher et al., 1999; Benson et al., 2003; Powell et al., 2014; Boutet et al., 2016; Kanehisa et al., 2008). Multiple sequences alignment (MSA) was performed with MAFFT software, and the ANI analysis between strain SJTG1 and the relatives was performed with JSpeciesWS software (Katoh et al., 2002; Richter et al., 2016).

2.4. Determination of the E2-degrading efficiency by strain SJTG1 under different culture and simulated soil

To study the E2-degrading efficiency, single colony of strain SJTG1 was cultured in LB medium overnight at 30 °C and cells were collected. The cells were inoculated into 100 mL BSM medium containing E2 of different concentrations (10, 25, or 50 mg/L) with an initial 0.05 OD_{600}, and cultured at 30 °C for seven days. The culture supplied with the

boiled cell inoculum was set as the control. To investigate the effect of various stresses (pH, salinity, heavy metals and surfactants) on the cell growth and E2-degrading efficiency of strain SJTG1, the BSM medium was adjusted into the gradual pH values (3.0–11.0), supplied with different concentrations of NaCl (10–40 g/L), Cu^{2+} (0.05–6.25 mg/L), Cd^{2+} (0.05–6.25 mg/L), Tween 80 [0.1–5.0 critical micelle concentration (CMC)] or Triton X-100 (0.1–5.0 CMC), respectively. The residual estrogens in the culture was sampled every two days and detected by the High Performance Liquid Chromatography (HPLC) system.

The E2 removal of strain SJTG1 in the simulated E2-polluted soil environment was analyzed as described before (Xiong et al., 2020a). The solid soil were collected, treated and amended with E2 (1 mg/kg or 10 mg/kg). The cells of strain SJTG1 were inoculated into soil samples with the final density of 0.1 OD_{600}/10 g soil. The soil samples were cultured at 30 °C for 15 days with every-day overturning and water replenishing. The soil adding the boiled cell inoculum was used as control. All the soil samples were taken every 2 days to extract E2 residues with acetonitrile for HPLC detection.

The HPLC detection of steroid chemicals was performed as described before (Wang et al., 2019). The Agilent 1260 Infinity LC system (Agilent, CA, USA) and a SB-C18 reverse-phase column (4.6 ×150 mm, 5 μm particle size, Agilent, CA, USA) was used. Steroids (E1, E2, and TES) dissolved by acetonitrile into different concentrations (0.1 μg/mL~25 μg/mL) to plot the standard curves. The column was maintained at 30 ℃, the mobile phase consisted of acetonitrile and water (55/45, v/v) with flow rate of 1.0 mL/min. The degrading metabolites and residual steroids were detected and calculated based on the standard curves with R^2 values over 0.99. The steroid recovery efficiency in all samples were over 95%. All data were the average values of five independent experiments.

2.5. Transcription analysis of the target genes in strain SJTG1 cultured with steroids

The transcription levels of genes in strain SJTG1 cultured in BSM medium with different carbon sources were detected by reverse transcription and quantitative PCR (RT-qPCR). Strain SJTG1 was cultured in BSM medium with 10 mg/L of E2, TES or 0.2% glucose for 24 h, and the total RNA was extracted for cDNA synthesis with PrimeScript RT reagent Kit (TaKaRa Biotechnolog, Dalian, China). The quantitative PCR was performed using Premix ExTaq (TaKaRa Biotechnolog, Dalian, China) in a qTOWER 3 Real-Time PCR Thermal Cycler (Analytik Jena AG, Jena, GER). The transcription levels of target genes (*hsd-G*129 and *lysR-G*128) were detected with the primers listed in Table S1. The *rpoD* gene was used as an internal control and the $2^{-\Delta\Delta Ct}$ algorithm was used to calculate the relative fold changes in transcription. Three parallels were set for one gene and five independent experiments were conducted in each treatment; all of the presented data were the average values with standard errors. Statistical analysis was performed with the relative mRNA quantities and the p value was calculated (p value < 0.05, significant).

2.6. Expression detection of gene hsd-G129 co-constructed with gene lysR-G128

The Green fluorescent protein (GFP) reporter assay was used to detect the expression of the *hsd-G*129 gene in strain SJTG1. The promoter region of *hsd-G*129 gene fused with *egfp* gene, was cloned under P_{BAD} promoter in plasmid pBAD18, generating plasmid pB-pG129-gfp. Then the gene *lysR-G*128 was inserted between P_{BAD} promoter and the promoter of *hsd-G*129 gene, forming plasmid pB-G128-pG129-gfp (Table S1). The two plasmids were transformed into *E. coli* (DH5α), respectively, and cultured in BSM medium with 0.2% of glucose. Then the culture were collected and induced with 0.1% L-arabinose for 4 h. The GFP fluorescence was measured with a Fluoroskan Ascent FL (Thermo Fisher Scientific, USA) with wavelength of 485 nm/527 nm. Five independent experiments were conducted and all data were the average values with standard errors. Statistical analysis was performed and the p value was calculated (p value < 0.05, significant).

2.7. Heterologous gene expression and recombinant protein purification

The *hsd-G*129 gene and *lysR-G*128 gene were amplified from the genome DNA of strain SJTG1 and cloned into the plasmid pET-28a, forming plamsids pET-hsd-G129 and pET-lysR-G128 (Table S1). The two plasmids were transformed into *E. coli* BL21(DE3) and the expression of two heterologous genes were induced by adding Isopropyl β-D-Thiogalactoside (IPTG). Affinity purification of two recombination proteins (HSD-G129 and LysR-G128) were performed with the Ni-NTA resin as described before (Wang et al., 2019). The purified proteins were analyzed with the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and its concentration was determined using a BCA protein assay kit (TaKaRa Biotechnolog, Dalian, China).

2.8. Detection of steroid transformation by the recombinant HSD-G129

The transformation ability of recombinant HSD-G129 to different steroids (E1, E2, TES, DHEA) was analyzed by the in vitro enzymatic assay.The residual substrate and the generated products was detected with HPLC. The reaction system contained 1 × reaction buffer (20 mM Tris-HCl, 25 mM NaCl, 200 μM NAD^+), 100 μM steroid, and 150 μM HSD-G129. The mixture with the boiled HSD-G129 or with no substrate were used as controls. The reactions were performed at 37 °C for 5 min. Five independent experiments were repeated to obtain average values with standard errors.

2.9. Electrophoretic mobility shift assay (EMSA)

The EMSA assay to detect the binding capability of recombinant protein LysR-G128 to the upstream fragment of *hsd-G*129 gene was performed as described before (Wang et al., 2019). The 5′-FAM-labeled DNA fragments of the *hsd-G*129 gene promoter were amplified from the genome DNA of strain SJTG1. The reaction system of 20 μL (containing the binding buffer, 5–10 nM DNA fragments of *hsd-G*129 gene and protein HSD-G128 in gradient amounts) was mixed and incubated at 37 °C for 30 min, with adding 5 μL of 30% glycerol for reaction stop. The mixture of the binding assay was analyzed with the 8% native polyacrylamide gel and visualized using an imaging system of Amersham Typhoon RGB (GE, CT, USA).

3. Results

3.1. Microbacterium hominis SJTG1 degraded E2 efficiently in various adverse conditions and in simulated solid soil

The isolated strain SJTG1 with the E2-degrading capability was aerobic, asporous, and rod-shaped. It can utilize sucrose, rhamnose, L-arabinose, glycogen, and citrate, and reacts positively to ONPG, CIT, TIDA, VP, resistant to kanamycin (Table S2). The phylogenetic tree showed that strain SJTG1 clustered with the members of *Microbacterium hominis* (Fig. 1). The ANI analysis indicated that the genome sequence of strain SJTG1 was close to those of *M. hominis* NBRC 15708 and *M. hominis* LCDC 84–0209, with ANIm/ANIb values of 98.88/ 98.45 and 98.63/ 98.19, respectively (Table 1). Therefore, strain SJTG1 was a member of *M. hominis*.

The E2-degrading efficiency of *M. hominis* SJTG1 was detected by monitoring cell growth and E2 residues in culture. Results showed that it removed nearly 100% of 10 mg/L E2 or over 85% of 50 mg/L E2 in BSM medium in 6 days, with the biomass accumulated from 0.1 to 0.3 (Fig. 2). No reduction of E2 was observed in the samples with the boiled cells (data not shown). The generation of E1 was also detected in the samples added with 25 mg/L of E2, with the amounts of E1 reached peak at about 36–72 hous and decreased gradually (Fig. S1). It also the period

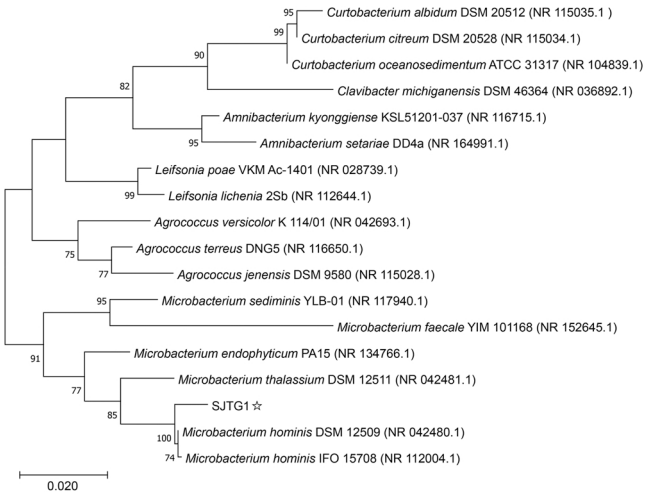

Fig. 1. Phylogenetic tree based on 16 S rRNA gene sequences of strain SJTG1 and the related strains. The phylogenetic tree was constructed using MEGA-X on te basis of 16 S rDNA gene sequences from different strains with the neighbor-joining method. The bootstrap consensus tree was performed with 1000 replications. The distances and similarities were shown.

Table 1
ANI analysis between strain SJTG1 and 14 most closely related strains.

Species	Strain SJTG1	
	ANIm (%)	ANIb (%)
M. hominis NBRC 15708	98.88 [86.08]	98.45 [83.33]
M. hominis LCDC 84–0209	98.63 [85.79]	98.19 [83.14]
M. hominis TPW29	86.69 [53.49]	83.80 [61.69]
M. telephonicum S2T63	85.40 [26.90]	78.21 [46.88]
M. oleivorans CD11_3	85.23 [20.76]	76.75 [43.86]
M. testaceum StLB037	84.77 [17.41]	75.41 [43.90]
M. aerolatum NBRC 103071	84.57 [12.94]	74.30 [36.76]
M. hydrocarbonoxydans DSM 16089	84.46 [13.14]	74.39 [38.72]
M. oxydans DSM 20578	84.31 [12.36]	74.03 [38.69]
M. humi DSM 21799	83.96 [6.48]	71.60 [30.73]
M. lindanitolerans DSM 22422	83.86 [3.39]	70.32 [27.67]
M. agarici DSM 21798	83.82 [3.35]	70.38 [27.66]
M. sorbitolivorans C1.15228	83.81 [8.81]	72.91 [29.32]
M. gubbeenense DSM 15944	83.66 [7.98]	72.69 [29.63]

with the highest E2 removal rate. The biodegradation kinetics of E2 by strain SJTG1 was fitted with the first-order kinetic equations. Its degrading half-lives to E2 of 10 mg/L, 25 mg/L, and 50 mg/L were 1.99, 4.69, and 6.76 days, respectively (Table 2).

Notably, strain SJTG1 showed great tolerance to various harsh conditions, with normal cell growth and stable E2-degrading efficiency with wide-range pH values (3−11) and high salinity (2% NaCl). Too much NaCl (40 g/L) inhibited the E2 degradation significantly due to great repression on cell growth (Fig. S2). Strain SJTG1 could endure high-concentration of copper and cadmium, with almost no effect of 6.25 mg/L $CuCl_2$ on growth and degradation. However, the effect of cadmium was more significant than that of copper (Fig. S2). The two surfactants (Tween 80, Triton X-100) showed diverse effect on strain SJTG1. Tween 80 had little influence on cell growth and E2-degrading efficiency; at high concentration, it promoted cell growth of strain SJTG1 to some extent, probably due to improved solubility and accessibility of substrate. Low concentration of Triton X-100 (0.1–1 CMC) hardly had effect on strain SJTG1, while 5 CMC of Triton X-100 impaired cell growth greatly probably by a complete cell lysis (Fig. S2).

Furthermore, strain SJTG1 exhibited stable suitability to the simulated E2-polluted soil environment, and presented effective removal of E2. The inoculation of strain SJTG1 removed 93% of 1 mg/kg E2 and 74% of 10 mg/kg E2 from solid soil in two weeks (Fig. 3). It was significantly higher than the removal rate of 10% in control with the boiled cells, which may come from the absorption of solid soil particles. The E2-degrading half-lives of strain SJTG1 in solid soil were 3.91 and 5.93 days, respectively, much longer than those in liquid medium. It may because of limited dissolved oxygen, restricted energy amount, and confined substrate accessibility. Taken together, *M. hominis* SJTG1 can tolerate various harsh conditions and degrade E2 in soil efficiently and promptly, implying its great application potential for soil bioremediation.

Table 2
First order kinetics of E2 degradation by *M. hominis* SJTG1.

Initial E2 conc. (mg/L)	Equation	R^2	E2 half-life (day)
1	$C = 0.8475e^{-0.265\,t}$	0.8082	1.99
10	$C = 13.54e^{-0.212\,t}$	0.9557	4.69
25	$C = 26.833e^{-0.113\,t}$	0.9863	6.76

C represents the E2 concentration (mg/L), t represents cultivation time (day).

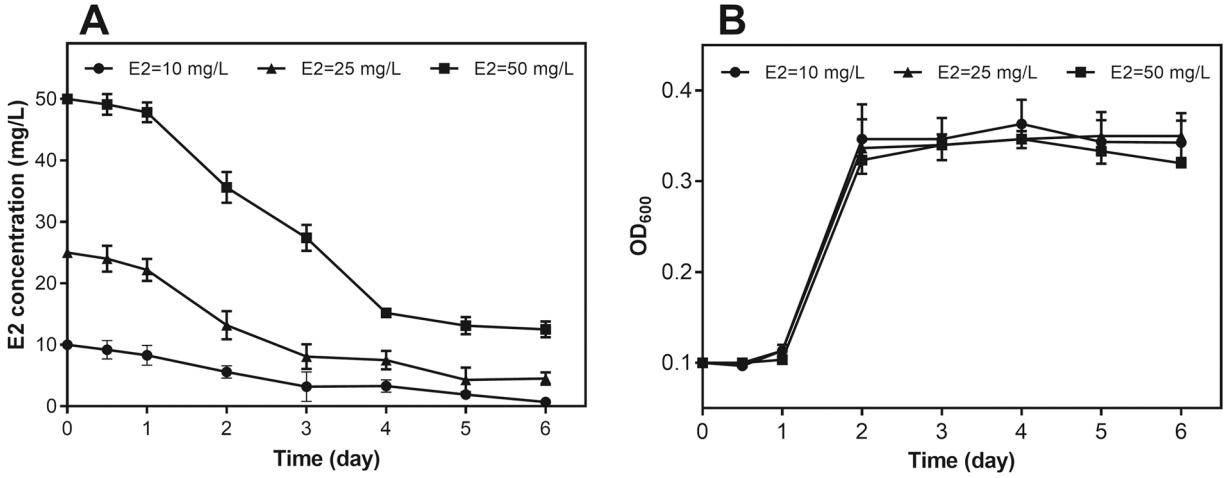

Fig. 2. The profiles of cell growth and E2 degradation of strain SJTG1. The growth curve (A) and the E2-degrading efficiency profile (B) of strain SJTG1 cultured in BSM medium with E2 of different concentrations (10, 25, and 50 mg/L) were plotted. The E2 residues and the cell growth were detected every day in the 6-day cultivation at 30 °C with constant shaking (220 r). The error bars represented the standard errors of five independent experiments.

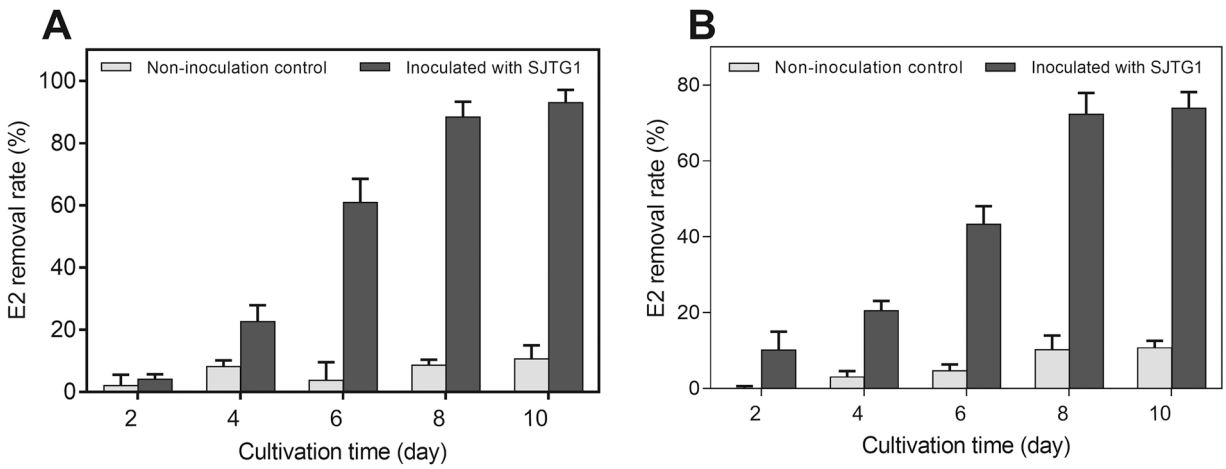

Fig. 3. Detection of E2 removal efficiency by strain SJTG1 in solid soil samples. The solid soil samples were amended with E2 of 1 mg/kg (A) or 10 mg/kg (B), and were inoculated with the live cells (black) or the boiled cells (gray) of strain SJTG1 in the initial cell density of 0.1 OD/10 g soil. The removal rates of E2 in the soil samples were detected every two days and were plotted. All the data was the mean values of five independent experiments.

3.2. Strain SJTG1 contained the intact pathway for E2 biodegradation

The whole genome sequence of strain SJTG1 was obtained to clarify the genetic basis and analyze the degrading mechanism. It is a 4.89 Mb circular chromosome (G+C content of 70.81%) and contains 3467 genes, with 3356 protein coding genes, 72 tRNA genes, 6 rRNA genes, and 3 ncRNAs (Fig. S3, Table S3). Among the predicted CDSs, 2496 ORFs were classified into 22 COG categories, and the five most abundant groups were group R (general function prediction, 307 ORFs), group E (amino acid transport and metabolism, 285 ORFs), group G (carbohydrate transport and metabolism, 267 ORFs), group K (transcription, 224 ORFs), and group P (inorganic ion transport and metabolism, 188 ORFs) (Fig. S3). Comparative genome analysis of strain SJTG1 and other six E2-degrading strains (*C. tardaugens* NBRC 16725, *S. denitrificans* DSM 18526, *C. testosteroni* TA441, *Novosphimgobium* sp. ES2–1, *Rhodococcus* sp. P14, and *Sphingomons* sp. KC8) revealed that these strains are quite diverse with ANIb values ranging from 62.7% to 71.1% (Fig. S4A) (Ye et al., 2019; Chen et al., 2017; Li et al., 2020; Ibero et al., 2020; Fahrbach et al., 2008; Arai et al., 2000). Many syntenic blocks were observed in *C. tardaugens* NBRC 16725, *Novosphimgobium* sp. ES2–1, *Sphingomonas* sp. KC8, *C. testosteroni* TA441 and *S. denitrificans* DSM 18526, while few blocks could be aligned in strain SJTG1 and *Rhodococcus* sp. P14. It implied a relatively far distance between strain SJTG1, *Rhodococcus* sp. P14, and the other four strains (Fig. S4B).

3.3. HSD-G129 functioned as a 3β/17β-HSD catalyzing the transformation of steroids

Based on the sequence alignment with the reported HSD genes, several genes encoding the short-chain dehydrogenase in strain SJTG1 were predicted (Table S4). Among them, gene *hsd-G*129 (CXR34_RS00710) shared 15% identity to *oecA* gene from *Sphingomonas* sp. KC8, 37% identity to 17*β-hsdx* gene from *Rhodococcus* sp. P14, and 38% identity to 3,17*β-hsd* gene from *C. testosteroni* ATCC 11996 (Ye et al., 2019; Chen et al., 2017; Benach et al., 2002). It encodes HSD-G129 (WP_005054998.1), which clusters with the 17β-HSDs from *Homo sapiens* (NP_000404.2 and NP_002144.1) and the SDR from *C. testosteroni* (WP 087085097.1) (Fig. S5). MSA analysis of HSD-G129 and other five HSDs from *C. testosteroni*, *H. sapiens*, *Rhodococcus*, and *Citreicella* species revealed that they all contained the N-terminal Rossmann-fold NAD(P)H/NAD(P)$^+$ binding (NADB) domain (GXGXXG) and the C-terminal conserved 3β/17β-HSD-like active site (YXXXK) (Fig. S5). The structures of HSD-G129 and 17β-HSDx were predicted by Alphafold2 and aligned to that of 3β/17β-HSD (1HXH). The mutual RMSD values were 0.732, 0.596, and 0.847, respectively, implying the high similarity in their structures (Fig. S6). The likeness of these proteins meant that they are likely to perform similar function. Further RT-qPCR results showed that after overnight incubation with E2, the transcription of *hsd-G*129 gene increased significantly (about 4.8 folds); TES also slightly induced the its transcription (1.6 folds) (Fig. 4A). The in vitro enzymatic assay showed that the recombinant HSD-G129 could not only efficiently catalyze the transformation of E2, but also oxidize TES, and DHEA, generating their oxidized products at C3- or C17-site, E1 or AD (Fig. 4B-D). Therefore, the HSD-G129 can be induced by steroids and function as a 3β/17β-HSD, participating in the steroid degradation by strain SJTG1.

3.4. LysR-G128 repressed the transcription of hsd-G129 by specific binding

In the genome of strain SJTG1, gene *hsd-G*129 was clustered with gene *lysR-G*128 (CXR34_RS00705) and gene *sdr*127 (CXR34_RS00700), encoding LysR-G128 (WP_017580937.1, a LysR-type transcriptional regulator) and SDR127 (WP_005249807.1, an alcohol dehydrogenase), respectively. These three genes were arranged in same direction, and their intergenic region was less than 103 bp (Fig. 5 A). The − 10 and − 35 regions and two transcriptional regulator binding sites were predicted locating in the 100–150 bp and the 150–200 bp upstream of *hsd-G*129 gene. RT-PCR analysis was performed to determine whether the three genes were monocistronically transcribed using primers spanning each adjacent gene. Results demonstrated that all the three primers successfully achieved positive amplifications, implying the three genes were in one transcript (Fig. 5 A). Results of the GFP fluorescence reporter assay demonstrated that overexpressed LysR-G128 dramatically repress the transcription of *egfp* gene fused with the promoter of *hsd-G*129 gene, exhibiting the significant reduction of GFP fluorescence (Fig. 5B). It meant that LysR-G128 could negatively regulate the transcription of gene *hsd-G*129 in strain SJTG1 by binding to the upstream region of its promoter.

Further to determine whether there existed the direct binding, the EMSA assay was performed using the amplified FAM-labeled upstream fragment (50–200 bp) of gene *hsd-G*129 and the recombinant protein LysR-G128 (Fig. S7). A significant gel-shift band was only observed in the mixture with 200-bp upstream fragment of gene *hsd-G*129 and protein LysR-G128, but not in groups using the fragments of other length (0, 50, 100, 150 bp fragments) (Fig. 5C). It meant that there existed a direct binding of LysR-G128 to the upstream region of gene *hsd-G*129, and the specific binding site located between 150 bp and 200 bp. Generally, a typical LTTR binding site is 5–15 bp long within the

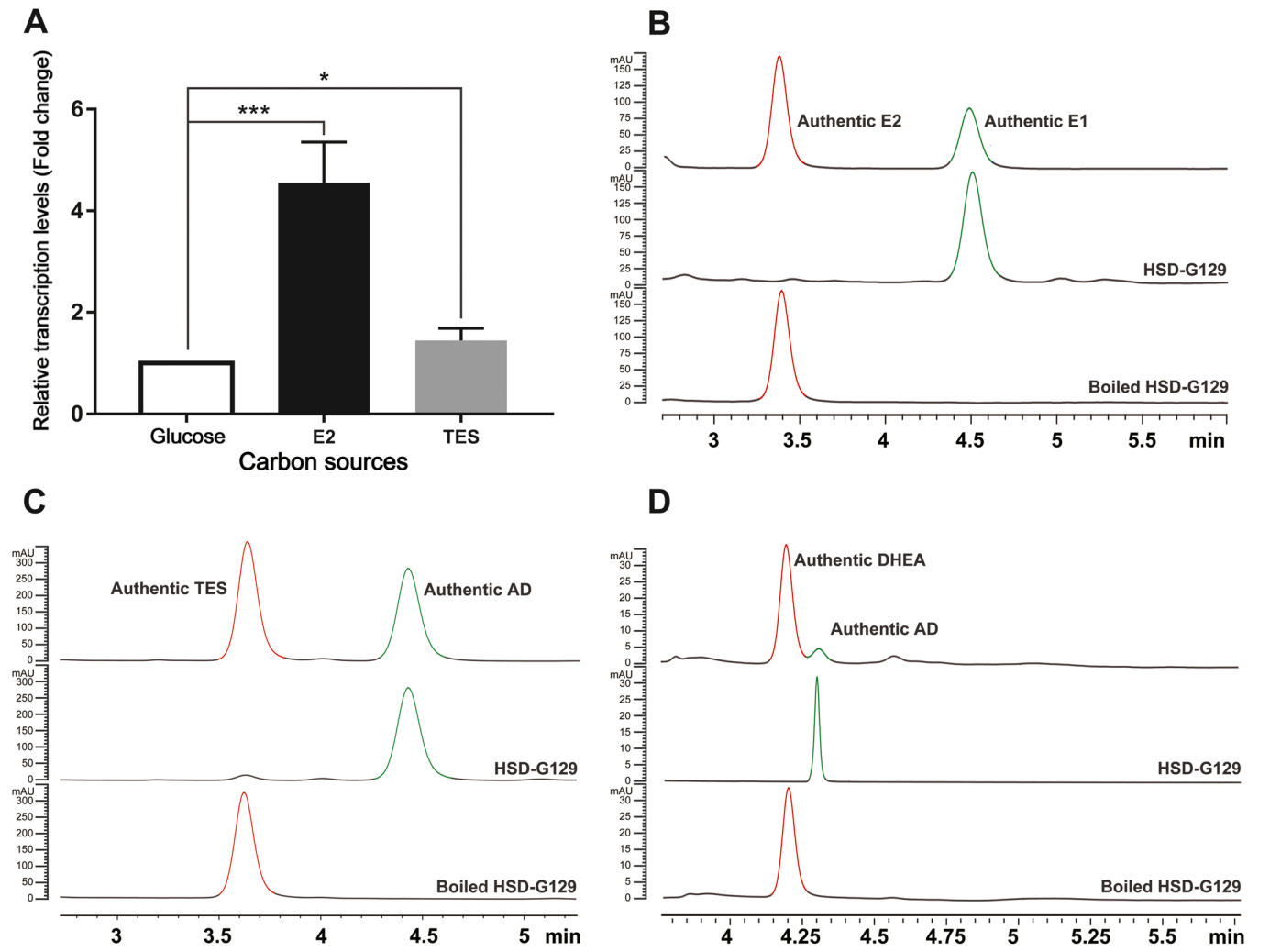

Fig. 4. Transcription analysis of gene *hsd-G*129 and the in vitro transformation of steroids of HSD-G129. (A) The transcription of *hsd-G*129 in strain SJTG1 cultured in BSM containing different carbon sources (25 mg/L of E2, TES or 0.2% of glucose) were detected with RT-qPCR. All the data were the mean values of five independent experiments. The statistical analysis was performed and marked with star (*, p value <0.05; **, p value <0.01). The profiles of HPLC detection for the reaction products in the in vitro reaction system with HSD-G129 and E2 (B), TES (C), or DHEA (D) as the substrates. The reaction mixture contained reaction buffer, 100 μM steroids, and 150 μM HSD-G129. Mixture with the boiled HSD-G129 was used as control, and the mixture without substrate was used as blank. The reactions were performed at 37 °C for 5 min and detected with HPLC.

promoter region of its target gene, as the known $TN_{11}A$ core binding motif typically located in the 50–100 bp upstream of the transcriptional start site (Schell, 1993; MacLean et al., 2008). Furthermore, the specific binding of LysR-G128 to the 200-bp upstream fragment of gene *hsd-G*129 was found in a concentration-dependent manner. Complete binding happened when the protein/DNA ration was 0.6:1, implying the binding was of high affinity. However, the addition of E2 into the reaction system relieved this binding directly and effectively. Only 5 μM of E2 could release most of the binding, and 15 μM of E2 disassociated the binding completely (Fig. 5D). These results indicated that LysR-G128 represses the transcription of gene *hsd-G*129 by specific binding to its promoter region, and the repression can be relieved by E2 via abolishing this binding.

4. Discussion

Steroidic chemicals as the pitavol members of endocrine disrupting chemicals (EDCs) pollutants, have drawn the widespread public attention since the last 20 years (Zhong et al., 2021). Among them, environmental estrogens (EEs) pollution becomes a concerning issues because of its wide distribution, long persistence and adverse effect (Adeel et al., 2017). Even at very low concentration (ng/L), estrogens can disrupt the endocrine systems in animals and human beings, resulting in various disorders and diseases in reproduction process and endocrine system (Adeel et al., 2017). Human activities and livestock poultry breeding are the major source of natural estrogens input into the ecological systems (Adeel et al., 2017; Zhao et al., 2019). Although most of the discharged estrogenic chemicals can be removed through wastewater treatment and waste compost, the EESs pollution has aggravated, with the vigorous growing in pharmaceutical industry and dramatic development in intensive livestock and poultry farms. EEs are detectable in wastewater, sludge, aquatic and soil environments (Zhao et al., 2019). Fortunately, most of estrogens can be used as energy sources and degraded by microorganisms.

However, due to stable chemical structures and low aqueous solubility of estrogens, it is not easy for bacteria to utilize these carbon-rich and highly reduced compounds. Some strains of bacteria, yeast, fungi, or microalgae, can degrade and mineralize the natural estrogens, and bacteria is the main estrogen degrader (Liu et al., 2018; Li et al., 2018, 2017; Wang et al., 2019; Xiong et al., 2020a, 2020b; Ye et al., 2019; Qiu et al., 2019). To meet the bioremediation requirement of actual estrogen-polluted environments, bacterial strains with high efficiency,

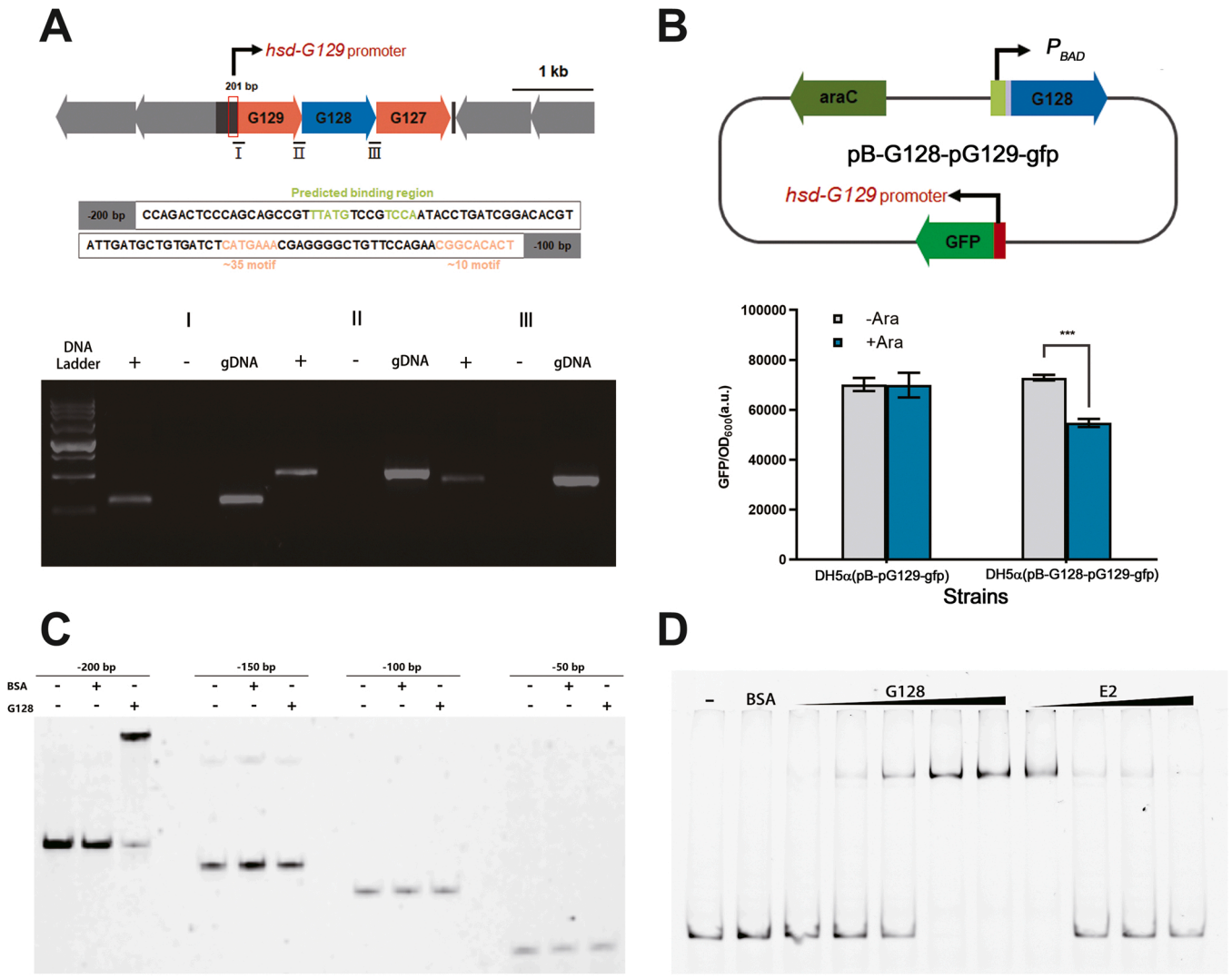

Fig. 5. Regulation analysis of LysR-G128 to *hsd-G*129 gene. (A) The genetic location of *hsd-G*129 gene and its promoter region were plotted, and the RT-PCR results about the polycistron detection of the gene cluster were shown. (B) The schemata and the results of the GFP fluorescence assay. The *lysR-G*128 gene was inserted between the P_{BAD} promoter and the promoter of *hsd-G*129. The *E. coli* DH5α cells containing plasmid pB-G128-pG129-gfp or pB-pG129-gfp were cultured and induced with 0.2% L-arabinose. The GFP fluorescences were detected and plotted. (C) The EMSA detection of LysR-G128 to the fragments of different promoter region of *hsd-G*129 gene. The EMSA detection was performed with the LysR-G128 protein and the 5′-FAM labeled fragments of *hsd-G*129 promoter region (50 bp, 100 bp, 150 bp, 200 bp). The reaction contained 2 μM labeled DNA for each reaction and 0.8 μM protein, with 10 μM BSA used as the negative control. (D) The analysis of binding properties and E2 effect on the binding of LysR-G128 to the labeled 200-bp fragment of *hsd-G*129 promoter. The first two lanes was the free labeled DNA and the mixture of labeled DNA with 10 μM BSA. Lane 3–7 were the labeled DNA supplied with the increased amounts of LysR-G128 protein, in the protein/DNA ratio from 0.1:1, 0.2:1,0.4:1, 0.6:1, 0.8:1. Lane 8 was same to Lane 7. Lane 9–11 were the binding mixture supplied with E2 (5, 10 and 15 μM).

great suitability and clear background are rare and in demand. Therefore, obtaining effective estrogen-degrading strains and understanding its degrading dynamics and genetic basis are crucial for environmental clean-up of estrogenic pollutants.

In this work, *M. hominis* SJTG1 was isolated and identified with high E2-degrading efficiency and great stress tolerance. Compared with other estrogen-degrading bacteria, strain SJTG1 can remove E2 from the medium culture and in the simulated solid soil. Its degradation half-life of 1 mg/L E2 was only 1.99 days in liquid culture and 3.91 days in solid soil, significantly higher than those of *Rhodococcus sp.* P14, *Stenotrophomonas. maltophilia* SJTH1, *S. maltophilia* SJTL3, and *R. equi* ATCC13557, and comparable to that of *C. tardaugens* NBRC 16725 (Xiong et al., 2020a, 2020b; Ye et al., 2019; Ibero et al., 2020; Harthern-Flint et al., 2021). What's more, due to the limited nutrients and fickle conditions in real environment, microorganisms have to dispose the effect of diverse environmental factors and diminish their restraints on cell growth and metabolic ability. The microbial strains with unexceptionable tolerance to stresses is in dire need for the waste treatment and environment remediation. Strain SJTG1 exhibited extraordinary environmental suitability and maintained stable E2-degrading efficiency under various harsh conditions like high salinity, heavy metals, and biosurfactants. These characteristics endow strain SJTG1 the bioremediation potentials in actual E2-polluted environment.

The genus *Microbacterium* belongs to the *Microbacteriaceae* family, a high GC actinobacterial taxon, and contains more than 90 recognized species that were isolated from a wide range of habitats (Park et al., 2008; Wang et al., 2014; Zhang et al., 2012; Kook et al., 2014). Members of the genus *Microbacterium* are Gram-positive, non spore-forming and rod-shaped bacteria. It has been reported that *Microbacterium* strains can degrade a wide range of pollutants, such as petroleum, polycyclic aromatic hydrocarbon (PAH), antibiotics, lignin and steroidic chemicals (Li et al., 2021; Zhao et al., 2021; Serrano-Gamboa et al., 2022). *Microbacterium* is also considered as estrogen-degrading microbes.

M. testaceum KC5 was firstly found able to degrade E2 to E1; however, no detailed studies on the degradation of E2 by this genus was conducted (Yu et al., 2007). In the pig manure composting, the abundance of *Microbacterium* were positively correlated with estrogen degradation (Sun et al., 2022). Notably, *Microbacterium* strains can survive in heavy metal contaminated environments, reduce specific metals (As, Cu, Zn, Cr, Cd) and change the mobility of heavy metals in contaminated soils (Brown et al., 2012; Kuffffner et al., 2010). Thus *Microbacterium* species have the potential as plant growth promoting bacteria (Sun et al., 2019). It is associated with the existence of many heavy-metal-resistant genes in the genome of *Microbacterium* strain. As to *M. hominis* strain SJTG1 reported in this work, it exhibited great tolerance to copper and cadmium of very high concentration (about 100–200 folds to concentration in real pollution site). In the genome DNA of strain SJTG1, there are about 15 genes and 22 genes related to the transportation, resistance and reduction of copper and zinc, and 2 genes are predicted encoding the cadmium transporters. In addition, *Microbacterium* can generate large amount of biosurfactants, which facilitate the substrate accessibility and the transmembrane transportation, and also enhance the strains' environment adaptability. This may be the reason that *Microbacterium* can effectively degrade polycyclic compounds and chemicals with complex structures (Tripathi et al., 2020; Kalami and Pourbabaee, 2021). This characteristics can not only promote the estrogen degradation rate of strain SJTG1, but also it may be the reason that addition of Tween 80 has little effect on the cell growth and E2-degrading efficiency.

Although series of degrading microorganisms have been identified, the bacterial estrogen-degrading mechanism are still indistinct. Based on the estrogenicities and structures of the isolated intermediate metabolites of E2, three estrogen-degrading patterns (patterns A-C) was proposed (Chiang et al., 2020). Thereinto, strains in pattern C could completely transform E2 into nonestrogenic compounds. *Sphingomonas* sp. KC8 is a typical pattern C strain in which the intermediate metabolites can be extracted via ethyl acetate extraction to forecast the estrogen-degrading pathway (Wu et al., 2019). Thereafter, similar estrogen-degrading pathways were also proposed in *Novosphingobium* sp. SLCC, *Novosphingobium* sp. ES2–1, and *C. tardaugens* NBRC 16725 (Li et al., 2018, 2020; Ibero et al., 2020). Strains in pattern B could quickly transform E2 into E1, and then degrade E1 slowly and partially with unknown mechanism. In this work, strain SJTG1 can oxidize E2 into E1 quickly, while no reasonable intermediate metabolites in E1 degradation were obtained despite a lot of hard work. A plausible explanation is that as the subsequent degradation of E1 is slow, the intermediates can not accumulate, easy to escape from the ethyl acetate extraction. Besides, it is possible that trace amounts of intermediates can bind to cell components and reduce detection likelihood (Swaneck and Fishman, 1988; Charneira et al., 2020).

In most microorganisms, the transformation from E2 to E1 is the initial and restricted step for E2 degradation, presumably as E1 is more suitable as a hydroxylation substrate of further degrading enzymes like CYP450 (Ibero et al., 2020). Meanwhile, as the estrogenic activity of E1 is about 10 times lower than that of E2, transformation of E2 into E1 is vital for estrogenicity reduction (Thorpe et al., 2003). Bacterial HSDs have been reported to be responsible for the dehydrogenation of E2 with varied characteristics, and multiple isoenzymes may participate in the in vivo transformation of E2 (Wang et al., 2019, 2018; Xiong et al., 2020a, 2020b; Ye et al., 2019, 2017; Chen et al., 2017; Yu et al., 2015). The 17β-HSDs catalyzes the dehydrogenation at C17 site of E2 and transformed E2 into E1; the 3,17β-HSD from *C. testosteroni* ATCC 11996 dehydrogenates steroid chemicals at C3 or C17 site (Ye et al., 2017; Yu et al., 2015). The enzymatic properties and regulatory mode of two 17β-HSDs in *P. putida* SJTE-1 also have been investigated (Wang et al., 2019, 2018). However, the enzymatic properties and the regulatory modes of these degrading elements from different strains are various and still unclear. In this work, 17 potential HSD encoding genes were aligned in strain SJTG1 based on genome mining and comparative genome analysis; and the *hsd-G*129 gene shared 38% identity with the 3β/17β-HSD gene from *C. testosteroni* ATCC 11996. HSD-G129 exhibits strong enzymatic activity and transformed E2 of high concentrations (> 10 mg/L) into E1 completely. It can also transform DHEA, TES, and pregnenolone efficiently in vitro, but can not use E3. This is quite similar to the HSD60 and HSD70 in *C. tardaugens* NBRC 16725 (Ibero et al., 2019). As the transcription of *hsd-G*129 gene was induced significantly by E2 and slightly by TES, HSD-G129 plays an important role in the steroid degradation of strain SJTG1.

In addition, only very few of regulators involved in steroid biodegradation have been reported and the regulatory networks were still unclear. Several regulators, PhaR, TetR, LuxR, and BRP proteins, were found to negatively regulate the transcription of 3,17*β-hsd* gene in *C. testosteroni* and influence the testosterone degradation (Ji et al., 2017; Li et al., 2013; Wu et al., 2015; Pan et al., 2015). However, these regulators not functioned in the regulation of estrogen degradation. Two transcriptional regulators (OxyR and CrgA) in *P. putida* SJTE-1 were found to be induced by E2, and able to repress or activate the transcription of 17β-*hsd* gene by binding to the specific sites of the target gene (Wang et al., 2019). In this work, LysR-G128 can repress the transcription of its adjacent *hsd-G*129 gene via the direct and specific binding to the conserved site in the promoter region. It is worth noting that the two genes are in the same gene cluster with the monocistronical transcription, implying that the transcription of *lysR-G*128 is also regulated in a self regulation mode. The self-inhibitory regulation of this gene cluster is probably accompanied by the elevated basic transcription of the genes in cluster, thus enabling a rapid response to E2 in environment. Although the solubility of E2 in water phase is normally quite low (< 3 ng/L), it still can trigger the release of the LysR-G128 binding from the promoter region of *hsd-G*129 gene and initiate the transformation of E2. The LysR-type transcription regulator (LTTR) family has many members with specific binding properties, acting as both auto repressers and activators of various target genes. They are normally 300 residues in length with high sequence similarity, and contain the N-terminal HTH motif for DNA binding and the C-terminal domain for ligand binding. The allosteric transcription factors (aTFs) can regulate gene expression in response to certain environmental or chemical stimuli. The dose-dependent release of the bound LysR-G128 from the promoter of *hsd-G*129 gene induced by E2, indicated that LysR-G128 was also an aTF, suggesting its potential to be designed as a simple and sensitive biosensor to monitor E2 concentrations in natural environments.

In conclusion, identification of the efficient and adaptable estrogen-degrading strain SJTG1 and interpretation of the functional elements for E2 degradation can not only replenish the estrogen-degrading strain pool for effective bioremediation, but also facilitate the mechanism study of estrogen biodegradation.

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CRediT authorship contribution statement

R L designed the experiments, wrote and modified the manuscript. W X performed most of the experiments and wrote the manuscript. W L and Y L performed and analyzed some experiments. S L and Z D gave advice to this work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that has been used is confidential.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.130371.

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