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

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Steroid estrogens modulate physiology and development of vertebrates. Conversion of C_{19} androgens into C_{18} estrogens is thought to be an irreversible reaction. Here, we report a denitrifying *Denitratisoma* sp. strain DHT3 capable of catabolizing estrogens or androgens anaerobically. Strain DHT3 genome contains a polycistronic gene cluster, *emtABCD*, differentially transcribed under estrogen-fed conditions and predicted to encode a cobalamin-dependent methyltransferase system conserved among estrogen-utilizing anaerobes; an *emtA*-disrupted DHT3 derivative could catabolize androgens but not estrogens. These data, along with the observed androgen production in estrogen-fed strain DHT3 cultures, suggested the occurrence of a cobalamin-dependent estrogen methylation to form androgens. Consistently, the estrogen conversion into androgens in strain DHT3 cell extracts requires methylcobalamin and is inhibited by propyl iodide, a specific inhibitor of cobalamin-dependent enzymes. The identification of the cobalamin-dependent estrogen methylation thus represents an unprecedented metabolic link between cobalamin and steroid metabolism and suggests that retroconversion of estrogens into androgens occurs in the biosphere.

estrogens | biocatalysis | cobalamin-dependent methyltransferase | microbial metabolism | steroids

Sex steroids, namely androgens and estrogens, modulate physiology, development, and reproduction of animals (1–4). While required by animals, estrogens are also classified as group 1 carcinogens by the World Health Organization (https://monographs.iarc.fr/list-of-classifications/) and are often detected in surface waters of industrialized countries (5, 6). Long-term exposure to estrogen-contaminated water at nanomolar levels can disrupt the endocrine system and sexual development in animals (7, 8).

Biosynthesis of C_{18} estrogens from C_{19} androgens proceeds through the removal of the C-19 angular methyl group, resulting in the formation of an aromatic A-ring (9). This aromatization proceeds through 2 consecutive hydroxylations of the C-19 methyl group and subsequent oxidative bond cleavage between steroidal C-10 and C-19, which is catalyzed by an aromatase (namely P450arom or CYP19) at the cost of 3 NADPH and 3 O_2 (*SI Appendix*, Fig. S1) (10). The reverse reaction (from estrogens to androgens) is thermodynamically challenging and has not been reported in any organisms.

Sex steroids are exclusively de novo synthesized by eukaryotes. However, bacteria are major consumers of steroids in the biosphere (11). Interestingly, recent studies suggested that sex steroids mediate bidirectional interactions between bacteria and their eukaryotic hosts (12, 13). Meanwhile, bacteria can also alter a host's sex steroid profile (14). For example, intestinal *Clostridium scindens* is capable of converting glucocorticoids into androgens (15); *Comamonas testosteroni*, an opportunistic human pathogen, is capable of using a host's androgens as the sole carbon source and electron donor (16). Furthermore, an earlier study of fecal microbiome suggested that the phylogenetic profile of gut microbiota likely affects endogenous estrogen metabolism in postmenopausal women (17).

Biochemical mechanisms involved in bacterial androgen catabolism have been studied extensively, which includes an O_2-dependent 9,10-*seco* pathway and an O_2-independent 2,3-*seco* pathway (Fig. 1) (18–22). In contrast, current knowledge of the mechanisms involved in estrogen catabolism is very limited. The low aqueous solubility of estrogens (~1.5 mg/L at room temperature) (23) and the stable aromatic A-ring render estrogen a difficult substrate. Therefore, aerobic bacteria employ O_2 as a cosubstrate of oxygenases to activate and to cleave the aromatic A-ring through the 4,5-*seco* pathway (Fig. 1) (24–26). In general, microorganisms degrade estrogens slowly under oxygen-limited or -fluctuating conditions (27). Thus, anaerobic environments, such as river sediments and marine sediments, are considered as the major reservoirs for estrogens (28). To date, only *Denitratisoma oestradiolicum* and *Steroidobacter denitrificans* have been reported to utilize estrogens under anaerobic conditions (29,

Significance

Steroids are mainly produced by animals, while bacteria are major steroid consumers in the biosphere. Anaerobic environments are major reservoirs for estrogens; however, prior to this study, biochemical mechanisms involved in anaerobic estrogen catabolism remained completely unknown. Here, we characterized anaerobic estrogen catabolic pathway in denitrifying *Denitratisoma* sp. strain DHT3, which includes the transformation of estrogens into androgens via a cobalamin-dependent methylation. Data presented here complete the central pathways for bacterial steroid catabolism and reveal an unprecedented role of cobalamin in microbial steroid metabolism. Sex steroids are involved in bidirectional metabolic interactions between bacteria and their eukaryotic hosts; the discovery of retroconversion of estrogens into androgens in bacteria portends unexplored microbe–host metabolic interdependencies via this cobalamin-dependent estrogen methylation reaction.

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The authors declare no competing interest.

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Data deposition: The genome sequence of the strain DHT3 has been deposited in the National Center for Biotechnology Information (NCBI) Genome database (accession no. CP020914). The transcriptomes of the strain DHT3 have been deposited in the NCBI database (accession nos. SRR10362955 and SRR10362956).

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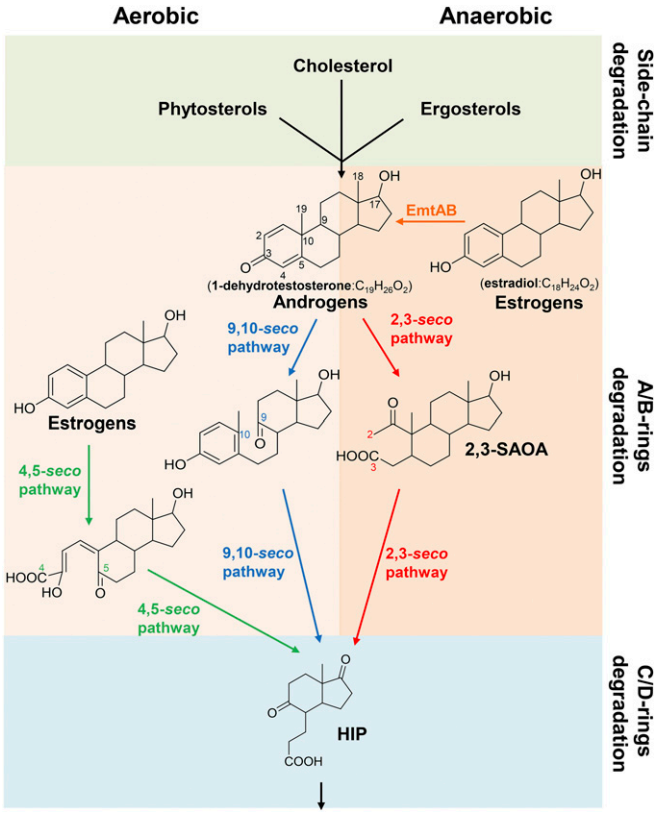

Fig. 1. Central pathways for bacterial steroid catabolism. Bacteria adopt a convergent pathway (the 2,3-*seco* pathway) to catabolize different steroids under anaerobic conditions and adopt divergent pathways to catabolize estrogens (the 4,5-*seco* pathway) and other steroids (sterols and androgens; the 9,10-*seco* pathway) under aerobic conditions. All of the 3 steroid catabolic pathways converge at HIP. 2,3-SAOA, 17β-hydroxy-1-oxo-2,3-*seco*-androstan-3-oic acid (2,3-SAOA); HIP, 3aα-H-4α(3′-propanoate)-7aβ-methylhexahydro-1,5-indanedione.

30). However, the biochemical mechanisms and catabolic genes involved in the anaerobic estrogen catabolism remain completely unknown.

In this study, we enriched an estrogen-degrading denitrifying bacterium *Denitratisoma* sp. strain DHT3 from a municipal wastewater treatment plant, which exhibits high efficiency in estrogen degradation under denitrifying conditions. We first characterized strain DHT3 and annotated its circular genome. Subsequently, we performed comparative transcriptomic analysis to identify the genes potentially involved in the anaerobic estrogen catabolism. The results along with bridging PCR analysis revealed a polycistronic gene cluster *emtABCD* (*emt*: estradiol methylation) that is differentially expressed in the strain DHT3 transcriptome under estrogen-fed conditions. Bioinformatic analysis predicted that the *emtABCD* gene cluster encodes a putative cobalamin-dependent methyltransferase, which is also present in *D. oestradiolicum* and *S. denitrificans* but not in other steroid-degrading anaerobes incapable of utilizing estrogens. Moreover, the *emtA*-disrupted strain DHT3, although incapable of growing on estrogens, is capable of growing on androgens. Finally, the ^{13}C metabolite profile revealed estrogen consumption followed by androgen production in estradiol-fed strain DHT3 cultures. These data indicate the involvement of a cobalamin-mediated conversion of estrogens into androgens in strain DHT3.

Results and Discussion

Enrichment and Characterization of *Denitratisoma* sp. Strain DHT3. The estrogen-degrading mixed culture was enriched from a denitrifying sludge that was collected from the Dihua Sewage Treatment Plant (Taipei, Taiwan). The estrogen-degrading denitrifier was highly enriched by repeating 10^{-8} dilution transfers in a chemically defined mineral medium containing estradiol as the sole substrate and nitrate as the terminal electron acceptor until a microscopically pure culture (vibrio-shaped cells) was obtained (*SI Appendix*, Fig. S2). Growth of the estradiol-degrading denitrifier on solid media (agar or gelrite plate) was not observed. The circular genome of strain DHT3 (3.66 Mb; 64.9% G + C; accession CP020914) has been sequenced and annotated (31). The phylogenetic analysis showed that the estradiol-degrading denitrifier shares a 97.5% 16S rRNA gene similarity with *D. oestradiolicum* DSM 16959, suggesting that it belongs to the genus *Denitratisoma* (31). Therefore, the estradiol-degrading denitrifier is named as *Denitratisoma* sp. strain DHT3 in this study.

Stoichiometric analysis suggested that estradiol was mineralized to CO_2 during the denitrifying growth of strain DHT3 (Fig. 2*A*). Strain DHT3's growth (measured based on the increasing protein concentration in culture over time) was in parallel to the consumption of estradiol (electron donor) and nitrate (electron acceptor) in culture (1 L). After 72 h of incubation, ∼0.5 g estradiol (∼1.8 mmol) and 1.3 g of nitrate (∼21 mmol) were consumed, and ∼380 mg dry cell mass was produced in the 1-L bacterial culture. The complete oxidation of estradiol with nitrate follows the dissimilation equation (30): $C_{18}H_{24}O_2 + 23NO_3^- + 23H^+ \rightarrow 18CO_2 + 11.5N_2 + 23.5H_2O$. Hence, based on nitrate consumption, ∼0.25 g of estradiol (∼0.9 mmol) was completely oxidized to CO_2, leaving ∼0.25 g estradiol (∼0.9 mmol) of estradiol to be assimilated into biomass. The amount of assimilated carbon from 0.9 mmol estradiol corresponds to ∼195 mg carbon. Assuming that carbon constitutes 50% of dry cell mass (32), the calculated dry cell mass produced from estradiol should be ∼390 mg. This value is close to the observed cell yield (380 mg).

Next, we characterized the substrate spectrum (*SI Appendix*, Table S1) and vitamin requirements of strain DHT3. Strain DHT3 is able to utilize estradiol, estrone, testosterone, acetate, fumarate, glycerol, and hexanoate as the sole substrate, but was not able to utilize other steroids, including synthetic estrogen 17α-ethynylestradiol, cholic acid, or cholesterol. The doubling time of strain DHT3 when it anaerobically grows on estrogens and testosterone ranged from 10 to 14 h and 8 to 10 h, respectively. No denitrifying growth with the following substrates was observed: yeast extract, peptone, formate, laureate, oleate, pimelate, 2-propanol, butanol, cyclohexanol, cyclopentanone, citrate, glutamate, glucose, fructose, sucrose, benzoate, toluene, or phenol. Moreover, strain DHT3 cannot utilize Fe^{3+}, sulfate, O_2, or perchlorate as the alternative electron acceptor to degrade estradiol. The vitamin requirements revealed by the growth experiments are consistent with the presence or absence of a complete set of biosynthetic genes for the given vitamin as annotated in the genome (31). Only cobalamin (namely cyanocobalamin; 20 μg/L) is required for strain DHT3's growth (Fig. 2*B*). Addition of other vitamins did not facilitate the growth of strain DHT3 on estradiol (*SI Appendix*, Fig. S3), suggesting that strain DHT3 is cobalamin auxotrophic.

The genomes of 3 characterized anaerobic estrogen utilizers do not possess a complete set of biosynthetic genes to produce cobalamin for estrogen utilization (31). Interestingly, a previous study on microbial cholesterol catabolism also observed common cobalamin auxotrophy among cholesterol-utilizing anaerobes (33). These findings suggest that 1) the occurrence of interspecies cobalamin transfer between the cobalamin producers and the anaerobic steroid utilizers in natural habitats and 2) estrogens and other steroids are prone to accumulating in O_2-limited ecosystems devoid of cobalamin or cobalamin-producing microbes. Therefore, cobalamin or cobalamin-producing anaerobes likely can be augmented into O_2-limited ecosystems contaminated by sex steroids to boost in situ bioremediation.

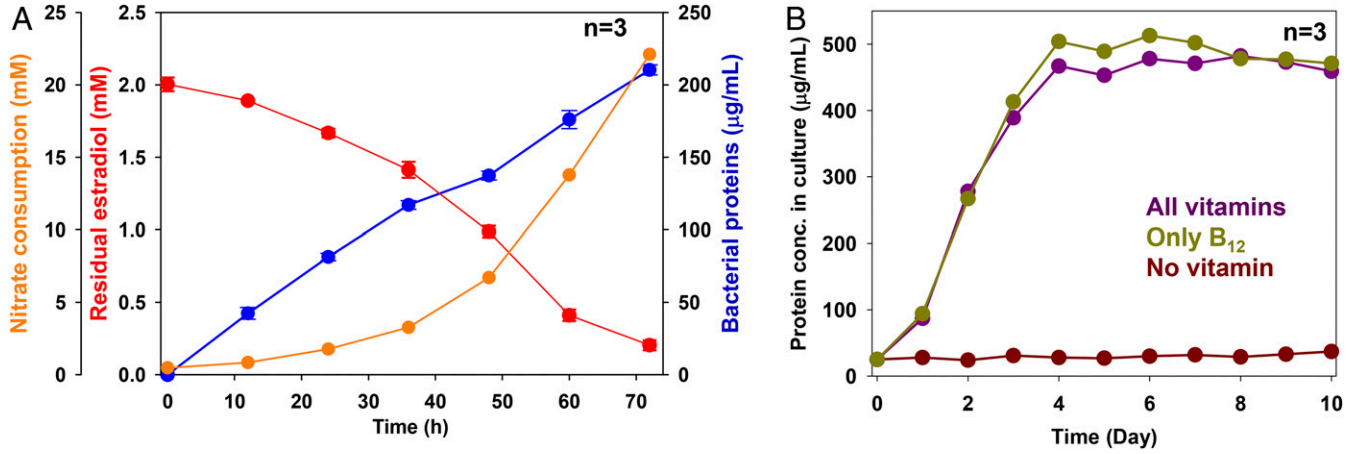

Fig. 2. Anaerobic growth of *Denitratisoma* sp. strain DHT3 with estradiol under denitrifying conditions and under different vitamin-supplementing conditions. (*A*) Anaerobic growth of strain DHT3 using estradiol as the sole substrate under denitrifying conditions. (*B*) Anaerobic growth of strain DHT3 on estradiol in the medium supplemented with different vitamins or without vitamins. Bacterial growth was measured based on the increasing total protein concentrations in the cultures. Results are representative of 3 individual experiments. Data shown are means ± SEM of 3 technical replicates.

Identification of Estrogen Catabolic Genes in Strain DHT3. We first performed the comparative genomic analysis of the strain DHT3 genome to identify genes potentially involved in the anaerobic estrogen catabolism (Dataset S1). Consistent with the observed phenotype, the strain DHT3 genome contains a complete set of androgen catabolic genes in the established 2,3-*seco* pathway, including the genes involved in steroidal A/B-ring degradation (B9N43_01910 to 1920) and C/D-ring degradation (B9N43_4420 to 4465) (Fig. 3*A*). Moreover, the strain DHT3 genome lacks most genes for anaerobic cobalamin biosynthesis (34, 35), while it possesses the genes for cobalamin transport (B9N43_10265 to 10280) and utilization (B9N43_10425 to 10440) (Fig. 3*A*).

Subsequently, we performed a comparative transcriptomic analysis to detect the genes up-regulated in the estradiol-fed cultures but not in the testosterone-fed cultures. Our data suggested that genes involved in the 2,3-*seco* pathway are expressed at similar levels (<4-fold difference) in both estradiol-fed and testosterone-fed cultures (Fig. 3*B*). In contrast, the genes involved in transport, salvage, and reductive activation of cobalamin are differentially up-regulated (>5-fold difference) in the estradiol-fed cultures (Fig. 3*B* and Dataset S1). Among them, B9N43_10285 and _10290 encode a putative methyltransferase-activating protein (36) and a RamA-like ferredoxin (37), respectively. Additionally, a gene cluster putatively encoding a methyltransferase system (B9N43_10310 to 10325; denoted as *emtABCD*) was up-regulated (>5-fold difference) in the estradiol-fed culture. Notably, the *emtABCD* gene cluster is also present in estrogen-degrading anaerobes *D. oestradiolicum* and *S. denitrificans* but not in other steroid-degrading bacteria that cannot utilize estrogens (Fig. 3*A*).

We then characterized whether the mRNA products of the *emtABCD* cluster are polycistronically transcribed. Bridging PCR reactions were performed using primers spanning the intergenic regions of these genes (see *SI Appendix*, Table S2 for individual sequences). The results suggested that B9N43_10310 to 10330 are transcribed polycistronically, including *emtABCD*, and B9N43_10330 that encodes a putative serine hydroxymethyltransferase (*SI Appendix*, Fig. S4). However, the B9N43_10330-coding protein is less likely a necessary component of the putative cobalamin-dependent methyltransferase since 1) the B9N43_10330 homolog is not cooperonic with the *emtABCD* gene cluster in the genome of estrogen-utilizing *S. denitrificans* (Fig. 3*A*) and 2) the expression of B9N43_10330 in the transcriptome of the estradiol-fed culture is significantly lower than that of *emtABCD* (Fig. 3*B* and Dataset S1).

Functional Validation and Phylogenetic Analysis of *emtA*. Our functional genomic analysis suggested that the polycistronic *emtABCD* is likely involved in the anaerobic estrogen catabolism. Thus, we disrupted the *emtA* gene in strain DHT3 using the TargeTron Gene Knockout System (with a group II intron and the kanamycin-resistant gene inserted) to validate the function of EmtABCD in the anaerobic estrogen catabolism in strain DHT3. We selected *emtA* for the gene disruption experiment since it is annotated as the catalytic subunit of EmtABCD. The *emtA*-disrupted mutant was isolated via 2 successive 10^{-8} dilution transfers in a defined mineral medium with testosterone as the sole substrate and kanamycin (20 μg/mL). PCR with primers flanking the *emtA* gene confirmed successful intragenic insertion of the group II intron into *emtA* in the mutant strain (Fig. 4*A*). The *emtA*-disrupted strain DHT3 can only utilize testosterone but not estradiol (Fig. 4*B* and *SI Appendix*, Fig. S5), revealing that *emtA* is involved in the anaerobic estrogen catabolism in strain DHT3.

Subsequently, we elucidated the phylogenetic relationship of EmtA and other cobamide-dependent methyltransferases (*SI Appendix*, Table S4). Based on sequence homology, the most EmtA-similar protein in other organisms is MtmB (identity of protein sequence ~30%), a catalytic subunit of the monomethylamine methyltransferase in methanogenic archaea (38). Moreover, pyrrolysine codons, a hallmark of archaeal methylamine:cobamide methyltransferases (39), are absent in *emtA* (*SI Appendix*, Fig. S6*A*). The phylogenetic tree showed that EmtA orthologs from the 3 estrogen-degrading anaerobes form a distinct lineage (Fig. 4*C*), separated from other experimentally characterized cobamide-dependent methyltransferases in prokaryotes. These EmtA orthologs were closely placed into the same clade with the MtmB in archaea *Methanosarcina* spp. (sequence accession nos. are provided in *SI Appendix*, Table S4), whereas other bacterial cobamide-dependent methyltransferases were phylogenetically distant from the EmtA orthologs (<30% sequence similarity) (Fig. 4*C*).

Initial Step of the Anaerobic Estrogen Catabolism in Strain DHT3 Proceeds through Estrogen Conversion into Androgens. Next, we managed to identify the estrogen-derived metabolites by analyzing the ^{13}C-labeled metabolite profile of the estrogen-fed strain DHT3 cultures (Fig. 5*A*). The strain DHT3 cultures were anaerobically incubated with a mixture of [3,4C-^{13}C]estrone and unlabeled estrone in a 1:1 molar ratio (^{13}C-labeled estradiol is not commercially available). The ultraperformance liquid chromatography–high resolution mass spectrometry (UPLC–HRMS) analysis revealed estrone consumption and sequential appearance of

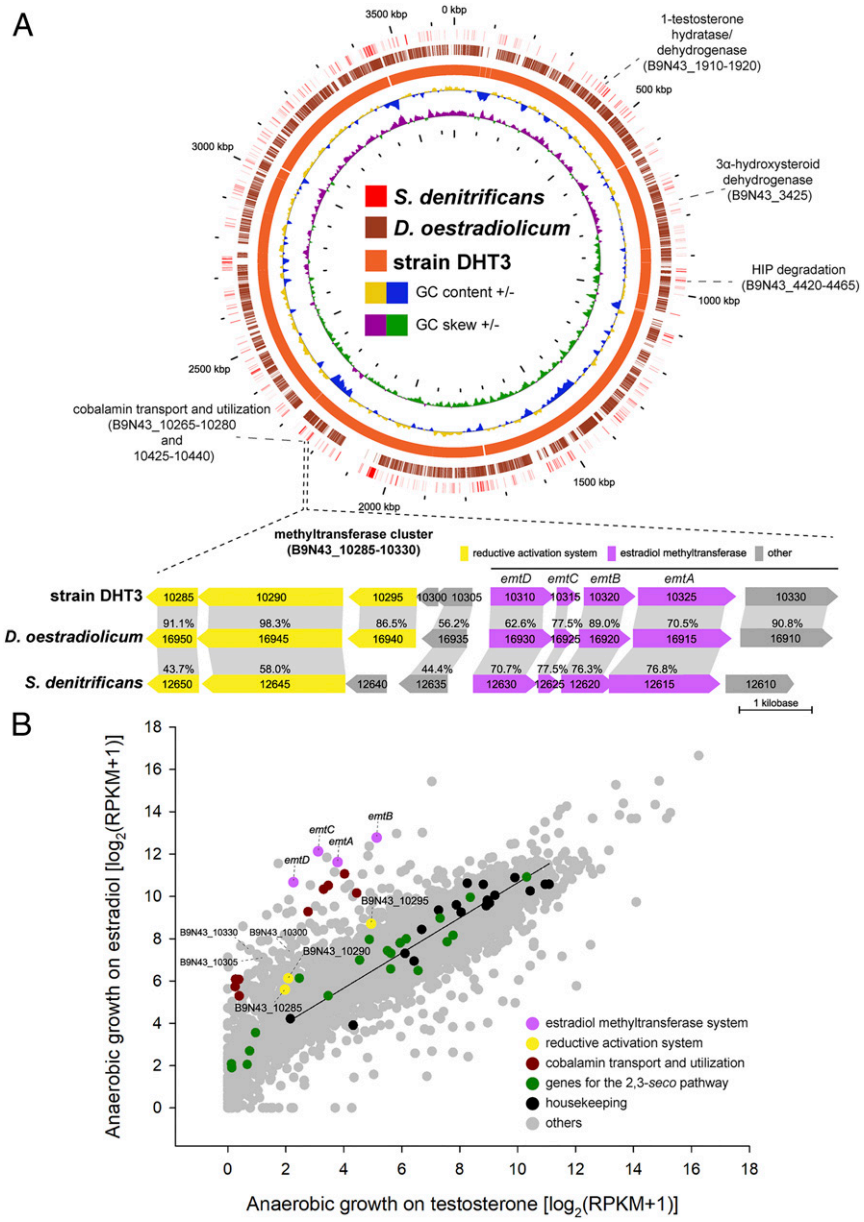

Fig. 3. Comparative genomic analysis and comparative transcriptomic analysis of *Denitratisoma* sp. strain DHT3. (*A*) Steroid catabolic genes and the putative estrogen catabolic genes on circular genomes of strain DHT3, *D. oestradiolicum* DSM 16959, and *S. denitrificans* DSM 18526. The gene cluster *emtABCD* encoding putative estradiol methyltransferase is polycistronically transcribed in strain DHT3 and is present in these 3 estrogen-degrading anaerobes. Homologous open reading frames (ORFs) (colored arrows) between different bacterial genomes are connected with gray-colored blocks. Percentage (%) indicates the shared identity of the deduced amino acid sequences. (*B*) Global gene expression profiles (RNA-Seq) of strain DHT3 anaerobically grown on estradiol or testosterone. Each spot represents a gene. The linear regression line is based on the data points of the selected housekeeping genes (*SI Appendix*, Table S3). Relative gene expression values were estimated by calculating reads per kilobase transcript per million mapped reads (RPKM).

several ^{13}C-labeled metabolites in the strain DHT3 cultures (Fig. 5*B*), including 6 androgenic metabolites (*SI Appendix*, Table S5). After 10 h of incubation, the amounts of the androgenic metabolites in the strain DHT3 cultures significantly decreased with a temporal spike of 17β-hydroxy-1-oxo-2,3-*seco*-androstan-3-oic acid (2,3-SAOA) (Fig. 5) and 3aα-H-4α(3′-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP) (*SI Appendix*, Fig. S7), 2 characteristic ring-cleaved intermediates in the 2,3-*seco* pathway (20, 22), revealing that the anaerobic estrogen catabolism in strain DHT3 proceeds via C_{18} estrogen conversion into C_{19} androgens. The androgens were further degraded to HIP via the established 2,3-*seco* pathway. Consistently, genes in the 2,3-*seco* pathway were also expressed in the estradiol-fed strain DHT3 cells (Fig. 3*B*).

Our data suggested that the observed estrogen conversion into androgens in the strain DHT3 cultures is a methylation reaction likely catalyzed by the putative cobalamin-dependent methyltransferase EmtAB. Methionine synthase MetH, the best characterized cobalamin-dependent methyltransferase, catalyzes the methyl transfer from 5-methyl-tetrahydrofolate to homocysteine (40). In the primary catalytic cycle (Fig. 6*A*), the cob(I)alamin prosthetic group of MetH is methylated to form the methylcobalamin using a 5-methyl-tetrahydrofolate as the methyl donor. Subsequently, the methyl group of methylcobalamin is transferred to homocysteine to produce a methionine (41). However, the cob(I)alamin prosthetic group is prone to undergoing single-electron oxidation during the catalytic cycle, yielding the inactive cob(II)alamin even

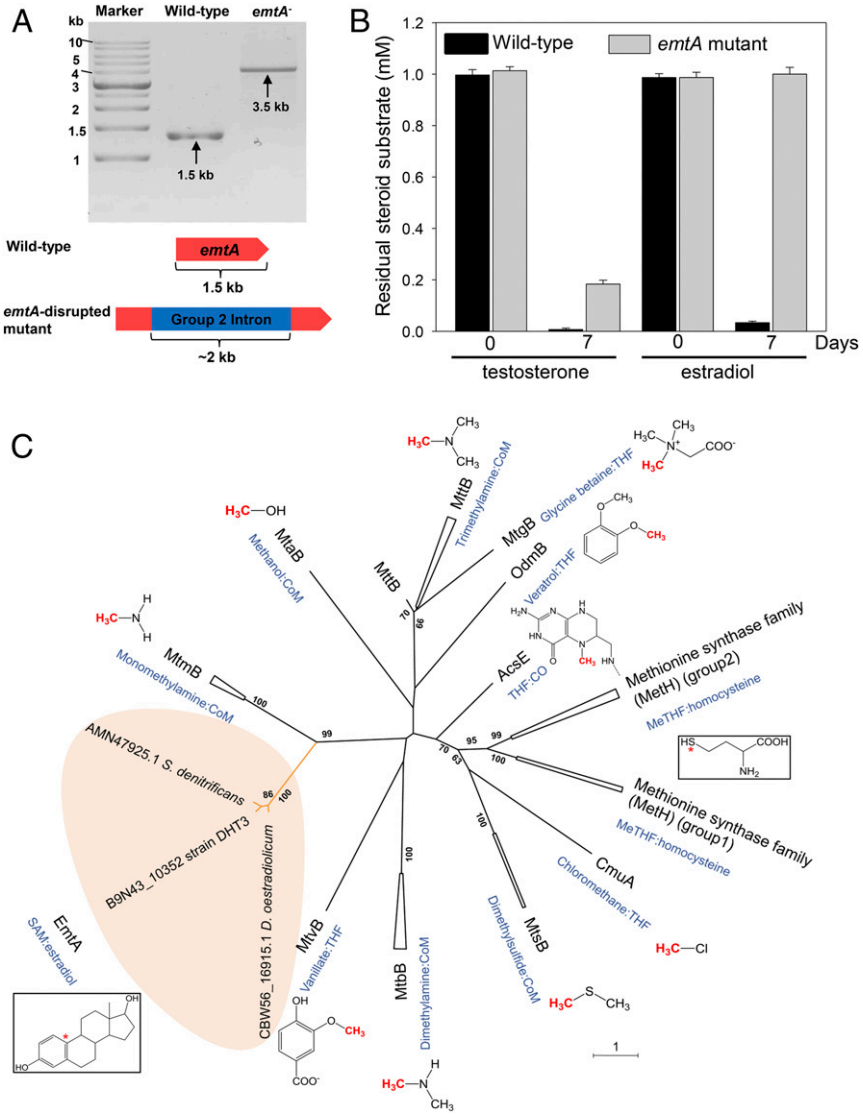

Fig. 4. EmtA is involved in the anaerobic estrogen catabolism in *Denitratisoma* sp. strain DHT3. (*A*) Confirmation of intragenic insertion of the group II intron (~2 kb) into the *emtA* of the *emtA*-disrupted mutant (*emtA*$^-$) using PCR with the primers flanking this gene. (*B*) Testosterone and estradiol utilizations by the wild type or *emtA*-disrupted mutant of strain DHT3 (*emtA*$^-$). (*C*) Phylogenetic relationship of EmtA and other cobamide-dependent methyltransferases. The phylogenetic tree was constructed using the neighbor-joining method with Jukes–Cantor parameter and a bootstrap value of 1,000. The asterisk (*) represents the terminal methyl acceptors for MetH and EmtA.

under anaerobic conditions (42); therefore, endergonic reductive activation of the cob(II)alamin prosthetic group is required for the reentry of MetH to the catalytic cycle (43), which consumes an NAD(P)H (electron donor) and a SAM (methyl donor) for the flavodoxin-mediated methylcobalamin salvage (41, 44–46) (Fig. 6*A*). On the other hand, reductive activation of the cob(II)amide (naturally occurring analogs of cobalamin) prosthetic group in methylamine methyltransferase in methanogenic archaea proceeds through an ATP-dependent mechanism catalyzed by RamA (37) (Fig. 6*B*). This ATP-dependent mechanism and RamA-like proteins also function in bacteria (47).

A Cobalamin-Mediated Estradiol Methylation to Form Androgens in Strain DHT3. We then managed to validate the occurrence of the cobalamin-mediated estradiol methylation in strain DHT3. Thus, we monitored the methylation of estradiol (mixture of ^{2}H-labeled estradiol and unlabeled estradiol in 1:1 molar ratio) in strain DHT3 cell extracts under anaerobic conditions, along with the addition of the reported methyl donors (SAM and methylcobalamin) and cofactors [cob(I)alamin, NAD(P)H, and ATP] required for the catalysis and reductive activation of cobalamin-dependent methyltransferases (37, 44, 45, 48, 49). After incubation, the estradiol-derived metabolites were extracted and analyzed using thin-layer chromatography (TLC) and UPLC–HRMS. We observed apparent production of 2 metabolites in the assay containing the strain DHT3 cell extracts, estradiol, ATP, and methylcobalamin, along with an obvious consumption of estradiol (lane 1 in Fig. 7*B*). The UPLC–HRMS analysis indicated that the 2 estradiol-derived metabolites AND1 and AND2 are both androgens (*SI Appendix*, Fig. S8). The ^{1}H- and ^{13}C-NMR spectra of AND2 (*SI Appendix*, Table S6) suggest the presence of 2 hydroxyl groups at C-3 and C-17 as well as 2 methyl groups (C-18 and C-19) in the steroidal structure, respectively. The fragment ion profile of the HRMS spectra (*SI Appendix*, Fig. S8) and the TLC characteristics of the 2 androgen metabolites were identical to those of the authentic standards of 17β-hydroxyandrostan-3-one ($[M+H]^+$ = 291.23; $C_{19}H_{30}O_2$; AND1) and 3β,17β-dihydroxyandrostane ($[M+H]^+$ = 293.25; $C_{19}H_{32}O_2$; AND2). The relative abundance

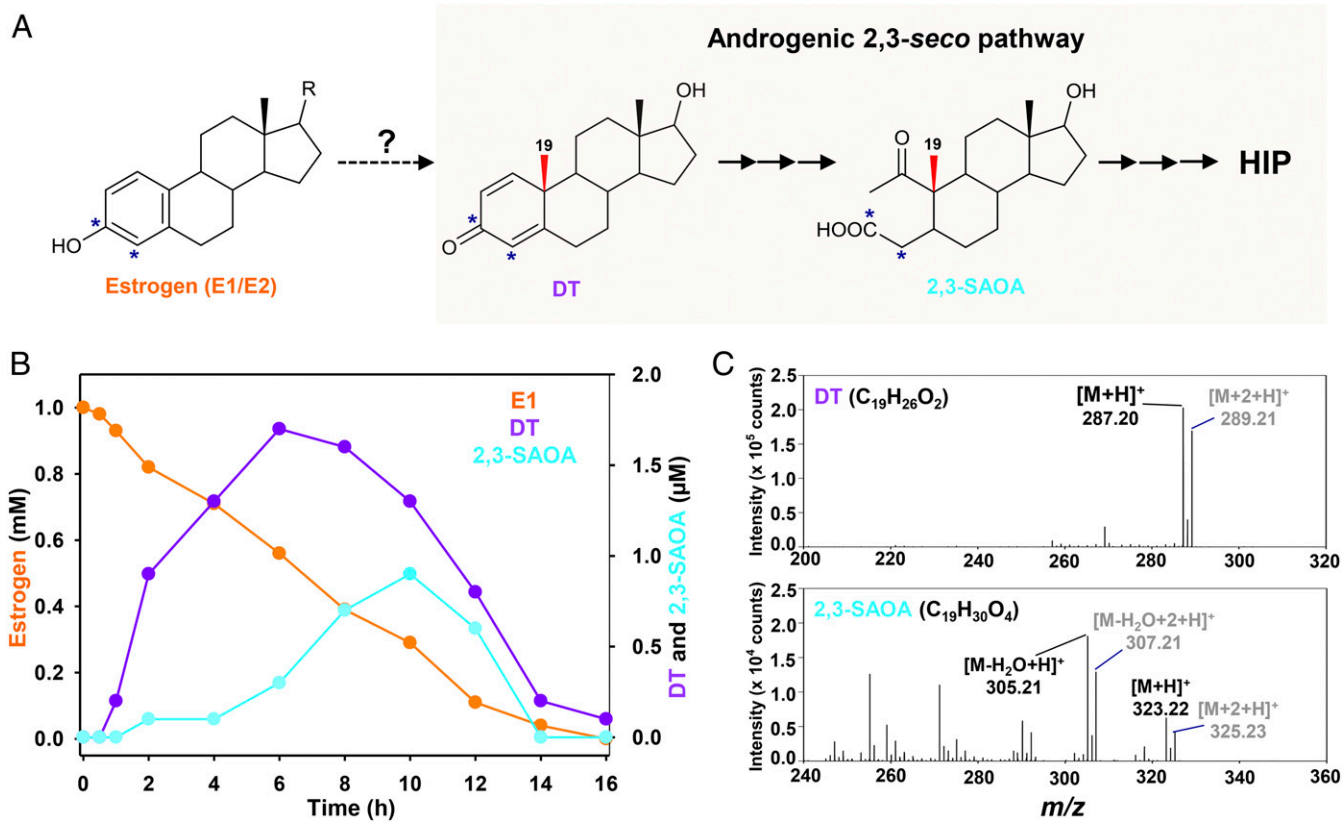

Fig. 5. Anaerobic estrogen catabolism by *Denitratisoma* sp. strain DHT3 via estrogen conversion into androgens. (*A*) Schematic of anaerobic estrogen catabolism in strain DHT3 through a step of androgen production and subsequent degradation via the established 2,3-*seco* pathway. *, ^{13}C-labeled carbon. (*B*) Time-dependent estrone (E1) consumption and intermediate production in the strain DHT3 cultures incubated with estrone (1 mM). Data are averages (deviations <5%) of 3 experimental measurements. (*C*) UPLC–HRMS-based identification of the androgenic metabolites in the estrone-fed strain DHT3 culture. The estrogen substrate contained unlabeled estrone and [3,4C-^{13}C]estrone mixed in a 1:1 molar ratio.

of the M1 isotopomers of the 2 androgenic metabolites in the MS spectra is highly enriched (*SI Appendix*, Fig. S8), suggesting that they are downstream metabolites of the ^{2}H-labeled estradiol mixture. The androgenic metabolites were not produced in the assays when estradiol, cell extracts, or ATP were excluded (lanes 2 through 4 in Fig. 7*B*). However, small amounts of AND2 were produced in the assays without the methylcobalamin addition (lanes 5 and 6), likely due to the presence of endogenous methylcobalamin in the strain DHT3 cell extracts. SAM addition significantly enhanced androgenic metabolite production in the strain DHT3 cell extracts in a dose-dependent manner (*SI Appendix*, Fig. S9). These results are analogous to the case of reductive activation of the cob(II)amide prosthetic group in MetH or in MtmC (Fig. 6).

Next, we managed to validate the involvement of cobalamin-dependent methyltransferase in the estradiol methylation by adding propyl iodide, a specific inhibitor for cobalamin-dependent enzymes, to the assays. Propyl iodide inactivates cobalamin-dependent methyltransferases by propylating the cob(I)alamin prosthetic group in the dark (50, 51). Nevertheless, cobalamin-dependent methyltransferases can regain activity upon exposure to daylight (52). Consistently, propyl iodide addition significantly inhibited the production of the androgenic metabolites in the assays; the inhibition by propyl iodide was much less effective in the daylight-exposed assays (Fig. 7*C*). Furthermore, the addition of exogenous methylcobalamin, estradiol, NADH, and ATP to the cell extracts of the *emtA*-disrupted strain DHT3 cultures did not result in the androgen production (Fig. 7*D*), as opposed to the apparent production of AND2 in the assays added with wild-type strain DHT3 cell extracts. The *emtA*-disrupted mutant is unable to grow on estrogens; thus, both the wild-type and mutant were grown on testosterone (2 mM), with estradiol (50 μM) as an inducer of the *emt* genes. The *emtA*-disrupted mutant grew slower than the wild type (*SI Appendix*, Fig. S5*A*) and was not able to exhaust the fed testosterone (Fig. 7*D*), likely related to the toxicity of kanamycin in the growth medium.

Altogether, our data confirm that the anaerobic estradiol conversion into androgens in strain DHT3 is a cobalamin-mediated methylation reaction catalyzed by EmtA, the catalytic subunit of a putative cobalamin-dependent methyltransferase. Nonetheless, the specific roles of the products of *emtBCD* remain to be elucidated. The most EmtB-similar protein is MtbC (identity of protein sequence ~42%), the cobamide-binding subunit of the dimethylamine methyltransferase in *Methanosarcina* spp (53). Pairwise alignment of the EmtB and MtbC sequences (*SI Appendix*, Fig. S6*B*) revealed that the EmtB homologs have the cobamide-binding motifs (D-x-H-x_2-G-x_{41-42}-S-x-L-x_{24-28}-G-G) conserved in cobamide-dependent methyltransferases (54). EmtC is a hypothetical protein; the most similar protein to EmtD is F420/FMN-dependent oxidoreductase (flavodoxin) involved in reductive activation of cobamide-dependent methyltransferases (46).

For most methyl transfer reactions catalyzed by the cobamide-dependent methyltransferases (e.g., monomethylamine:coenzyme M [CoM] methyltransferase), 3 components are required to complete the methyl transfer cycle (Fig. 6*B*) (38, 45, 49, 55). First, the catalytic subunit MtmB (i.e., the MT1 component) transfers the methyl group from the monomethylamine to the highly reduced cob(I)amide prosthetic group in the cobamide-binding protein MtmC, followed by a second methyl transfer from methylcobamide to the final methyl acceptor CoM by the MtbA

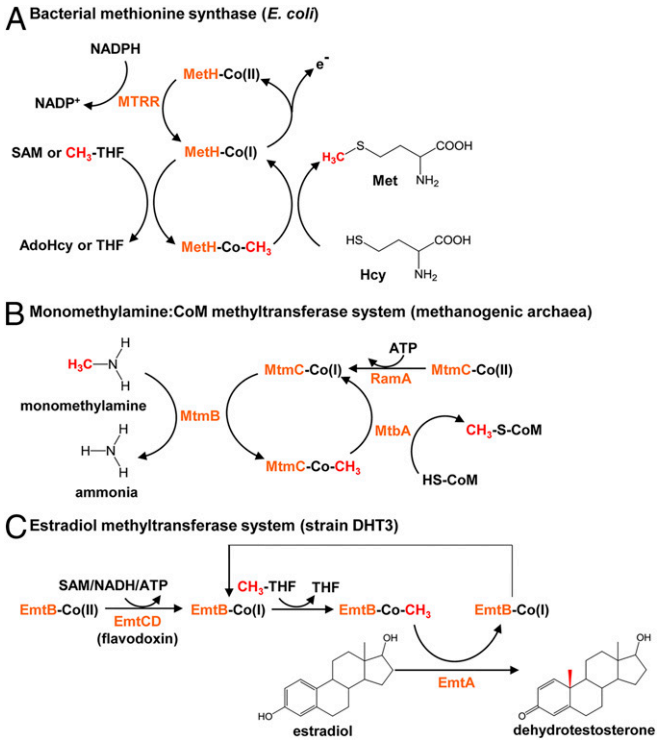

Fig. 6. Proposed mechanisms involved in the catalytic cycles of EmtABCD based on the mechanisms of other characterized cobamide-dependent methyltransferases. (*A*) Methionine synthase MetH: In the catalytic cycle, the cobalamin prosthetic group is methylated by 5-methyl-tetrahydrofolate (CH_3-THF), followed by the methyl transfer to homocysteine. For reductive activation of the cob(II)alamin prosthetic group, NAD(P)H and SAM serve as the electron donor and the methyl donor, respectively. AdoHcy, *S*-adenosylhomocysteine. (*B*) Monomethylamine:CoM methyltransferase MtmBC: the cobamide-binding subunit MtmC forms a heterotrimeric complex with MtbA (the CoM-binding subunit) and MtmB (the catalytic subunit). Reductive activation of the co(II)bamide prosthetic group proceeds through an ATP-dependent reduction catalyzed by RamA. (*C*) Proposed mechanism for the estradiol methylation to form 1-dehydrotestosterone by estradiol methyltransferase EmtABCD in strain DHT3. The cobalamin-binding subunit EmtB and the catalytic subunit EmtA are involved in the catalytic cycle of the estradiol methylation. Reductive activation of the co(II)balamin prosthetic group likely catalyzed by EmtCD (flavodoxin) at the cost of SAM, ATP, or NADH.

(i.e., the MT2 component). Accordingly, EmtA and EmtB are functionally analogous to MtmB and MtmC, respectively. However, the MT2 component-coding gene is missing from the strain DHT3 genome. The results of the cell-extract assays also suggested that SAM or methylcobalamin can serve as the direct methyl donor for the Emt-mediated estradiol methylation. Therefore, the Emt-mediated estradiol methylation resembles a reverse reaction of the MtmB-catalyzed methylamine demethylation and seems to employ a hybrid mechanism of RamA (ATP dependent) and MetH [NAD(P)H- and SAM-dependent] to reductively activate the cob(II)alamin prosthetic group (Fig. 6). Nevertheless, purification of the estradiol methyltransferase and further biochemical investigations are required to elucidate the specific functions of each Emt subunit in the estradiol methylation.

In this study, we found that strain DHT3 converts estrogens into androgens through a cobalamin-mediated methylation at C-10 on estrogens (Fig. 6*C*). However, activation of the phenolic A-ring of estrogens is a prerequisite for this methylation reaction. Given that the activated methyl group (CH_3^+) of methylcobalamin serves as an electrophile, the estradiol methylation in strain DHT3 likely includes the formation of a nucleophilic carboanion at C-10 on the phenolic A-ring (Fig. 7*A*). This catalytic strategy has been reported in many studies of anaerobic aromatic catabolism in denitrifying bacteria (56–59), which first proceeds through the deprotonation of the phenolic hydroxyl group to form a phenolate anion. Subsequently, the lone pair electrons on the deprotonated hydroxyl group are migrated to the *para* carbon atom through resonance of the *pi* system, forming a transient quinonic ring. Similarly, in the case of the estradiol methylation, the formation of a quinonic A-ring would come along with the formation of a C-10 nucleophilic carboanion, enabling the electrophilic attack by the methyl cation (CH_3^+) on methylcobalamin, yielding the androgenic products with a quinonic A-ring (Fig. 7*A*). Consistently, the ^{13}C metabolite profile also showed that 1-dehydrotestosterone with a quinonic A-ring was produced in the strain DHT3 cultures following estradiol consumption (Fig. 5*B*). In the cell extracts, the produced 1-dehydrotestosterone was further converted into AND1 and AND2 via the NADH-dependent reduction of both the C-1 double bond and the C-3 keto group (Fig. 7*A*) by 3-ketosteroid Δ^1-reductase and 3β-hydroxysteroid dehydrogenase that are constitutively expressed in strain DHT3 cells (Dataset S1). This claim is supported by the observed transformation of 1-dehydrotestosterone into AND2 and 2,3-SAOA in the strain DHT3 cell extracts with and without addition of NADH (2 mM), respectively (*SI Appendix*, Fig. S10).

Conclusion

In this study, we demonstrated that strain DHT3 converts estrogens into androgens via a cobalamin-mediated methylation and subsequently catabolizes the androgenic intermediates to HIP through the established 2,3-*seco* pathway. The discovery completes central pathways for bacterial steroid catabolism (Fig. 1). Briefly, anaerobic bacteria utilize a convergent catabolic pathway (the 2,3-*seco* pathway) to catabolize sterols, androgens, and estrogens, while aerobic bacteria adopt divergent pathways to catabolize 1) sterols and androgens (9,10-*seco* pathway) and 2) estrogens (4,5-*seco* pathway). Nevertheless, all 3 steroid catabolic pathways finally converge at HIP (Fig. 1) and HIP catabolic genes are conserved in the genomes of all characterized steroid-utilizing bacteria (*SI Appendix*, Fig. S11) (11, 60).

Cobamides such as cobalamin are a family of cobalt-containing tetrapyrrole biomolecules with essential biochemical functions in all 3 domains of life, serving as the prosthetic group for various methyltransferases, isomerases, and reductive dehalogenases (61). Before this study, the known methyl acceptors for cobalamin-dependent methyltransferases included tetrahydrofolate and homocysteine in most organisms (43, 48, 62) as well as CoM and tetrahydromethanopterin in methanogenic archaea (38, 53). Here, we demonstrated that estrogens are terminal methyl acceptors of a cobalamin-dependent methyltransferase in a denitrifying proteobacterium, revealing an unexpected role of cobalamin in steroid metabolism. Given that sex steroids are involved in bidirectional metabolic interactions between bacteria and their eukaryotic hosts, finding retroconversion of estrogens into androgens in bacteria portends unexplored microbe–host metabolic interdependencies via this Emt-mediated estrogen methylation reaction. Therefore, the *emtABCD* gene cluster can serve as a biomarker to elucidate the occurrence of retroconversion of estrogens in eukaryotic microbiota.

Materials and Methods

The estrogen-degrading denitrifying betaproteobacterium strain DHT3 was isolated from the estradiol-spiked anoxic sludge collected from the Dihua Sewage Treatment Plant (Taipei, Taiwan). Isolation and routine cultivation of strain DHT3 was performed at 28 °C in the dark with a headspace consisted of N_2/CO_2 (80:20, vol/vol). The resting cells of strain DHT3 were incubated with ^{13}C-labeled estrone under denitrifying conditions. The cultural samples were withdrawn at different time intervals. The estrone-derived metabolites

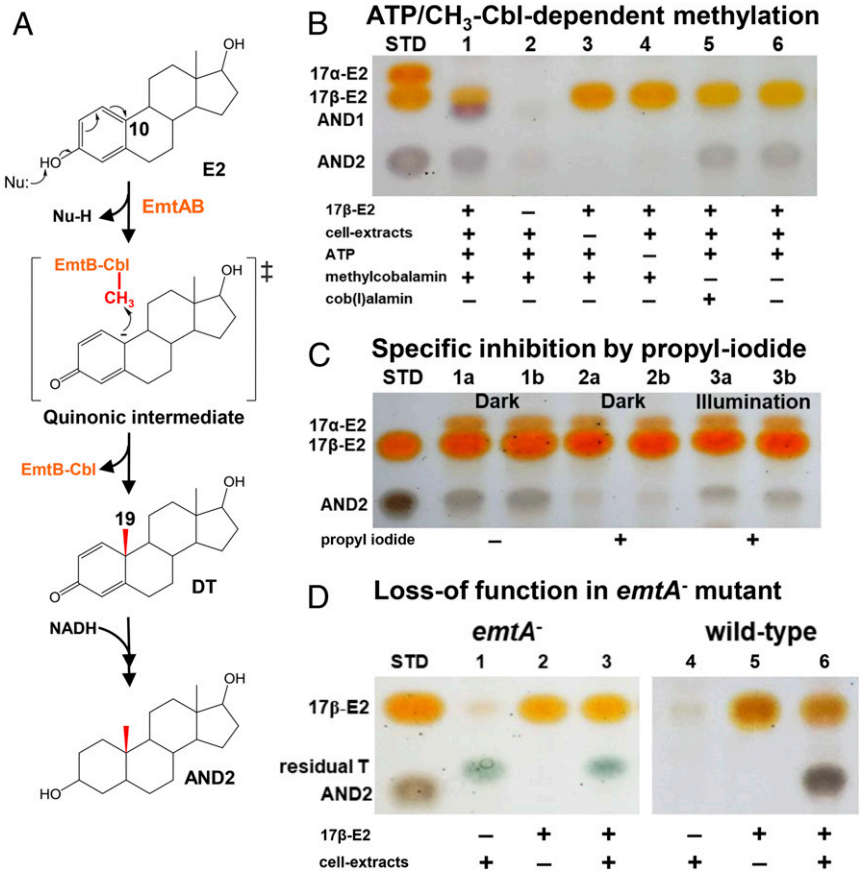

Fig. 7. Proposed mechanism for the Emt-catalyzed, cobalamin-mediated estradiol methylation. (*A*) Proposed mechanism involved in estrogenic A-ring activation and subsequent cobalamin-mediated C-10 methylation to form androgens. (*B–D*) TLC analysis of the cobalamin-mediated estradiol methylation in the strain DHT3 cell extracts. (*B*) ATP (lane 4) and methylcobalamin (lanes 5 and 6) are required for the estradiol (E2) methylation. (*C*) Specific inhibition of the E2 methylation in the strain DHT3 cell extracts by propyl iodide (lanes 2a/2b) in a reversible manner with daylight (lanes 3a/3b). Assays a and b are technical replicates in each treatment. All assays in *C* contain E2, cell extracts, ATP, NADH, and with or without propyl iodide. (*D*) Loss of E2 methylation activity in the cell extracts of the *emtA*-disrupted strain DHT3 mutant (lane 3). Assays 3 and 6 in *D* contain E2, cell extracts, ATP, NADH, and methylcobalamin. Abbreviations: AND1, 17β-hydroxyandrostan-3-one; AND2, 3β,17β-dihydroxyandrostane; DT, 1-dehydrotestosterone; Nu, nucleophile; STD, steroidal standards; and T, testosterone.

were extracted using ethyl acetate and analyzed through UPLC–atmospheric pressure chemical ionization (APCI)–HRMS. Cell extracts of strain DHT3 were prepared using a French pressure cell. After removing the membrane proteins through ultracentrifugation, the soluble proteins were anaerobically incubated with ^{2}H-labeled estradiol (0.25 mM), ATP (5 mM), and methylcobalamin (1 mM) overnight. The resulting androgen metabolites were separated through liquid–liquid partition, TLC, and HPLC. The chemical structure of a HPLC-purified product was elucidated using NMR spectroscopy. Total RNA was extracted from strain DHT3 cells anaerobically grown with estradiol or testosterone. rRNA was removed from the total RNA samples, and cDNA samples were prepared using the RNA fragments as reverse-transcription templates. The constructed DNA libraries were sequenced using the Illumina HiSEq. 2000 system (Illumina). The *emtA* gene of strain DHT3 was disrupted using the TargeTron Gene Knockout System kit (Sigma-Aldrich, St. Louis, MO). Detailed materials and methods are described in *SI Appendix, Supplemental Materials and Methods*.

Data Availability. Oligonucleotide primers used in this study are listed in *SI Appendix*, Table S2. The NMR spectral data of AND2 are provided in *SI Appendix*, Table S6. Nucleotide sequences of the 16S rRNA and *emtABCD* genes of *Denitratisoma* sp. strain DHT3 are shown in *SI Appendix*, Appendices S1–S5. Transcriptomic data of the strain DHT3 are available in *SI Appendix*, Dataset S1. Genome sequence of the strain DHT3 has been deposited in the National Center for Biotechnology Information (NCBI) Genome database, accession no. CP020914. The transcriptomes of the strain DHT3 have been deposited in the NCBI database [accession nos. SRR10362955 (testosterone-grown condition) and SRR10362956 (estradiol-grown condition)].

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