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Supplementary Information for

Retroconversion of estrogens into androgens by bacteria via a cobalamin-mediated methylation

Po-Hsiang Wang, Yi-Lung Chen, Sean Ting-Shyang Wei, Kan Wu, Tzong-Huei Lee, Tien-Yu Wu, and Yin-Ru Chiang

Correspondence to Yin-Ru Chiang
Email: yinru915@gate.sinica.edu.tw

This PDF file includes:

Other supplementary materials for this manuscript include the following:

Supplemental Materials and Methods

Chemicals and Bacterial Strains

[3,4C-^{13}C]estrone (99%) was purchased from Cambridge Isotope Laboratories (Tewksbury, USA). [16,16,17-D3]17β-estradiol (98 %), 5α-androstan-17β-ol-3-one (=17β-hydroxyandrostan-3-one) and 3aα-H-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5α-androstan-3β,17β-diol (=3β,17β-dihydroxyandrostane) was purchased from Steraloids Inc (Newport, RI, USA). Other chemicals were analytical grade and purchased from Mallinckrodt Baker (Phillipsburg, USA), Merck Millipore (Burlington, USA), and Sigma-Aldrich unless specified otherwise. *Denitratisoma oestradiolicum* strain AcBE2-1 (=DSM 16959) and *Steroidobacter denitrificans* strain FS (=DSM 18526) were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany).

Enrichment of *Denitratisoma* sp. strain DHT3 from an anoxic sludge

Strain DHT3 was enriched from an anoxic sludge collected from the Dihua Sewage Treatment Plant, Taipei, Taiwan (DHSTP; 25°4'20.97"N, 121°30'36.17"E). To enrich the anaerobic estrogen utilizer, the sludge sample (100 mL) was spiked with estradiol (1 mM) and nitrate (10 mM), and was incubated at 28˚C under a N_2/CO_2 (80:20, v/v) atmosphere in the dark (to avoid growth of phototroph) for 14 days. Later, twenty milliliters of the estradiol-spiked sludge sample was transferred to a chemically defined mineral medium (500 mL) (1) with estradiol (1 mM) as the sole substrate and nitrate as the terminal electron acceptor. The final pH was adjusted to 6.5 with HCl to alleviate the gradual pH increase in culture caused by denitrification. After the added estradiol was mostly consumed, the estradiol-spiked mixed culture (1 mL) was transferred to the same defined medium (200 mL). This step was repeated ten times to selectively enrich the anaerobic estrogen utilizer. The 10^{th} transfer culture was serially diluted from 10^1 to 10^9 times in the same defined medium to eliminate the non-estrogen degraders, and incubated under the same conditions. After the 10^9 times-diluted culture consumed most of the added estradiol, the dilution-to-elimination transfers were repeated again. After that, the culture purity was validated microscopically and by growth tests of the enrichment culture in liquid R2A rich medium and in the defined medium added with yeast extracts. The 16S rRNA gene sequence was amplified from the total genomic DNA extracted from the enrichment culture using universal primers 27F and 1492R (2), and the taxonomy of strain DHT3 was determined using BLAST. Since the growth of strain DHT3 in the solid medium (agar or gelrite plate) was not observed, we do not exclude the possibility that trace contaminated microbes are present in the microscopically pure strain DHT3 enrichment culture.

Anaerobic growth of strain DHT3 with and without vitamins

To test the vitamin requirements of strain DHT3, we anaerobically cultivated strain DHT3 in the defined mineral media containing estradiol (3 mM), nitrate (initially 10 mM), and individual vitamins [cyanocobalamin (20 μg/L), biotin (10 μg/L), calcium pantothenate (25 μg/L), thiamine (50 μg/L), *p*-aminobenzoic acid (50 μg/L), nicotinic

acid (100 μg/L), or pyridoxamine (250 μg/L)]. The growth of strain DHT3 with and without the mixed vitamin solution VL-7 (3) was served as the positive and negative controls, respectively. The cultures were sampled daily, and the samples (0.5 mL each) were centrifuged at 10,000 × g for 10 min at 4°C. After centrifugation, the bacterial pellets were stored at -20°C before protein content determination. Proteins were extracted from the frozen pellets using B-PER Bacterial Cell Lysis Reagents (Thermo Fisher Scientific; Waltham, MA, USA). The protein content in the samples was estimated using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to manufacturer's instructions, with a standard curve generated using analytical-grade bovine serum albumin. The nitrate content was measured using Spectroquant Nitrate Test Kit HC707906 (Merck, Germany) according to manufacturer's instructions.

Analytical methods

(a) Thin-layer Chromatography (TLC)

The steroid standards and products were separated on silica gel-coated aluminum TLC plates (Silica gel 60 F_{254}: thickness, 0.2 mm; 20 × 20 cm; Merck) using dichloromethane:ethyl acetate:ethanol (14:4:0.05, v/v/v) as the developing phase. The steroids were visualized under UV light at 254 nm or by spraying the TLC plates with 30% (v/v) H_2SO_4, followed by an incubation for 1 min at 100°C (in an oven).

(b) High-Performance Liquid Chromatography (HPLC)

A reversed-phase Hitachi HPLC system equipped with an analytical RP-C_{18} column (Luna 18(2), 5 μm, 150 × 4.6 mm; Phenomenex) was used for separating steroidal metabolites in this study. The separation was achieved isocratically using a mobile phase of 45% methanol (v/v) at 35°C at a flow rate of 0.5 mL/min. The steroidal metabolites were detected using a photodiode array detector (200–450 nm). In some studies, HPLC was also used for quantifying steroids present in the strain DHT3 cultures. The quantity of steroids was measured using a standard curve generated from individual commercial steroid standards. The R^2 values for the standard curves were at least > 0.98. The presented data are the average values of three experimental measurements.

(c) Ultra-Performance Liquid Chromatography–High Resolution Mass Spectrometry (UPLC–HRMS)

Steroids were analyzed using UPLC–HRMS on a UPLC system coupled to either an Electric Spray Ionization–Mass Spectrometry (ESI–MS) system or an Atmosphere Pressure Chemical Ionization–Mass Spectrometry (APCI–MS) system. Steroids were firstly separated using a reversed-phase C_{18} column (Acquity UPLC® BEH C18; 1.7 μm; 100 × 2.1 mm; Waters) at a flow rate of 0.4 mL/min at 35°C (oven temperature). The mobile phase comprised a mixture of two solutions: solution A (0.1% formic acid (v/v) in 2% acetonitrile (v/v)) and solution B (0.1% formic acid (v/v) in methanol). Separation was achieved using a gradient of solvent B from 10% to 99% in 8 min. ESI–MS analysis was performed using a Thermo Fisher Scientific™ Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Waltham, MA, USA). Mass spectrometric data in positive ionization mode were collected. The source voltage was set at 3.2 kV; the capillary and source heater temperatures were 360°C and 350°C, respectively; the sheath, auxiliary, and sweep gas flow rates were 30, 15 and 2 arb units, respectively. APCI–MS analysis

was performed using a Thermo Fisher Scientific™ Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Waltham, MA, USA) equipped with a standard APCI source. Mass spectrometric data in positive ionization mode (parent scan range: 50–600 *m/z*) were collected. The capillary and APCI vaporizer temperatures were 120°C and 400°C, respectively; the sheath, auxiliary, and sweep gas flow rates were 40, 5 and 2 arbitrary units, respectively. The source voltage was 6 kV and current 15 μA. The elemental composition of individual adduct ions was predicted using Xcalibur™ Software (Thermo Fisher Scientific).

(d) Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H- and ^{13}C-NMR spectra were recorded at 27°C using a Bruker AV600_GRC 600MHz NMR. Chemical shifts (δ) were recorded and presented in parts per million with deuterated methanol (99.8%) as the solvent and internal reference.

General molecular biological methods

The strain DHT3 genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). PCR mixtures (50 μL) contained nuclease-free H_2O, 2 × PCR buffer (25 μL), dNTPs (2 mM), *Taq* polymerase (1.2 U) (BioTherm; NatuTec GmbH; Frankfurt am Main, Germany), forward and reverse primers (each 200 nM), and template DNA (30 ng). The PCR products were verified using standard TAE-agarose gel (1.5%) electrophoresis with the SYBR® Green I nucleic acid gel stain (Thermo Fisher Scientific), and the PCR products were purified using the GenepHlow Gel/PCR Kit (Geneaid).

RNA isolation and bridging PCR

Bridging PCR was applied to determine the polycistronism of the *emt* gene cluster. Total RNA was extracted from the estradiol-grown strain DHT3 cells using the Direct-zol RNA MiniPrep Kit (Zymo Research; Tustin, CA, USA). The crude total RNA was further purified using Turbo DNA-free Kit (Thermo Fisher Scientific) to eliminate DNA. The DNA-free total RNA was reversely transcribed to cDNA using the SuperScript® IV First-Strand Synthesis System (Thermo Fisher Scientific). Random hexamer primers were used for cDNA synthesis. The polycistronism of the *emt* gene cluster was analyzed using intergenic PCR with total cDNA as the template and the following PCR program was used: 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 75 s, and finally 72°C for 3 min. The primers are shown in **Table S2**. The strain DHT3 genomic DNA and total RNA were used as the positive and negative control, respectively.

RNA extraction and RNA-Seq

The strain DHT3 transcriptome was extracted from the strain DHT3 cells anaerobically cultivated with estradiol (2 mM) or testosterone (2 mM) as the sole substrate. The transcriptomes were extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research), and were further purified using Turbo DNA-free Kit (Thermo Fisher Scientific) to eliminate DNA. rRNA was removed from the transcriptome samples using the Ribo-Zero rRNA Removal Kit (Epicentre Biotechnologies; Madison, WI, USA). The quality of the resulting RNA library was assessed using the Experion RNA Analysis Kit (Bio-Rad; Hercules,CA, USA), and only the samples with an integrity number 8-10 were

selected for transcriptomic analysis. First-strand cDNA was synthesized using the purified transcriptomes as the templates. Second-strand cDNA was synthesized in a reaction mixture containing Second-Strand Synthesis Kit (New England Biolabs; Ipswich, MA, USA). cDNA was purified using the Qiagen purification kit, followed by end repair using NEBNext® End Repair Module (New England Biolabs). Antisense strand DNA was digested in the samples using the Uracil-Specific Excision Reagent Enzyme Kit (New England Biolabs), followed by a PCR to amplify the cDNA. The constructed sequencing libraries were sequenced as paired-end reads (with 126-bp read length) on the Illumina HiSeq 2000 system (Illumina; San Diego, CA). Adapter of the RNA-Seq reads were removed with cutadapt (Version 1.4.2) and the remaining sequences were trimmed by using Seqtk (Version 1.2-r94). The maximum ambiguous nucleotide number was set to 2; the lasting sequences (min length = 35-bp; error probability < 0.05) were included for subsequent analysis. The constructed sequencing libraries were mapped to the coding DNA sequences in the strain DHT3 genome using Bowtie2 (Version 2.2.3). The mapping results were quantified using eXpress (Version 1.5.1) with software's default setting and expressed in terms of Reads Per Kilobase of transcript per Million mapped reads (RPKM).

emtA gene disruption in strain DHT3

The *emtA* gene of strain DHT3 was disrupted by an intragenic insertion of a group II intron into this gene using the TargeTron Gene Knockout System Kit (Sigma-Aldrich, St. Louis, MO). The intron insertion sites for *emtA* were predicted using online intron design software (TargeTron gene knockout system; Sigma-Aldrich), and the primer sequences were generated for intron retargeting. The intron PCR template from the TargeTron Gene Knockout System Kit and the following four primers were used for the construction: *emtA*-773|774 -IBS, *emtA*-773|774s -EBS1d, *emtA*-773|774s -EBS2, and EBS universal primer (see **Table S2** for individual sequences). The PCR was performed according to the manufacturer's instructions. The 350-bp PCR product was purified and subsequently ligated into the pACD4K-C Linear Vector. This resulted in a plasmid, pACD4K-C::*emtA*, specific for *emtA* knockout by insertion of the intron fragment between the 773th and 774th base pairs in the sense direction. The construct mixture was firstly transformed into the ECOS™ 101 *E. coli* strain DH5α competent cells via heat-shock incubation (Yeastern Biotech, Taipei, Taiwan). The pACD4K-C::*emtA* purified from the correct clones (sequences verified using Sanger Sequencing) was then transformed to strain DHT3 through electroporation (2,500 V, 25 μF, and 200 Ω). Strain DHT3 does not carry a copy of T7 RNA Polymerase on the chromosome. Therefore, a source of T7 RNA Polymerase was provided by co-transforming another plasmid pAR1219 (Sigma-Aldrich, St. Louis, MO). The plasmids pACD4K-C::*emtA* and pAR1219 were maintained by selection on chloramphenicol and ampicillin, respectively. Strain DHT3 containing the two plasmids was anaerobically incubated in a chemically defined medium containing estradiol (2 mM), chloramphenicol (100 μg/mL) and ampicillin (100 μg/mL). The bacterial culture was cultivated at 28°C under a N_2/CO_2 atmosphere (80:20, v/v). When the cultures reached a cell density of ~8×10^8/mL, isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 mM) was added to the bacterial culture. After overnight incubation, the strain DHT3 cells were harvested through a centrifugation at 3,500 × g for 10 min at 20°C. The *emtA*-disruption was then selected using a kanamycin marker that was activated upon

chromosomal insertion. Therefore, the resulting strain DHT3 cell pellet was resuspended and was cultivated in a chemically defined medium containing testosterone (2 mM) and kanamycin (20 μg/mL). Kanamycin-resistant strain DHT3 cells were then isolated by two subsequent serial dilution series (10^1 to 10^9 times) using the same medium. Successful *emtA* disruption was confirmed using PCR with primers flanking the *emtA* gene (see **Table S2** for individual sequences).

Analysis of the steroidal metabolite profile of the estrogen-grown strain DHT3

Strain DHT3 cells in the exponential growth phase (with a cell density of approximately 8×10^8/mL) were harvested through a centrifugation at 8,000 × g for 10 min at 25 °C. After decanting the supernatants, the cell pellets were immediately re-suspended in the same chemically defined medium. The concentrated strain DHT3 suspensions (5×10^9/mL; 10 mL) were spiked with 1 mM of estrone (unlabeled estrone and [3,4C-^{13}C]estrone mixed in a 1:1 molar ratio) and was incubated under a N_2/CO_2 (80:20, v/v) atmosphere at 28°C with continuous stirring. 17α-ethinylestradiol (final concentration 50 μM), which cannot be utilized by strain DHT3, was added to the concentrated strain DHT3 cell suspensions to serve as an internal control. The cell suspension samples (0.5 mL) were withdrawn every 30–120 min, and were extracted with equal volumes of ethyl acetate three times. The ethyl acetate fractions containing the estrone-derived metabolites were pooled, vacuum-dried, and analyzed using UPLC–HRMS.

Preparation of cell-extracts

Frozen strain DHT3 cell pellets (5 g) were suspended in 15 mL of HEPES-K^+ buffer (pH 8.0; 100 mM). Strain DHT3 cells were broken by passing the cell suspensions through a French pressure cell (Thermo Fisher Scientific) twice at 137 megapascals. The cell lysates were fractionated using two steps of centrifugation steps. First, the cell-lysates were centrifuged at 20,000 × g for 30 min to get rid of most cell debris, unbroken cells and residual estradiol. Second, the supernatants containing the crude cell-extracts were centrifuged at 100,000 × g for 1.5 h to fractionate the soluble proteins from the membrane-bound proteins. All steps used for preparation of cell-extracts were performed at 4°C under anaerobic conditions.

Cell-extract estradiol methylation activity assays

Assays were routinely performed in the dark (prevent abiotic cob(I)alamin oxidation) at 30°C for 1.5 h under an N_2 atmosphere. The assay mixtures (1 mL) contained HEPES-K^+ buffer (pH 8.0; 100 mM), soluble protein fraction (5 mg) extracted from strain DHT3 cells, estradiol (0.25 mM; [16,16,17-D3]estradiol and unlabeled estradiol in a 1:1 molar ratio), ATP (5 mM), $MgSO_4$ (10 mM), and methylcobalamin (1 mM). In some assays, Ti(III) citrate-reduced cob(I)alamin (1 mM), SAM (0.25 mM), NADH (2 mM), and propyl-iodide (2.5 mM) were added to the assay mixtures. After 1.5 hours of incubation, the assays were extracted twice with the same volume of ethyl acetate. The ethyl acetate fractions containing estradiol-derived compounds were pooled, vacuum-dried, and stored at -20°C before further analytical analysis.

Phylogenetic analysis

(a) cobalamin-dependent estradiol methyltransferase system EmtABCD

The amino acid sequences of B9N43_10325 (*emtA*) from strain DHT3, *emtA* orthologs CBW56_16915 from *D. oestradiolicum* DSM 16959 and AMN47925.1 from *S. denitrificans* DSM 18526, and sequences of cobalamin-dependent methyltransferases searched with EmtA as query in UniProt (manually reviewed) were selected. The amino acid sequences of other cobalamin-dependent methyltransferases that have been functionally characterized (4) were also used for this phylogenetic analysis (**Table S4**). Fifty protein sequences were aligned using MUSCLE (5) in MEGA X (6) without truncation. The unrooted maximum likelihood tree was constructed using the LG model for amino acid substitution plus Gamma distribution rates (G) after the Model Test in MEGA X. The NNI for ML heuristic method and NJ/BioNJ for tree initiation were used for tree inference. Branch support was determined by bootstrapping 1,000 times. The maximum likelihood tree was then visualized in MEGA X.

(b) 16S rRNA genes of selected steroid-degrading bacteria and their organization of genes involved in HIP catabolism

The steroid-degrading bacteria with the corresponding sequenced genomes were chosen for this analysis. Protein sequences deduced from the previously characterized HIP degradation genes were used as queries to identify the orthologs in these genomes (**Dataset S2**). The deduced protein sequences from *Comamonas testosteroni* strain CNB-2 (a model organism for aerobic testosterone catabolism) and from *Mycobacterium tuberculosis* strain H37Rv (a model organism for aerobic cholesterol catabolism) were used as the queries for the BLASTP analysis. Ortholog identification in each selected genomes was based on the criteria (e-value < 1e-5; pairwise identify ≥ 40%; query coverage ≥ 80%) of the BLASTP results.

Supplemental Figures

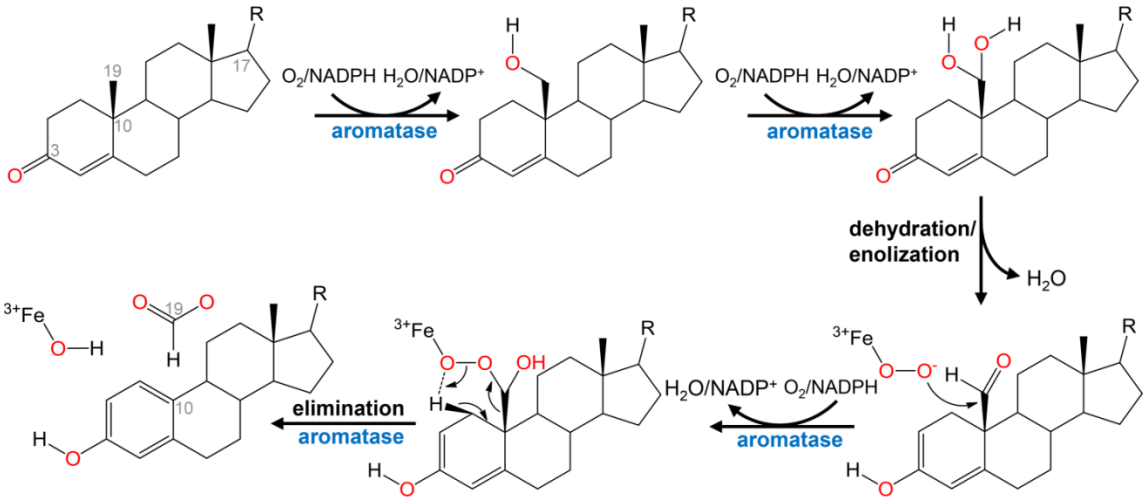

Fig. S1. Possible mechanisms involved in the aromatase-mediated conversion of C_{19} androgens into C_{18} estrogens.

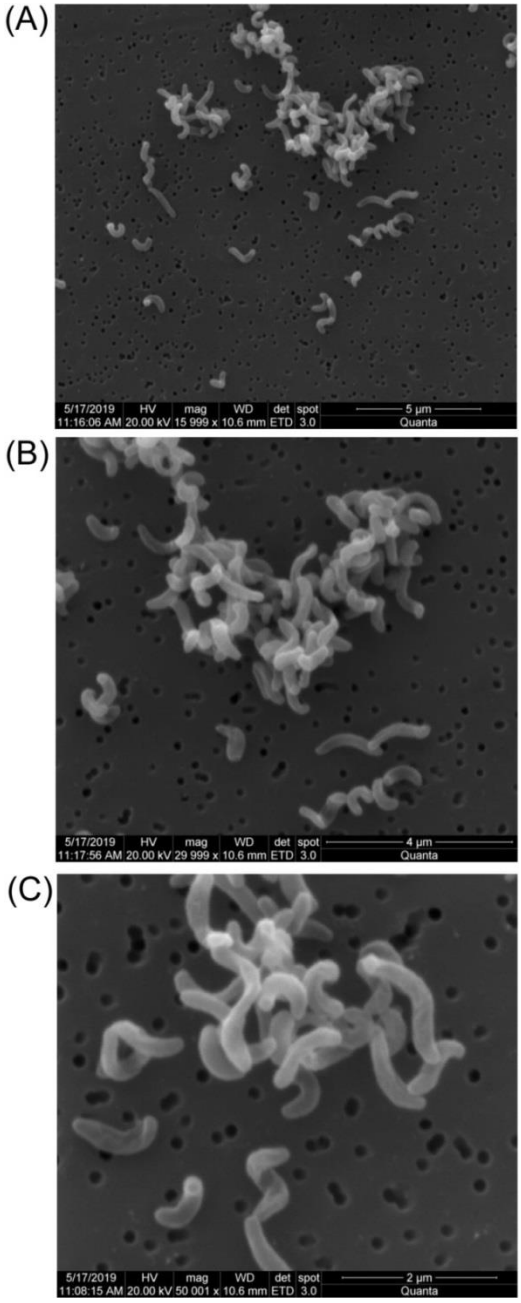

Fig. S2. Scanning electron micrographs of the strain DHT3 cells. The cells were fixed in 0.1 M sodium phosphate buffer (pH 7.0) containing 2.5 % (w/v) glutaraldehyde and 4 % (w/v) paraformaldehyde at room temperature for 1 hour. The sample was post-fixed in 1 % (w/v) OsO_4 in the same buffer at room temperature for 1 hour. The sample was then dehydrated using ethanol. Critical point drying was achieved using Hitachi HCP-2 critical point dryer. After coating using the Hitachi E-1010 ion sputter, a FEI Quanta 200 scanning electron microscope at 20 KV was used for viewing and imaging.

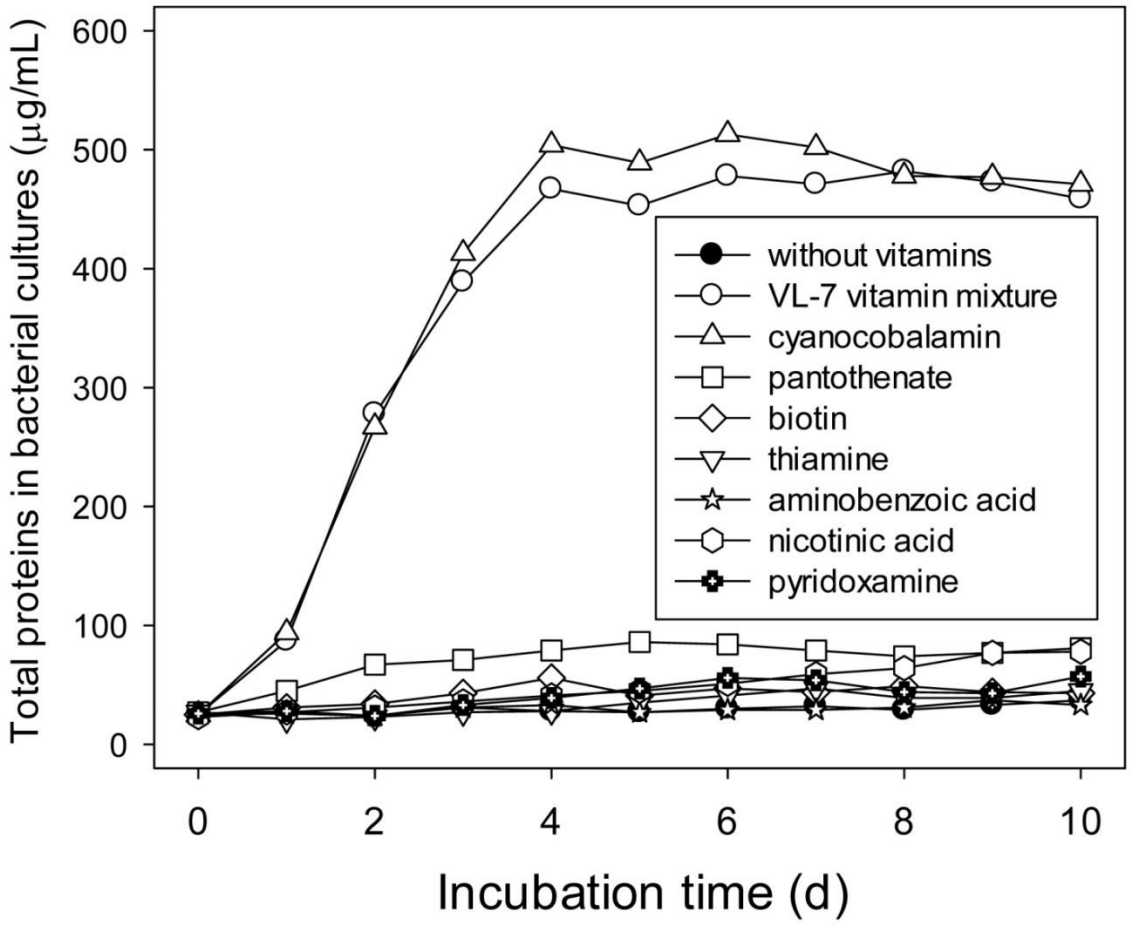

Fig. S3. Cobalamin as an essential vitamin during the anaerobic growth of strain DHT3 on estradiol. The fed-batch cultures (100 mL) of strain DHT3 were treated with or without vitamins. In all of the treatments, estradiol (3 mM) served as the sole carbon and energy source, whereas nitrate (initially 10 mM) served as terminal electron acceptor. Bacterial growth was measured as the total protein concentration in the cultures, and the data shown are averages (deviations <5%) of three experimental measurements.

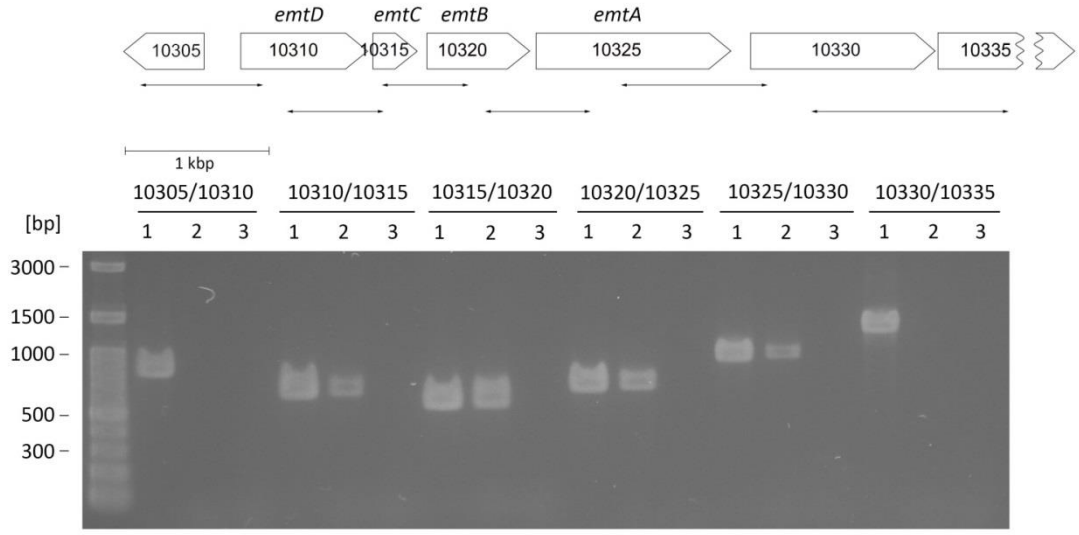

Fig. S4. Arrangement and expression analysis of the *emt* genes in strain DHT3. The *emt* operon of strain DHT3 comprises five genes (B9N43_10310~10330). For operon analysis, total RNA from strain DHT3 was extracted and the contaminating DNA was removed. cDNA was synthesized by reverse transcriptase using random hexamers as primers. To analyze the transcriptional unit, cDNA was used as template for PCR to bridge the intergenic regions of the *emt* genes. Each primer set was applied to 3 different PCR templates, including genomic DNA (lane 1), cDNA synthesized using random hexamers (lane 2), and total RNA (lane 3). Genomic DNA and total RNA were used as positive and negative controls, respectively. Oligonucleotides used in this study are listed in **Table S1** and their location is indicated by black arrows.

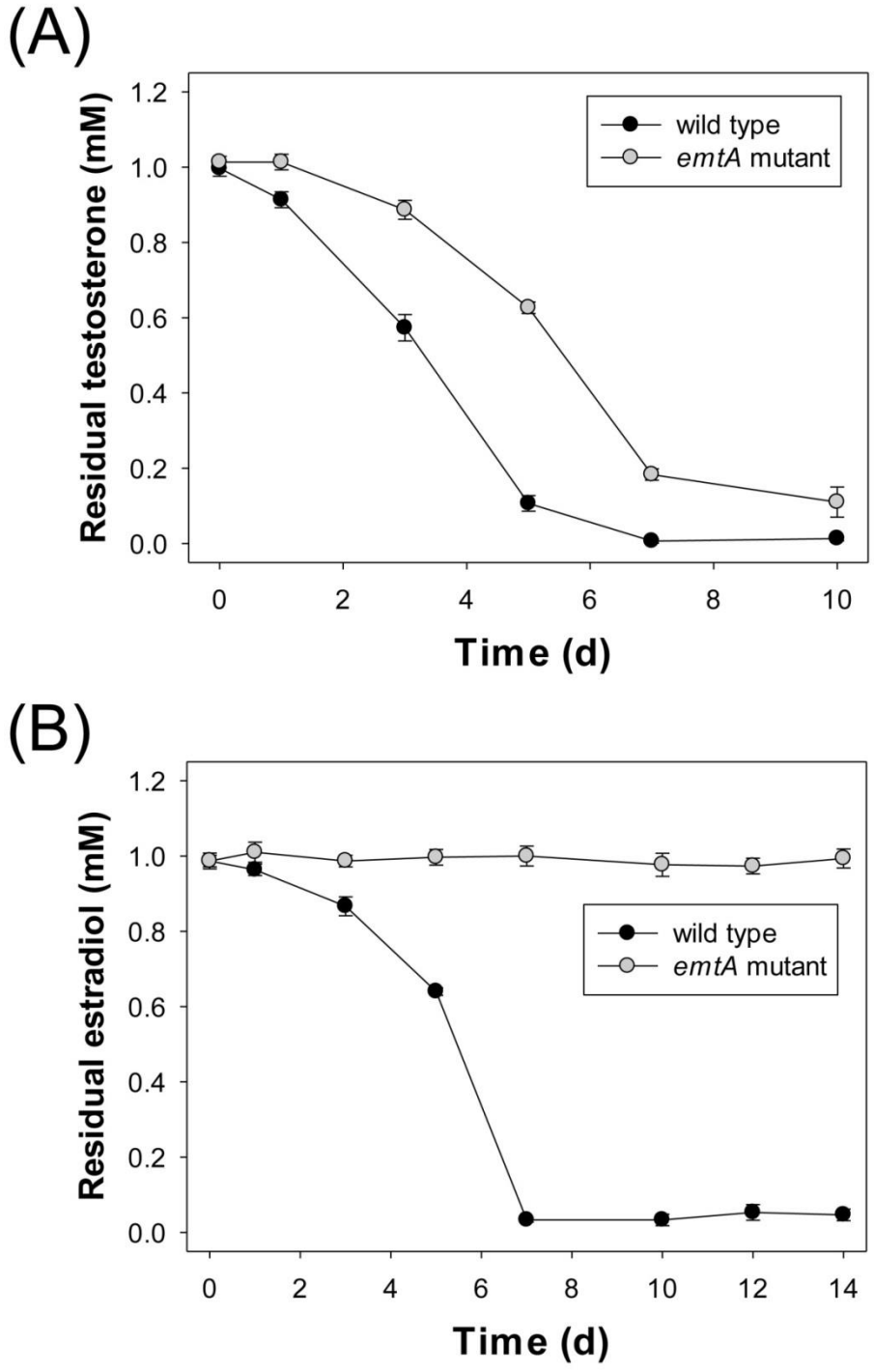

Fig. S5. The anaerobic growth of the wild type and the *emtA*-disrupted mutant of strain DHT3 with testosterone (A) and estradiol (B).

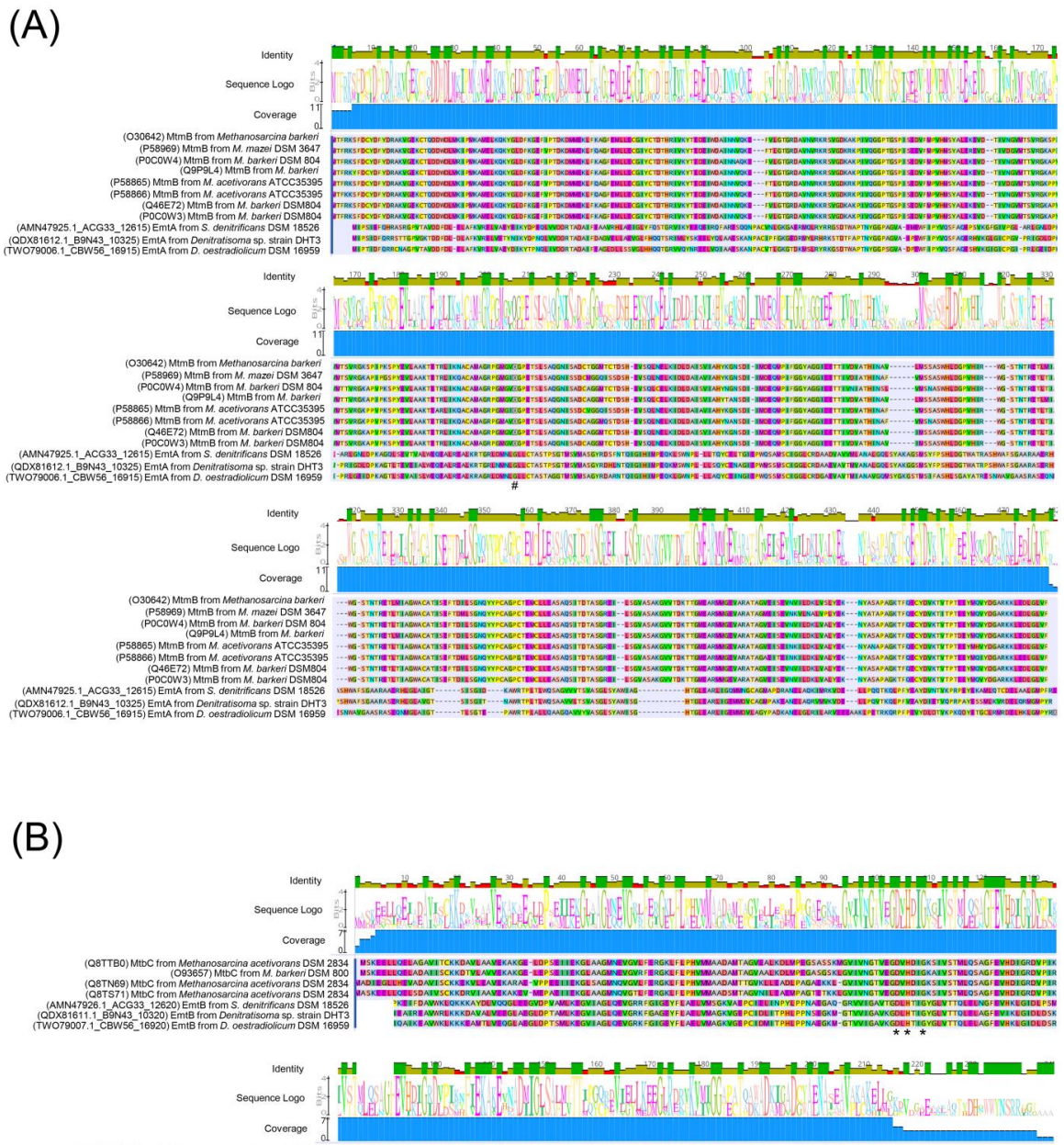

Fig. S6. Sequence alignments of EmtAB and their similar proteins via MUSCLE algorithm. (A) The sequence alignment of EmtA and MtmB. The pyrrolysine (1-letter abbreviation: O; marked with #) is identified in all the MtmB sequences but not in the EmtA sequences. These sequences are also used for the phylogenetic tree construction in **Fig. 4*C***. (B) The sequence alignment of EmtB and MtbC. The MtbC sequences are obtained from Uniport database using EmtB as a query. All MtbC used for this analysis are manually reviewed. The cobamide-binding motifs (D-x-H-x_2-G-$x_{41–42}$-S-x-L-$x_{24–28}$-G-G; the conserved amino acid residues are marked with *) are identified in all the MtbC and EmtB sequences.

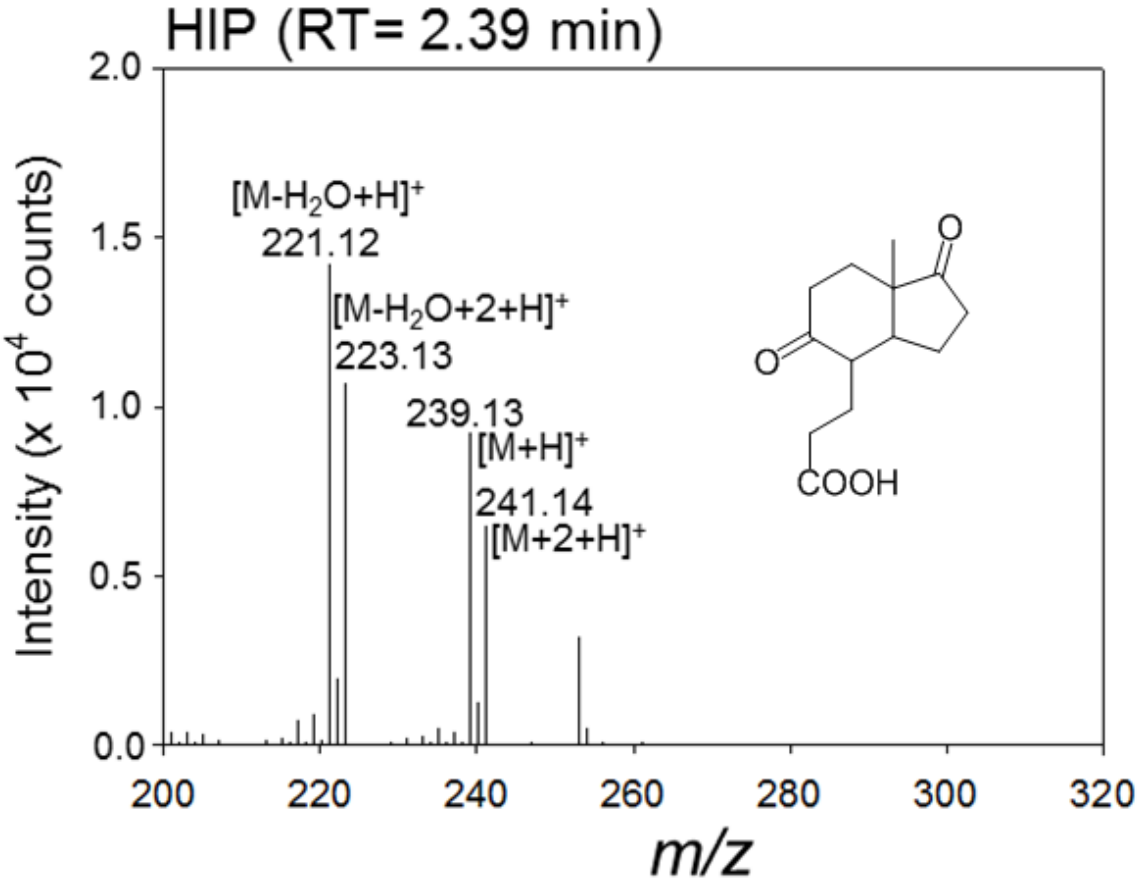

Fig. S7. APCI–HRMS spectrum of the HIP produced by the estrone-fed strain DHT3. The steroid substrate was composed of unlabeled estrone and [3,4C-^{13}C]estrone (mixed in a 1:1 molar ratio). The bacterial culture was extracted using ethyl acetate, and the metabolites were analyzed through UPLC–APCI–HRMS.

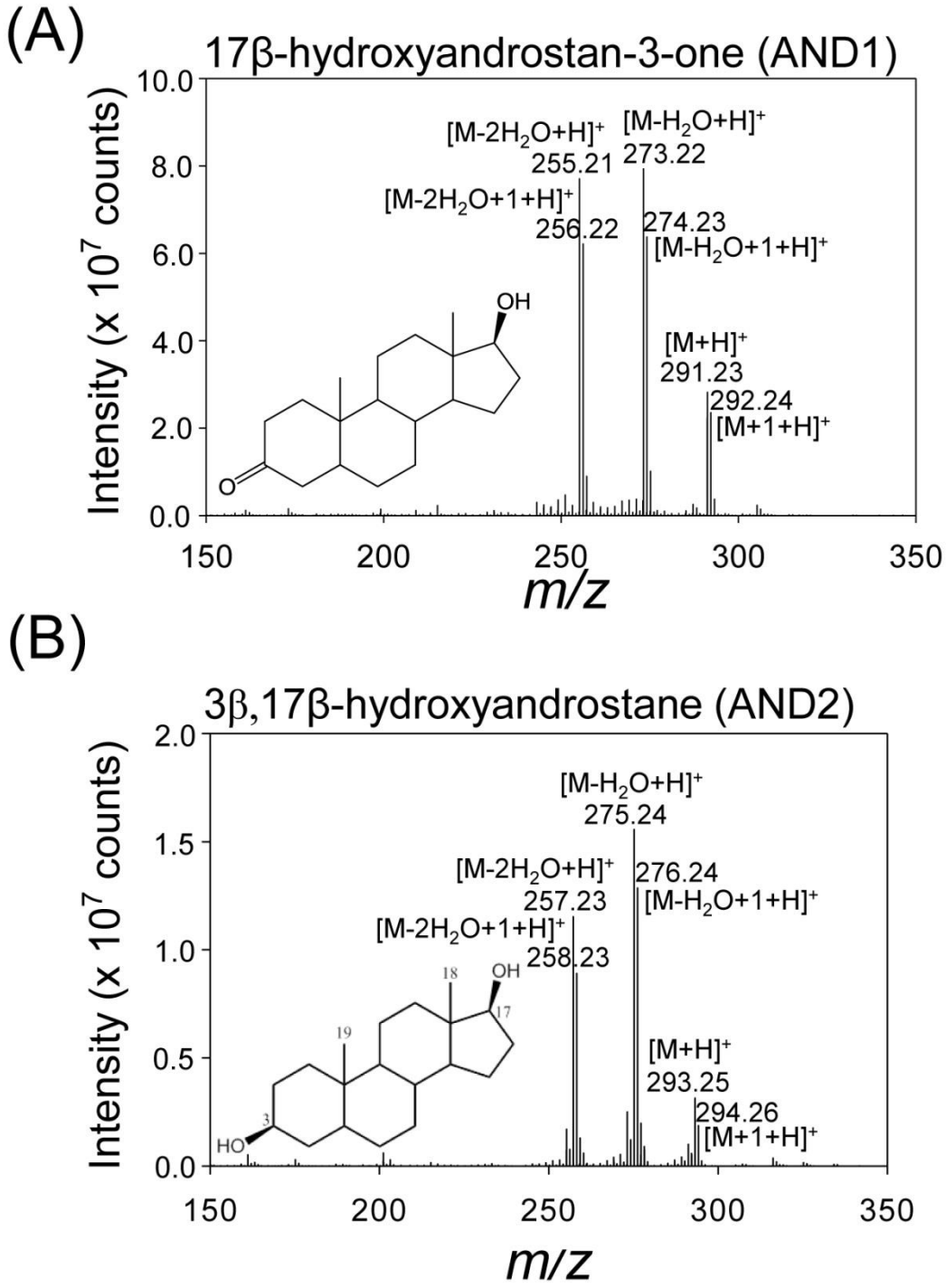

Fig. S8. UPLC–APCI–HRMS spectra of two TLC-purified androgen metabolites, 17β-hydroxyandrostan-3-one (A) and 3β,17β-dihydroxyandrostane (B). The androgens were produced by the strain DHT3 cell extract incubated with estradiol ([16,16,17-D3]17β-estradiol and unlabeled estradiol in a 1:1 molar ratio). See **Fig. 7*B1*** for the chemical composition of the reaction mixture. The predicted elemental composition of individual intermediates was calculated using Xcalibur™ Software (Thermo Fisher Scientific).

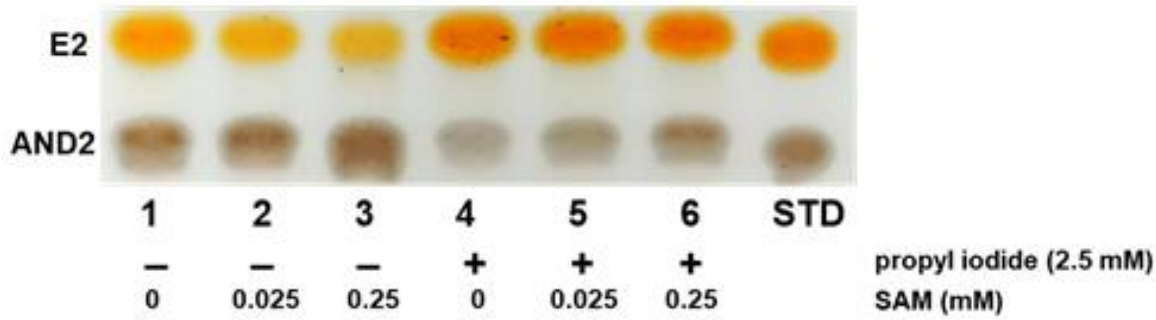

Fig. S9. SAM addition facilitates the estradiol methylation to form AND2 in the strain DHT3 cell-extracts. The androgen production exhibited a dose-dependent manner with SAM addition. The assay mixtures (1 mL) contained 100 mM HEPES-K^+ buffer (pH 8.0), strain DHT3 cell-extracts (5 mg), estradiol (0.25 mM), ATP (5 mM), $MgSO_4$ (10 mM), NADH (2 mM), SAM (0–0.25 mM), and with or without propyl iodide (2.5 mM). Abbreviations: E2, 17β-estradiol.

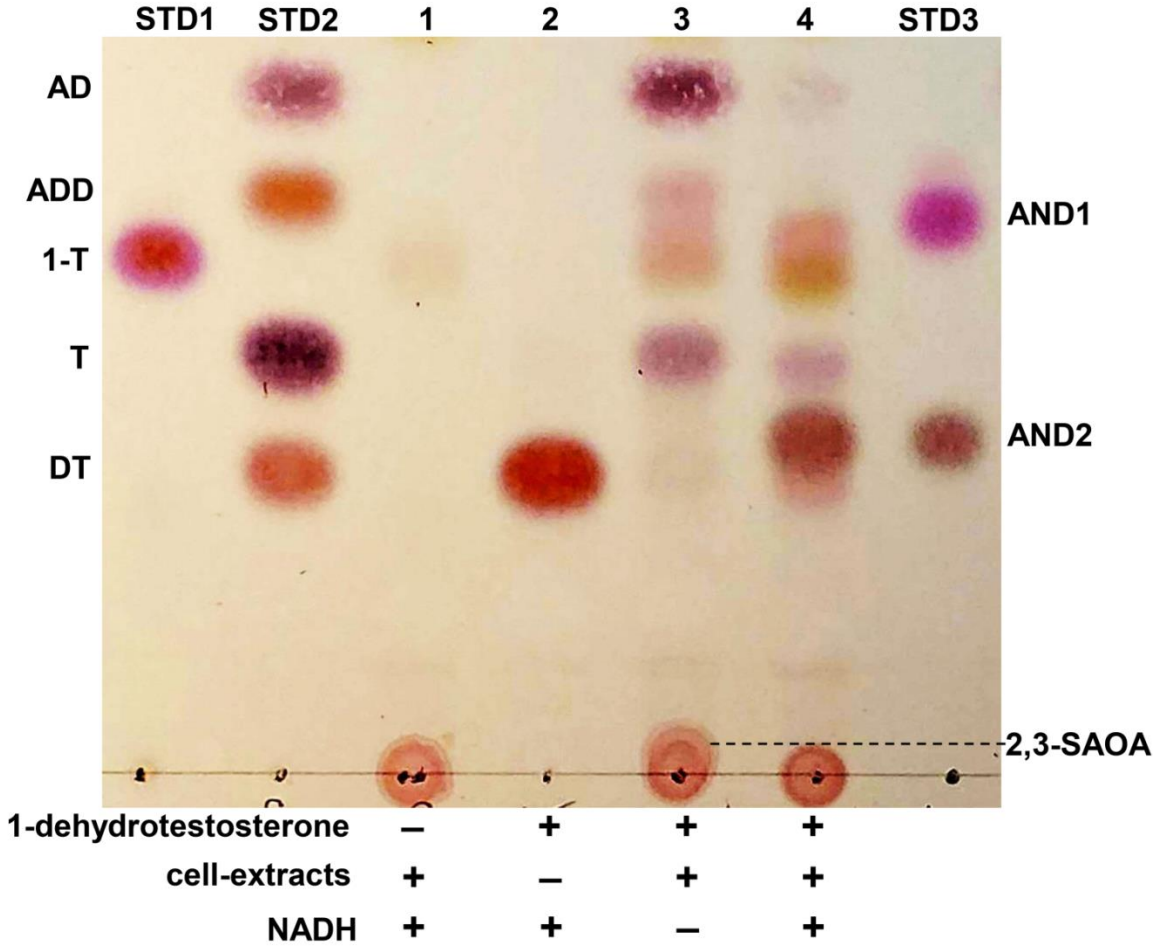

Fig. S10. TLC analysis indicated the addition of NADH (2 mM) to the strain DHT3 cell-extracts facilitates the transformation of 1-dehydrotestosterone (0.5 mM) into the reduced steroid metabolite AND2. The strain DHT3 cells were anaerobically grown with estradiol (2 mM). The identities of the AND2 and 2,3-SAOA on the TLC were validated through UPLC–HRMS. Abbreviations: AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; AND1, 17β-hydroxyandrostan-3-one; AND2, 3β,17β-dihydroxyandrostane; DT, 1-dehydrotestosterone; 2,3-SAOA, 17β-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid; STD, steroidal standards; T, testosterone; 1-T, 1-testosterone.

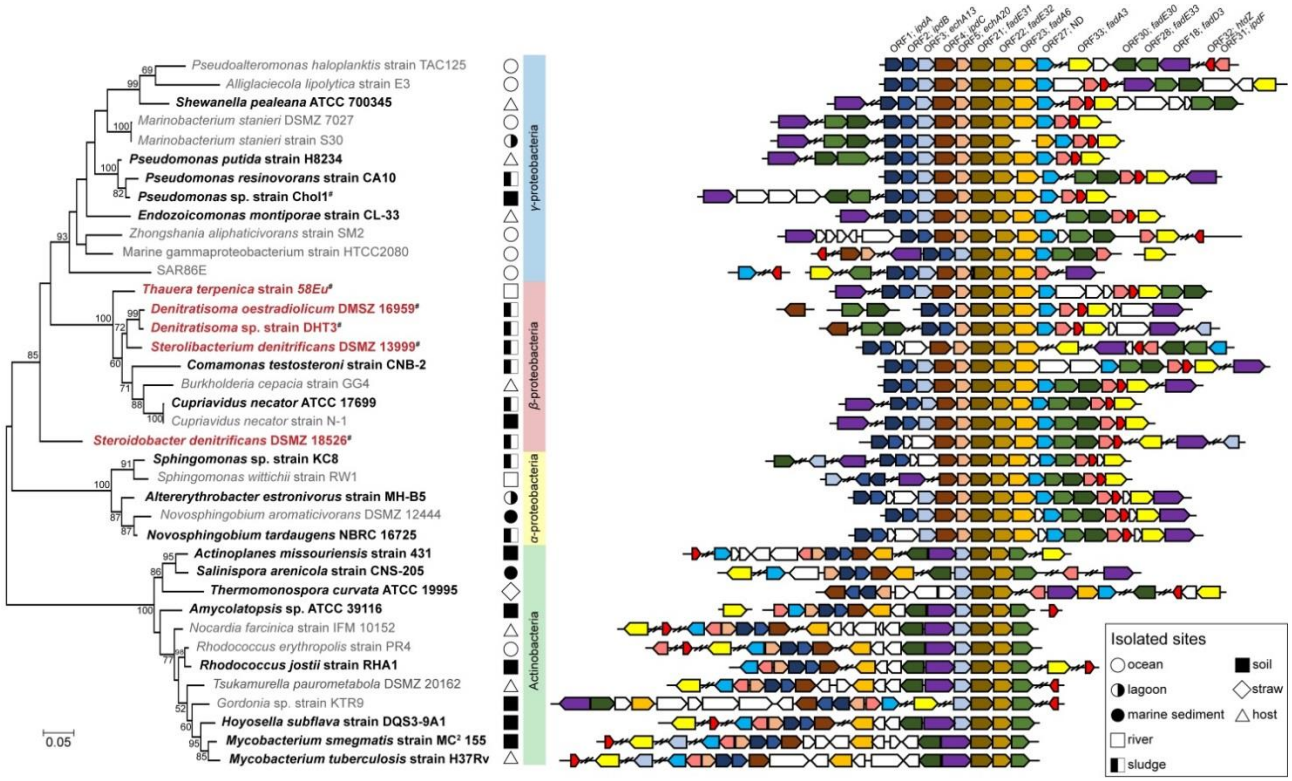

Fig. S11. Phylogenetic tree of the selected steroid-degrading bacteria and the distinguishable organization of HIP degradation genes in proteobacteria and actinobacteria (see **Dataset S2** for the detailed information of the selected bacterial strains). The phylogenetic trees were constructed using the neighbor-joining method with Jukes–Cantor parameter and a bootstrap value of 1000. Bacterial strains in bold: the steroid degradation capabilities have been experimentally verified. Bacterial strains in red: able to anaerobically degrade steroids.

Supplemental Tables

Table S1. Morphological, physiological, and cytological properties of strain DHT3. +, apparent growth; -, no apparent growth was observed within 1 month.

Characteristic	Phenotypes of strain DHT3
Cell morphology	curved rod
Motility	no
Growth on agar	no
DNA G+C content (mol%)	64.9
Temperature range for growth (°C)	10~32
Electron donors[a]	
Steroids	cholesterol (-),cholic acid (-), 17α-ethynylestradiol (-), estradiol (+), estrone (+), testosterone (+),
Alcohols and ketones	2-propanol (-), butanol (-), cyclohexanol (-), cyclopentanone (-), glycerol (+),
Aromatics	benzoate (-), toluene (-), phenol (-)
Organic acids	acetate (+),citrate (-), glutamate (-), formate (-), fumarate (+), hexanoate (+), laureate (-), oleate (-), pimelate (-)
Sugars	glucose (-), fructose (-), sucrose (-)
Extracts	yeast extract (-), peptone (-)
Electron acceptors[b]	ferric ion (-), nitrate (+), oxygen (-), perchlorate (-), sulfate (-)
Vitamins requirements[c]	*p*-aminobenzoic acid (-), biotin (-), calcium pantothenate (-), cyanocobalamin (+), nicotinic acid (-), pyridoxamine (-), thiamine (-)

[a]Substrates tested with nitrate as the electron acceptor.

[b]Electron acceptors tested with estradiol as the electron donor.

[c]Vitamins tested with estradiol and nitrate as the electron donor and acceptor, respectively.

Table S2. Oligonucleotides used in this study.

Primer	Sequence	Usage
10305_emtD_1F	AGACGTTCCTTGCCTAGTGC	Operon analysis: intergenic region B9N43_10305/*emtD*
10305_emtD_1R	GGAGGTTTTCCATCAGCCGA	
emtD_emtC_2F	GCCTATTTCAGCAATGGCCG	Operon analysis: intergenic region *emtD* /*emtC*
emtD_emtC_2R	ATCCTCTTCCCAGCTCTCGT	
emtC_emtB_3F	ACGAGAGCTGGGAAGAGGAT	Operon analysis: intergenic region *emtC*/*emtB*
emtC_emtB_3R	TTTGACTGCACCGATCACGA	
emtB_emtA_4F	AAAAAGCGGTCGAGCACAAC	Operon analysis: intergenic region *emtB* /*emtA*
emtB_emtA_4R	AGCAGGTCCACCGTAGTTTG	
emtA_emt10330_5F	AACCTGGGACTGCTATGCAC	Operon analysis: intergenic region *emtA*/ B9N43_10330
emtA_emt10330_5R	GAACCCGGGGACTTGCATAA	
10330_10335_6F	CGCCATTCAATACGGGCTTG	Operon analysis: intergenic region B9N43_10330/_10335
10330_10335_6R	TGTCCACCAGTTCGTCGAAG	
emtA_F	ATGATTCCAAGCATTGATTTCCAGC	*emtA*-specific primers (expected product size = 1.4 kb)
emtA_R	TTAGCGATAGGGCATGCC	
emtA_773|774s IBS	AAAAAAGCTTATAATTATCC TTACTGGACGAGTTCGTGCG CCCAGATAGGGTG	TargeTron Gene Knockout system: *emtA*-specific primer
emtA_773|774s EBS1d	CAGATTGTACAAATGTGGTG ATAACAGATAAGTCGAGTTC GATAACTTACCTTTCTTTGT	TargeTron Gene Knockout system: *emtA*-specific primer
emtA_773|774s EBS2	TGAACGCAAGTTTCTAATTTC GGTTTCCAGTCGATAGAGGAA AGTGTCT	TargeTron Gene Knockout system: *emtA*-specific primer
EBS Universal	CGAAATTAGAAACTTGCGTTCA GTAAAC	TargeTron Gene Knockout system: universal primer

Table S3. Selection of housekeeping genes of strain DHT3 used for constructing the linear regression line in the global gene expression profiles (RNA-Seq) in **Figure 3*B***. Strain DHT3 cells were anaerobically grown with estradiol or testosterone.

Locus tag	Gene name	Definition	Testosterone [log2(X+1)]	Estradiol [log2(X+1)]
B9N43_01505	*aceE*	pyruvate dehydrogenase (acetyl-transferring), homodimeric type	8.05	9.25
B9N43_03845	*gltA*	citrate (Si)-synthase	6.42	6.94
B9N43_03880	-	malate dehydrogenase	8.92	9.56
B9N43_04270	-	pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha	2.16	4.21
B9N43_07665	-	transcription termination factor Rho	8.27	10.62
B9N43_08640	-	DNA gyrase subunit A	8.96	9.81
B9N43_09345	*ftsZ*	cell division protein FtsZ	8.82	10.57
B9N43_09365	-	preprotein translocase subunit SecA	9.91	10.89
B9N43_11395	-	RNA polymerase sigma factor RpoD	11.09	10.58
B9N43_12040	*recA*	recombinase RecA	10.95	10.57
B9N43_12290	*gyrB*	DNA topoisomerase (ATP-hydrolyzing) subunit B	6.69	8.43
B9N43_12535	*glnA*	type I glutamate--ammonia ligase	7.27	9.35
B9N43_15425	*gap*	type I glyceraldehyde-3-phosphate dehydrogenase	9.22	10.05
B9N43_15595	-	isocitrate dehydrogenase (NADP(+))	9.03	9.69
B9N43_15770	*tuf*	elongation factor Tu	10.43	10.26
B9N43_15810	*rpoB*	DNA-directed RNA polymerase subunit beta	5.95	7.80
B9N43_15815	*rpoC*	DNA-directed RNA polymerase subunit beta'	7.88	9.60
B9N43_15830	*fusA*	elongation factor G	6.11	7.30
B9N43_16390	*gap*	type I glyceraldehyde-3-phosphate dehydrogenase	4.33	3.91

Table S4. Selection of the cobalamin-dependent methyltransferases used for the un-rooted maximum likelihood tree construction. The functions of listed proteins were manually reviewed (Swiss-Port) or experimentally characterized except AMN47925.1.

Enzyme	Subunit	Microorganism	Taxonomy (Class)	Accession No.
Estradiol methyltransferases from denitrifying proteotacteria				
estradiol methyltransferase		*Denitratisoma* sp. strain DHT3	Betaproteobacteria	This study
estradiol methyltransferase		*Denitratisoma oestradiolicum* DSM 16959	Betaproteobacteria	This study
estradiol methyltransferase		*Steroidobacter denitrificans* DSM 18526	Gammaproteobacteria	GeneBank AMN47925.1
Methyltetrahydrofolate:homocysteine methyltransferases (methionine synthases) from bacteria				
Methionine synthase (Group 2)	MetH	*Escherichia coli* K12	Gammaproteobacteria	UniPort P13009
Methionine synthase (Group 1)		*Mycobacterium tuberculosis* ATCC 25618	Actinobacteria	UniPort O33259
Methionine synthase (Group 2)		*Salmonella typhimurium* ATCC 700720	Gammaproteobacteria	UniPort P37586
Methionine synthase (Group 2)		*Vibrio cholerae* serotype O1 ATCC 39315	Gammaproteobacteria	UniPort Q9KUW9
Methionine synthase (Group 1)		*Mycobacterium leprae* TN	Actinobacteria	UniPort Q49775
Methionine synthase (Group 2)		*Pseudomonas aeruginosa* ATCC 15692	Gammaproteobacteria	UniPort Q9I2Q2
Methionine synthase (Group 1)		*Synechocystis* sp. PCC 6803	Cyanophyceae	UniPort Q55786
Methionine synthase (Group 2)		*Aliivibrio fischeri* ATCC 700601	Gammaproteobacteria	UniPort Q5E814
Methionine synthase (Group 2)		*Vibrio parahaemolyticus* serotype O3:K6 RIMD 2210633	Gammaproteobacteria	UniPort Q87L95
Methionine synthase (Group 2)		*Vibrio vulnificus* YJ016	Gammaproteobacteria	UniPort Q7MHB1
Methionine synthase (Group 2)		*Vibrio vulnificus* CMCP6	Gammaproteobacteria	UniPort Q7MHB1
Methionine synthase (Group 2)		*Aliivibrio fischeri*	Gammaproteobacteria	UniPort Q9AJQ8
Coenzyme M (CoM) methyltransferases from methanogenic archaea				
Monomethylamine:CoM methyltransferase	MtmB	*Methanosarcina barkeri*	Methanomicrobia	UniPort O30642
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort P58969
		Methanosarcina barkeri DSM 804	Methanomicrobia	UniPort P0C0W4
		Methanosarcina barkeri	Methanomicrobia	UniPort Q9P9L4
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort P58865
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort P58866
		Methanosarcina barkeri DSM 804	Methanomicrobia	UniPort Q46E72
		Methanosarcina barkeri DSM 804	Methanomicrobia	UniPort P0C0W3
Dimethylamine:CoM methyltransferase	MtbB	*Methanosarcina barkeri* DSM 804	Methanomicrobia	UniPort P0C0W5
		Methanosarcina barkeri DSM 804	Methanomicrobia	UniPort P0C0W6
		Methanosarcina barkeri	Methanomicrobia	UniPort O93661
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort P58971
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort P58970
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort P58972
		Methanosarcina barkeri	Methanomicrobia	UniPort Q9P9N0
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort Q8TS72
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort Q8TN68
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort Q8TTA5
		Methanosarcina barkeri	Methanomicrobia	UniPort Q9P9M9
Trimethylamine:CoM methyltransferase	MttB	*Methanosarcina barkeri*	Methanomicrobia	UniPort O93658
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort Q8TTA9
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort P58973
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort P58974
		Methanosarcina thermophila	Methanomicrobia	UniPort Q9P995
		Methanosarcina barkeri DSM 804	Methanomicrobia	UniPort P0C0W7
		Methanosarcina acetivorans ATCC 35395	Methanomicrobia	UniPort Q8TS73
		Methanococcoides burtonii DSM 6242	Methanomicrobia	UniPort Q12TR2
Methanol:CoM methyltransferase	MtaB	*Methanosarcina barkeri* DSM 804	Methanomicrobia	UniPort Q46EH3
Dimethylsulfide:CoM methyltransferase	MtsB	*Methanosarcina barkeri*	Methanomicrobia	UniPort Q48925
		Methanosarcina mazei DSM 3647	Methanomicrobia	UniPort Q8PUA7
Tetrahydrofolate (THF) methyltransferases from acetogenic or methylotrophic bacteria				
Veratrol:THF *O*-demethylase	OdmB	*Acetobacterium dehalogenans*	Clostridia	GeneBank AAC83696.2
Vanillate:THF *O*-methyltransferase	MtvB	*Moorella thermoacetica* ATCC 39073	Clostridia	GeneBank YP429263.1
glycine betaine:THF methyltransferase	MtgB	*Desulfitobacterium hafniense* Y51	Clostridia	UniPort Q24SP7
Acetyl-CoA synthase/CO dehydrogenase	AcsE	*Moorella thermoacetica*	Clostridia	UniPort Q46389
Chloromethane:THF methyltransferase	CmuA	*Methylobacterium extorquens* CM4	Alphaproteobacteria	GeneBank CAB39403.1

Table S5. UPLC–HRMS data of the intermediates involved in anaerobic estrone catabolism by strain DHT3. The steroid substrate was composed of unlabeled estrone and [3,4C-^{13}C]estrone (mixed in a 1:1 molar ratio). The bacterial culture was extracted using ethyl acetate, and the metabolites were analyzed through UPLC–APCI–HRMS. MS data shown are from the unlabeled metabolites.

Compound ID	UPLC behavior (RT[a], min)	Molecular formula (predicted molecular mass)[b]	Dominant ion peaks	Identification of product ions
testosterone	6.01	$C_{19}H_{28}O_2$ 288.21	289.21	$[M+H]^+$
androst-4-en-3,17-dione	5.74	$C_{19}H_{26}O_2$ 286.19	287.19	$[M+H]^+$
DT	5.57	$C_{19}H_{26}O_2$ 286.19	287.20	$[M+H]^+$
androsta-1,4-diene-3,17-dione	5.30	$C_{19}H_{24}O_2$ 284.18	285.18	$[M+H]^+$
1-testosterone	6.32	$C_{19}H_{28}O_2$ 288.21	289.21	$[M+H]^+$
17-hydroxy-androstan-1,3-dione	5.15	$C_{19}H_{28}O_3$ 304.21	287.20 305.21	$[M-H_2O+H]^+$ $[M+H]^+$
2,3-SAOA	5.08	$C_{19}H_{30}O_4$ 322.22	305.21 323.22	$[M-H_2O+H]^+$ $[M+H]^+$
HIP	2.39	$C_{13}H_{18}O_4$ 238.12	221.12 239.13	$[M-H_2O+H]^+$ $[M+H]^+$

[a]RT, retention time.

[b]The predicted molecular mass was calculated using the atom mass of ^{12}C (12.0000), ^{16}O (15.9949), and ^{1}H (1.0078).

Table S6. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz) spectral data of the HPLC-purified androgen metabolite (AND2**)** and the authentic standard 5α-androstan-3β,17β-diol (=3β,17β-dihydroxyandrostane) purchased from Steraloids, Inc.

Positions	AND2			5α-androstan-3β,17β-diol	
	^{1}H [a, b]	^{13}C		^{1}H [a, b]	^{13}C
3	3.51 (1H, m)	72.0		3.50 (1H, m)	72.0
17	3.55 (1H, t, *J* = 8.7)	82.7		3.55 (1H, t, *J* = 8.7)	82.7
18	0.85 (3H, s)	11.8		0.85 (3H, s)	11.8
19	0.72 (3H, s)	12.9		0.72 (3H, s)	12.9

[a] Measured in methanol-d_4.
[b] δ in ppm, mult. (*J* in Hz).

Legends for Datasets

Dataset S1 (separate file). Genome annotation of strain DHT3 and transcriptomic analysis (RNA-Seq) of bacterial cells grown anaerobically with testosterone or estradiol.

Dataset S2 (separate file). Selection of the bacteria used for comparative analysis of the gene organization for HIP degradation in **Fig. S11**.

Supplemental Appendix

Appendix S1. Nucleotide sequence of the 16S rRNA gene (B9N43_08010) of strain DHT3.

AGAGATTGAACTGAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGAATG
CTTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAG
TGGCGAACGGGTGAGTAATACATCGGAACGTGCCCAGTAGAGGGGGATAACC
GTCCGAAAGGATGGCTAATACCGCATACGCCCTGAGGGGGAAAGCGGGGGAC
CGCAAGGCCTCGCGTTATTGGAGCGGCCGATGTCGGATTAGCTAGTTGGTGGG
GTAAAGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATTTTGGACAATGGGGGCAACCCTGATCCAGCCATTCCGCGTGAGTGAAGAAG
GCCTTCGGGTTGTAAAGCTCTTTCGGCAGGAACGAAAAGGTGGTCACTAATAC
TGGCTACTGATGACGGTACCTGAAGAAGAAGCACCGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAA
GCGTGCGCAGGCGGTTTTGTAAGACAGCTGTGAAATCCCCGGGCTTAACCTGG
GAACTGCGGTTGTGACTGCAAGACTGGAGTGTGGCAGAGGGGGGTGGAATTC
CACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGG
CAGCCCCCTGGGTTAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCCAACTAGGTGTTGG
GGAAGGAGACTTCCTTAGTACCGTAGCTAACGCGTGAAGTTGGCCGCCTGGG
GAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAG
CGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTG
ACATGCCAGGAACTTTCCAGAGATGGATTGGTGCCCGAAAGGGAGCCTGGAC
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTTAGTTGGGCACTCTA
ATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTC
ATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGGT
TGCCAAGCCGCGAGGTGGAGCCAATCCCAGAAAGCCGATCGTAGTCCGGATT
GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAG
CATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTTCACCAGAAGTAGGTAGTCTAACCGCAAGGAGGGCGCTTAC
CACGGTGGGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGA
AGGTGCGGCTGGATCACCTCCTTTCTA

Appendix S2. Nucleotide sequence of the *emtA* gene (B9N43_10325) of strain DHT3.

ATGATTCCAAGCATTGATTTCCAGCGGCGCTCAACCACCGGACCTGTTGGCAA
GACCGACGACTTTGACCTGGATCTGGCCTTCAAGGTCAGGGAACTGGTGGAG
ACCTACAACATCAAGTACGACCCCAACCAACTTGTGGTGGATGACCGCACGG
CAGATGCAATTTTTGATGCGGGCGTCGAATTGCTGGCCGAGGTCGGCCTGTTT
CATCAGCAGACCTCGCGCATCATGCTGTACTCCAAGGAGGAACTCTATCAACT
GGCGGCCGAGTCCAAGGCGAACCCAGCCTGCATTCCCTTTGGAAAAGGCGAG
GACCGGATGTATCTGCGGCATCGCAAGAGTACAGACACCTTTGCCCCGACAAA
CTACGGTGGACCTGCTGGAGTCGCTGAACCGGAGTGGTTCATTCCCTATGTTC
AGTCCTTTGCCCAGGAGCGTCATGTAAAGGGCCTGGGGATTTGTCCGGGCGTA
CCGCGCATTGGCGATCTTGACCCGAAGGCCGGTACCCTGACCGAAGTGGAAAT
CGCCCTTTGGGAGCAGGAAGCCCTGCGTGAGGCGCTGAAGCGCACTGGCCGT
CTGAACATGAACCTGGGACTGCTATGCACCGCCAGCACCCCCTCGGGCACCAT
GTCGGTCATGGCCAGTGGGTATCGGGACCATCTGAATACCCAGATCGGCATTC
ACATCATGCCCGAACAGAAAATGAGCTGGAATCCGCTGCTGCTTTCCCAGTAC
TGCGAGAACGCCGGGATCGAGCCCTGGATGAGTTCGATGTCCTGCATCGGCGG
TTTGTGTCGGGATGCCGCCGAGGTCGCAGTAACAATGGTGGCTAACGCGTTGG
GGCAACTCAGCTATGCCAAGGGCGGCTCCATGAGCTACTTCCCCAGCCATCTG
GATGGGACATGGGCAACGCGACCTTCTCATTGGGCATTCAGTGGCGCAGCCCG
TGCTTCTGAACGTCACCTTGGACTGGCTGTGGGAACCTCTATCTCGGGCATCA
CCAATGCCTGGCGCACTCCCTTGACCCTGTGGCAGTCGGCGGCGGTCGTGTTG
ACCTCCGTGGCCAGCGGATTGTCCTATGCCTGGATTTCCGGACATACCGGCCTG
GAGGCGCGACTGATCGGAGAAATGATGGATGTTTGCGCTGGCATGCCGGCAA
AGGAAGCCAATGAACTGGCCCAGCGGGTTATGGTCAAGGTTGATGAGCTTCTG
CCTCAGGTAACGAAGCAGTTGCCCTTCGTCGAAGCTTATGACATCGAAACCGT
TCAGCCCCGTCCGGCATACGAGTCTTCCATGCTGAAGGTGCGGGATGAACTGC
AGCGTATGGGCATGCCCTATCGCTAA

Appendix S3. Nucleotide sequence of the *emtB* gene (B9N43_10320) of strain DHT3.

ATGAGCAGCATTGAGGCAATCCGTGAGGCCGTCTGGCGCCTGAAGAAAAAGG
ATGCCGTTGCCCTGGTGGAAGAAGGCCTTGCAGAAGGGCTTGATCCTACTGCG
ATGTTGAAGGAAGGGGTCATTGCCGGTTTGCAGGAGGTCGGGCGCAAGTTTG
GTGCCGGCGAGTATTTTCTGGCAGAACTGGTGATGGCTGGCAAGGTCGGTGA
GCCCTGTATCGACTTGATCACGCCTCACTTACCGCCGAATTCGGAAGGGAAGA
TGGGAACGGTCGTGATCGGTGCAGTCAAAGGCGATCTGCACACCATTGGCTAC
GGGCTGGTGACAACCCAACTGGAGTTGGCGGGATTCGAAGTCATCAAGCTGG
GCATCGACCTGGACAGCAAGTACTTCATCGAAAAAGCGGTCGAGCACAACGC
CGACATCATCGGCCTGTCCGCCTTCCTGGTGACGACCATTCCCTATTGCCCAGA
AGTCTTGGGTTATTTGAAGGACATGGGGCTGCGGGATCGCTTCAAGGTCATCA
TCGGCGGCACGGAATGTACCGCCGACAAGGCGGACGCCATGGATGCCGATGG
CTGGGCACTCAATGCAATCTCAGCGGTGCCACTGTGCAAGCGCCTGATGGGCA
AGGACGTGGGTGAGGAGGCCAAGCTCGCGCAAACCTACGACCACGGCTGGT
GGTACAACTCCCGCCGCCATGGCACCTGA

Appendix S4. Nucleotide sequence of the *emtC* gene (B9N43_10315) of strain DHT3.

ATGAAGCTCGAAGAATTAAAAACGCAGTTCCCGGATCTGACATTTGAGGAGGT
CGGTCCTGACGAGAGCTGGGAAGAGGATGGCGCCGGATATTGCTTCGTTATAG
AGGCGATGATCAAGGACAAGCGTGTTCGCGCTACCGTCATGGTGGATGACTTG
CCGCGGGTGGCCTTCACGGGTGACTCTGTGGGCATCCGCAAGATGCTGGTGA
GGTGGGCAGTGGGGCGGGATCACTTGGCATATCTGGCCTATGAGCTGGGCCGG
GCCGAGATGGCGATCCGTCACGGCGTCCCCTTCCTTCAGGAGTGA

Appendix S5. Nucleotide sequence of the *emtD* gene (B9N43_10310) of strain DHT3.

ATGTTCATGCAGTTCTGGCAATCACTGTTTCTGACGGAAGGGGACCAGATACT
TGATGTGACCAAGATATGCGATGGTCTTGGTTTCCACGGGATGCTGTTTCCCGA
TCATCTGATCCACCCCGAGAAGCAGGACTCCACCTACCTGTACTCGGCTGATG
GAAAACCTCCATCATTTACGGAGGACACAGTATGGCCTGAGTGTTGGTCGCTA
TTTGCGACGTTGGCGGCTATGACCAAGAATCTGCATTTCTGCACATGCGTTTTC
ATTCTTCCCTTACGCAATCCGATTGAACTGGCCAAGGCAACGTCAAGCGTTGC
CTATTTCAGCAATGGCCGTATCCATCTGGGCGCCGGGGCCGGCTGGATGAAGG
AAGAGTTCGAGATGCTCGGCGTGGATTGGGCCACGCGCGGAAAGCGATATGA
CGAGTGTATCGAGGTGATGCGCAAGCTCTATACGGGGCAGTACGTCGAACACC
ACGGCGAATTCTTCGATTTCCCACGCATCATGATGACGCCGGTTCCAGAGAAA
CCCGTGCCCATCCTGATCGGCGGCATCAGCGGCCCCGCCTTGCGTCGAGCTGC
CCGTATTGGCGATGGTTGGATCGGGCCGGGACAGAGTGTCGATGCGGCCCTGC
AAACCCTGAGTACCCTGAATAAGTTGCGGACCGAGTACGGAACGCAGAACAA
GGAATTCAACAACATCGTTCCTATCTACGGGGATGTCAGCATCGACGACATCA
AGCGACTGGAGGACGCTGGGGCTACAGGGATGGTCAGCTTGCCCTTTGCTTTC
ACAATCAAACCAGGAACGACCTTGGAGGAAAAGCGTGCCTATCTGGAGCGCT
ATTCCCAGGAAGTCATTGCGAAATTCAGGTGA

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