microbial biotechnology

Open Access

Mechanistic and phylogenetic insights into actinobacteria-mediated oestrogen biodegradation in urban estuarine sediments

Tsun-Hsien Hsiao,[1,†] Yi-Lung Chen,[2,†] Menghsiao Meng,[3] Meng-Rong Chuang,[1] Masae Horinouchi,[4] Toshiaki Hayashi,[5] Po-Hsiang Wang[6,7,] and Yin-Ru Chiang[1,*]**

[1]*Biodiversity Research Center, Academia Sinica, Taipei, 115, Taiwan.*

[2]*Department of Microbiology, Soochow University, Taipei, 111, Taiwan.*

[3]*Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, 402, Taiwan.*

[4]*Condensed Molecular Materials Laboratory, RIKEN, Saitama, 351-0198, Japan.*

[5]*Environmental Molecular Biology Laboratory, RIKEN, Saitama, 351-0198, Japan.*

[6]*Gradaute Institute of Environmental Engineering, National Central University, Taoyuan, 320, Taiwan.*

[7]*Earth-Life Science Institute (ELSI), Tokyo Institute of Technology, Tokyo, 145-0061, Japan.*

P450-type monooxygenase. We also detected the accumulation of two extracellular oestrogenic metabolites, including pyridinestrone acid (PEA) and 3aα-H-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP), in the oestrone-fed strain B50 cultures. Since actinobacterial *aedB* and proteobacterial *edcB* shared < 40% sequence identity, 4-hydroxyestrone 4,5-dioxygenase genes (namely *aedB* and *edcB*) could serve as a specific biomarker to differentiate the contribution of actinobacteria and proteobacteria in environmental oestrogen degradation. Therefore, 4-hydroxyestrone 4,5-dioxygenase genes and the extracellular metabolites PEA and HIP were used as biomarkers to investigate oestrogen biodegradation in an urban estuarine sediment. Interestingly, our data suggested that actinobacteria are active oestrogen degraders in the urban estuarine sediment.

Summary

Steroidal oestrogens are often accumulated in urban estuarine sediments worldwide at microgram per gram levels. These aromatic steroids have been classified as endocrine disruptors and group 1 carcinogens. Microbial degradation is a naturally occurring mechanism that mineralizes oestrogens in the biosphere; however, the corresponding genes in oestrogen-degrading actinobacteria remain unidentified. In this study, we identified a gene cluster encoding several putative oestrogen-degrading genes (*aed*; actinobacterial oestrogen degradation) in actinobacterium *Rhodococcus* sp. strain B50. Among them, the *aedA* and *aedB* genes involved in oestrogenic A-ring cleavage were identified through gene-disruption experiments. We demonstrated that actinobacterial oestrone 4-hydroxylase (AedA) is a cytochrome

Received 11 January, 2021; revised 24 February, 2021; accepted 28 February, 2021.

For correspondence. *E-mail yinru915@gate.sinica.edu.tw; Tel. (+886) 2 27872251; Fax (+886) 2 27899624. **E-mail pohsiang@ncu.edu.tw; Tel. (+886) 3 422 7151 #34665; Fax (+886) 3 422 1602.

[†]These authors contributed equally to this work.

Microbial Biotechnology (2021) **14**(3), 1212–1227
doi:10.1111/1751-7915.13798

Introduction

Oestrogens are steroid hormones that regulate the development of the reproductive system and secondary sex characteristics of vertebrates. Natural oestrogens include oestrone (E1), 17β-oestradiol (E2) and oestriol (E3). The synthesis and secretion of oestrogens exclusively occur in animals, especially in vertebrates (Matsumoto *et al.*, 1997; Tarrant *et al.*, 2003). In the animal liver, oestrogens undergo structural modifications (e.g., glucuronidation) and are converted into more soluble metabolites to be excreted through urine and faeces (Harvey and Farrier, 2011). While required by animals, chronic exposure to trace oestrogens at sub-nanomolar levels can disrupt the endocrine system and sexual development in animals (Belfroid *et al.*, 1999; Baronti *et al.*, 2000; Huang and Sedlak, 2001; Kolodziej *et al.*, 2003; Lee *et al.*, 2006). For example, an E2 concentration of 54 ng l^{-1} caused severe abnormal development among eelpout embryos (Morthorst *et al.*, 2014). Similarly, the EC50 of E2 causing infertility of fathead minnows (*Pimephales promelas*) was 120 ng l^{-1} (Kramer *et al.*, 1998). In addition to being endocrine disruptors, oestrogens have been classified as group 1 carcinogens by the World Health Organization (IARC Monographs-Classifications).

Oestrogen pollution has become a global concern and challenge due to the increased human population and mounting demand for livestock products. Livestock manure (Hanselman *et al*., 2003) and municipal sewage-derived fertilizers (Hamid and Eskicioglu, 2012; Lorenzen *et al*., 2004) have been a major source of environmental oestrogens. Natural oestrogens have been considered the most significant contributor to the endocrine-disrupting activity of the swine manure (Noguera-Oviedo and Aga, 2016). However, anaerobic digestion did not alter total oestrogen concentrations in livestock manure (Noguera-Oviedo and Aga, 2016), and the oestrogens could be released to aquatic ecosystems *via* rainfall and leaching (Hanselman *et al*., 2003; Kolodziej *et al*., 2004). Although oestrogens could be photodegraded in surface water ecosystems with a degradation half-live ranging from days to weeks (Jurgens *et al*., 2002; Lin and Reinhard, 2005), photodegradation is hardly occurred in the light-limited environments such as aquatic sediments. As a result, oestrogens are often accumulated in urban estuarine sediments downstream to industrialized areas due to their low solubility in water (e.g., 1.5 mg per litre for oestradiol) (Shareef *et al*., 2006) and chemical recalcitrance (Griffith *et al*., 2016; Wise *et al*., 2011).

Mineralization of natural oestrogens is only accomplished by microorganisms (Thayanukul *et al*., 2010; Chen *et al*., 2017, 2018; Wang *et al*., 2020; Chiang *et al*., 2020). Complete oestrogen mineralization by bacteria was first described by Coombe *et al*. (1966) in actinobacterium Nocardia sp. strain E110. Additionally, Rhodococcus isolates (e.g., R. equi and R. zopfii) (Yoshimoto *et al*., 2004; Kurisu *et al*., 2010), Novosphingobium tardaugens NBRC 16725 (Fujii *et al*., 2002) and Sphingomonas spp. (Ke *et al*., 2007; Yu *et al*., 2007) were also capable of mineralizing oestrogens. According to current literature, several putative oestrogen biodegradation pathways have been proposed (Yu *et al*., 2013), suggesting that different bacterial taxa likely adopt different degradation strategies to degrade oestrogens. Recently, the aerobic 4,5-*seco* pathway for oestrogen degradation and the corresponding enzymes in proteobacteria have been studied in some detail (Chen *et al*., 2017; Wu *et al*., 2019; Ibero *et al*., 2019a, 2019b, 2020). Ibero *et al*., (2020) revealed the essential role of 3 *edc* genes [*edcA*, oestrone 4-hydroxylase gene; *edcB*, 4-hydroxyestrone 4,5-dioxygenase gene; *edcC*, an indolepyruvate ferredoxin oxidoreductase gene responsible for the oxidative decarboxylation and subsequent coenzyme A (CoA) conjugation of the *meta*-cleavage product of E1] in the proteobacterial oestrogen degradation using the gene knockout mutants. However, homologous genes in the 4,5-*seco* pathway are not found in the genomes of the oestrogen-degrading actinobacteria based on sequence homology.

In this study, we used actinobacterium *Rhodococcus* sp. strain B50 isolated from the soil as the model microorganism to study actinobacterial oestrogen degradation due to its outstanding efficiency in oestrogen degradation and its compatibility with common genetic manipulation techniques: (i) forming independent colonies on agar-based solid media; (ii) incorporating commercial vectors *via* electroporation; and (iii) sensitivity to commercial antibiotics (e.g., chloramphenicol). We applied an integrated approach including genomics, metabolomics and gene-disruption experiments to elucidate the oestrogen degradation pathway in actinobacteria. Subsequently, we used the extracellular metabolites and 4-hydroxyestrone 4,5-dioxygenase genes as biomarkers to investigate oestrogen biodegradation in urban estuarine sediment.

Results

Isolation and characterization of the oestrogen-degrading Rhodococcus sp. strain B50

Strain B50 was isolated from a garden soil sample in Tokyo, Japan. The E1-degrading actinobacterium was highly enriched by repeating 10^8 dilution transfers. The highly enriched culture was then spread on an agar-based solid medium. Subsequently, independent colonies were selected for incubation in a chemically defined mineral medium containing E1 (270 mg l^{-1}) as the sole carbon source and electron donor to confirm their capability to degrade oestrogens. We isolated four oestrogen-degrading *Rhodococcus* spp. strains, including strain B50, from the soil samples (see Fig. S1 for the morphology of bacterial cells and colonies of strain B50). Among them, strain B50 is inherently sensitive to various antibiotics including ampicillin, kanamycin and chloramphenicol (Table S1), as opposed to the other three strains. Thus, strain B50 was selected as the model microorganism to study actinobacterial oestrogen biodegradation for its convenience in antibiotics-based genetic manipulation. All of the *Rhodococcus* spp. isolates are resistant to the quinolone antibiotic nalidixic acid, which enables plasmid transfer from *E. coli* (nalidixic acid-sensitive) through conjugation and allows the selective growth of *Rhodococcus* strains. Next, we characterized the substrate spectra of strain B50. Strain B50 can utilize E1, E2, E3, testosterone or cholesterol as the sole carbon source and electron donor (Fig. S2). The doubling time of strain B50 which grows on E1, testosterone and cholesterol ranges from 3 – 4, 6 – 8 and 12 – 14 h respectively.

Identification of oestrogen metabolites

The growth experiments suggested that strain B50 can degrade oestrogens. To characterize the oestrogen

degradation pathway in strain B50, strain 50 resting cells ($\sim 10^9$ cells ml^{-1}) were aerobically incubated with E1 (10 mg l^{-1}), sampled hourly and extracted using ethyl acetate, and the metabolite profile was analysed through UPLC–APCI–HRMS. The metabolite profile analysis revealed at least four E1-derived metabolites, including PEA and HIP in the established 4,5-*seco* pathway (Table S2). The retention time of the detected metabolites in the UPLC and their HRMS behaviours was identical to those of the corresponding authentic standards (Fig. 1B and Table S2), suggesting that strain B50 adopts the 4,5-*seco* pathway to degrade oestrogens. Moreover, we observed the accumulation of both PEA and HIP in the supernatants of strain B50 cultures in a dose-dependent manner depending on added E1 (Fig. 1C).

Identification of the oestrogen-degrading genes via comparative genomic analysis

Metabolite profile analysis suggested that strain B50 degrades oestrogens *via* the 4,5-*seco* pathway established in proteobacteria. However, the homologous genes involved in the proteobacterial 4,5-*seco* pathway were not annotated in the strain B50 genome, likely due to distant phylogeny between proteobacteria and actinobacteria. Therefore, we compared the strain B50 genome to the genomes of the reported oestrogen-degrading actinobacteria in the database. Through the comparative genomic analysis, we identified a putative oestrogen-degrading gene cluster (GMFMDNLD _05329 to 05349; Dataset S1) on a circular genetic element (i.e., megaplasmid; GMFMDNLD 3) of strain B50 (accession no.: WPAG00000000.1), which is also present in the genome of oestrogen-degrading *Rhodococcus* sp. strain DSSKP-R-001 (Zhao *et al*., 2018), but not in other *Rhodococcus* members incapable of degrading oestrogen. Moreover, the two homologous oestrogen-degrading gene clusters are both located on their megaplasmids (Fig. 2; Dataset S1). Among them, the gene cluster (*aed*, actinobacterial oestrogen degradation) of strain B50 is surrounded by a transcriptional regulator and a transposase gene (GMFMDNLD _05329 and 05330). In the putative oestrogen-degrading gene cluster, GMFMDNLD _05338 encodes a putative *meta*-cleavage enzyme, which likely functions as the 4-hydroxyestrone 4,5-dioxygenase (AedB). Moreover, GMFMDNLD_05336 encodes a member of the cytochrome P450 protein family and thus likely functions as an oxygen-dependent oestrone 4-hydroxylase (AedA). The nucleotide sequences of 16S rRNA, and the *aedA* and *aedB* genes of strain B50 are shown in Appendices S1-S3, respectively.

In addition to the oxygenase genes [(*aedA* (GMFMDNLD_05336)] and [*aedB* (GMFMDNLD _05338)], we identified a putative medium-chain fatty acid:CoA ligase gene [GMFMDNLD _05341]. Moreover, genes encoding two sets of β-oxidation enzymes, including acyl-CoA dehydrogenase (GMFMDNLD _05345 and 05347), enoyl-CoA hydratase (GMFMDNLD _05333 and 05344), β-hydroxyacyl-CoA dehydrogenase (GMFMDNLD _05334 and 05337), and thiolase (GMFMDNLD _05335 and 05342), are present in the *aed* gene cluster (Fig. 2). Highly similar oxygenase and β-oxidation genes (with the deduced amino acid sequence identities $> 60\%$) are also present in the megaplasmid (plasmid plas2; NCBI Reference Sequence: NZ_CP027795; 95.13 kb) of oestrogen-degrading *Rhodococcus* sp. strain DSSKP-R-001 (Fig. 2). Interestingly, the comparative genomic analysis indicated that only 20 coding sequences, including 18 *aed* genes responsible for the oestrogenic A- and B-rings degradation, are homologous between the two megaplasmids from strains B50 and DSSKP-R-001 (Fig. S3). The deduced amino acid sequences of actinobacterial *aed* genes exhibit low sequence identity ($< 40\%$) to those of the proteobacterial *edc* (Ibero *et al*., 2020) or *oec* genes (Chen *et al*., 2017), as opposed to their functional similarity.

In addition to degrade oestrogens including E1 and E2, the strain B50 is also capable of degrading other steroids such as testosterone and cholesterol. Consistent with the observed phenotype, the strain B50 linear chromosome (GMFMDNLD 2) contains a complete set of cholesterol/androgen degradation genes in the established 9,10-*seco* pathway (Holert *et al*., 2018; Crowe *et al*., 2018), including the genes involved in the cholesterol uptake (*mce4* genes; GMFMDNLD_02935 to 02949), steroidal side-chain degradation (GMFMDNLD_02968 to 02992 and GMFMDNLD_03076 to 03082), androgenic A/B-ring degradation (GMFMDNLD _03002 to 03014 and GMFMDNLD _03061 to 03069) and C/D-ring degradation (GMFMDNLD _03019 to 03022 and GMFMDNLD _03039 to 03047) (Dataset S1). Among them, we identified the *ipdAB* [GMFMDNLD_03020 (*ipdA*) and _03021 (*ipdB*)] and *echA20* (GMFMDNLD_03019) responsible for steroidal C- and D-rings degradation respectively (Fig. 2). Moreover, the observation of the temporary HIP production and subsequent depletion in the E1-fed strain B50 cultures is consistent with the presence of HIP-CoA ligase gene *fadD3* (GMFMDNLD_03043) responsible for the HIP activation in the strain B50 chromosome.

Functional validation of actinobacterial aedA and aedB in oestrogenic A-ring degradation

Next, we aimed to confirm the function of the putative oxygenase genes *aedA* and *aedB* involved in

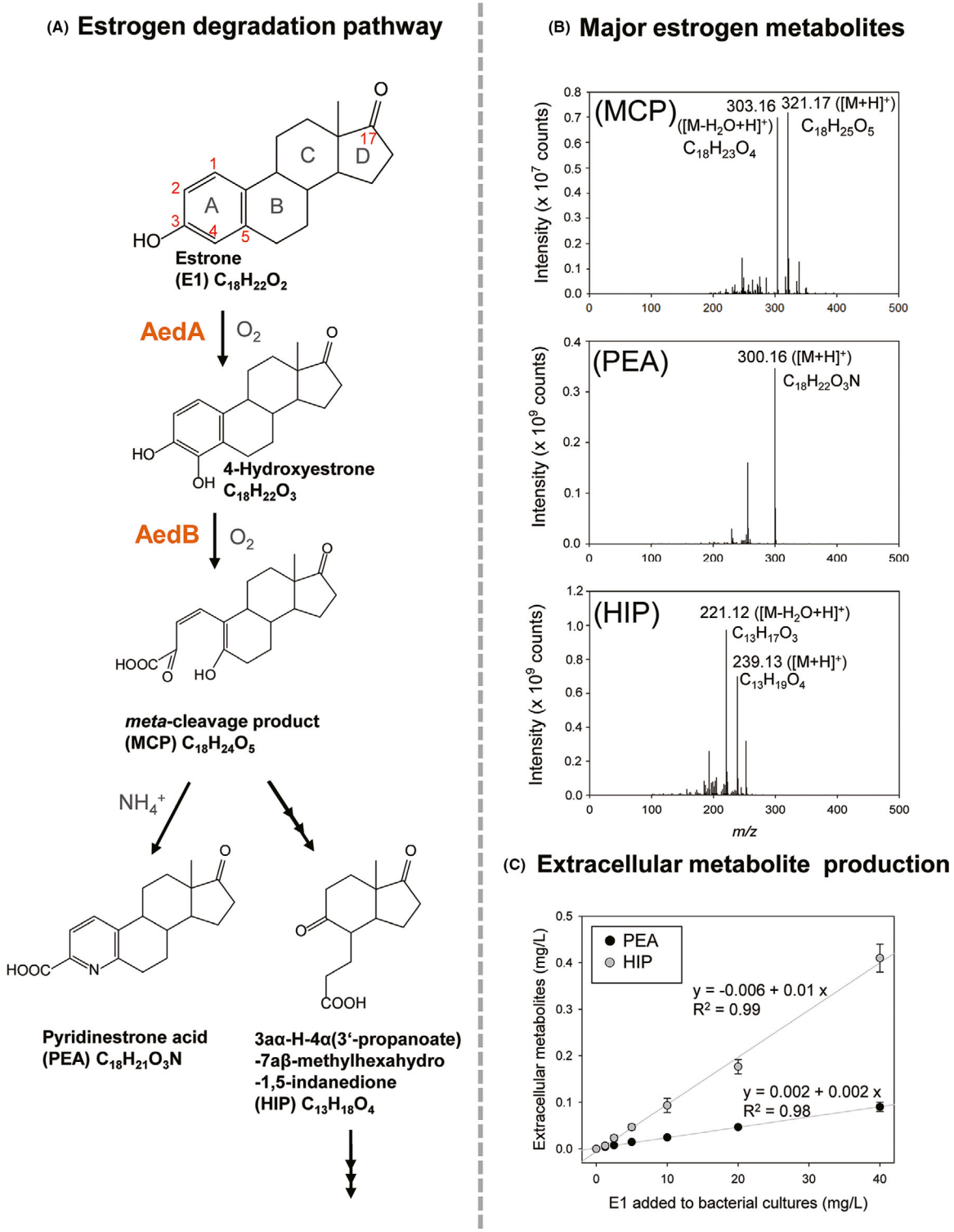

Fig. 1. Metabolites and genes involved in oestrogen degradation of *Rhodococcus* sp. strain B50.
A. The proposed pathway for oestrogen degradation of strain B50. AedA, oestrone 4-hydroxylase; AedB, 4-hydroxyestrone-4,5-dioxygenase.
B. Mass spectrometry spectra of major oestrogen metabolites of strain B50 incubated with E1 (100 mg l^{-1}). The UPLC and MS behaviours of these metabolites are shown in Table S2.
C. The dose-dependent manner of the extracellular metabolite production in the E1-grown strain B50 cultures.

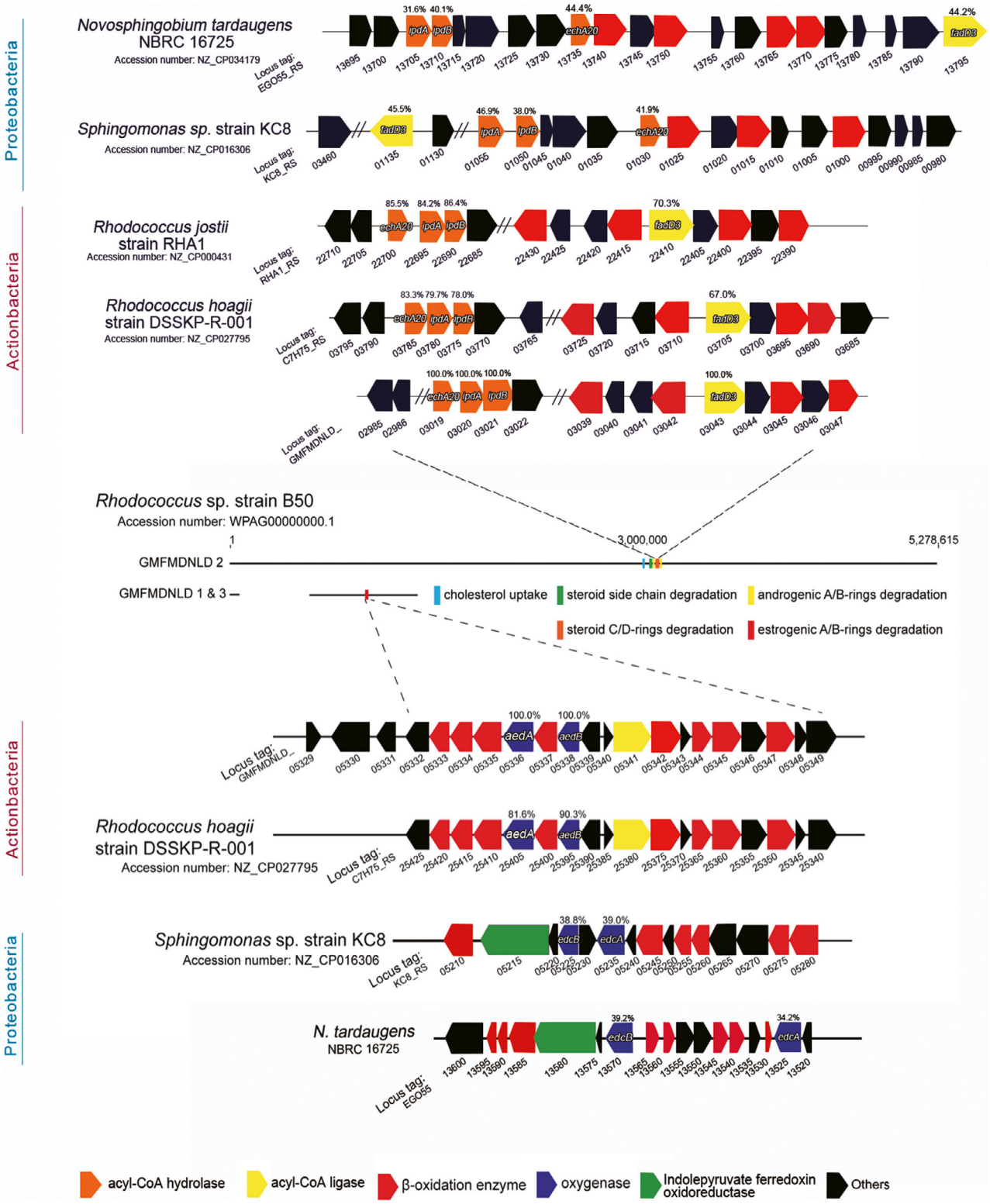

Fig. 2. Comparative genomic analysis of *Rhodococcus* sp. strain B50. Gene clusters for cholesterol uptake (blue), cholesterol side-chain degradation (green), androgenic A/B-rings degradation (yellow), and HIP degradation (orange) are located in the linear chromosome, whereas the gene cluster specific for oestrogenic A/B-rings degradation (red) is present in the megaplasmid (GMFMDNLD 3). Percentage (%) indicates the shared identity of the deduced amino acid sequences of the oestrogen degradation genes.

actinobacterial oestrogen degradation. Thus, we disrupted the putative *aedA* (GMFMDNLD_05336) and *aedB* (GMFMDNLD_05338) in strain B50 using site-directed mutagenesis (insertion of a chloramphenicol resistance gene (Cm^R) and *pheS*** cassette). The plasmid was transferred from *E. coli* (nalidixic acid-sensitive) to strain B50 (nalidixic acid-resistant) through conjugation. Then, the *aedA*- and *aedB*-disrupted strain B50 mutants were selected and maintained on Lysogeny broth (LB) agar containing two antibiotics: chloramphenicol (25 μg ml^{-1}) and nalidixic acid (12.5 μg ml^{-1}). PCR with primers flanking the *aedA* and the Cm^R genes confirmed successful insertion of the chloramphenicol resistance cassette into the *aedA* in the mutated strain (Fig. 3B). The *aedA*-disrupted mutant can utilize testosterone but not E1 (Fig. 3Ci), while the wild-type strain B50 apparently degraded testosterone and E1 within 4 h. Moreover, we did not observe any oestrogenic metabolites [e.g., 4-hydroxyestrone, the *meta*-cleavage product (MCP), PEA or HIP] in the *aedA*-disrupted strain B50 mutant incubated with E1 (Fig. 4), suggesting that *aedA* is involved in the transformation of E1 into 4-hydroxyestrone. Applying the same gene-disruption approach (insertion of the Cm^R and *pheS*** cassette), we obtained an *aedB*-disrupted strain B50 mutant (Fig. 3B). Similarly, the *aedB*-disrupted mutant can only utilize testosterone but failed to utilize E1 (Fig. 3Cii). Moreover, we observed 4-hydroxyestrone accumulation but no downstream products in the *aedB*-disrupted strain B50 mutant cultures incubated with E1 (Fig. 4), revealing that *aedB* is involved in oestrogenic A-ring cleavage.

Alignment of the 4-hydroxyestrone 4,5-dioxygenase genes from actinobacteria and proteobacteria

Our data suggest that both proteobacteria and actinobacteria adopt the *meta*-cleavage dioxygenase to cleave the A-ring of natural oestrogens. The phylogenetic tree shows that 4-hydroxyestrone 4,5-dioxygenase orthologues from all known oestrogen-degrading bacteria in the database form a distinct lineage (Fig. S4), separated from the *hsaC* and *tesB*, which are involved in androgenic A-ring cleavage in bacteria (Fig. S5). Proteobacteria-specific *edcB* primers have been designed and examined in our previous study (Chen *et al.*, 2018). In the present study, we aimed to design specific primers for actinobacterial *aedB*. The phylogenetic divergence of 4-hydroxyestrone 4,5-dioxygenase gene sequences between actinobacteria and proteobacteria allows the design of taxa-specific primers for environmental studies (Fig. 5A). The designed actinobacterial primers were validated using chromosomal DNA of the three other oestrogen-degrading *Rhodococcus* spp. strains isolated as mentioned above. To test primer specificity, gDNA from an oestrogen-degrading proteobacterium *Sphingomonas* sp. strain KC8 and from a testosterone-degrading actinobacterium *Gordonia cholesterolivorans* incapable of degrading oestrogens was used as negative controls. PCR products with an expected size of approximately 800 base pairs were only amplified from gDNA of the oestrogen-degrading *Rhodococcus* spp. but not from gDNA of *G. cholesterolivorans* or strain KC8 (Fig. 5B), suggesting that the degenerate primer is highly specific to actinobacterial *aedB* and cannot be used to amplify the androgenic *meta*-cleavage dioxygenase gene *hsaC* and proteobacterial *edcB*.

The metabolite profile and 4-hydroxyestrone 4,5-dioxygenase gene-based functional analyses reveal actinobacteria as active oestrogen degraders in urban estuarine sediment

Subsequently, the actinobacterial and proteobacterial degenerate primers were used to study oestrogen biodegradation in the urban estuarine sediment of the Tamsui River, a river passing through the Taipei metropolitan area in Taiwan. [3,4C-^{13}C]E1 (100 μg g^{-1} sediment) was spiked into the urban estuarine sediment samples. Metabolite profile analysis revealed time-dependent PEA and HIP accumulation in the supernatants of the sediment samples, suggesting the occurrence of oestrogen degradation in the sediment samples (Fig. 6). Moreover, a higher concentration of HIP (2 μg g^{-1} sediment) was produced by sediment microbiota after 8 days of incubation with [3,4C-^{13}C]E1, compared with that of PEA (0.2 μg g^{-1} sediment).

Total RNA was extracted and purified from the [3,4C-^{13}C]E1-spiked sediment samples hourly. Reverse-transcribed cDNA was used as the template for the degenerate primers in the PCR-based assays. After an 8-h incubation with [3,4C-^{13}C]E1, we detected the 4-hydroxyestrone 4,5-dioxygenase gene amplicons in the PCR experiment using the actinobacterial *aedB* primers but not in the experiment using the proteobacterial *edcB* primers (Fig. 7A). Next, the actinobacterial *aedB* amplicons were cloned into *E. coli* strain DH5α. Ten clones (sediment cDNA #1–10) were randomly selected for sequencing (Appendix S4). Notably, all of the ten *aedB* amplicon sequences obtained from the [3,4C-^{13}C]E1-spiked sediment samples were highly similar to that of strain B50 *aedB* (Fig. 7B) but were distant from the proteobacterial *edcB* sequences. Altogether, our E1-spiked mesocosm experiments and PCR-based functional assays suggest that actinobacteria are active oestrogen degraders in urban estuarine sediment.

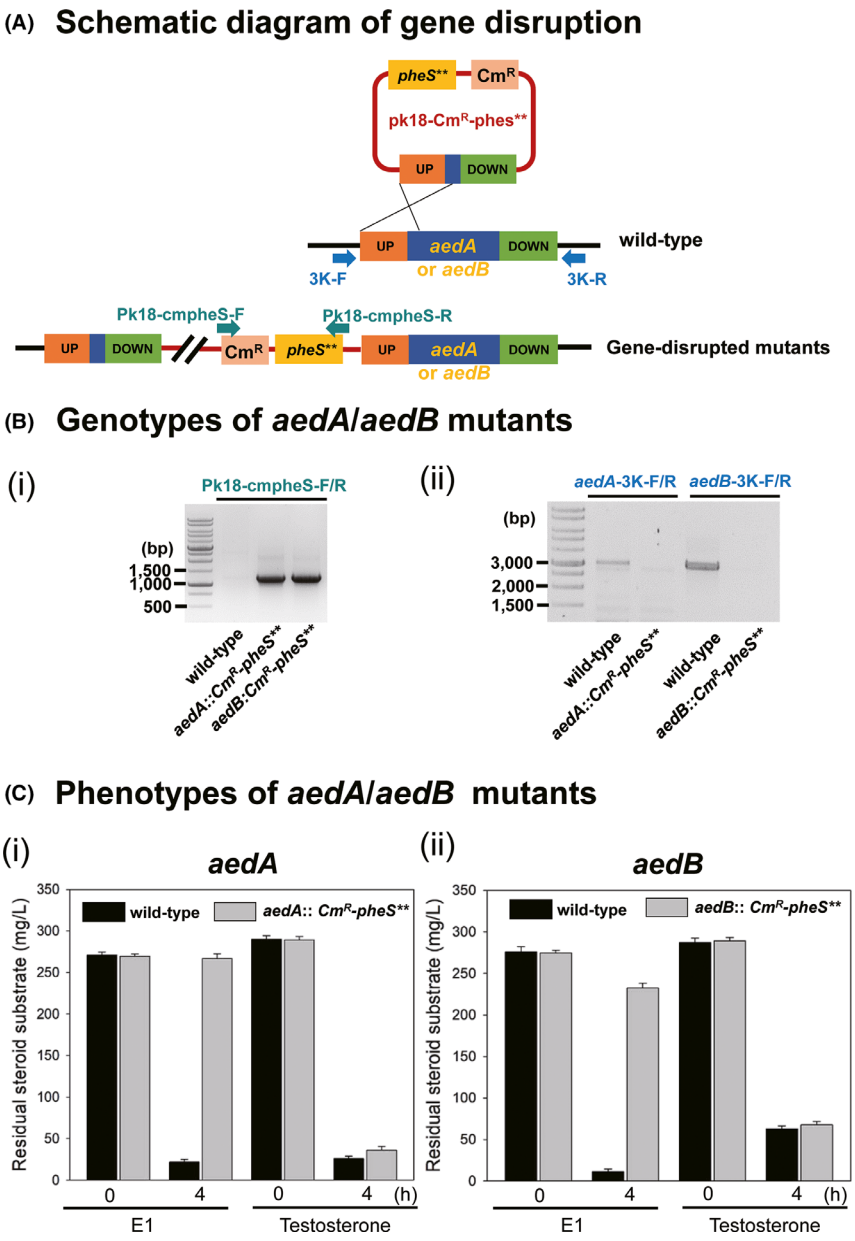

Fig. 3. Disruption of *aedA* and *aedB* in strain B50.
A. Schematic diagram of homologous recombination-mediated gene disruption.
B. Genotype examinations of *aedA*- and *aedB*-disrupted strain B50 mutants. (Bi) Agarose gel electrophoresis indicated the insertion of a chloramphenicol-resistant gene (Cm^R) and *pheS*** cassette into the target genes. (Bii) Agarose gel electrophoresis confirmed the gene disruption of *aedA* and *aedB*.
C. Phenotypes of *aedA*- and *aedB*-disrupted strain B50 mutants. The wild-type strain B50 was also tested for a comparison. Data shown are the means ± SD of three experimental replicates.

Discussion

A shared oestrogen degradation pathway in both actinobacteria and proteobacteria

Several lines of evidence suggested that strain B50 is capable of completely degrade E1 under aerobic conditions: (i) the identification of gene clusters responsible for the degradation of oestrogenic A/B- and C/D-rings in the strain B50 chromosome; (ii) the temporary production of HIP in the E1-fed strain B50 cultures, albeit ~ 1% of HIP are excreted into extracellular environments, escaping further bacterial degradation; (iii) no oestrogenic metabolites are apparently accumulated in the E1-fed wild-type strain B50 cultures. However, the possibility of

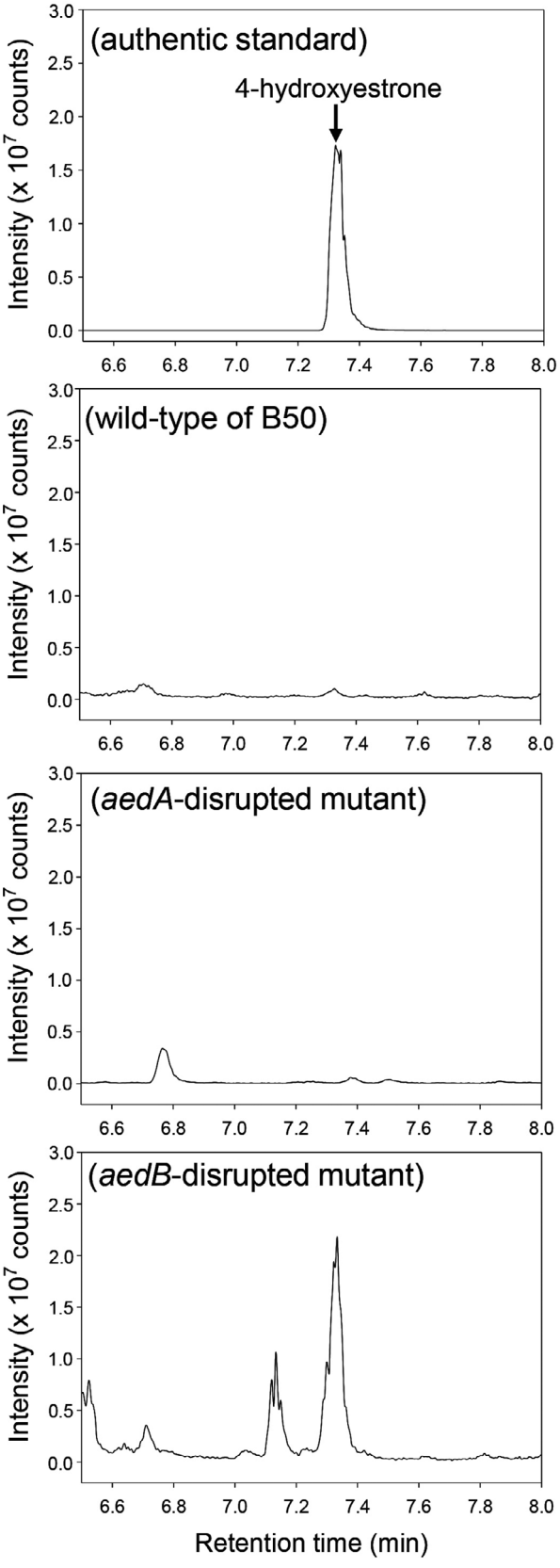

Fig. 4. Validation of the phenotype of the gene-disrupted strain B50 mutants. Liquid chromatography (LC) analysis of (A) an authentic 4-hydroxyestrone standard (B) the metabolite profile of the wild-type B50 strain, (C) the metabolite profile of the *aedA*-disrupted mutant and (D) the metabolite profile of the *aedB*-disrupted mutant.

partial E1 degradation by the strain B50 cannot be excluded due to the lack of stoichiometric evidence.

In the present study, the identification of the oestrogenic metabolites PEA and HIP, along with the identification of degradation genes *aedA* and *aedB*, in strain B50 reveals that actinobacteria also adopt the 4,5-*seco* pathway to degrade natural oestrogens. Actinobacteria such as *Mycobacterium* spp. and *Rhodococcus* spp. use the flavin-dependent monooxygenase *hsaAB*, with *hsaA* and *hsaB* as the oxygenase and reductase subunit, respectively, to add a hydroxyl group to the C-4 of the androgenic metabolite 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3-HSA) (Fig. S5) (Dresen *et al*., 2010; Bergstrand *et al*., 2016; Holert *et al*., 2018). Therefore, in a previous study using the *Sphingomonas* sp. strain KC8 as the model organism (Chen *et al*., 2017), we speculated that a similar *hsaA*-type gene might be responsible for the transformation of E1 into 4-hydroxyestrone in strain KC8. By using E1-degrading *Novosphingobium tardaugens* NBRC 16725 as the model organism, Ibero *et al*., (2020) provided convincing evidence that a cytochrome P450-type monooxygenase (CYP450) encoded by the *edcA* catalyses the 4-hydroxylation of E1. A highly similar gene is also present in the *Sphingomonas* sp. strain KC8 as well as other oestrogen-degrading proteobacteria. In the present study, we functionally validated that *aedA* in strain B50 also encodes a CYP450-type monooxygenase but not a flavin-dependent monooxygenase like HsaA in the 9,10-*seco* pathway. Together, these data suggest that both proteobacteria and actinobacteria employ the haem-dependent CYP450 to transform E1 into 4-hydroxyestrone.

Identification of the specific gene cluster for aerobic degradation of oestrogenic A/B-rings

In the strain B50 genome, we identified a gene cluster responsible for oestrogen degradation. The disruption of the oxygenase genes *aedA* and *aedB* in this gene cluster apparently aborted oestrogen degradation by strain B50, indicating direct involvement of *aedA* and *aedB* in the activation and cleavage of the oestrogenic A-ring. The *aedA* is a typical cytochrome P450 monooxygenase which requires O_2 and NADPH as the co-substrate and reductant respectively. Only a minor amount of the expected metabolite 4-hydroxyestrone was produced from E1 in the bacterial cultures of the *aedB* mutant,

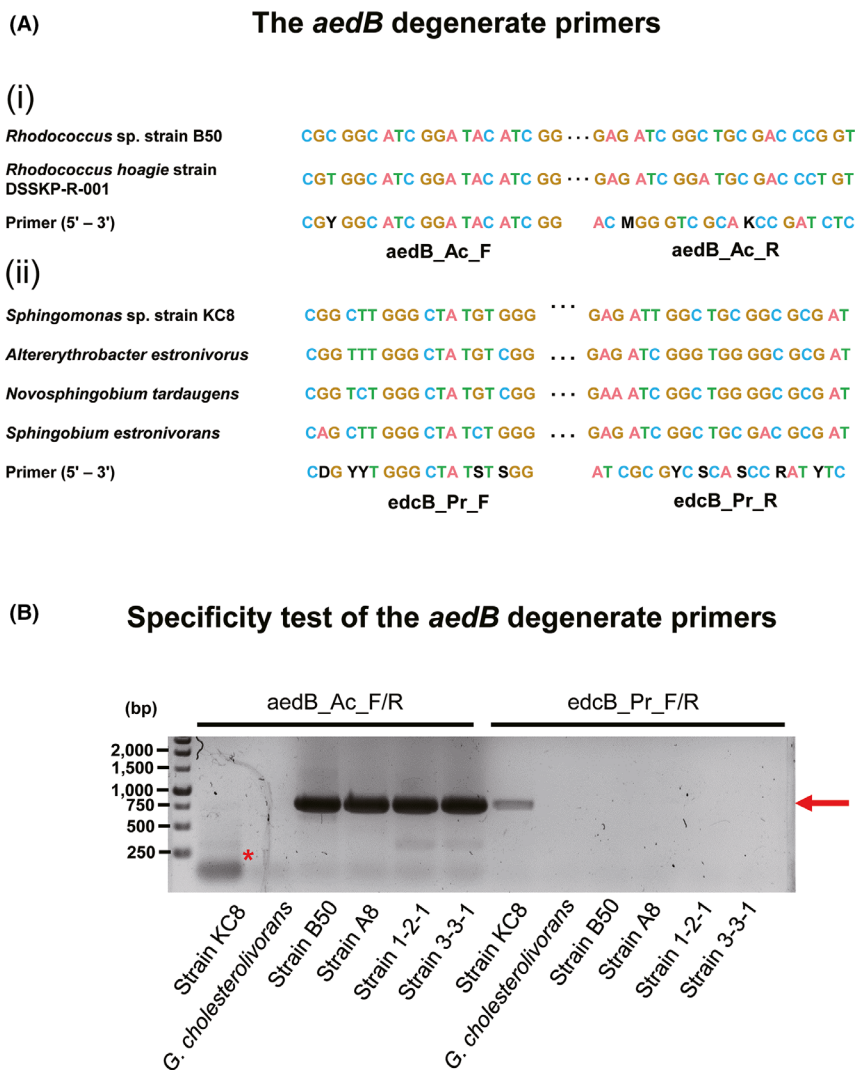

Fig. 5. PCR-based functional assay using degenerate primers derived from 4-hydroxyestrone 4,5-dioxygenase genes.
A. Highly conserved nucleotide sequence regions of 4-hydroxyestrone 4,5-dioxygenase genes derived from (Ai) actinobacteria and (Aii) proteobacteria used to deduce degenerate primers.
B. Agarose gel electrophoresis indicated that actinobacteria-type *aedB* is harboured only by oestrogen-degrading *Rhodococcus* spp. but not by testosterone-degrading actinobacterium *Gordonia cholesterolivorans* strain DSM 45229 or oestrogen-degrading alpha-proteobacterium *Sphingomonas* sp. strain KC8. R = A + G, S = G + C, and Y = C + T. Arrow indicates the expected fragment of *aedB or edcB*; asterisk demonstrates the primer dimer.

likely related to the shortage of NADPH regeneration in strain B50 cells as E1 cannot be oxidized by the *aedB*-disrupted mutant to generate reducing equivalents. However, the function of the other genes in this gene cluster remains to be investigated through gene-disruption experiments and phenotype analysis. For example, after the oestrogenic A-ring cleavage, the *meta*-cleavage product should be further degraded by the gene products in this gene cluster, such as the putative CoA ligase and two sets of β-oxidation enzymes. Proteobacteria including *Novosphingobium tardaugens* NBRC 16725 (Ibero *et al.*, 2020) and *Sphingomonas* sp. strain KC8 (Wu *et al.*, 2019) employ an indolepyruvate ferredoxin oxidoreductase-like EdcC to activate the *meta*-cleavage product of E1 through a coupled oxidative decarboxylation and CoA conjugation. However, members of the indolepyruvate ferredoxin oxidoreductase family did not present in the *aed* gene clusters of strain B50 or *Rhodococcus* sp. strain DSSKP-R-001. Therefore, we speculate that actinobacteria may depend on acyl-CoA ligase but not indolepyruvate ferredoxin oxidoreductase-like EdcC to activate the *meta*-cleavage product. Considering the different biochemical mechanisms involved in the *meta*-cleavage product activation, actinobacteria might produce different CoA-ester intermediates during the degradation of oestrogen A-ring and B-ring.

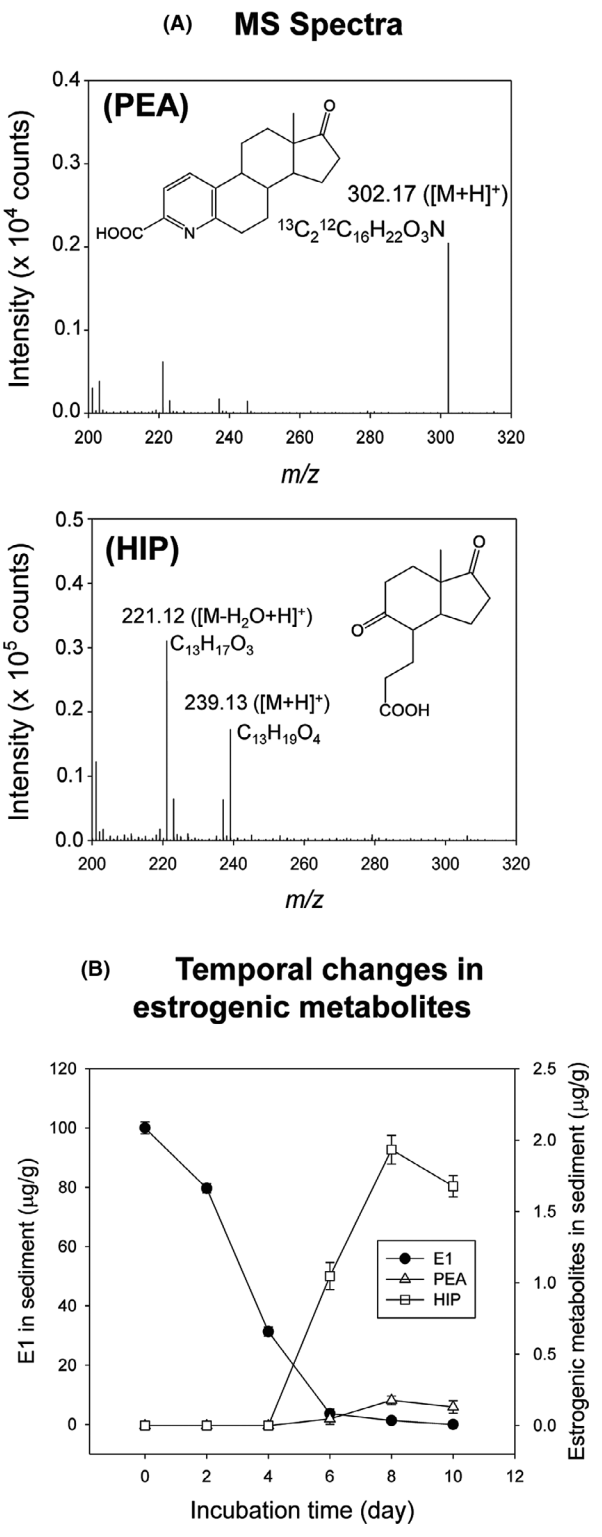

Fig. 6. UPLC–APCI–HRMS detection of PEA and HIP in [3,4C-^{13}C] E1 (100 μg g^{-1} sediment)-spiked estuarine sediment.
A. MS spectra of the oestrogenic metabolites PEA and HIP.
B. Temporal change in oestrogenic metabolite production. Measurements of PEA and HIP were based on the adducts corresponding to the two compounds using UPLC–APCI–HRMS. Data shown are the means ± S.D. of three experimental replicates.

The *aed* gene cluster is located in the megaplasmid of strain B50 and is surrounded by transposon elements and transposases. In parallel, a highly similar gene cluster (C7H75_25375~_25425) is also present in a plasmid (plas2; 95 132 bp) of the oestrogen-degrading *Rhodococcus* sp. strain DSSKP-R-001 (Zhao *et al*., 2018). The coincidence raises speculation that the oestrogen-degrading capacity in the environment may be conferred among actinobacteria via horizontal gene transfer. This gene cluster may thus be used as a biomarker to identify actinobacteria capable of oestrogen degradation. Therefore, one may assess the oestrogen degradation potential of various actinobacterial strains in different environments by probing this gene cluster in the metagenomic and/or metatranscriptomic data.

The extracellular metabolites PEA and HIP are competent biomarkers for assessing the occurrence and fate of oestrogen in environmental samples

A highlight in this study is the detection of two extracellular metabolites (i.e., PEA and HIP) in strain B50 cultures with added E1. Bacteria depend on HIP-CoA ligase (FadD3) to activate HIP, enabling further degradation of the oestrogenic C/D-rings (Crowe *et al*., 2018; Wu *et al*., 2019). CoA is an essential cofactor in numerous biosynthetic and energy-yielding metabolic pathways (Boll *et al*., 2020). When CoA is required in other metabolic pathways, the CoA-esters in the 4,5-*seco* pathway (e.g., HIP-CoA) can be deconjugated (Takamura and Nomura, 1988; Lin *et al*., 2015). The deconjugated metabolites like HIP are often toxic to bacterial cells and are therefore excreted to the medium (Wu *et al*., 2019). Our data revealed that approximately 0.2–0.5% and 1–2% of E1 molecules are transformed to PEA and HIP during oestrogen degradation by strain B50 respectively. We thus propose that PEA and HIP may be suitable biomarkers for monitoring environmental oestrogen biodegradation because (i) these two metabolites are produced by two major oestrogen-degrading bacterial taxa, namely actinobacteria and proteobacteria; (ii) PEA and HIP are critical metabolites for oestrogenic A-ring and B-ring degradation, respectively; (iii) the extracellular accumulation of these two metabolites exhibited an oestrogen dose-dependent manner; (iv) these two metabolites can be easily detected using UPLC-HRMS (detection limits at picomolar level); and (v) PEA is exclusively produced during bacterial oestrogen degradation.

Conclusion

In summary, we identified extracellular metabolites (PEA and HIP) and two essential genes (*aedA* and *aedB*)

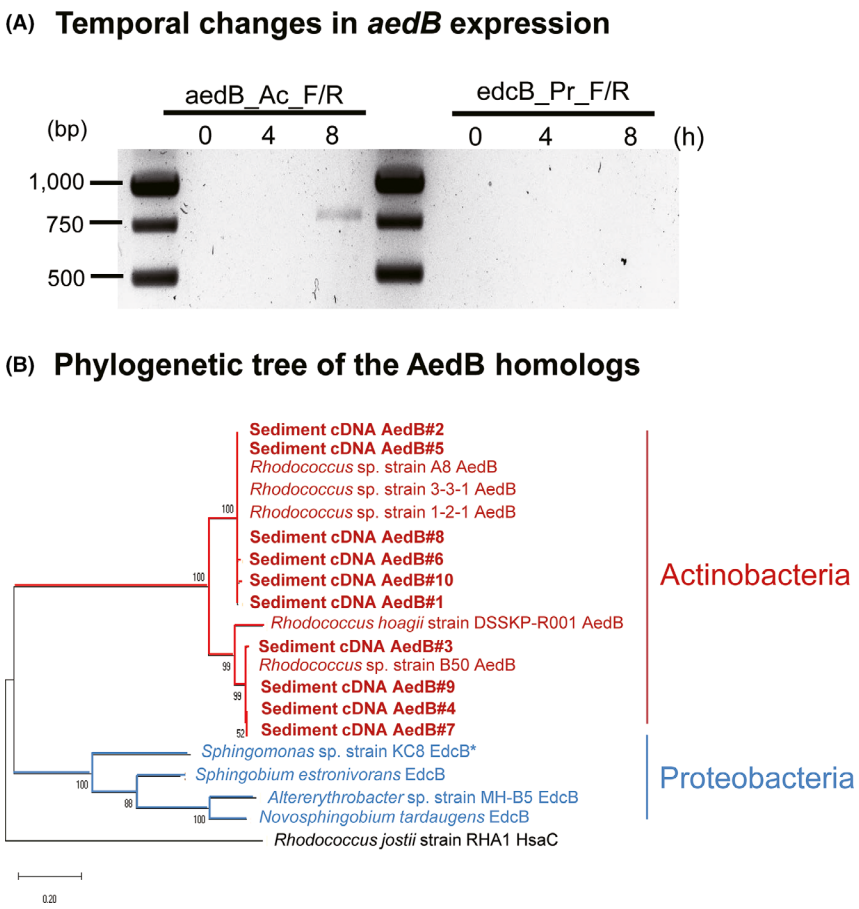

Fig. 7. Phylogenetic identification of 4-hydroxyestrone 4,5-dioxygenase genes expressed in the [3,4C-^{13}C]E1-spiked estuarine sediments.
A. RT-PCR indicated temporal changes in the expression of the actinobacterial and proteobacterial 4-hydroxyestrone 4,5-dioxygenase genes in [3,4C-^{13}C]E1-spiked estuarine sediment.
B. Phylogenetic tree of deduced amino acid sequences of the 4-hydroxyestrone 4,5-dioxygenase genes from oestrogen-degrading bacterial isolates and *aedB* fragments obtained from the cDNA of the [3,4C-^{13}C]E1-spiked estuarine sediment. *, EdcB was alternatively named as the OecC in Chen *et al.* (2017).

involved in actinobacterial oestrogen degradation. Since the phenolic A-ring of steroidal oestrogens directly interacts with the oestrogen receptor (Baker and Lathe, 2018), the cleavage of the phenolic A-ring eliminates their oestrogenic activity (Chen *et al.*, 2017). Therefore, PEA and HIP, along with *aedB*, are suitable biomarkers for monitoring the water quality of environments contaminated by oestrogens. We designed and tested specific primers for 4-hydroxyestrone 4,5-dioxygenase genes of proteobacteria (Chen *et al.*, 2018) and actinobacteria (this study). Interestingly, while previous studies suggested that proteobacteria are the major oestrogen consumers in wastewater treatment plants, our PCR-based functional assays demonstrate that actinobacteria are active oestrogen degraders in urban estuarine sediments. The combination of targeted metabolites analysis with PCR-based functional assays thus represents a simple, cost-effective and rapid approach to gain a holistic view of the fate of steroidal oestrogens in the environment. Nevertheless, the contribution of proteobacteria in this oestrogen-contaminated aquatic ecosystem could be underestimated due to the potential bias (e.g., annealing efficiency or the template bias derived from cDNA construction) produced during the PCR using the *aedB*-specific primers. Finally, the gene cluster containing the oestrogen-degrading geneses in strain B50 and strain DSSKP-R-001 is all present in their plasmids. Therefore, the *aedA*- and *aedB*-containing plasmids could also be used to transform other actinobacteria into efficient oestrogen degraders or even with a broader substrate spectrum via gene knock-in.

Experimental procedures

Enrichment and isolation of strain B50

Soil samples were collected from Dr. Hayashi's garden in Kodaira, Tokyo, Japan, in 2004. To enrich the oestrogen-degrading actinobacteria, the soil samples (2 g)

were incubated in a rich growth medium (100 ml in a 0.5-l flask) containing $(NH_4)_2HPO_4$ (0.12 g), KCl (0.25 g), Bacto yeast extract (0.02 g), E1 (0.2 g), and soil extract (20 ml). Medium pH was adjusted to 7.1 with HCl before autoclaving. To prepare the soil extract, soil (500 g) was suspended in double-distilled water (ddH_2O) (2.4 L) and the soil suspension was autoclaved. After that, the autoclaved soil suspension was centrifuged at 1,000 × *g* for 10 min and the resulting supernatant was defined as the soil extract. The bacterial cultures were incubated at 28°C with continuous shaking (150 rpm) in the dark (to avoid the growth of phototrophs) for 14 days. The E1-spiked enrichment cultures were diluted (10^{-4}-fold) and spread on E1-coated agar plates containing $(NH_4)_2HPO_4$ (0.12 g), KCl (0.25 g), Bacto yeast extract (0.02 g), and soil extract (20 ml). E1 was dissolved in methanol (10 mg l^{-1}) and was spread onto the surface of each agar plate; the plates were placed in laminar flow for 2 days at room temperature to remove methanol before inoculation. The plates were then incubated at 28°C for an additional 10 days. Bacterial colonies with a clear zone (in which E1 was exhausted) were selected and streaked on agar plates to obtain single colonies. After a three-day incubation at 28°C, single colonies with a clear zone were further selected and incubated with E1 (1 mM) as the sole carbon and electron donor in a chemically defined mineral medium. The basal medium used for the isolation and routine cultivation of strain B50 contained NH_4Cl (2.0 g l^{-1}), KH_2PO_4 (0.67 g l^{-1}), and K_2HPO_4 (3.95 g l^{-1}). After autoclaving, this basal medium was supplemented with $MgSO_4$ (2 mM), $CaCl_2$ (0.7 mM), filtered vitamin mixture (1000x; the stock solution contained cyanocobalamin (50 mg), pantothenic acid (50 mg), riboflavin (50 mg), pyridoxamine (10 mg), biotin (20 mg), folic acid (20 mg), nicotinic acid (25 mg), nicotine amide (25 mg), α-lipoic acid (50 mg), *p*-aminobenzoic acid (50 mg), and thiamine (50 mg) per litre), ethylenediaminetetraacetic acid (EDTA)-chelated trace elements (1,000 x) (Rabus and Widdel, 1995), and sodium selenite (4 μg l^{-1}). Dimethyl sulfoxide (DMSO)-dissolved E1 (stock concentration = 125 mM) was added to the mineral medium to a final concentration of 1 mM E1. The 16S rRNA gene sequence was amplified from the total genomic DNA extracted from the bacterial culture using universal primers 27F and 1492R, and the taxonomy of strain B50 was determined using the Nucleotide Basic Local Alignment Search Tool (BLASTn) from the National Center for Biotechnology Information (NCBI).

Aerobic incubation of strain B50 with sex steroids

The wild-type and the gene-disrupted mutants of strain B50 were used in the resting cell biotransformation assays. Bacteria were first aerobically grown in LB broth (40 ml in a 200-ml Erlenmeyer flask) containing E1 (50 μM) as an inducer at 28°C with continuous shaking (150 rpm). Cells were collected through centrifugation (8000 *g*, 20 min, 15°C) at the exponential growth phase with an optical density at 600 nm (OD_{600}) of 0.5 (optical path, 1 cm), followed by removal of the supernatant. The cell pellet was resuspended in a chemically defined mineral medium as mentioned above. The cell suspension (OD_{600} = 1; 10 ml) was incubated with E1 or other sex steroids (100 mg l^{-1}) and was aerobically incubated at 28°C with continuous shaking (150 rpm) and was sampled (1 ml) hourly. The resulting samples were extracted twice using equal volumes of ethyl acetate. The ethyl acetate fractions were evaporated, and the pellets containing oestrogen metabolites were stored at −20°C until further analysis.

Extracellular production of PEA and HIP

The preparation of the resting cell assays of strain B50 was the same as described above. The cell suspensions (OD_{600} = 1; 10 ml) were fed with E1 (twofold serial dilution; from 40 to 1.25 mg ml^{-1}) and then aerobically incubated at 28°C with shaking (150 rpm). The cell suspensions were sampled (1 ml) after 24 h of aerobic incubation with E1. Cells were separated from the growth medium through centrifugation (10 000 *g*, 10 min) followed by isolation of the supernatant, and the cell pellet was discarded. To facilitate the extraction of PEA and HIP, the samples were acidified using 30 μL of 6N HCl. The resulting samples were extracted twice using ethyl acetate (1 ml), and the extracted oestrogen metabolites were stored at −20°C until further analysis.

The aedA and aedB disruption in strain B50

The disruption of individual oxygenase genes (*aedA* or *aedB*) in strain B50 were performed using homologous recombination by a pK18-Cm^R-pheS** plasmid. This plasmid with the UP and DOWN sequences was designed for the markless gene deletion through a double crossing-over of recombination sites. The double crossing-over is absolutely required only when the multiple genes are managed to be deleted in the target bacterial strain. Thus, we used the gene-disrupted mutants (via single crossing-over) but not gene-deleted mutants (via double crossing-over) in the current study.

First, gene-specific DNA fragments, including the 900 base pair flanking region and the 100 base pair coding sequence, of the target genes were cloned through Platinum polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and purified with GenepHlow™ Gel/PCR kit (Geneaid, New Taipei City, Taiwan). The upstream,

downstream and plasmid backbone fragments were assembled *via* an In-Fusion® HD Cloning Kit (TAKARA Bio; Kusatsu, Shiga, Japan) to generate the plasmid (*aedA*- or *aedB*-pK18-Cm^R-pheS**). This plasmid was electroporated into *E. coli* strain S17-1 using a Gene Pulser Xcell™ (Bio-Rad, Hercules, CA, USA) with the conditions of 2.5kV, 25 μF and 200Ω. The transformed *E. coli* strain S17-1 was co-incubated with wild-type *Rhodococcus* sp. strain B50 at 30°C overnight for horizontal gene transfer through conjugation. Successfully transformed colonies of *Rhodococcus* sp. strain B50 were selected with nalidixic acid and chloramphenicol. The insertion of the chloramphenicol-resistant gene into the strain B50 genome was confirmed using the plasmid-specific primer pairs 5′-TTCATCATGCCGTTTGTG AT-3′ (Pk18-cmpheS-F) and 5′-ATCGTCAGACCCTTG TCCAC-3′ (Pk18-cmpheS-R). The genotypes of the *aedA*- and *aedB*-disrupted mutants were further examined using the gene-specific primer pairs [5′-AGGTCGA TGTCCTCGACACCGAGG-3′ (*aedA*-3k-F) and 5′-CGCA TCCTCAGTCACCTCGGCG- 3′ (*aedA*-3k-R)] and [′′-AA CCATGATCTTCACCATCG- 3′ (*aedB*-3k-F) and 5′-TCA GTAGCCGTGCACGAG- 3′ (*aedB*-3k-R)] respectively.

Sampling site and estuarine sediment sample collection

Approximately six million people reside in the basin of the Tamsui River, Taipei, Taiwan. The Tamsui River estuary receives sewage discharge and waste effluent from the Taipei metropolitan area (Kao *et al.*, 2013). This includes effluent from the Dihua Sewage Treatment Plant, which contains oestrogens (approximately 1 ng l^{-1}) (Chen *et al.*, 2018). Our sampling site, Guandu (25°6′59.56″N, 121°27′46.99″E), with a salinity of 5–22 parts per thousand (ppt) (Kao *et al.*, 2013; Shih *et al.*, 2017), is located in the upper estuary where the Keelung River meets the main channel of the Tamsui River, and the sewage discharge and seawater intrusion are mixed in Guandu (Kao *et al.*, 2013). In the current study, four sediment cores were collected from Guandu. The sediment samples were collected using polyvinyl chloride corers (7.5-cm diameter). During low tide on 20 May 2019, the corers were pressed down approximately 30 cm into the sediments and sealed with a rubber stopper immediately after collection. Estuarine water samples (20 l) were collected from Guandu on the same day. The sediment and estuarine water samples were carried to the laboratory within 1 h after sampling and were processed immediately.

Spiking the estuarine sediments with E1

Each sediment core was fractionated into three sections: a subsurface layer (0–5 cm depth), a middle layer (6–10 cm depth) and a bottom layer (11–15 cm depth). Vertical sectioning of the sediment cores was based on the vertical distributions of chemicals and bacteria in the Guandu sediments (Shih *et al.*, 2017). The subsurface layer sediment (1 g) was added to 100-ml Erlenmeyer flasks containing river water (9 ml). The microcosms (10 ml) were then spiked with [3,4C-^{13}C]E1 (10 μg ml^{-1}) and incubated in the dark at 30°C with stirring (150 rpm). Oestrogen metabolites in the microcosms were sampled (1 ml) every 2 days (0–10 days) and were detected using ultra-performance liquid chromatography–atmospheric pressure chemical ionization–high-resolution mass spectrometry (UPLC − APCI−HRMS). The microcosms were also sampled (1 ml) every 4 h (0–8 h) and stored at − 80°C before the RNA extraction. Oestrogen metabolites in the samples were detected using UPLC − APCI−HRMS. The functional *aedB* genes in the microcosm samples were analysed through PCR-based functional assays as described below.

RNA isolation and cDNA preparation

Total RNA was extracted from the E1-spiked estuarine sediment sample using the RNeasy® PowerSoil® total RNA kit (Qiagen, Hilden, Germany). The crude total RNA was further purified using Turbo DNA-free Kit (Thermo Fisher Scientific) to remove DNA. The DNA-free total RNA was reverse-transcribed to cDNA using the SuperScript® IV First-Strand Synthesis System (Thermo Fisher Scientific) with random hexamer primers (Thermo Fisher Scientific).

Amplification of 4-hydroxyestrone 4,5-dioxygenase genes from the estuarine sediment samples using degenerate primers

Multiple alignments of 4-hydroxyestrone 4,5-dioxygenase genes from oestrogen-degrading actinobacteria or alphaproteobacteria were conducted with Geneious® 11.1.5 (Biomatters; Auckland, New Zealand). Degenerate primer pairs were designed according to the conserved regions of actinobacteria (forward: 5′-CGYGGCATCGG ATACATCGG-3′; reverse: 5′-ACMGGGTCGCAKCCGA TCTC-3′) or alpha-proteobacteria (forward: 5′-CDG YYTGGGCTATSTSGG-3′; reverse: 5′-ATCGCGYCSC ASCCRATYTC-3′) respectively. The *aedB* fragments were amplified with PCR with a program of 95°C for 1 min, followed by 30 cycles at 95°C for 30 s, 64°C for 30 s, 72°C for 60 s and finally 72°C for 5 min. Amplified *aedB* sequences were cloned into *E. coli* DH5α-derived ECOS™ 101 competent cells (Yeastern Biotech; Taipei, Taiwan) using the yT&A Cloning Kit (Yeastern Biotech; Taipei, Taiwan). The *aedB* fragments (approximately 800 bp) were sequenced on an ABI 3730xl DNA

Analyzer (Applied Biosystems; Waltham, MA, USA) with the BigDye Terminator kit according to the manufacturer's instructions by the DNA Sequencing Core Facility at Academia Sinica.

Other materials and methods for general chemical analyses and molecular biological manipulation are described in Supporting information.

Acknowledgements

This study is supported by the Ministry of Science and Technology of Taiwan (109-2221-E-001-002; 110-2222-E-008-002; 109-2811-B-001-513). Po-Hsiang Wang is also supported by the Research and Development Office as well as Research Center for Sustainable Environmental Technology, National Central University, Taiwan. Yi-Lung Chen is supported by the Research Grants for New Teachers of College of Science, Soochow University, Taiwan. We thank the Institute of Plant and Microbial Biology, Academia Sinica, for providing access to the Small Molecule Metabolomics Core Facility (for UPLC–HRMS analyses).

Conflict of interest

The authors have no conflicts of interest to declare.

Author Contributions

Y.-R.C. and P.-H.W. designed the research. T.-H. H., Y.-L.C. and M.-R.C. performed the research. M..M., M.H. and T.H. contributed new reagents and analytic tools. Y.-L.C., T.-H.H. and Y.-R.C analysed the data. Y.-R.C and P.-H.W. drafted the manuscript. All authors reviewed the manuscript.

References

Baker, M.E., and Lathe, R. (2018) The promiscuous estrogen receptor: Evolution of physiological estrogens and response to phytochemicals and endocrine disruptors. *J Steroid Biochem Mol Biol* **184:** 29–37.

Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., and Samperi, R. (2000) Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water. *Environ Sci Technol* **34:** 5059–5066.

Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schäfer, A.J., Rijs, G.B., Wegener, J., and Cofino, W.P. (1999) Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci Total Environ* **225:** 101–108.

Bergstrand, L.H., Cardenas, E., Holert, J., Van Hamme, J.D., and Mohn, W.W. (2016) Delineation of steroid-degrading microorganisms through comparative genomic analysis. *mBio* **7:** e00166-16

Boll, M., Geiger, R., Junghare, M., and Schink, B. (2020) Microbial degradation of phthalates: biochemistry and environmental implications. *Environ Microbiol Rep* **12:** 3–15.

Chen, Y.-L., Fu, H.-Y., Lee, T.-H., Shih, C.-J., Huang, L., Wang, Y.-S., *et al*. (2018) Estrogen Degraders and Estrogen Degradation Pathway Identified in an Activated Sludge. *Appl Environ Microbiol* **84:** e00001–18.

Chen, Y.L., Yu, C.P., Lee, T.H., Goh, K.S., Chu, K.H., Wang, P.H., *et al*. (2017) Biochemical Mechanisms and Catabolic Enzymes Involved in Bacterial Estrogen Degradation Pathways. *Cell Chem Biol* **24:** 712–724.

Chiang, Y.R., Wei, S.T.S., Wang, P.H., Wu, P.H., and Yu, C.P. (2020) Microbial degradation of steroid sex hormones: implications for environmental and ecological studies. *Microb Biotechnol* **13:** 926–949.

Coombre, R.G., Tsong, Y.Y., Hamilton, P.B., and Sih, C.J. (1966) Mechanisms of steroid oxidation by microorganisms. X. Oxidative cleavage of estrone. *J Biol Chem* **241:** 1587–1595.

Crowe, A.M., Workman, S.D., Watanabe, N., Worrall, L.J., Strynadka, N.C.J., and Eltis, L.D. (2018) lpdAB, a virulence factor in Mycobacterium tuberculosis, is a cholesterol ring-cleaving hydrolase. *Proc Natl Acad Sci USA* **115:** E3378–E3387.

Dresen, C., Lin, L.Y.C., D'Angelo, I., Tocheva, E.I., Strynadka, N., and Eltis, L.D. (2010) A flavin-dependent monooxygenase from Mycobacterium tuberculosis involved in cholesterol catabolism. *J Biol Chem* **285:** 22264–22275.

Fujii, K., Kikuchi, S., Satomi, M., Ushio-Sata, N., and Morita, N. (2002) Degradation of 17β-Estradiol by a Gram-Negative Bacterium Isolated from Activated Sludge in a Sewage Treatment Plant in Tokyo, Japan. *Appl Environ Microbiol* **68:** 2057–2060.

Griffith, D.R., Kido Soule, M.C., Eglinton, T.I., Kujawinski, E.B., and Gschwend, P.M. (2016) Steroidal estrogen sources in a sewage-impacted coastal ocean. *Environ Sci: Processes Impacts* **18:** 981–991.

Hamid, H., and Eskicioglu, C. (2012) Fate of estrogenic hormones in wastewater and sludge treatment: A review of properties and analytical detection techniques in sludge matrix. *Water Res* **46:** 5813–5833.

Hanselman, T.A., Graetz, D.A., and Wilkie, A.C. (2003) Manure-borne estrogens as potential environmental contaminants: a review. *Environ Sci Technol* **37:** 5471–5478.

Harvey, R.A., and Ferrier, D.R. (2011) *Biochemistry*. Baltimore, MD: Lippincott Williams & Wilkins.

Holert, J., Cardenas, E., Bergstrand, L.H., Zaikova, E., Hahn, A.S., Hallam, S.J., and Mohn, W.W. (2018) Metagenomes reveal global distribution of bacterial steroid catabolism in natural, engineered, and host environments. *mBio* **9:** e02345-17.

Huang, C.-H., and Sedlak, D.L. (2001) Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. *Environ Toxicol Chem* **20:** 133–139.

Ibero, J., Galán, B., Díaz, E., and García, J.L. (2019a) Testosterone degradative pathway of *Novosphingobium tardaugens*. *Genes* **10:** 871.

Ibero, J., Galán, B., Rivero-Buceta, V., and García, J.L. (2020) Unraveling the 17β-estradiol degradation pathway in *Novosphingobium tardaugens* NBRC 16725. *Front Microbiol* **11.588300**

Ibero, J., Sanz, D., Galán, B., Díaz, E., and García, J.L. (2019b) High-quality whole-genome sequence of an estradiol-degrading strain, *Novosphingobium tardaugens* NBRC 16725. *Microb Resour Annouc* **8:** e01715–e01718.

Jurgens, M.D., Holthaus, K.I.E., Johnson, A.C., Smith, J.J.L., Hetheridge, M., and Williams, R.J. (2002) The potential for estradiol and ethinylestradiol degradation in English Rivers. *Environ Toxicol Chem* **21:** 480–488.

Kao, Y.H., Wang, S.W., Maji, S.K., Liu, C.-W., Wang, P.L., Chang, F.J., and Liao, C.M. (2013) Hydrochemical, mineralogical and isotopic investigation of arsenic distribution and mobilization in the Guandu wetland of Taiwan. *J Hydrol* **498:** 274–286.

Ke, J., Zhuang, W., Gin, K.Y.H., Reinhard, M., Hoon, L.T., and Tay, J.H. (2007) Characterization of estrogen-degrading bacteria isolated from an artificial sandy aquifer with ultrafiltered secondary effluent as the medium. *Appl Microbiol Biotechnol* **75:** 1163–1171.

Kolodziej, E.P., Gray, J.L., and Sedlak, D.L. (2003) Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. *Environ Toxicol Chem* **22:** 2622–2629.

Kolodziej, E.P., Harter, T., and Sedlak, D.L. (2004) Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. *Environ Sci Technol* **38:** 6377–6384.

Kramer, V.J., Miles-Richardson, S., Pierens, S.L., and Giesy, J.P. (1998) Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. *Aquat Toxicol* **40:** 335–360.

Kurisu, F., Ogura, M., Saitoh, S., Yamazoe, A., and Yagi, O. (2010) Degradation of natural estrogen and identification of the metabolites produced by soil isolates of *Rhodococcus* sp. and *Sphingomonas* sp. *J Biosci Bioeng* **109:** 576–582.

Lee, Y.C., Wang, L.M., Xue, Y.H., Ge, N.C., Yang, X.M., and Chen, G.H. (2006) Natural Estrogens in the Surface Water of Shenzhen and the Sewage Discharge of Hong Kong. *Hum Ecol Risk Assess Int J* **12:** 301–312.

Lin, A.Y., and Reinhard, M. (2005) Photodegradation of common environmental pharmaceuticals and estrogens in river water. *Environ Toxicol Chem* **24:** 1303–1309.

Lin, C.W., Wang, P.H., Ismail, W., Tsai, Y.W., El Nayal, A., Yang, C.Y., *et al.* (2015) Substrate uptake and subcellular compartmentation of anoxic cholesterol catabolism in *Sterolibacterium denitrificans*. *J Biol Chem* **290:** 1155–1169.

Lorenzen, A., Hendel, J.G., Conn, K.L., Bittman, S., Kwabiah, A.B., Lazarovitz, G., *et al.* (2004) Survey of hormone activities in municipal biosolids and animal manures. *Environ Toxicol* **19:** 216–225.

Matsumoto, T., Osada, M., Osawa, Y., and Mori, K. (1997) Gonadal Estrogen Profile and Immunohistochemical Localization of Steroidogenic Enzymes in the Oyster and Scallop during Sexual Maturation. *Comp Biochem Physiol B: Biochem Mol Biol* **118:** 811–817.

Morthorst, J.E., Brande-Lavridsen, N., Korsgaard, B., and Bjerregaard, P. (2014) 17β-estradiol causes abnormal development in embryos of the viviparous eelpout. *Environ Sci Technol* **48:** 14668–14676.

Noguera-Oviedo, K., and Aga, D.S. (2016) Chemical and biological assessment of endocrine disrupting chemicals in a full scale dairy manure anaerobic digester with thermal pretreatment. *Sci Total Environ* **550:** 827–834.

Rabus, R., and Widdel, F. (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol* **163:** 96–103.

Shareef, A., Angove, M.J., Wells, J.D., and Johnson, B.B. (2006) Aqueous solubilities of estrone, 17β-estradiol, 17α-ethynylestradiol, and bisphenol A. *J Chem Eng Data* **51:** 879–881.

Shih, C.J., Chen, Y.L., Wang, C.H., Wei, S.T.S., Lin, I.T., Ismail, W.A., and Chiang, Y.R. (2017) Biochemical Mechanisms and Microorganisms Involved in Anaerobic Testosterone Metabolism in Estuarine Sediments. *Front Microbiol* **8:** 1520.

Takamura, Y., and Nomura, G. (1988) Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in relation to the carbon and energy metabolism of *Escherichia coli* K12. *J Gen Microbiol* **134:** 2249–2253.

Tarrant, A.M., Blomquist, C.H., Lima, P.H., Atkinson, M.J., and Atkinson, S. (2003) Metabolism of estrogens and androgens by scleractinian corals. *Comp Biochem Physiol B Biochem Mol Biol* **136:** 473–485.

Thayanukul, P., Zang, K., Janhom, T., Kurisu, F., Kasuga, I., and Furumai, H. (2010) Concentration-dependent response of estrone-degrading bacterial community in activated sludge analyzed by microautoradiography-fluorescence in situ hybridization. *Water Res* **44:** 4878–4887.

Wang, P.H., Chen, Y.L., Wei, S.T.S., Wu, K., Lee, T.H., Wu, T.Y., and Chiang, Y.R. (2020) Retroconversion of estrogens into androgens by bacteria via a cobalamin-mediated methylation. *Proc Natl Acad Sci USA* **117:** 1395–1403.

Wise, A., O'Brien, K., and Woodruff, T. (2011) Are oral contraceptives a significant contributor to the estrogenicity of drinking water? *Environ Sci Technol* **45:** 51–60.

Wu, K., Lee, T.H., Chen, Y.L., Wang, Y.S., Wang, P.H., Yu, C.P., *et al.* (2019) Metabolites Involved in Aerobic Degradation of the A and B Rings of Estrogen. *Appl Environ Microbiol* **85:** e02223–e2318.

Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., *et al.* (2004) Degradation of estrogens by Rhodococcus zopfii and Rhodococcus equi isolates from activated sludge in wastewater treatment plants. *Appl Environ Microbiol* **70:** 5283–5289.

Yu, C.P., Deeb, R.A., and Chu, K.H. (2013) Microbial degradation of steroidal estrogens. *Chemosphere* **91:** 1225–1235.

Yu, C.P., Roh, H., and Chu, K.H. (2007) 17β-Estradiol-Degrading Bacteria Isolated from Activated Sludge. *Environ Sci Technol* **41:** 486–492.

Zhao, H., Tian, K., Qiu, Q., Wang, Y., Zhang, H., Ma, S., et al. (2018) Genome Analysis of *Rhodococcus* Sp.

DSSKP-R-001: A Highly Effective β-Estradiol-Degrading Bacterium. *Int J Genomics* **2018**: 3505428.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Morphology of *Rhodococcus* sp. strain B50 colonies and cells. (A) Photograph of strain B50 colonies grown on Lysogeny Broth (LB) agar. The grown colonies are smooth and contain orange pigments. (B) Light micrograph of strain B50 cells (1,000 x). Scalebar is 10 μm.

Fig. S2. Growth of strain B50 on several common steroids: (**A**) estrone (E1), (**B**)17β-estradiol (E2), (**C**) estriol (E3), (**D**) 17α-ethynylestradiol (EE2), (**E**) testosterone, and (**F**) cholesterol. The strain B50 cultures (50 ml) were aerobically incubated with different steroids in a chemically defined mineral medium. In all of the treatments, individual steroids (100 mg l^{-1}; ●) served as the sole carbon source and electron donor. Bacterial growth was measured based on the increasing total protein concentrations (▲) in the cultures. Results are representative of 3 individual experiments. Data shown are mean values with standard errors of 3 technical replicates.

Fig. S3. Comparing genomic characteristics of the two megaplasmids from strains B50 and DSSKP-R-001. (A) DNA sequence alignment of the two megaplasmids carried out through progressiveMauve incorporated into the software Geneious Prime. Colored blocks indicate areas of sequence that are presumably homologous and internally free from genomic rearrangement. Among them, the bright green blocks include 18 locus tags of GMFMDNLD_05332~05459 (namely the *aed* gene cluster) and C7H75_RS25340~25425 from strain B50 and strain DSSKP-R-001, respectively. (B) Coding sequence homology between the two megaplasmids evaluated using a web platform OrthVenn2 (https://orthovenn2.bioinfotoolkits.net/home; E-value: 1e-10; inflation value: 1.5). The Vann diagram indicated that only 20 coding sequences are homologous between these two megaplasmids. In addition to the 18 locus tags within bright green blocks in the Fig. S3A, the consensus genes include the locus tags (GMFMDNLD_05329 and C7H75_RS25325; encoding a putative TetR family transcriptional regulator) within green blocks and locus tags (GMFMDNLD_04997 and C7H75_RS25540; encoding a putative peptidoglycan endopeptidase) within blue blocks.

Fig. S4. Phylogenetic analysis of the dioxygenases involved in the *meta*-cleavage of the steroidal A-ring, including 4-hydroxyestrone 4,5-dioxygenase (EdcB and AedB; estrogenic A-ring), proteobacterial TesB (androgenic A-ring), and actinobacterial HsaC (androgenic A-ring).

Fig. S5. A comparison of estrogen (A) and androgen (B) degradation pathways in actinobacteria. Characterized oxygenases are marked in orange. AedA estrone 4-hydroxylase; AedB, 4-hydroxyestrone 4,5-dioxygenase; KshAB, 3-ketosteroid 9α-hydroxylase; HsaAB, 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione 4-hydroxylase; HsaC, 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase. Protein nomenclature is based on that of *Rhodococcus* sp. strain B50 (estrogen degradation) and *R. jostii* strain RHA1 (androgen degradation). The proposed structure in the bracket (9α-hydroxy-androsta-1,4-diene-3,17-dione) is very unstable and has never been detected.

Fig. S6. The uncropped full-range agarose gel (1%) of Fig. 3B(i).

Fig. S7. The uncropped full-range agarose gel (1%) of Fig. 3B(ii).

Fig. S8. The uncropped full-range agarose gel (1%) of Fig. 5B.

Fig. S9. The uncropped full-range agarose gel (1%) of Fig. 7A.

Table S1. Antibiotic test of the *Rhodococcus* spp. reported in this study.

Table S2. MS analysis of detected E1 metabolites in strain B50 cultures.

Table S3. Oligonucleotides used in this study.

Appendix S1. Nucleotide sequence (5'-3' direction) of the 16S rRNA gene of strain B50.

Appendix S2. Nucleotide sequence (5'-3' direction) of the *aedA* gene (GMFMDNLD_3_05336) of strain B50.

Appendix S3. Nucleotide sequence (5'-3' direction) of the *aedB* gene (GMFMDNLD_3_05338) of strain B50.

Appendix S4. Nucleotide sequences (5'-3' direction) of the *aedB* amplicons identified in the E1-spiked sediment.

Data S1. Genome annotation of strain B50.

