The ISME Journal (2016) 10, 1967–1983
© 2016 International Society for Microbial Ecology All rights reserved 1751-7362/16
www.nature.com/ismej

ORIGINAL ARTICLE

Integrated multi-omics analyses reveal the biochemical mechanisms and phylogenetic relevance of anaerobic androgen biodegradation in the environment

Fu-Chun Yang[1,6], Yi-Lung Chen[1,2,3,6], Sen-Lin Tang[1,2,3], Chang-Ping Yu[4], Po-Hsiang Wang[1,7], Wael Ismail[5], Chia-Hsiang Wang[1], Jiun-Yan Ding[1], Cheng-Yu Yang[1], Chia-Ying Yang[1] and Yin-Ru Chiang[1,2,3]

[1]*Biodiversity Research Center, Academia Sinica, Taipei, Taiwan;* [2]*Department of Life Science, National Taiwan Normal University, Taipei, Taiwan;* [3]*Biodiversity Program, Taiwan International Graduate Program, Academia Sinica and National Taiwan Normal University, Taipei, Taiwan;* [4]*Graduate Institute of Environmental Engineering, National Taiwan University, Taipei, Taiwan and* [5]*Environmental Biotechnology Program, Life Sciences Department, College of Graduate Studies, Arabian Gulf University, Manama, Kingdom of Bahrain*

Steroid hormones, such as androgens, are common surface-water contaminants. However, literature on the ecophysiological relevance of steroid-degrading organisms in the environment, particularly in anoxic ecosystems, is extremely limited. We previously reported that *Steroidobacter denitrificans* anaerobically degrades androgens through the 2,3-*seco* pathway. In this study, the genome of *Sdo. denitrificans* was completely sequenced. Transcriptomic data revealed gene clusters that were distinctly expressed during anaerobic growth on testosterone. We isolated and characterized the bifunctional 1-testosterone hydratase/dehydrogenase, which is essential for anaerobic degradation of steroid A-ring. Because of apparent substrate preference of this molybdoenzyme, corresponding genes, along with the signature metabolites of the 2,3-*seco* pathway, were used as biomarkers to investigate androgen biodegradation in the largest sewage treatment plant in Taipei, Taiwan. Androgen metabolite analysis indicated that denitrifying bacteria in anoxic sewage use the 2,3-*seco* pathway to degrade androgens. Metagenomic analysis and PCR-based functional assays showed androgen degradation in anoxic sewage by *Thauera* spp. through the action of 1-testosterone hydratase/dehydrogenase. Our integrative 'omics' approach can be used for culture-independent investigations of the microbial degradation of structurally complex compounds where isotope-labeled substrates are not easily available.
The ISME Journal (2016) **10,** 1967–1983; doi:10.1038/ismej.2015.255; published online 12 February 2016

Introduction

Steroids are ubiquitous and abundant in nature. In contrast to the structural function of sterols in cell membranes, steroid hormones are extremely bioactive and regulate various physiological processes, including development and reproduction, in animals (Ghayee and Auchus, 2007). The increasing number of reports on intersex organisms found in global aquatic ecosystems has raised concerns regarding the occurrence and persistence of steroid hormones in the environment (Safe, 2004; Wise *et al.*, 2011). Several studies have reported the masculinization of freshwater wildlife exposed to androgens in polluted rivers (Howell *et al.*, 1980; Bortone *et al.*, 1989; Parks *et al.*, 2001; Orlando *et al.*, 2004). Furthermore, steroids were identified as pheromones and adversely affect fish behavior even at extremely low concentrations (Kolodziej *et al.*, 2003). For example, androgens at picomolar concentrations elicit both odorant and pheromonal responses in fish (Adams *et al.*, 1987; Moore and Scott, 1991; Serrano *et al.*, 2008; Katare *et al.*, 2011).

Steroid hormones are discharged into the environment through various routes, including the agricultural application of livestock manure and municipal sewage biosolids as fertilizers (Hanselman *et al.*, 2003;

Correspondence: Y-R Chiang, Biodiversity Research Center, Academia Sinica, 128 Academia Road Sec. 2, Nankang, Taipei 115, Taiwan.
E-mail: yinru915@gate.sinica.edu.tw
[6]These authors contributed equally to this work.
[7]Current address: Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada.
Received 31 August 2015; revised 30 November 2015; accepted 4 December 2015; published online 12 February 2016

Lorenzen *et al.*, 2004). Moreover, phytosterols in pulp and paper mill effluents were transformed to androgens by microorganisms in river sediments, consequently influencing fish development (Jenkins *et al.*, 2001, 2003). Steroid hormones have been frequently detected in various environmental matrices, including soil, groundwater, surface water and sediments (Ying *et al.*, 2002). These substances were often found at nanograms to micrograms per liter concentrations in surface water (Ternes *et al.*, 1999; Baronti *et al.*, 2000; Kolodziej *et al.*, 2003; Yamamoto *et al.*, 2006; Chen *et al.*, 2010; Chang *et al.*, 2011; Fan *et al.*, 2011). Quantifying five classes of steroid hormones in the surface waters of Beijing (China) revealed that androgens (up to $1.9\,\mu g\,l^{-1}$) were the most abundant steroids in urban rivers (Chang *et al.*, 2009).

Biodegradation has been considered a crucial mechanism for removing steroids from natural and engineered ecosystems (Johnson and Sumpter, 2001; Andersen *et al.*, 2003; Khanal *et al.*, 2006). Sewage treatment plants are essential in removing steroid hormones, as shown by a comparison between sewage influents and effluents (Fan *et al.*, 2011). Several androgen-degrading aerobes have been isolated and characterized (Donova, 2007; Yang *et al.*, 2011; Horinouchi *et al.*, 2012). Among them, *Comamonas testosteroni* has received special attention, and catabolic intermediates, genes, and enzymes have been identified (see Horinouchi *et al.* (2012) for a review). By contrast, literature on the biochemical mechanisms and the phylogenetic diversity involved in anaerobic steroid biodegradation is extremely limited. Hanselman *et al.* (2003) indicated that anoxic sediments and soil may be reservoirs for steroids because microbial degradation occurs slowly in these matrices. So far, two anaerobic species (*Steroidobacter (Sdo.) denitrificans* and *Sterolibacterium denitrificans*) capable of growing on androgens have been reported (Tarlera and Denner, 2003; Fahrbach *et al.*, 2008). We recently elucidated the anaerobic catabolic pathway (2,3-*seco* pathway) of testosterone by using *Sdo. denitrificans* DSMZ 18526 (gammaproteobacterium) and *Sterolibacterium* (*Stl.*) *denitrificans* DSMZ 13999 (betaproteobacterium) as model microorganisms (Wang *et al.*, 2013b, 2014) (Figure 1b). However, information regarding the catabolic genes and enzymes involved in the anaerobic catabolic pathway is scant.

The biochemical mechanisms of androgen biodegradation have been studied using a few pure cultures. However, whether these established catabolic pathways are functional in the environment remains unknown. In addition, the distribution, abundance, and ecological relevance of culturable steroid-degraders in the ecosystem remain unexplained. Conventional culture-independent approaches, such as stable-isotope probing and fluorescence *in situ* hybridization-microautoradiography, are rarely used in the studies of microbial steroid degradation because (i) completely ^{13}C-labeled steroids are not commercially available and (ii) hydrophobic compounds, such as steroids, easily attach to or pass through bacterial membranes. Therefore, distinguishing the metabolic activities

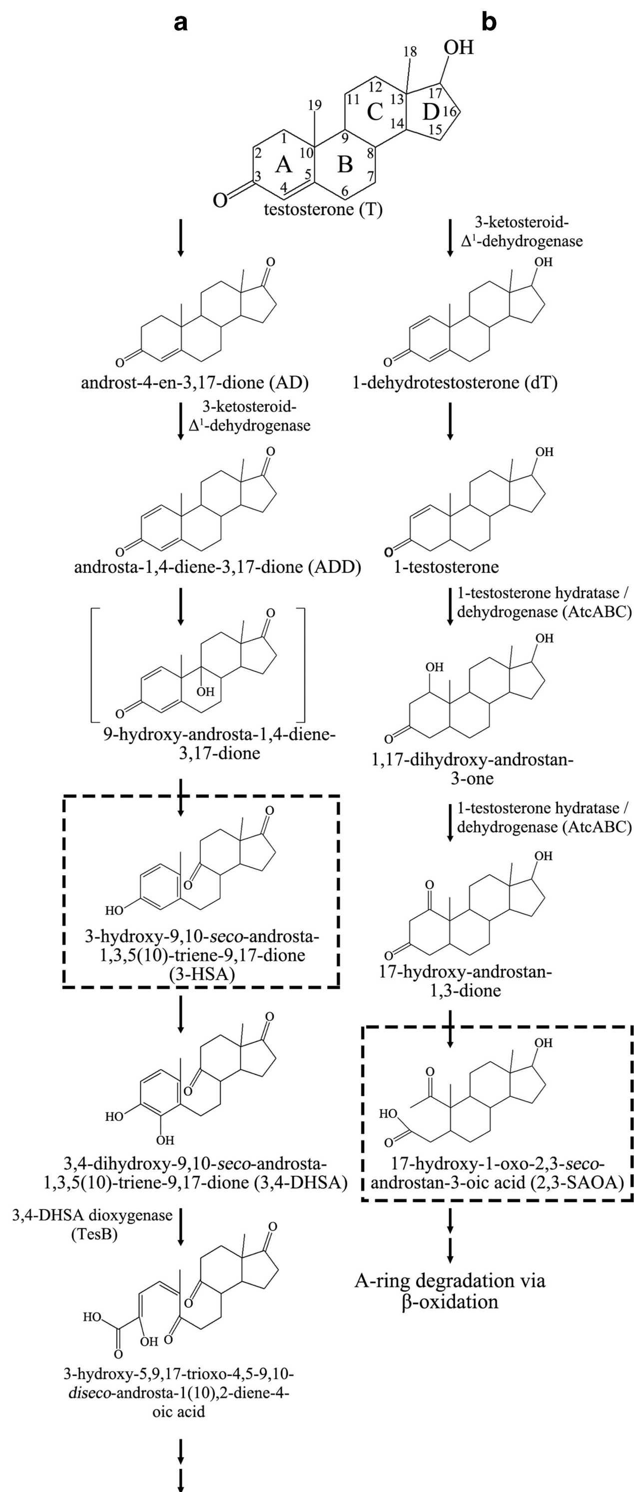

Figure 1 Proposed catabolic pathways of testosterone. (**a**) Aerobic 9,10-*seco* pathway established in *Comamonas testosteroni* TA441. (**b**) Anaerobic 2,3-*seco* pathway established in *Steroidobacter denitrificans* DSMZ18526. The compound in bracket is presumed. Suggested signature metabolites are enclosed in boxes.

(passive diffusion or active uptake; redox transformation or complete degradation) of radiolabeled bacterial cells is difficult. The lack of molecular markers and suitable culture-independent techniques has impeded *in situ* investigations of microbial steroid degradation, thus necessitating efficient and specific tools for monitoring the environmental fate of steroids.

In this study, we first identified the androgen catabolic genes and enzymes of *Sdo. denitrificans*. We then investigated androgen biodegradation in the anoxic sewage of a municipal sewage treatment plant designed to treat domestic wastewater produced by three million residents of Taipei City, Taiwan, for which we used the following culture-independent approaches: (i) ultra-performance liquid chromatography—tandem mass spectrometry identification of signature metabolites; (ii) identification of major catabolic players through next-generation sequencing techniques; and (iii) PCR-based identification of functional genes. This study is the first integrated 'omics' investigation on the biochemical mechanisms and phylogenetic diversity of steroid biodegradation in the environment.

Materials and methods

General materials and methods

The materials and methods section in the Supplementary Information provide complete descriptions of the following: bacterial strains and bacterial cultivation; steroid extraction, detection, and quantification; DNA/RNA extraction; basic molecular biological methods; genome sequencing, assembly and gene prediction; RNA-Seq; Illumina MiSeq sequencing of 16S rRNA gene amplicons; 16S rDNA-based taxonomic analysis; *lacZ*-based yeast androgen bioassay; and the purification and characterization of 1-testosterone hydratase/dehydrogenase (AtcABC: anaerobic testosterone catabolism).

DCPIP-based spectrophotometric assays

AtcABC activity was spectrophotometrically assayed (SpectraMax M2e spectrophotometer; Molecular Devices, Sunnyvale, CA, USA) using 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor. The decrease in absorption caused by the substrate-dependent reduction of DCPIP occurred at 578 nm ($\varepsilon = 16.8\ cm^{-1} mmol^{-1}$ (Jin *et al.*, 2011)). To determine the hydratase/dehydrogenase activity of individual protein fractions, assay mixtures (0.9 ml) containing 20 mM Tris-HCl (pH 7.5), 50 μM DCPIP, 125 μM 1-testoterone and 1.25% (v/v) 2-propanol were aerobically incubated at 30 °C. The reaction was initiated by adding 0.1 ml of enzyme solution (0–500 μg of proteins).

Sewage sample collection

Dihua sewage treatment plant (DHSTP) is the largest municipal wastewater treatment plant (500 000 $m^3\ day^{-1}$) in Taipei. Along with domestic water, DHSTP receives industrial, medical and livestock wastewater as well as groundwater (Lin *et al.*, 2009). The hydraulic retention time is ~ 10 h, and the effluent is discharged into Tamsui River, the largest in Taipei and its suburbs (Lin *et al.*, 2010). The plant is designed as an anoxic/oxic process for carbon and nitrogen removal (see Lin *et al.* (2009 and 2010) for detailed information). Influent and effluent water samples (5 l) were collected in triplicate in June 2014. Formaldehyde (final concentration = 1% v/v) was added to the glass bottles before sample collection. After 10 min of formaldehyde fixation, 5 μg of [2,3,4C-^{13}C]testosterone (Isosciences, King of Prussia, PA, USA) was added to the samples. Androgens in water samples were quantified using liquid chromatography-mass spectrometry as described in Supplementary Information. Sewage samples (20 l) were collected from the anoxic tank (denitrification tank; range of dissolved oxygen levels = 0.2–0.5 $mg\ l^{-1}$) of DHSTP in April and June 2014. A sterilized 20 l glass bottle was completely filled with the anoxic sewage and was tightly capped to avoid headspace formation. The samples were delivered to the laboratory within 30 min.

Incubation of anoxic sewage with testosterone

Anoxic sewage (1 l) was transferred into 1.2 l glass bottles in a laminar flow hood. The bottles containing sewage were sealed with a rubber stopper, and sterile nitrogen gas (~1200 ml; passed through a sterile cotton plug) was used as the carrier gas (flow rate, 40 $ml\ min^{-1}$) to expel residual oxygen from the bottles. Anoxic sewage was treated under the following conditions: autoclaved sewage with testosterone (1 mM) and nitrate (10 mM), native sewage with nitrate and without testosterone, native sewage with testosterone and without nitrate and native sewage with testosterone and nitrate. Ascorbate (a reducing agent) and resazurin (a redox indicator) were not added to the anoxic sewage treatments because these compounds could be used as carbon and energy sources for sewage bacteria. Sewage treatments were performed in duplicate and the bottles were incubated at 25 °C with stirring at 160 r.p.m. for 2 weeks. Sewage samples (10 ml) were withdrawn from the bottles by using a 10 ml sterile syringe every 12 h and were stored at − 80 °C before use. Androgenic activity and metabolites in sewage samples were detected using the yeast androgen assay and ultra-performance liquid chromatography (UPLC)—atmospheric pressure chemical ionization (APCI)—MS/MS, respectively. Bacterial 16S rRNA gene and the androgen catabolic gene (*atcA*) in sewage samples were analyzed using the Illumina (San Diego, CA, USA) MiSeq platform and PCR-based functional assay, respectively.

PCR-based functional assay by using the atcA gene probe

Search for *atcA*-related gene sequences in the GenBank Database was conducted on protein–protein

BLAST (http://www.ncbi.nlm.nih.gov/blast/) with *Sdo. denitrificans* AtcA as a query. Multiple alignment of the *atcA*-related gene sequences was performed using Align/Assemble (Geneious 8.1.4). A degenerate primer pair was deduced from regions conserved in the *atcA* genes but not in the gene coding for the large subunit of 3-hydroxycyclohexanone dehydrogenase (MhyADH) from *Alicycliphilus denitrificans* (Jin *et al.*, 2011) (Supplementary Figure S1). The *atcA* fragments were amplified using the degenerate primers (forward primer: 5′–GGCASCGYYSAGTTCATCGAC AA–3′ and reverse primer: 5′–GCCGCTGTCRTAYT CRTTSCCGCTSGG–3′) through PCR (94 °C for 2 min; 25 cycles: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; and 72 °C for 10 min). The *atc*A fragments (~1100 bp) amplified from the anoxic DHSTP sewage were cloned in *Escherichia coli* (One Shot TOP10; Invitrogen, Waltham, MA, USA) by using the pGEM-T Easy Vector Systems (Promega, Fitchburg, WI, USA).

Results

Sdo. denitrificans genome

The 3.47 Mb chromosome (61.7% G+C; accession number: CP011971) of *Sdo. denitrificans* contains 2986 predicted protein-coding genes (Supplementary Table S1), 1 rRNA operon (5S, 16S and 23S), 47 tRNA genes, 1 CRISPR gene cluster and 62 pseudogenes. Altogether, 2022 protein-coding genes were classified into COG categories, and lipid metabolism genes (197 genes; COG I; Supplementary Table S1) were the most abundant group of metabolism-related genes. A high correlation was observed between the phenotype and genotype of *Sdo. denitrificans*. First, this denitrifying bacterium cannot use ferric or perchlorate as the alternative electron acceptor. Second, *Sdo. denitrificans* has a narrow substrate spectrum and cannot utilize carbohydrates, monoaromatic compounds and long-chain fatty acids (Fahrbach *et al.*, 2008). Third, this bacterium depends on the supply of various vitamins (for example, cobalamin, pyridoxamine and thiamine) for growth (DSMZ Medium 1116). No genes related to these metabolic features were found in the genome of *Sdo. denitrificans* (Supplementary Table S1). We identified the genes encoding the ABC transporters for the uptake of ferric and molybdate ions (Figure 2a; Supplementary Table S1). Molybdenum serves as a cofactor of the essential enzymes for the denitrification and anaerobic degradation of hydrocarbons (Dermer and Fuchs, 2012). The genes (ACG33_02275 ~ ACG33_02290) encoding the molybdopterin biosynthesis proteins (Moe) were identified (Figure 2a). In addition, genes for the bacterial secretion systems, including type II, type IV, Sec and Tat, are present on the *Sdo. denitrificans* chromosome (Supplementary Table S1; highlighted in green).

The androgen metabolite analysis of aerobically and anaerobically grown *Sdo. denitrificans* cells suggested the involvement of various redox enzymes in the initial steps of both catabolic pathways (Wang *et al.*, 2013B). In the *Sdo. denitrificans* chromosome, the genes ACG33_00240, ACG33_09105 and ACG33_10715, respectively coding for 3-ketosteroid Δ^1-dehydrogenase, 3α(20β)-hydroxysteroid dehydrogenase and 3β(17β)-hydroxysteroid dehydrogenase, were identified. The other putative catabolic genes involved in steroid degradation were also identified in this genome. For example, four clustered genes (ACG33_10670 ~ ACG33_10685) whose products were similar (>38% sequence identity) to the steroid C25 dehydrogenase of *Stl. denitrificans* (Figure 2b). This molybdoenzyme catalyzes the anaerobic hydroxylation of steroid substrates (Dermer and Fuchs, 2012). In addition, a cluster of 14 genes (ACG33_00310 ~ ACG33_00375) was identified within a 15 kb stretch of the circular chromosome. The encoding proteins shared up to 88% amino acid sequence identity with the β-oxidation enzymes involved in the degradation of steroid C/D rings by *C. testosteroni* (Figure 2c). In the genome of *C. testoteroni* TA441, *tesB* encoding a meta-cleavage dioxygenase is located next to ORF1. Similar gene organization was observed in the genomes of *C. testosteroni* strains CNB-2 (Figure 2c) and KF1 (Horinouchi *et al.*, 2012). However, a *tesB*-like gene was not present in the chromosomes of *Sdo. denitrificans* and *Stl. denitrificans* (Figure 2c). *Stl. denitrificans* uses the 2,3-*seco* pathway (see Figure 1b for detailed information) to degrade androgens regardless of oxygen conditions (Wang *et al.*, 2013a; Wang *et al.*, 2014), and no genes encoding steroid catabolic oxygenases are found in the *Stl. denitrificans* genome (Dermer and Fuchs, 2012). Therefore, the β-oxidation genes (ACG33_00310 ~ ACG33_00375) of *Sdo. denitrificans* may be involved in the degradation of steroid C/D rings during anaerobic growth on testosterone. In the genome of *C. testosteroni*, the genes responsible for the oxygenase-dependent cleavage of the steroid A/B rings are organized into a single cluster (ORF18,17, tesIHA2A1DEFG). By contrast, the corresponding genes of *Sdo. denitrificans* are scattered throughout its chromosome (Figure 2a). The putative steroid catabolic genes are highlighted in red in Supplementary Table S1.

Comparative transcriptomics of Sdo. denitrificans and the identification of genes expressed during anaerobic testosterone degradation

Depending on oxygen availability, *Sdo. denitrificans* degrades testosterone through two distinct pathways (Wang *et al.*, 2013B). Therefore, we proposed that testosterone catabolic genes were regulated on the basis of the prevailing oxygen tension. To identify the genes involved in anaerobic testosterone catabolism, transcriptomic investigations were conducted on *Sdo. denitrificans* grown under three conditions: (i) aerobic growth with testosterone (2.5 mM);

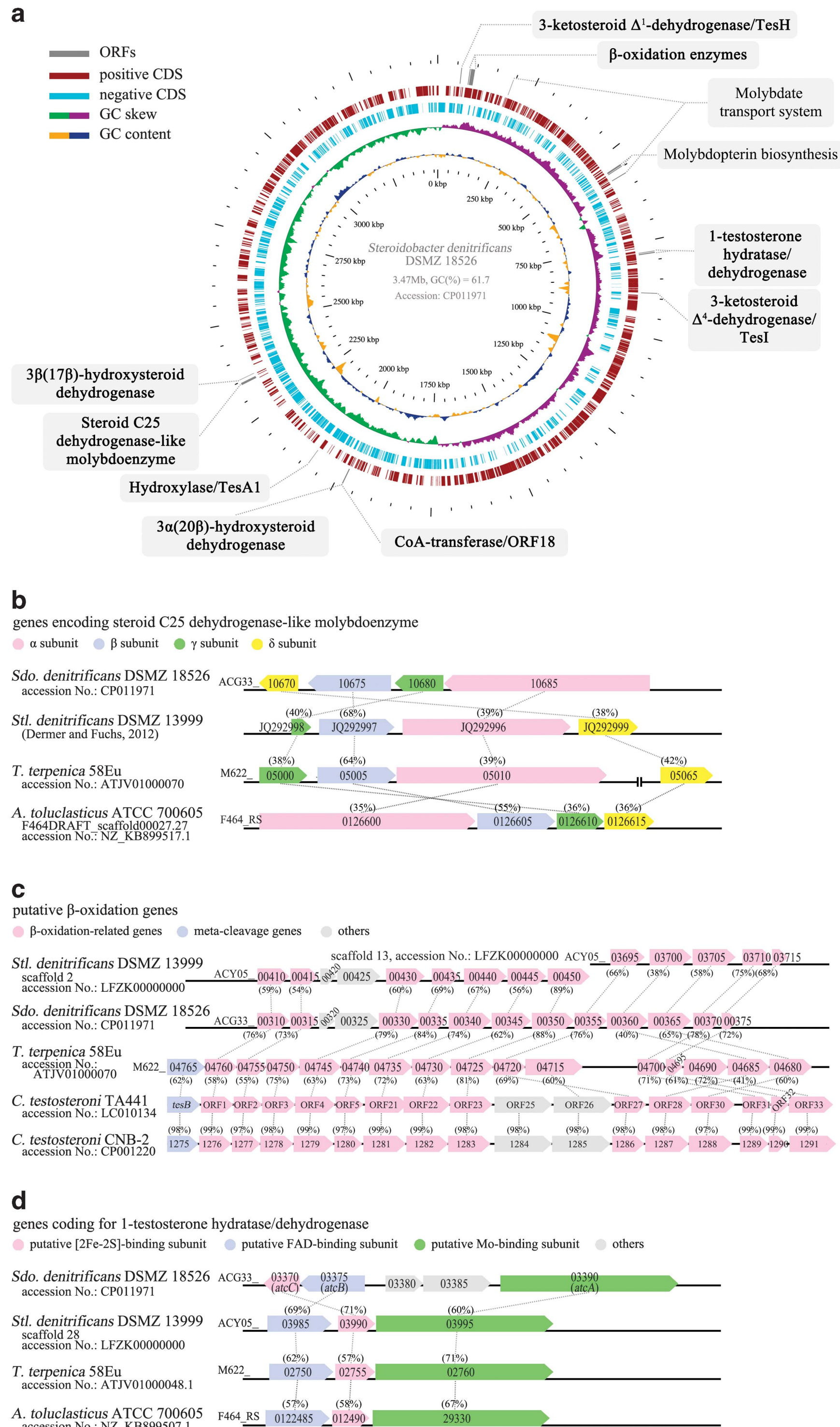

Figure 2 (**a**) Structure and distribution of the putative catabolic genes for androgen degradation on the chromosome of *Sdo. denitrificans*. The genes involved in molybdopterin biosynthesis and molybdate transport are also shown. Proposed gene products are enclosed in gray boxes. (**b**) Genes coding for a steroid C25 dehydrogenase-like enzyme are found in the genomes of *Sdo. denitrificans*, *Stl. denitrificans* and *Thauera terpenica*. The latter was identified as an anaerobic androgen-degrader in this study. Homologous open reading frames (colored arrows) between different bacterial strains are connected with dotted lines. Numbers (%) indicate the identity of the deduced amino acid sequences with those of the genes of *Sdo. denitrificans*. (**c**) Genes encoding β-oxidation enzymes are conserved in androgen-degrading anaerobes and aerobes (e.g., *C. testosteroni*). The numbers (%) indicate the identity of the amino acid sequences with those of the functionally confirmed genes of *C. testosteroni* TA441. For the detailed catabolic functions of individual genes of the strain TA441, refer Horinouchi *et al*. (2012). (**d**) Genes coding for the molybdoproteins of the xanthine oxidase family. The numbers (%) indicate the identity of deduced amino acid sequences with those of the corresponding genes of *Sdo. denitrificans*, whose product was characterized as 1-testosterone hydratase/dehydrogenase in this study.

(ii) anaerobic growth with testosterone; and (iii) anaerobic growth with heptanoic acid (10 mM). *Sdo. denitrificans* transcriptomes were sequenced using RNA-Seq (Supplementary Table S2). Transcriptomic analysis revealed 232 genes (highlighted in orange in Supplementary Table S2) that were at least twofold upregulated during anaerobic growth on testosterone, compared with anaerobic growth on heptanoic acid and aerobic growth on testosterone (Figure 3a). Many of the upregulated genes were involved in transcription (COG K; 14 genes), translation (COG J; 14 genes) and post-translational modification (COG O; 18 genes). Moreover, we detected the upregulation of 11 genes involved in secondary metabolites biosynthesis and catabolism (COG Q), 12 in lipid metabolism (COG I); 12 in energy production (COG C), 15 genes in cell wall and membrane biogenesis (COG M), and 21 associated with coenzyme transport and metabolism (COG H). This reflects a general physiological adaptation of *Sdo. denitrificans* to anaerobically grow on a hydrophobic compound with a complex structure like

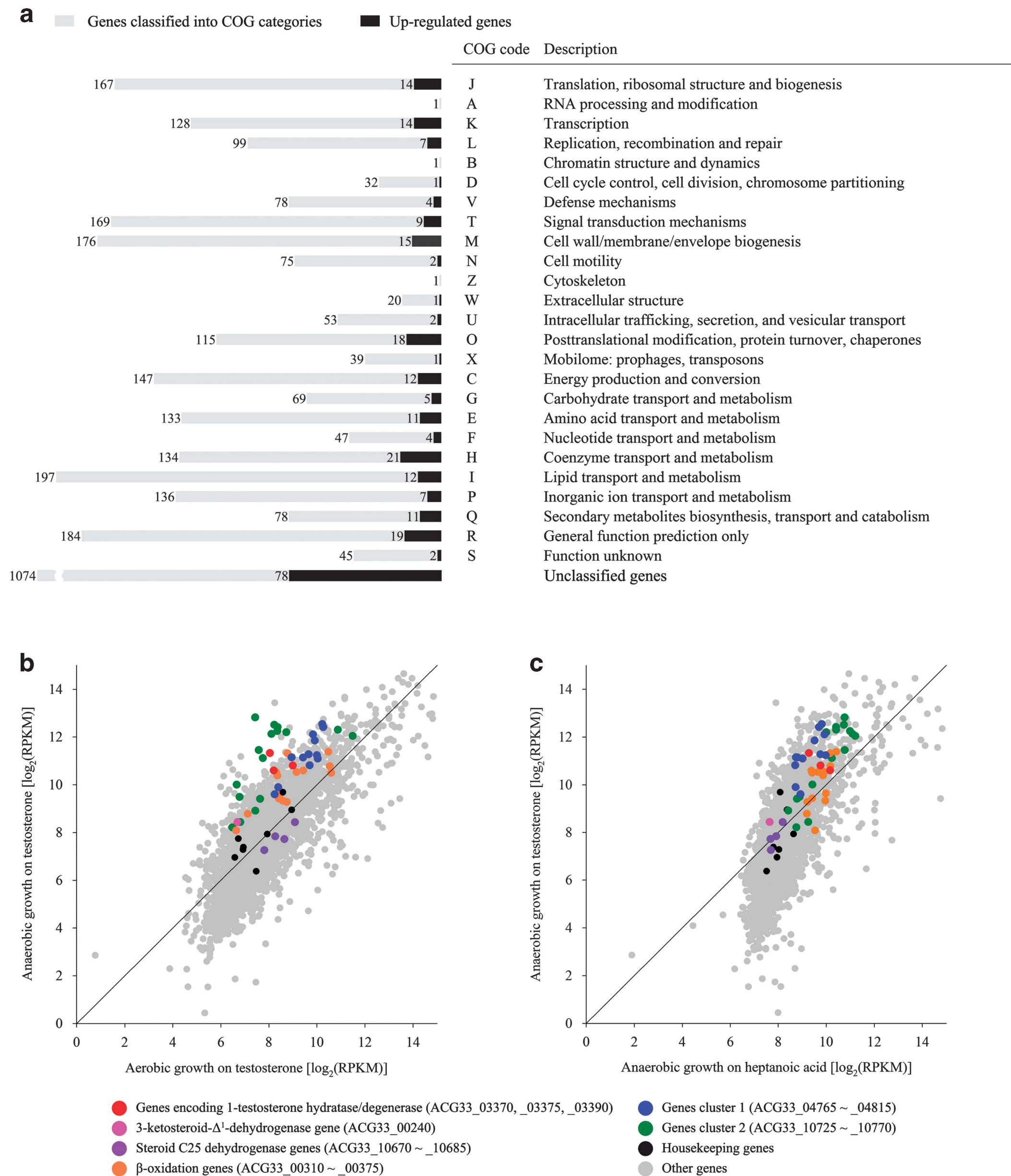

Figure 3 Global gene expression profiles (RNA-Seq) of *Sdo. denitrificans* grown under different conditions. (**a**) The COG classification of the upregulated (greater than twofold) genes during anaerobic growth on testosterone, compared with anaerobic growth on heptanoic acid and aerobic growth on testosterone. Note that some genes are assigned into multiple COG groups. (**b**) Aerobic versus anaerobic growth on testosterone. (**c**) Anaerobic growth on testosterone and heptanoic acid. Each spot represents a gene. Diagonal lines indicate the identical thresholds. The house keeping genes include glucose-6-phosphate 1-dehydrogenase (ACG33_00890), chaperone htpG (ACG33_01365), isocitrate dehydrogenase (ACG33_02930), enolase (ACG33_09515), pyruvate dehydrogenase (ACG33_11195), malate dehydrogenase (ACG33_14070) and type II citrate synthase (ACG33_14495).

testosterone. In addition, 78 upregulated genes coded for hypothetical proteins and could not be classified into any COG categories. We observed the apparent upregulation (threefold) of most genes in cluster 1 (ACG33_4765 ~ ACG33_4815; 11 genes) and cluster 2 (ACG33_10725 ~ ACG33_10770; 10 genes) during anaerobic growth with testosterone (Figure 3b). Most genes in cluster 1 encoded proteins involved in membrane and cell wall biogenesis. Most genes in cluster 2 exhibited high sequence identity (65–86%) to the uncharacterized proteins of *Azoarcus toluclasticus*, and some genes in this cluster (for example, ACG33_10730 encoding an acetyl-CoA acetyltransferase and ACG33_10735 coding for a short-chain dehydrogenase) were proposed to be involved in lipid metabolism (Table 1). Furthermore, the specific expression of genes involved in the uptake and biosynthesis of the enzyme cofactors, for instance, genes encoding a TonB-dependent receptor (for siderophore uptake; ACG_02125), cobalamin-bind protein (ACG_04760), and Fe-S cluster assembly protein (ACG_06585) was observed. Moreover, genes encoding the molybdopterin biosynthesis proteins MoeA (ACG_02290) and MoeC (ACE_2280) and the molybdenum ABC transporter (ACG_10875) were upregulated in cells anaerobically grown on testosterone (Supplementary Table S2).

The transcriptomic analysis revealed the apparent upregulation (greater than threefold), compared with aerobic growth on testosterone, of three clustered genes (ACG_03370, 3375 and 3390) during anaerobic growth on testosterone (Figure 3b). Compared with *Sdo. denitrificans* grown anaerobically on heptanoic acid (Figure 3c), these three genes were upregulated, albeit to a lesser extent, in anaerobically testosterone-grown cells. Deduced amino acid sequences of these upregulated genes exhibited sequence identities of 38–64% to those of the 3-hydroxycyclohexanone dehydrogenase genes of the cyclohexanol-degrading *Ali. denitrificans* (Jin *et al.*, 2011). We previously hypothesized that a MhyADH-like molybdoenzyme might catalyze the hydration of 1-testosterone (Wang *et al.*, 2013B). Similar genes were harbored by *Stl. denitrificans* and closely related betaproteobacteria (Figure 2d). The majority of the clustered β-oxidation genes (ACG33_00310 ~ ACG33_00375) were slightly upregulated (less than twofold) in the anaerobically testosterone-grown cells, compared with those aerobically grown on testosterone. We suggest that *Sdo. denitrificans* uses the β-oxidation enzymes to degrade the C- and D-rings of testosterone regardless of the oxygen conditions. This suggestion is based on (i) the highly conserved primary structures (up to 88%) of the β-oxidation enzymes between the denitrifying *Sdo. denitrificans* and the strictly aerobic *C. testosteroni*, and (ii) the common expression of these β-oxidation genes under both anoxic and oxic conditions. The expression profile of the putative steroid C25 dehydrogenase genes and the 3-ketosteroid Δ^1-dehydrogenase gene in the *Sdo. denitrificans* cells aerobically and anaerobically grown on testosterone was indistinguishable (less than twofold) (Figure 3b).

Purification and characterization of 1-testosterone hydratase/dehydrogenase (AtcABC) from Sdo. denitrificans

The androgen metabolite profile (Wang *et al.*, 2013B) and genomic and transcriptomic (this study) analyses suggested the involvement of a MhyADH-like molybdoenzyme in anaerobic testosterone catabolism by *Sdo. denitrificans*. To prove our hypothesis, AtcABC was purified from the cell extract of testosterone-grown *Sdo. denitrificans* in three chromatographic steps (Figure 4a). A spectrophotometric assay with 1-testosterone as substrate and DCPIP as artificial electron acceptor was developed for activity measurements during purification of this bifunctional enzyme. The enzyme assay was performed aerobically at 30 °C and the transformation of the substrate to 17-hydroxy-androstan-1,3-dione was monitored at 578 nm. The identity of the reaction product was confirmed through UPLC-APCI-MS (Figure 4b). SDS–polyacrylamide gel electrophoresis analysis of the active gel filtration pool showed three protein bands with the following molecular masses: large protein (AtcA), ~85 kDa; medium protein (AtcB), ~30 kDa; and small protein (AtcC), ~18 kDa (Figure 4a). Liquid chromatography-tandem mass spectrometry analysis revealed that tryptic peptides originating from the protein bands AtcA, AtcB and AtcC are identical to the predicted tryptic products of genes ACG33_03390, ACG33_03375 and ACG33_03370, respectively.

Purified AtcABC had a pH optimum of 6.5 (Supplementary Figure S2A) and an apparent *Km* of 52 ± 9 μM for the natural substrate 1-testosterone. The V_{max} of 1-testosterone oxidation was 169 ± 12 nmol min^{-1} mg protein^{-1} (see Supplementary Figure S2B for the Michaelis–Menten plot). The substrate preference of AtcABC was tested by screening various compounds (125 μM) with an α,β-unsaturated ketone structure (Figure 4c). The molybdoenzyme exhibited the highest dehydrogenation activity toward 3-keto-1-en-steroids. The 3-keto-4-en-steroids could not serve as substrates, suggesting that an unsaturated double bond at the C-4/C-5 positions of steroids hinders dehydrogenase activity. Unlike MhyADH, AtcABC could not recognize the monocyclic substrate 2-cyclohexenone (Figure 4c). The apparent substrate preference of AtcABC enables the application of *atcABC* genes as molecular biomarkers to assess anaerobic androgen biodegradation in the environment.

Phylogenetic analyses of essential catabolic enzyme involved in the anaerobic pathway

We used the AtcA sequence of *Sdo. denitrificans* as a query to blast the protein database of NCBI. The most

Table 1 Expression profile of the *Sdo. denitrificans* genes which are probably involved in anaerobic testosterone degradation

Locus tag	*Length*	*Definition*	*Target species*	*E-value*	*Identity (%)*	*COG*	*RPKM value*		
							Heptanoic acid (anaerobic)	*Testosterone (anaerobic)*	*Testosterone (aerobic)*
240	1671	3-Ketosteroid-delta1-dehydrogenase	*Stl. denitrificans* DSM13999	0	54	C	200	346	103
310	876	CoA-transferase alpha subunit (ORF1)	*C. testosteroni* TA441	3.00e−173	76	I	1153	1759	1486
315	768	CoA transferase beta subunit (ORF2)	*C. testosteroni* TA441	1.00e−141	72	I	681	1449	1545
320	435	Subunit of benzoylsuccinyl-CoA thiolase	Gamma proteobacterium HdN1	1.00e−56	58	R	1363	2666	1422
325	1185	Lipid-transfer protein	*Nevskia soli*	0	85	I	1013	798	359
330	1083	Enoyl-ACP reductase (ORF4)	*C. testosteroni* TA441	0	79	R	995	645	384
335	756	Enoyl-CoA hydratase/isomeras family (ORF5)	*C. testosteroni* TA441	1.00e−149	84	I	969	1477	569
340	1155	Acyl-CoA dehydrogenase (ORF21)	*C. testosteroni* TA441	0	75	I	666	1544	689
345	1074	Acyl-CoA dehydrogenase (ORF22)	*C. testosteroni* TA441	4.00e−148	62	I	932	1337	327
350	1155	Thiolase (ORF23)	*C. testosteroni* TA441	0	88	I	803	1483	315
355	876	Short-chain dehydrogenase/reductase (ORF27)	*C. testosteroni* TA441	6.00E-170	77	I, Q, R	1139	2557	437
360	1119	Acyl-CoA dehydrogenase (ORF28)	*C. testosteroni* TA441	4.00e−77	41	I	740	271	100
365	1170	Acyl-CoA dehydrogenase (ORF30)	*C. testosteroni* TA441	3.00e−180	64	I	593	621	429
370	786	Short-chain dehydrogenase/reductase (ORF31)	*C. testosteroni* TA441	3.00e−148	79	I, Q, R	584	440	139
375	459	MaoC domain protein (ORF32)	*C. testosteroni* TA441	9.00e−82	72	I	684	690	340
3370	492	1-Testosterone hydratase/dehydrogenase (AtcC)	*Sdo. denitrificans* DSM 18526	0	100	C	868	1791	511
3375	888	1-Testosterone hydratase/dehydrogenase (AtcB)	*Sdo. denitrificans* DSM 18526	0	100	C	621	2571	264
3390	2418	1-Testosterone hydratase/dehydrogenase (AtcA)	*Sdo. denitrificans* DSM 18526	0	100	C	1144	1552	294
4765	711	Methyltransferase type 12	*Microgenomates (Amesbacteria) bacterium*	2.48e−11	17	C	900	5957	1193
4770	912	LPS biosynthesis protein	*Chloroherpeton thalassium* ATCC 35110	4.56e−49	37	M	1006	2411	1024
4775	1134	Hypothetical protein	*Paenibacillus ginsengihumi*	1.04e−21	22	—	517	2188	1047
4780	618	Imidazole glycerol phosphate synthase	*Aquimarina muelleri*	4.80e−68	48	E	829	5472	1227
4785	1071	ExsB family transcriptional regulator	*Bordetella trematum*	8.00e−148	54	J	972	4420	913
4790	1152	Glycoside hydrolase	*Achromobacter* sp.	2.00e−82	37	M	731	3695	964
4795	1116	Hypothetical protein	*Bacillus ligniniphilus*	1.55e−113	47	M	865	2479	807
4800	855	Hypothetical protein	*Bdellovibrio* sp. ArHS	9.10e−64	26	M	427	2268	494
4805	768	Putative methyltransferase	*Candidatus* Scalindua brodae	2.00e−95	52	H	487	777	303
4810	1140	Cell wall biogenesis protein	*Burkholderiales bacterium* JOSHI_001	2.01e−168	63	M	422	955	336
4815	765	dTDP-6-deoxy-L-hexose 3-*o*-methyltransferase	*Sphingomonas* sp. UNC305MFCol5.2	2.00e−135	73	—	418	1802	831
10 670	531	Putative ethylbenzene dehydrogenase (S25dD4)	*Stl. denitrificans* DSM 13999	9.00e−37	38	I	241	229	308
10 675	1134	Putative steroid C25 dehydrogenase (S25dB4)	*Stl. denitrificans* DSM 13999	0	68	I	205	211	400
10 680	660	Putative steroid C25 dehydrogenase (S25dC4)	*Stl. denitrificans* DSM 13999	7.00e−19	40	I	291	345	543
10 685	2796	Putative steroid C25 dehydrogenase (S25dA4)	*Stl. denitrificans* DSM 13999	0	39	I	207	153	224
10 725	2130	Hypothetical protein	*Ottowia thiooxydans*	0	49	C	474	719	110
10 730	2361	Acetyl-CoA acetyltransferase	*Burkholderia* sp. MSh1	0	59	I	686	1028	101
10 735	759	Short-chain dehydrogenase	*Cyanothece* sp. PCC 8801	1.46e−70	47	I, Q, R	1201	2222	215
10 740	996	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	0	81	H, R	2032	4863	326
10 745	930	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	2.00e−144	68	H, R	1359	5473	329
10 750	1155	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	2.00e−146	63	I	1735	7240	172
10 755	1176	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	0	78	I	1746	2805	191
10 760	1074	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	0	78	H, R	2183	4481	276
10 765	1017	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	0	88	H, R	1708	5837	299
10 770	1656	Hypothetical protein	*Azo. toluclasticus* ATCC 700605	0	80	I, Q, R	1027	4709	423

Abbreviations: LPS, lipopolysaccharide; RPKM, reads per kilobase per million mapped reads.
The genes in cluster 1 (ACG33_4765 ~ 4815) and cluster 2 (ACG33_10725 ~ 10770) may be accessory genes that are activated during anaerobic growth on testosterone.

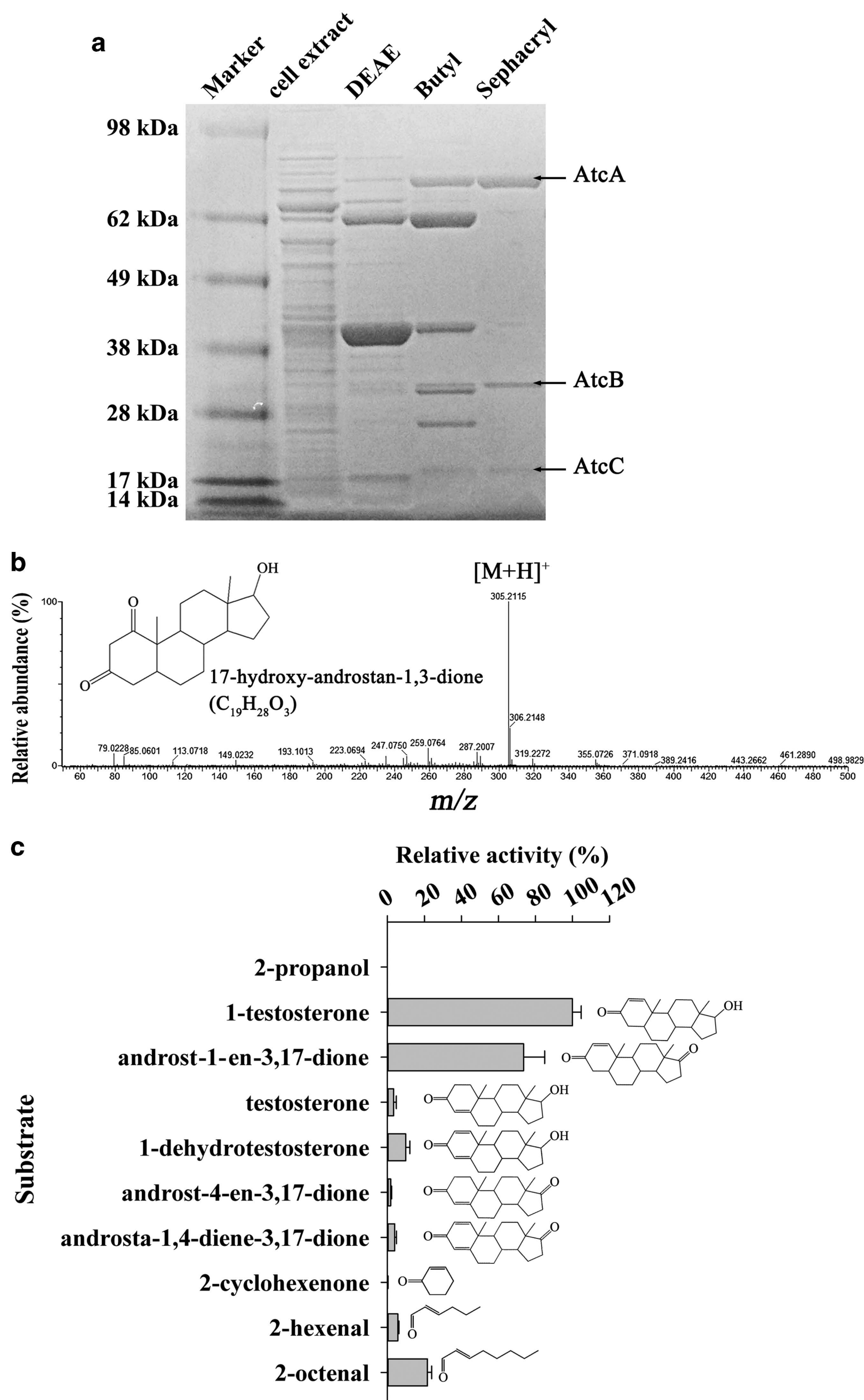

Figure 4 Purification and characterization of 1-testosterone hydratase/dehydrogenase (AtcABC) from *Sdo. denitrificans* grown anaerobically on testosterone. (**a**) SDS–polyacrylamide gel electrophoresis (4% to 12%) of active pools during the fast protein liquid chromatography purification of AtcABC. (**b**) APCI-MS spectrum of the reaction product 17-hydroxy-androstan-1,3-dione. (**c**) Substrate preference of AtcABC determined using a DCPIP-based enzyme assay. The assay mixtures (0.9 ml) contained 20 mM Tris-HCl (pH 7.5), 5 μg purified enzyme, 50 μM DCPIP, 125 μM of individual substrates and 1.25% 2-propanol. 2-Propanol was used as the solvent. Abbreviations: DEAE, DEAE sepharose; Butyl, butyl sepharose; Sephacryl, Sephacryl S-300.

similar sequences were harbored by denitrifying betaproteobacteria (Figure 5 and Supplementary Table S3), which showed an identity of ~60% with the *Sdo. denitrificans* sequence. The AtcA-like proteins of *Thauera terpenica* and *Azo. toluclasticus* are completely uncharacterized. By contrast, the activity of 1-testosterone hydratase/dehydrogenase was detected in the cytoplasm of *Stl. denitrificans* (Lin *et al*., 2015), although the corresponding genes were not identified. The *Sdo. denitrificans* AtcA shows a low sequence identity (38%) with the large subunit of 3-hydroxycyclohexanone dehydrogenase

Figure 5 Phylogenetic tree of the members of the xanthine oxidase family based on the amino acid sequences of molybdopterin-containing subunits. Supplementary Table S3 provides detailed information on these molybdoenzymes. The phylogenetic trees were constructed using the neighbor-joining method with Jukes–Cantor parameter and a bootstrap value of 1000. Abbreviations: CHO, aldehyde; CO, carbon monoxide; DH, dehydrogenase; 1-t, 1-testosterone.

of *Ali. denitrificans*; this result is consistent with the substrate preference of AtcABC (Figure 4c). Moreover, we used the sequence of *Sdo. denitrificans* AtcA as a query to blast UniProtKB/Swiss-Prot, in which the protein functions are experimentally confirmed. Members of the xanthine oxidase family were the most similar proteins extracted from this database (see Supplementary Table S3 for detailed information). These proteins introduce a hydroxyl group into substrates and use water as the oxygen atom source (Leimkühler *et al.*, 2004; Okamoto *et al.*, 2004). Phylogenetic analysis (Figure 5) revealed that AtcABC and its highly similar proteins formed a distinct clade and were separated from other members of this enzyme family. The distribution of AtcABC-like proteins in bacteria therefore suggested that the anaerobic androgen degradation ability is conserved in proteobacteria (mainly beta-subgroup).

UPLC-MS/MS identification of androgenic signature metabolites in DHSTP sewage

The water and sewage samples were collected in June 2014. Four androgens (testosterone; androst-4-en-3,17-dione; androsta-1,4-diene-3,17-dione; and epiandrosterone) in the influent and effluent water of DHSTP were quantified through liquid chromatography-mass spectrometry (Supplementary Table S4). Our data indicated that androgens (approximately 10 μg l^{-1}, mainly testosterone) in the domestic wastewater of Taipei City were efficiently removed (> 99%). DHSTP sewage incubated under different conditions was sampled every 12 h, extracted using ethyl acetate, and the androgenic metabolites were identified through UPLC-APCI-MS/MS (Figure 6 and Supplementary Figure S3). The androgenic activity of the ethyl acetate extracts was determined using the *lacZ*-based yeast androgen assay (Supplementary Figure S4).

Testosterone transformation was not observed in autoclaved anoxic sewage incubated with testosterone and nitrate (Supplementary Figure S3). The biotransformation of testosterone to androst-4-en-3,17-dione was observed in anoxic sewage incubated with testosterone (1 mM) but without nitrate (Figure 6a). However, androgens in this treatment were not apparently consumed. By contrast, testosterone was exhausted after 72 h of incubation under denitrifying conditions (Figure 6b). The drastic decrease in androgenic activity with time confirmed androgen biodegradation in the denitrifying sewage (Supplementary Figure S4). UPLC-APCI-MS analysis revealed the biotransformation of testosterone to other androgens through redox reactions within the first 2 days. Unlike androgenic metabolites with a sterane structure, the ring-cleavage product 17-hydroxy-1-oxo-2,3-*seco*-androstan-3-oic acid (2,3-SAOA) cannot be easily ionized. Thus, we employed extracted ion current for *m/z* 305.21 (the most dominant ion peak of 2,3-SAOA) to detect 2,3-SAOA (Figure 6c). The UPLC retention time (5.0 min) and the MS/MS spectrum of the extracted ion were the same as those of the authentic standard (Figure 6d). In addition, 2,3-SAOA was detected in anoxic sewage collected in April 2014 (Supplementary Figure S5) during denitrifying incubation with

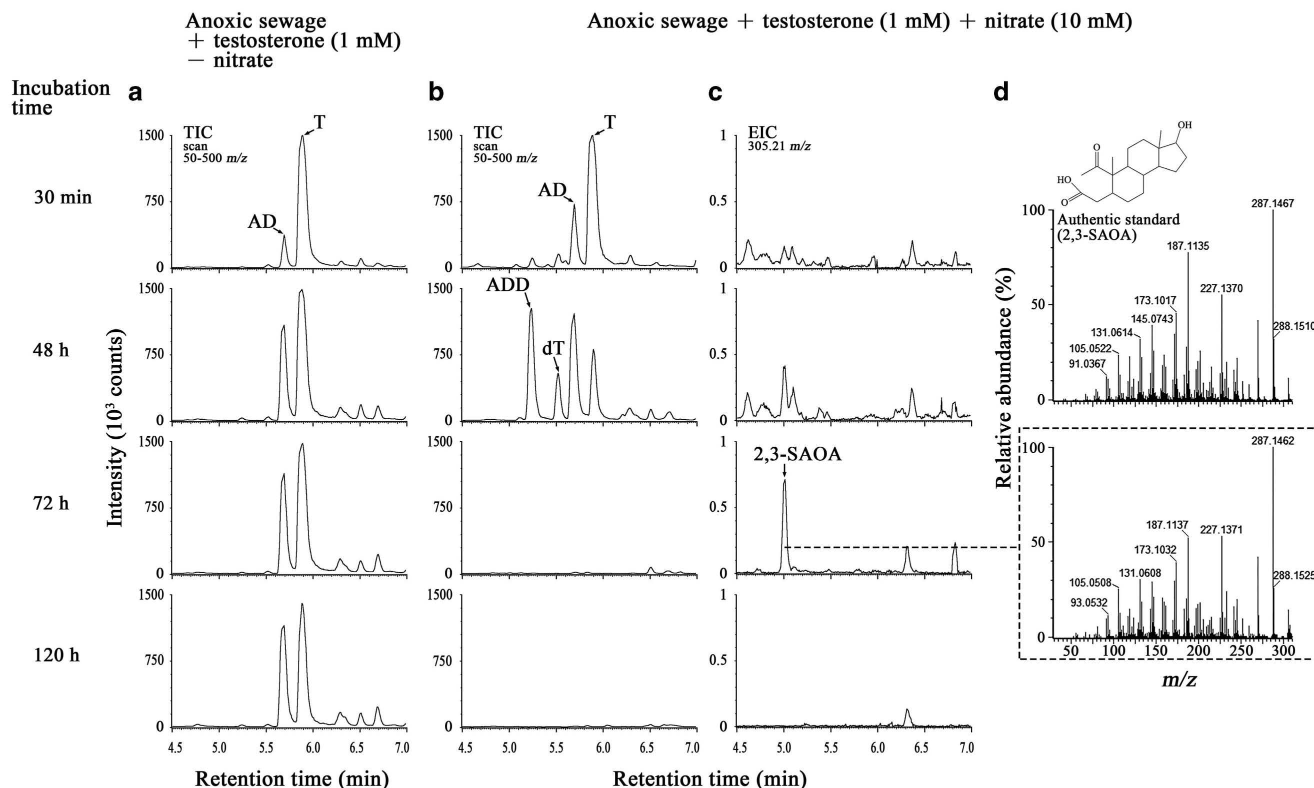

Figure 6 UPLC-APCI-MS/MS analysis of the ethyl acetate extracts of the anoxic DHSTP sewage treatments. (**a**) Total ion chromatograms of anoxic sewage incubated with testosterone but without nitrate. (**b**) Total ion chromatograms of anoxic sewage incubated with testosterone and nitrate. (**c**) Extracted ion chromatograms ($m/z = 305.21$ for 2,3-SAOA) of anoxic sewage incubated with testosterone and nitrate. (**d**) MS/MS spectra of the authentic standard (top) and 2,3-SAOA extracted from anoxic sewage incubated with testosterone and nitrate (bottom). See Figure 1 for the abbreviations of androgens.

testosterone. In contrast, the signature metabolite of the aerobic 9,10-*seco* pathway, 3-hydroxy-9,10-*seco*-androsta-1,3,5(10)-triene-9,17-dione (3-HSA), was not detected in anoxic sewage. UPLC-APCI-MS/MS analysis indicated that denitrifying bacteria in anoxic sewage used the 2,3-*seco* pathway to degrade testosterone.

Phylogenetic identification of androgen-degrading bacteria in anoxic sewage

DNA was extracted from sewage samples, and the V3-V4 hypervariable region of bacterial 16S rDNA sequences was amplified through PCR. The resulting amplicons were sequenced using an Illumina MiSeq sequencer. For each sewage treatment, an average of 341 585 reads was obtained and the sequences were analyzed at the genus level by using the BaseSpace 16S Metagenomics app V1.01 (Illumina). The nucleotide sequence data set was deposited in the NCBI Sequence Read Archive under the accession number SRP062202. Except for the unclassified and other (individual genus with a relative percentage of $<1\%$) organisms, overall, 35 genera were identified in anoxic DHSTP sewage (Supplementary Figure S6). Among them, *Lewinella* was common in all sewage treatments, regardless of the incubation conditions. The duplicates of individual sewage treatments exhibited high similarity in bacterial community structures (Supplementary Figure S6).

The relative abundance of *Thauera* spp. was extremely low (~0.5%) in the initial anoxic sewage. During denitrifying incubation with testosterone, *Thauera* was apparently enriched with time in anoxic sewage (14% abundance after 96 h) (Figure 7a). The growth of *Thauera* spp. did not improve in anoxic sewage incubated with testosterone or nitrate alone.

PCR amplification of atcA-like genes in anoxic sewage

Degenerate gene-specific primers were used for detecting *atcA* in anoxic sewage. Chromosomal DNA was isolated from androgen-degrading anaerobes, *Sdo. denitrificans*, *Stl. denitrificans* and *T. terpenica* 58Eu. We found that *T. terpenica* is indeed able to degrade testosterone under denitrifying conditions (Supplementary Figure S7). The negative controls were DNA from strictly aerobic androgen degraders, *C. testosteroni*, *Gordonia cholesterolivorans* (Wang *et al.*, 2013a), and *Sphingomonas* sp. KC8 (Roh and Chu, 2010). PCR products with the expected size of ~1100 bp were amplified from the androgen-degrading anaerobes but not from the androgen-degrading aerobes (Figure 7bI). Note that the *atcA* gene probe

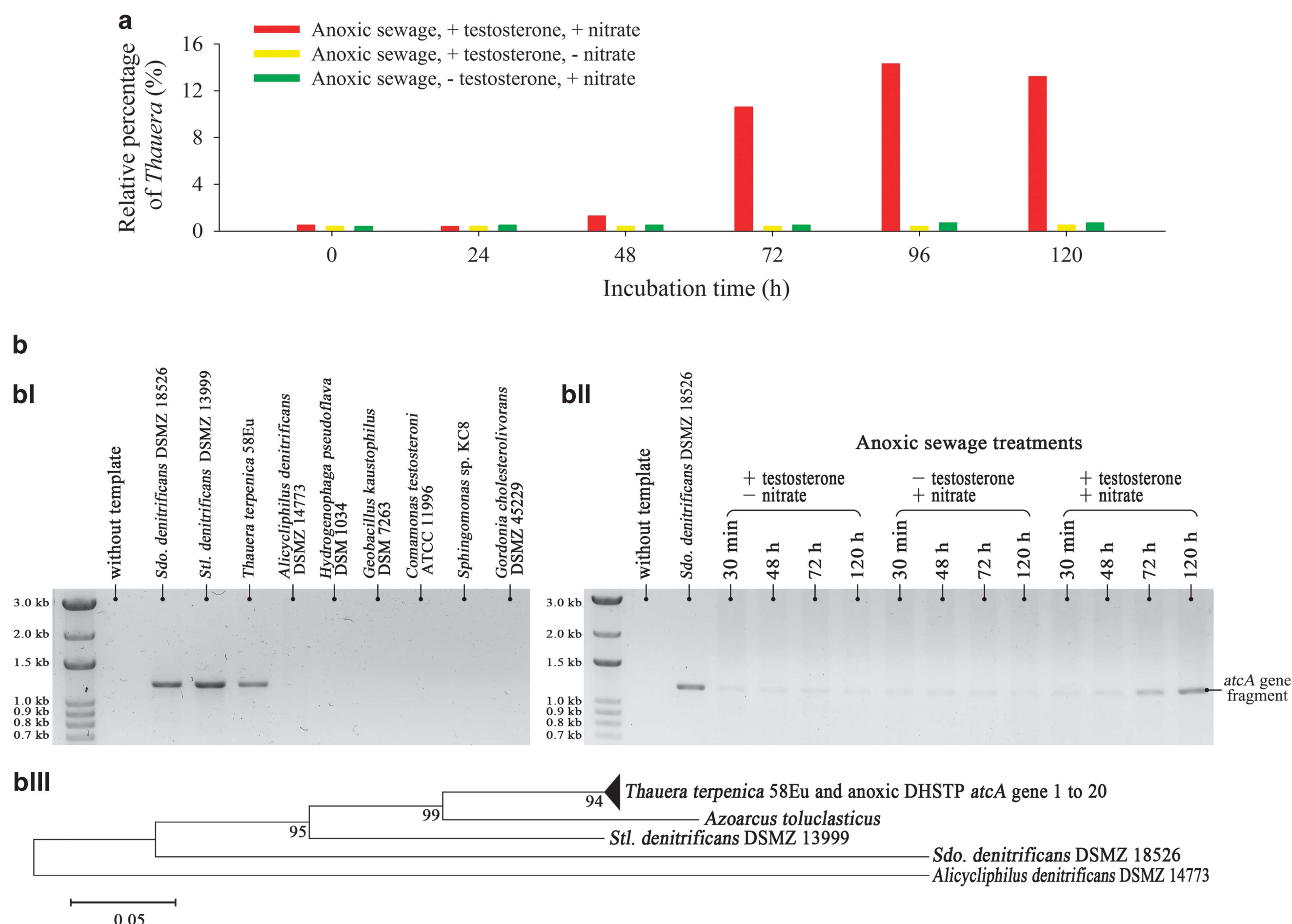

Figure 7 (**a**) Genus-level phylogenetic analysis (Illumina MiSeq) revealed the enrichment of *Thauera* spp. in anoxic sewage incubated with testosterone and nitrate. See Supplementary Figure S6 for detailed information. (**b**) PCR-based functional assay using degenerate primers (see Supplementary Figure S1 for sequences) derived from the *atcA* genes. (bI) Agarose gel electrophoresis showed that the *atcA* genes are harbored only by androgen-degrading anaerobes. (bII) Temporal increase in *atcA*-specific PCR products was observed only in anoxic DHSTP sewage incubated with testosterone and nitrate. (bIII) Neighbor-joining tree of *atcA* gene fragments obtained from anoxic DHSTP sewage incubated with testosterone and nitrate for 120 h. *atcA*-like sequences are shown in Supplementary Figure S8. The gene encoding the large subunit (MhyADHL) of 3-hydroxycyclohexanone dehydrogenase from *Ali. denitrificans* served as an outgroup sequence.

could not be used to detect the related genes (33–39% sequence identity), including the 3-hydroxycyclohexanone dehydrogenase gene of *Ali. denitrificans*, the gene encoding the molybdopterin-binding protein (accession number: WP 011229495) of *Geobacillus kaustophilus*, and the gene coding for the carbon monoxide dehydrogenase (accession number: P19913) of *Hydrogenophaga pseudoflava*, under the PCR conditions used.

To investigate the phylogeny of the *atcA*-harboring bacteria enriched in anoxic sewage incubated with testosterone and nitrate, DNA extracted from three treatments of anoxic DHSTP sewage was used as the nucleotide template. During denitrifying incubation with testosterone, the amount of PCR products in anoxic sewage increased with time (Figure 7bII). By contrast, the amount of PCR products did not increase in anoxic sewage incubated with testosterone or nitrate alone. PCR products amplified from denitrifying sewage (120 h of incubation with testosterone) were cloned in *E. coli*. Twenty clones were randomly selected for sequencing. The DNA fragments revealed minor differences in nucleotide sequences (Supplementary Figure S8). According to the results of phylogenetic analysis, all partial *atc*A sequences showed the highest similarities to that from *T. terpenica* (Figure 7bIII).

Discussion

We adopted a holistic approach to investigate anaerobic steroid biodegradation in the environment. This included the detection of signature metabolites, profiling of the community structure, and detection of specific functional genes. It was thus necessary to begin by investigating the genome and transcriptome of *Sdo. denitrificans* anaerobically grown on testosterone. This enabled the identification of relevant biomarkers that could be used in our environmental study. We purified and characterized 1-testosterone hydratase/dehydrogenase

(AtcABC) and the corresponding genes were identified in the *Sdo. denitrificans* genome. The phylogenetic analysis of the sequences of AtcABC suggested that this enzyme belongs to the xanthine oxidase family containing molybdopterin, FAD, and iron-sulfur clusters. Nonetheless, the prosthetic groups of AtcABC are yet to be experimentally confirmed. The transcriptomic analysis revealed the upregulation of the *atc* genes during anaerobic growth on testosterone, indicating that AtcABC is crucial for anaerobic testosterone catabolism. We also identified the genes coding for a putative molybdoenzyme that is highly similar (39–68%) to a putative steroid C25 dehydrogenase (S25dA4B4C4D4) of *Stl. denitrificans*. This bacterium exhibits an extremely narrow substrate spectrum (limited to a few sterols, androgens, and long-chain fatty acids) and its genome harbors genes coding for eight C25 dehydrogenase-like enzymes, suggesting their roles in the anaerobic hydroxylation of different intermediates of anaerobic steroid catabolism (Dermer and Fuchs, 2012). Moreover, highly similar genes were found in the *T. terpenica* genome, implying their catabolic role in anaerobic testosterone degradation. However, we could not detect the specific expression of the putative steroid C25 dehydrogenase genes in the *Sdo. denitrificans* cells anaerobically grown on testosterone. Nevertheless, the catabolic function of this gene cluster remains to be unraveled. The genes encoding the redox enzymes (for example, 3-ketosteroid Δ^1-dehydrogenase) were also found on the *Sdo. denitrificans* chromosome. Their similar expression during aerobic and anaerobic growth may indicate that these genes function in both aerobic and anaerobic testosterone catabolism. The *tesB* gene was not be found in the chromosome of *Sdo. denitrificans*. The detection of 3,4-DHSA in the aerobically grown cells (Wang *et al*., 2013b) suggested that *Sdo. denitrificans* adopts a *meta*-cleavage dioxygenase to open the steroid A-ring. This bacterium might use an enzyme exhibiting a low sequence similarity with the *tesB* gene product of *C. testosteroni* to aerobically cleave the steroid A-ring. Nonetheless, the possibility that a *tesB*-like gene is harbored in an extra-chromosomal genetic element of *Sdo. denitrificans* cannot be excluded. The presence of catabolic genes in plasmids has been reported in numerous bacteria (Roselli *et al*., 2013; Stolz, 2014).

In the chromosome of *Sdo. denitrificans* (and other androgen-degrading anaerobes, *Stl. denitrificans* and *T. terpenica*), we identified β-oxidation genes whose products were highly similar (62–88% sequence identity) to the enzymes of *C. testosteroni* involved in the degradation of the C- and D-rings of testosterone (Horinouchi *et al*., 2014). A similar β-oxidative mechanism was demonstrated in the degradation of steroid C/D rings by actinobacteria (Casabon *et al*., 2013a, b). β-oxidative enzymes are active under both oxic and anoxic conditions, and a relevant example is the side-chain degradation of cholesterol by *Stl. denitrificans* (Lin *et al*., 2015). Moreover, the β-oxidation genes of *Sdo. denitrificans* were expressed during anaerobic and aerobic growth with testosterone, but they were slightly upregulated under anoxic conditions. One may thus envisage that *Sdo. denitrificans* uses the products of the β-oxidation genes to degrade the C/D rings of testosterone, regardless of oxygen availability. The biochemical mechanism of anaerobic cleavage of the B-ring by *Sdo. denitrificans* is completely unknown. The androgen metabolite profiles (Wang *et al*., 2013B) and genomic and transcriptomic analyses suggested that in the anaerobic pathway of androgen catabolism by *Sdo. denitrificans*, the cleavage of the B-ring may precede the degradation of C/D rings.

The transcriptomic analysis revealed the apparent upregulation of various membrane transporters and at least one gene cluster involved in cell wall and membrane biogenesis during the anaerobic growth of *Sdo. denitrificans* on testosterone. The lipopolysaccharide leaflet on the outer surface of the outer membrane impedes steroid uptake by proteobacteria through passive diffusion (Plésiat and Nikaido, 1992). The biochemical mechanisms involved in susbstrate accession and uptake are thus crucial for the survival of steroid-degrading proteobacteria in the environment. The purification and characterization of the membrane transporters for the uptake of hydrophobic compounds is difficult, and the alternative strategy includes the identification of the transporter genes through mutagenesis. For example, by mutational analysis, Mohn *et al.* (2008) identified the actinobacterial genes encoding the cholesterol transporter. *Sdo. denitrificans* and *Stl. denitrificans* cannot not grow on the media solidified with agar or gelrite, therefore preventing the use of conventional molecular biological techniques. Molecular biology experiments have been performed using various *Thauera* strains (Bhandare *et al*., 2006). Thus, *T. terpenica* 58Eu can be used as another model organism for anaerobic steroid degradation.

The signature metabolite 2,3-SAOA and the 1-testosterone hydratase/dehydrogenase genes were identified in anoxic sewage incubated with testosterone and nitrate, indicating that denitrifying bacteria in anoxic sewage adopt the 2,3-*seco* pathway to degrade testosterone. Although the relative abundance of *Steroidobacter* spp. did not apparently increase during the denitrifying incubation of DHSTP sewage with testosterone (1 mM), our data based on the enrichment experiments cannot exclude the role of *Steroidobacter* in *in situ* androgen degradation. Considering the highly conserved catabolic strategy for anaerobic androgen degradation, the genetic information obtained from this model microorganism serves as a basis for environmental investigations.

The quantification of androgens in the influent and effluent water samples from DHSTP suggested that most androgens (> 99%) were degraded by the

sewage microorganisms. Bacteria belonging to Saprospiraceae (mainly *Lewinella*) were dominant microorganisms in the anoxic sewage of DHSTP; however, their abundance appeared stable in different treatments. Although most members of Saprospiraceae are marine species, some strains were isolated from activated sludge (Kragelund *et al.*, 2008; Xia *et al.*, 2008; Nielsen *et al.*, 2009). The members of Saprospiraceae are capable of hydrolyzing complex carbon sources, including carbohydrates and proteins (Kämpfer, 1995; Xia *et al.*, 2007). Based on our results, we posit that the members of Saprospiraceae may be involved in the degradation of organic compounds other than androgens in sewage.

The redox transformations of testosterone to other androgens were observed in denitrifying sewage incubated with testosterone for 30 min. However, *Thauera* was not enriched until 72 h of the treatment. In addition, the transformation of testosterone to 1-dehydrotestosterone was detected in anoxic sewage incubated without nitrate, where the abundance of *Thauera* spp. was extremely low. Our data thus suggested that in the denitrifying DHSTP sewage, testosterone was first transformed to other androgens via redox reactions by various microorganisms, including the initial *Thauera* spp. The redox transformations of steroids have been reported in numerous microorganisms, including bacteria (Kisiela *et al.*, 2012) and fungi (Kristan and Rižner, 2012). For example, the 3-ketosteroid Δ^1-dehydrogenase and cholesterol dehydrogenase of *Stl. denitrificans* can function under both oxic and anoxic conditions (Chiang *et al.*, 2008a, b). After the initial redox transformations of testosterone, *T. terpenica* cleaved the steroidal core ring, extracted the carbons and energy from steroids, and began proliferating. *T. terpenica* can degrade bicyclic terpenoids (Foss and Harder, 1998); however, its ability to degrade steroids has not been reported. Our data indicated that *T. terpenica* 58Eu (DSMZ 12139) anaerobically degrades testosterone also through the 2,3-*seco* pathway (Supplementary Figure S7). Moreover, the genome of *T. terpenica* harbors the clustered genes that are highly similar to those involved in anaerobic testosterone catabolism by *Sdo. dentirificans*. Although *T. terpenica* is not a dominant organism in the DHSTP sewage, which usually contains androgens at approximately 10 μg l^{-1} (35 nM), this bacterium can efficiently respond to the pulsed androgen input (1 mM in this study), suggesting that *T. terpenica* can be used in the bioremediation of androgen-contaminated ecosystems.

A noticeable feature of *Sdo. denitrificans* is its narrow substrate spectrum, which is restricted to a few steroids (natural estrogens and androgens), fatty acids (C2–C7), and glutamate (Fahrbach *et al.*, 2008). Moreover, it cannot grow on complex media composed mainly of peptides and carbohydrates. Analyzing the *Sdo. denitrificans* genome reveals the lack of catabolic genes of alkanes, aliphatic alcohols, long-chain fatty acids, monoaromatic compounds and carbohydrates, which are abundant in sewage (Harrison *et al.*, 2006). By contrast, *T. terpenica* can utilize a number of organic compounds as carbon and energy sources (Foss and Harder, 1998), which is reflected by its ability to grow on complex media. The versatile metabolic capability of *T. terpenica* may explain why this organism, but not *Sdo. denitrificans*, was apparently enriched in the testosterone-incubated anoxic sewage. Investigating the utilization kinetics of *Sdo. denitrificans* and *T. terpenica* toward testosterone may also provide an explanation for the overwhelming enrichment of *T. terpenica* in the anoxic sewage incubated with testosterone.

Catabolic genes involved in the biodegradation of complex compounds have been used as molecular markers in environmental investigations (Kuntze *et al.*, 2011). We propose the use of the *atcA* gene as the biomarker for anaerobic steroid biodegradation because (i) AtcABC catalyzes a crucial hydration/dehydrogenation reaction in anaerobic steroid catabolism, including androgens and cholesterol, (ii) AtcABC exhibits a narrow substrate spectrum and (iii) the large subunit (AtcA) contains the unusual molybdopterin-binding domain. Although AtcABC exhibits some sequence similarity ($>38\%$) to 3-hydroxycyclohexanone dehydrogenase from *Ali. denitrificans*, it cannot transform the monocyclic compounds. This observation is reflected by the inability of *Sdo. denitrificans* to grow on cyclohexanol (Fahrbach *et al.*, 2008). We observed a temporal increase in *atcA* PCR products during the denitrifying incubation of sewage samples with testosterone. This increase was absent in sewage incubated with testosterone or nitrate alone. These results show that AtcABC is involved in anaerobic testosterone biodegradation. Furthermore, they validated *atcA* as a competent and reliable biomarker for monitoring anaerobic steroid biodegradation. Androgens serve as intermediates in both aerobic and anaerobic catabolism of cholesterol (Van der Geize *et al.*, 2007; Wang *et al.*, 2013a). For example, in the anaerobic catabolic pathway, *Stl. denitrificans* degrades the side chain of cholesterol to produce androgens, whose A-ring is then cleaved through the 2,3-*seco* pathway (Lin *et al.*, 2015). Cholesterol and other sterols are highly abundant in the environment. The determination of steroid contents and substrate utilization patterns of the microorganisms in the environmental samples may help to elucidate the metabolic capacity and ecological roles of *atcA*-harboring microorganisms.

Isotope-independent approaches based on the UPLC—tandem mass spectrometry detection of signature metabolites, community structure analysis, and PCR-based functional assays can be used for investigating the bacterial biodegradation of complex and hydrophobic compounds like steroids, for which conventional culture-independent techniques, such as stable-isotope probing and fluorescence *in situ*

hybridization-microautoradiography, are unavailable. Our integrative omics approach is particularly useful for compounds that are highly abundant (for example, sterols, alkanes and aromatic compounds) in the environment because then substrates with a concentration close to detection levels in most natural environments can be used to enrich the potential degraders.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Ministry of Science and Technology of Taiwan (104-2311-B-001-023 -MY3). We thank the Institute of Plant and Microbial Biology, Academia Sinica, for access to the Small Molecule Metabolomics Core Facility (for UPLC-MS/MS analyses) and the Proteomics Core Laboratory (for mass spectrometric protein identification). In addition, we thank the High Throughput Genomics Core Facility of the Biodiversity Research Center in Academia Sinica for index library construction and next-generation sequencing experiments.

References

Adams MA, Teeter JH, Katz Y, Johnson PB. (1987). Sex-pheromones of the sea lamprey (*Petromyzon marinus*) - steroid studies. *J Chem Ecol* **13**: 387–395.

Andersen H, Siegrist H, Halling-Sorensen B, Ternes TA. (2003). Fate of estrogens in a municipal sewage treatment plant. *Environ Sci Technol* **37**: 4021–4026.

Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, 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.

Bhandare R, Calabro M, Coschigano PW. (2006). Site-directed mutagenesis of the *Thauera aromatica* strain T1 tutE tutFDGH gene cluster. *Biochem Biophys Res Commun* **346**: 992–998.

Bortone SA, Davis WP, Bundrick CM. (1989). Morphological and behavioral characters in mosquitofish as potential bioindication of exposure to kraft mill effluent. *Bull Environ Contam Toxicol* **43**: 370–377.

Casabon I, Crowe AM, Liu J, Eltis LD. (2013a). FadD3 is an acyl-CoA synthetase that initiates catabolism of cholesterol rings C and D in actinobacteria. *Mol Microbiol* **87**: 269–283.

Casabon I, Zhu SH, Otani H, Liu J, Mohn WW, Eltis LD. (2013b). Regulation of the KstR2 regulon of *Mycobacterium tuberculosis* by a cholesterol catabolite. *Mol Microbiol* **89**: 1201–1212.

Chang H, Wan Y, Hu J. (2009). Determination and source apportionment of five classes of steroid hormones in urban rivers. *Environ Sci Technol* **43**: 7691–7698.

Chang H, Wan Y, Wu S, Fan Z, Hu J. (2011). Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. *Water Res* **45**: 732–740.

Chen TS, Chen TC, Yeh KJ, Chao HR, Liaw ET, Hsieh CY *et al.* (2010). High estrogen concentrations in receiving river discharge from a concentrated livestock feedlot. *Sci Total Environ* **408**: 3223–3230.

Chiang YR, Ismail W, Gallien S, Heintz D, Van Dorsselaer A, Fuchs G. (2008a). Cholest-4-en-3-one-Δ^1-dehydrogenase: a flavoprotein catalyzing the second step in anoxic cholesterol metabolism. *Appl Environ Microbiol* **74**: 107–113.

Chiang YR, Ismail W, Heintz D, Schaeffer C, Van Dorsselaer A, Fuchs G. (2008b). Study of anoxic and oxic cholesterol metabolism by *Sterolibacterium denitrificans*. *J Bacteriol* **190**: 905–914.

Dermer J, Fuchs G. (2012). Molybdoenzyme that catalyzes the anaerobic hydroxylation of a tertiary carbon atom in the side-chain of cholesterol. *J Biol Chem* **287**: 36905–36916.

Donova MV. (2007). Transformation of steroids by actinobacteria: a review. *Appl Biochem Microbiol* **43**: 1–14.

Fahrbach M, Kuever J, Remesch M, Huber BE, Kämpfer P, Dott W *et al.* (2008). *Steroidobacter denitrificans* gen. nov., sp. nov., a steroidal hormone-degrading gammaproteobacterium. *Int J Syst Evol Microbiol* **58**: 2215–2223.

Fan Z, Wu S, Chang H, Hu J. (2011). Behaviors of glucocorticoids, androgens and progestogens in a municipal sewage treatment plant: comparison to estrogens. *Environ Sci Technol* **45**: 2725–2733.

Foss S, Harder J. (1998). *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, menthol, and eucalyptol) nitrate. *Syst Appl Microbiol* **21**: 365–373.

Ghayee HK, Auchus RJ. (2007). Basic concepts and recent developments in human steroid hormone biosynthesis. *Rev Endocr Metab Disord* **8**: 289–300.

Hanselman TA, Graetz DA, Wilkie AC. (2003). Manure-borne estrogens as potential environmental contaminants: a review. *Environ Sci Technol* **37**: 5471–5478.

Harrison EZ, Oakes SR, Hysell M, Hay A. (2006). Organic chemicals in sewage sludges. *Sci Total Environ* **367**: 481–497.

Horinouchi M, Hayashi T, Kudo T. (2012). Steroid degradation in *Comamonas testosteroni*. *J Steroid Biochem Mol Biol* **129**: 4–14.

Horinouchi M, Hayashi T, Koshino H, Malon M, Hirota H, Kudo T. (2014). Identification of 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid in steroid degradation by *Comamonas testosteroni* TA441 and its conversion to the corresponding 6-en-5-oyl coenzyme A (CoA) involving open reading frame 28 (ORF28)- and ORF30-encoded acyl-CoA dehydrogenases. *J Bacteriol* **196**: 3598–3608.

Howell WM, Black DA, Bortone SA. (1980). Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: Evidence for environmentally-induced masculinization. *Copeia* **4**: 676–681.

Jenkins R, Angus R, McNatt H, Howell W, Kemppainen J, Kirk M *et al.* (2001). Identification of androstenedione in a river containing paper mill effluent. *Environ Toxicol Chem* **20**: 1325–1331.

Jenkins R, Wilson E, Angus R, Howell W, Kirk M. (2003). Androstenedione and progesterone in the sediment of

a river receiving paper mill effluent. *Toxicol Sci* **73**: 53–59.
Jin J, Straathof AJJ, Pinkse MWH, Hanefeld U. (2011). Purification, characterization, and cloning of a bifunctional molybdoenzyme with hydratase and alcohol dehydrogenase activity. *Appl Microbiol Biotechnol* **89**: 1831–1840.
Johnson AC, Sumpter JP. (2001). Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environ Sci Technol* **35**: 4697–4703.
Kämpfer P. (1995). Physiological and chemotaxonomic characterization of filamentous bacteria belonging to the genus *Haliscomenobacter*. *Syst Appl Microbiol* **18**: 363–367.
Katare YK, Scott AP, Laframboise AJ, Li W, Alyasha'e Z, Caputo CB *et al.* (2011). Release of free and conjugated forms of the putative pheromonal steroid 11-oxo-etiocholanolone by reproductively mature male round goby (*Neogobius melanostomus* Pallas, 1814). *Biol Reprod* **84**: 288–298.
Khanal SK, Xie B, Thompson ML, Sung S, Ong SK, van Leeuwent J. (2006). Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ Sci Technol* **40**: 6537–6546.
Kisiela M, Skarka A, Ebert B, Maser E. (2012). Hydroxysteroid dehydrogenases (HSDs) in bacteria: a bioinformatic perspective. *J Steroid Biochem Mol Biol* **129**: 31–46.
Kolodziej EP, Gray JL, Sedlak DL. (2003). Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. *Environ Toxicol Chem* **22**: 2622–2629.
Kragelund C, Levantesi C, Borger A, Thelen K, Eikelboom D, Tandoi V *et al.* (2008). Identity, abundance and ecophysiology of filamentous bacteria belonging to the Bacteroidetes present in activated sludge plants. *Microbiology* **154**: 886–894.
Kristan K, Rižner TL. (2012). Steroid-transforming enzymes in fungi. *J Steroid Biochem Mol Biol* **129**: 79–91.
Kuntze K, Vogt C, Richnow HH, Boll M. (2011). Combined application of PCR-based functional assays for the detection of aromatic-compound-degrading anaerobes. *Appl Environ Microbiol* **77**: 5056–5061.
Leimkühler S, Stocker AL, Igarashi K, Nishino T, Hille R. (2004). The role of active site glutamate residues in catalysis of *Rhodobacter capsulotus* xanthine dehydrogenase. *J Biol Chem* **279**: 40437–40444.
Leu YL, Wang PH, Shiao MS, Ismail W, Chiang YR. (2011). A novel testosterone catabolic pathway in bacteria. *J Bacteriol* **193**: 4447–4455.
Lin AYC, Yu TH, Lateef SK. (2009). Removal of pharmaceuticals in secondary wastewater treatment processes in Taiwan. *J Hazard Mater* **167**: 1163–1169.
Lin AYC, Lin CF, Tsai YT, Lin HHH, Chen J, Wang XH *et al.* (2010). Fate of selected pharmaceuticals and personal care products after secondary wastewater treatment processes in Taiwan. *Water Sci Technol* **62**: 2450–2458.
Lin CW, Wang PH, I, smail W, Tsai YW, El Nayal A, Yang CY *et al.* (2015). Substrate uptake and subcellular compartmentation of anoxic cholesterol catabolism in *Sterolibacterium denitrificans*. *J Biol Chem* **290**: 1155–1169.
Lorenzen A, Hendel JG, Conn KL, Bittman S, Kwabiah AB, Lazarovitz G *et al.* (2004). Survey of hormone activities in municipal biosolids and animal manures. *Environ Toxicol* **19**: 216–225.
Mohn WW, van der Geize R, Stewart GR, Okamoto S, Liu J, Dijkhuizen L. (2008). The actinobacerial *mce4* locus encodes a steroid transporter. *J Biol Chem* **283**: 35368–35374.
Moore A, Scott AP. (1991). Testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L) parr. *Proc R Soc Lond B* **332**: 241–244.
Nielsen PH, Kragelund C, Seviour RJ, Nielsen JL. (2009). Identity and ecophysiology of filamentous bacteria in activated sludge. *FEMS Microbiol Rev* **33**: 969–998.
Okamoto K, Matsumoto K, Hille R, Eger BT, Pai EF, Nishino T. (2004). The crystal structure of xanthine oxidoreductase during catalysis: implications for reaction mechanism and enzyme inhibition. *Proc Natl Acad Sci USA* **101**: 7931–7936.
Orlando EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambright CS *et al.* (2004). Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environ Health Perspect* **112**: 353–358.
Parks LG, Lambright CS, Orlando EF, Guillette Jr LJ, Ankley GT, Gray LE Jr. (2001). Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol Sci* **62**: 257–267.
Plésiat P, Nikaido H. (1992). Outer membranes of Gram-negative bacteria are permeable to steroid probes. *Mol Microbiol* **6**: 1323–1333.
Roh H, Chu KH. (2010). A 17beta-estradiol-utilizing bacterium, *Sphingomonas* strain KC8: part I - characterization and abundance in wastewater treatment plants. *Environ Sci Technol* **44**: 4943–4950.
Roselli S, Nadalig T, Vuilleumier S, Bringel F. (2013). The 380 kb pCMU01 plasmid encodes chloromethane utilization genes and redundant genes for vitamin B12- and tetrahydrofolate-dependent chloromethane metabolism in *Methylobacterium extorquens* CM4: a proteomic and bioinformatics study. *PLoS One* **8**: e56598.
Safe S. (2004). Endocrine disruptors and human health: is there a problem? *Toxicology* **205**: 3–10.
Serrano RM, Lopes O, Hubbard PC, Araújo J, Canário AV, Barata EN. (2008). 11-Ketotestosterone stimulates putative sex pheromone production in the male peacock blenny, *Salaria pavo* (Risso 1810). *Biol Reprod* **79**: 861–868.
Stolz A. (2014). Degradative plasmids from sphingomonads. *FEMS Microbiol Lett* **350**: 9–19.
Tarlera S, Denner EBM. (2003). *Sterolibacterium denitrificans* gen. nov., sp. nov., a novel cholesterol-oxidizing, denitrifying member of the β-Proteobacteria. *Int J Syst Evol Microbiol* **53**: 1085–1091.
Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos M. (1999). Behavior and occurrence of estrogens in municipal sewage treatment plants - I. investigations in Germany, Canada, and Brazil. *Sci Total Environ* **225**: 81–90.
Van der Geize R, Yam K, Heuser T, Wilbrink MH, Hara H, Maarten H *et al.* (2007). A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival

in macrophages. *Proc Natl Acad Sci USA* **104**: 1947–1952.

Wang PH, Lee TH, Ismail W, Tsai CY, Lin CW, Tsai YW *et al.* (2013a). An oxygenase-independent cholesterol catabolic pathway operates under oxic conditions. *PLoS ONE* **8**: e66675.

Wang PH, Leu YL, Ismail W, Tang SL, Tsai CY, Chen HJ *et al.* (2013b). The anaerobic and aerobic cleavage of the steroid core ring structure by *Steroidobacter denitrificans*. *J Lipid Res* **54**: 1493–1504.

Wang PH, Yu CP, Lin CW, Ismail W, Wey SP, Kuo AT *et al.* (2014). Anoxic androgen degradation by denitrifying *Sterolibacterium denitrificans* via the 2,3-*seco*-pathway. *Appl Environ Microbiol* **80**: 3442–3452.

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

Xia Y, Kong Y, Nielsen PH. (2007). *In situ* detection of protein-hydrolysing microorganisms in activated sludge. *FEMS Microbiol Ecol* **60**: 156–165.

Xia Y, Kong Y, Thomsen TR, Nielsen PH. (2008). Identification and ecophysiological characterization of epiphytic protein hydrolyzing *Saprospiraceae* (''*Candidatus* Epiflobacter'' spp.) in activated sludge. *Appl Environ Microbiol* **74**: 2229–2238.

Yang YY, Pereyra LP, Young RB, Reardon KF, Borch T. (2011). Testosterone-mineralizing culture enriched from swine manure: characterization of degradation pathways and microbial community composition. *Environ Sci Technol* **45**: 6879–6886.

Yamamoto A, Kakutani N, Yamamoto K, Kamiura T, Miyakoda H. (2006). Steroid hormone profiles of urban and tidal rivers using LC/MS/MS equipped with electrospray ionization and atmospheric pressure photoionization sources. *Environ Sci Technol* **40**: 4132–4137.

Ying GG, Kookana RS, Ru YJ. (2002). Occurrence and fate of hormone steroids in the environment. *Environ Int* **28**: 545–551.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)