Minireview

Microbial degradation of steroid sex hormones: implications for environmental and ecological studies

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Summary
Steroid hormones modulate development, reproduction and communication in eukaryotes. The widespread occurrence and persistence of steroid hormones have attracted public attention due to their endocrine-disrupting effects on both wildlife and human beings. Bacteria are responsible for mineralizing steroids from the biosphere. Aerobic degradation of steroid hormones relies on O2 as a co-substrate of oxygenases to activate and to cleave the recalcitrant steroidal core ring. To date, two oxygen-dependent degradation pathways – the 9,10-seco pathway for androgens and the 4,5-seco pathways for oestrogens – have been characterized. Under anaerobic conditions, denitrifying bacteria adopt the 2,3-seco pathway to degrade different steroid structures. Recent meta-omics revealed that microorganisms able to degrade steroids are highly diverse and ubiquitous in different ecosystems. This review also summarizes culture-independent approaches using the characteristic metabolites and catabolic genes to monitor steroid biodegradation in various ecosystems.

Introduction
Thus far, more than 1000 different steroids are found to naturally occur (Haubrick and Assmann, 2006; Hannich et al., 2011; Valitova et al., 2016; Zubair et al., 2016; Staley et al., 2017; Stonik and Stonik, 2018), including commonly distributed sterols (e.g. cholesterol, phytosterols and ergosterol), steroid hormones (e.g. 17β-oestradiol, progesterone and testosterone) and bile acids (e.g. cholic acid) (see Fig. 1 for the common steroid structures). A remarkable characteristic of steroids is their extremely low aqueous solubility; that is, cholesterol has a maximum solubility of 4.7 μM (= 1.8 mg l−1) in aqueous solutions (Haberland and Reynolds, 1973). The aqueous solubility of steroid hormones is also extremely low; for example, in neutral aqueous solutions, the solubility of natural oestrogens [e.g. oestrone (E1) and 17β-oestradiol (E2)] is approximately 1.5 mg l−1 at room temperature (Shareef et al., 2006), whereas the experimental aqueous solubility of testosterone can reach 23 mg l−1 at 25°C (Barry and El Eini, 1976). Similarly, the synthetic 17α-ethynylestradiol (EE2) also has a low solubility in water (4.8 mg l−1 at 20°C) (Aris et al., 2014).

In animals, cholesterol is the precursor of all classes of steroid hormones, namely glucocorticoids, mineralocorticoids and sex hormones (androgens, oestrogens and progesterogens). The biosynthesis of steroid hormones involves the elimination of the cholesterol side chain and hydroxylation of the steroid nucleus (Ghayee and Auchus, 2007). All these hydroxylation reactions require NADPH and molecular oxygen; thus, steroid biosynthesis only occurs in the aerobic biosphere. Among sex steroids, progesterone (such as progesterone) function in preparing the lining of the uterus for implantation of an ovum and are also essential for maintaining pregnancy. The biotransformation of progesterone into androgens includes a hydroxylation at C-17 and the subsequent cleavage of the side chain. Androgens regulate the development and maintenance of male characteristics in vertebrates, and the major androgens naturally produced in males are testosterone, dihydrotestosterone and androstenedione (also named androst-4-en-3,17-dione, AD) (see Fig. 1 for structures) (O’Connor et al., 2011). Oestrogens are responsible for...
developing and regulating the reproductive system and secondary sex characteristics of female vertebrates. Major endogenous oestrogens in females include E1, E2 and estriol (E3) (see Fig. 1 for structures). Oestrogens are synthesized from androgens by the loss of the C-19 angular methyl group and the formation of an aromatic A-ring. The aromatization proceeds with three consecutive oxidative steps (Miyairi and Fishman, 1985). Aromatase (namely P450arom or CYP19) catalyses the sequential hydroxylations of a C19 substrate using three molecules of NADPH and three molecules of molecular oxygen to produce one molecule of oestrogen (Praporski et al., 2009).

Numerous steroids are used for medical purposes, such as in treatments for cancer, arthritis and allergies, as well as birth control (Woutersz, 1991; Peter et al., 1994; Merz et al., 2010; Dokras, 2016). A variety of synthetic hormones are commonly used as medications for humans as well as livestock and aquaculture. Synthetic androgens (anabolic steroids) are ester derivatives of androgens known as 19-nortestosterone or nandrolone (see Fig. 1 for structures) are often used to treat anemias, cachexia, osteoporosis and breast cancer. EE2 is a synthetic oestrogen widely used in oral contraceptives in combination with progestin, a synthetic progestogen (Wise et al., 2011). EE2 is also used to improve productivity by promoting growth and preventing and treating reproductive disorders in livestock (Liu et al., 2014; Xu et al., 2018). In aquaculture, EE2 is often used to develop single-sex populations of fishes to optimize growth (Aris et al., 2014).

### The impact, occurrence and fates of steroid sex hormones in environments

**Steroid sex hormones are pheromones and endocrine disruptors**

Some steroid hormones are noted as pheromones in animals, including fish, amphibians and mammals (Doyle and Meeks, 2018). Studies on fish olfactory systems indicated that most fishes use steroids for chemical communication (Moore and Scott, 1991; Baza’s es and Schnachtenberg, 2012). Sulphated steroids (e.g. 17β-oestradiol disulphate) are potent olfactory chemosignals.

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**Fig. 1.** The chemical structures of prevalent natural and synthetic steroid hormones. The ring identification (A-D) and carbon numbering (1-27) systems for steroids are shown on cholesterol. Underlined compounds are synthetic steroids.
in larval amphibians (Houck, 2009). Androstenone, 17β-oestradiol disulphate, estratetraenol and testosterone sulphate are considered sex pheromones in some mammals, such as mice and pigs (Doyle and Meeks, 2018). As pheromones, steroids can affect fish behaviour in a variety of ways, even at extremely low concentrations (Adams et al., 1987; Kolodziej et al., 2003; Kolodziej et al., 2004; Serrano et al., 2008).

The potential for sex hormones to disrupt endocrine functions in various organisms via direct or indirect exposure has been extensively investigated (Aris et al., 2004). Several studies showed that oestrogens cause endocrine disruption in fishes (Jobling et al., 2006; Morthorst et al., 2014), frogs (Lambert et al., 2015; Regnault et al., 2018) and invertebrates (Oetken et al., 2004). Notably, both natural and synthetic oestrogens displayed endocrine-disrupting activities at concentrations as low as nanograms per litre (Robinson and Helou, 2009). The potencies of these oestrogens are measured in relation to E2 (set at 100) – EE2: 246; E1: 2.54; and E3: 17.6 (Pillon et al., 2005). On the other hand, some studies have reported that exposure to androgens in polluted rivers leads to the masculinization of freshwater wildlife (Howell et al., 1980; Bortone et al., 1989; Parks et al., 2001; Orlando et al., 2004). In addition, the endocrine-disrupting effects of synthetic progestogens on aquatic species have been documented (Zeilinger et al., 2009; Cardoso et al., 2017).

Potential sources of steroid hormones in environments
Steroid sex hormones may originate from agriculture, industry, humans, household products and other pharmaceuticals (Wise et al., 2011). Human excreta have been considered a major source of steroid hormones in aquatic environments (Johnson et al., 2000; Chang et al., 2011). Livestock also excrete large amounts of sex steroids into the environment (Maier et al., 2000; Lange et al., 2002). Manure used as fertilizers has also been a major source of steroid hormones released into the environment (Hanselman et al., 2003; Kjaer et al., 2007). Steroid hormones can also end up in aquatic ecosystems via rainfalls and leaching from livestock wastes (Hanselman et al., 2003; Kolodziej et al., 2004). In addition, steroid hormones can be discharged into environments through agricultural applications of municipal sewage biosolids as fertilizers (Lorenzen et al., 2004; Hamid and Eskicioglu, 2012). Moreover, steroid hormones in the environment may partially be the result of microbial activities (Mendelski et al., 2019). For example, phytosterols in pulp and paper mill effluents can be transformed into androgens by microorganisms in river sediments (Jenkins et al., 2003; Orrego et al., 2009).

Environmental levels of steroid sex hormones
Global urbanization has led to the widespread occurrence of steroid hormones becoming a concern worldwide. Both biogenic (natural) and anthropogenic steroid hormones are frequently detected in soils and aquatic environments in the United Kingdom, United States, Japan, Korea, Denmark, Spain, Taiwan, France, China, Swaziland, Czech Republic and Slovak Republic (Belfroid et al., 1999; Ternes et al., 1999; Baronti et al., 2000; Hashimoto et al., 2000; Huang and Sediak, 2001; Kolodziej et al., 2003; Shore and Shemesh, 2003; Labadie and Budzinski, 2005; Bjerregaard et al., 2006; Chen et al., 2007, 2007; Fan et al., 2011; Vajda et al., 2008; Li et al., 2009; Wise et al., 2011; Orlando and Ellesstad, 2014; Gorga et al., 2015; Shih et al., 2017; Sauer et al., 2018; Shen et al., 2018; Zhang et al., 2018; Zhang and Fent, 2018). In surface water, the concentration of steroid hormones ranges from nanograms to micrograms per litre (Ternes et al., 1999; Baronti et al., 2000; Kolodziej et al., 2003; Labadie and Budzinski, 2005; Yamamoto et al., 2006; Chen et al., 2010; Chang et al., 2011; Fan et al., 2011; Sauer et al., 2018; Shen et al., 2018; Zhang et al., 2018; Zhang and Fent, 2018). For example, oestrogens, androgens, progestogens, glucocorticoids and mineralocorticoids were detected in the surface water of urban rivers in Beijing (China), with androgens (0.48–1.9 µg l−1) being the most abundant (Chang et al., 2009). In addition, concentrations of natural oestrogens (E1, E2 and E3) in the Wulo Creek of southern Taiwan were as high as 1.3 µg l−1 due to the livestock feedlot nearby (Chen et al., 2010). The content of steroid hormones in river and marine sediments is often detected at ng g−1 levels (Huang et al., 2013; Gorga et al., 2015; Praveena et al., 2016; Tiwari et al., 2016).

Fate of steroid sex hormones in engineered and natural ecosystems
Sex hormones can be removed or transformed by engineered ecosystems such as activated sludge in wastewater treatment plants (Andersen et al., 2003; Chang et al., 2009; Yu and Chu, 2009; Chang et al., 2011; Fan et al., 2011), constructed wetlands (Song et al., 2009), microalgae systems (Lai et al., 2002; Solé and Matamoros, 2016), sludge-amended soils (Albero et al., 2013), swine manure, poultry litter, dairy waste disposal systems and compost (Hutchins et al., 2007; Liu et al., 2012; Lin et al., 2015). Wastewater treatment plants are crucial for removing steroid hormones via physical adsorption and biodegradation, although the latter is considered the major mechanism (Joss et al., 2004; Yu et al., 2013). Chang et al. (2011) investigated the removal of androgens, oestrogens and progestogens in seven wastewater treatment plants. Their study indicated...
Microorganisms involved in the degradation of steroid sex hormones

Steroids are carbon-rich and highly reduced compounds that are abundant and ubiquitous in the environment; thus, they are attractive carbon and energy sources for microorganisms. Certain microorganisms, including bacteria (Fernandes et al., 2003; Donova and Egorova, 2012), yeasts (Liu et al., 2017), fungi (Kristan and Rižner, 2012) and microalgae (Pollio et al., 1994), are able to transform steroids, but the ability to mineralize steroids (complete degradation of steroids to CO$_2$) has only been identified in certain bacteria (Bergstrand et al., 2016; Yang et al., 2016; Holert et al., 2018). The major focus of recent research has been elucidating the diversity of steroid degraders and their degradation mechanisms. Several studies have used culture-dependent approaches to isolate steroid hormone-degrading bacteria from different engineered and natural ecosystems, and diverse degraders classified as actinobacteria and proteobacteria have been reported (Bergstrand et al., 2016). The significance of investigating steroid-microorganism interactions has increased for four main reasons. First, microbial degradation is crucial for removing steroid sex hormones from polluted ecosystems. Second, microbial transformation of steroids has been exploited in the pharmaceutical industry to produce high-value steroid drugs through biotechnology processes. Third, steroid degradation is important for the virulence of some bacterial pathogens. Fourth, recent studies suggest that steroid hormones mediate bidirectional interactions between bacteria and their eukaryotic hosts (vom Steeg and Klein, 2017). In this review, we summarize the important steroid hormone-degrading bacteria.

Aerobic steroid hormone-degrading bacteria

Talalay et al. (1952) were the first to isolate an unidentiﬁed Gram-negative bacterium from soils capable of growing on an agar plate containing testosterone as the sole carbon source. This bacterium – reclassiﬁed as *Comamonas testosteroni* DSM 50244 (Betaproteobacteria) (Tamaoka et al., 1987) – was able to degrade testosterone completely, according to a stoichiometry ratio of testosterone, O$_2$ and CO$_2$. Later, another betaproteobacterium isolated from soils, *Alcaligenes* sp. strain M21, was shown to be capable of growing on testosterone or E2 as its sole carbon source (Payne and Talalay, 1985). Thus far, *C. testosteroni* strains ATCC 11996 and TA441 have been the model microorganism for studying the testosterone catabolic pathway (Zhang et al., 2011; Horinouchi et al., 2012). Interestingly, some testosterone-degrading proteobacterial isolates originated from marine environments (Zhang et al., 2011; Sang et al., 2012); for example, *Endozoicomonas montiporae* BCRC 17933, a gammaproteobacterium isolated from the encrusting pore coral *Montipora aequituberculata* (Yang et al., 2010), is capable of using testosterone as sole carbon source (Ding et al., 2016). In addition to the proteobacteria (Horinouchi et al., 2012), most other testosterone-degrading bacteria belong to the phylum Actinobacteria (Bergstrand et al., 2016). Among them, *R. ruber* DSM 43338, *R. aetherivorans* DSM 44752 and *R. rhodochrous* DSM 43269 can only grow on a single type of steroid hormone (testosterone), whereas *R. ruber* strain Chol-4 (= DSM 45280) displayed a broad range of catabolic capacities for cholesterol, testosterone and progesterone (Fernández de las Heras et al., 2009). This strain and *R. rhodochrous* DSM 43269 have been used to identify degradation genes responsible for different steroid substrates in actinobacteria (Petrusma et al., 2009, 2011, 2014; Fernández de las Heras et al., 2017). On the other hand, information on progesterone degraders is relatively scant. Apart from the above-mentioned *R. ruber* strain Chol-4, only a few studies have reported transformation of progesterone via certain kinds of bacteria (Donova, 2007; Donova and Egorova, 2012).

The complete microbial degradation of oestrogens was first described by Coombe et al. (1966) in the actinobacterium *Nocardia* sp. E 110, isolated from soil. Some *Rhodococcus* isolates from soil or activated sludge (e.g. *R. equi* and *R. zopfi*) were capable of degrading oestrogens completely (Yoshimoto et al., 2004; Kurisu et al., 2010). On the other hand, the oestrogen-degrading alphaproteobacterial *Novosphingobium tarda* DSM 16725 (Fujii et al., 2003) and *Sphingomonas* spp. (Ke et al., 2007; Yu et al., 2007) were isolated and characterized, and the mechanisms involved in the oestrogen catabolism have been identified recently (Chen et al., 2017; Wu et al., 2019). A list of the bacterial strains capable of aerobic steroid degradation is given in Table 1. To our knowledge, no microorganisms
are able to utilize the synthetic oestrogen EE2 as sole carbon and energy source. However, strains \textit{R. equi} ATCC 13557 and \textit{R. erythropolis} ATCC 4277 displayed partial EE2 degradation activity when co-incubated with glucose and adipic acid, respectively (O’Grady et al., 2009).

\textbf{Anaerobic steroid hormone-degrading bacteria}

The aerobic steroid degraders have been extensively isolated; by contrast, the number of anaerobic steroid-degrading bacterial isolates is relatively limited. The complete degradation (mineralization) of steroids in sediments under denitrifying conditions was first reported by Taylor et al. (1981). To date, only a few anaerobic steroid-degrading bacteria have been isolated and characterized (Harder and Probian, 1997; Fahrbach et al., 2008). \textit{Sterolibacterium denitrificans} strain Chol-1S (= DSM 13999), isolated from denitrifying sludge in a wastewater treatment plant (Tarlera and Denner, 2003), is able to grow with various sterols (Warnke et al., 2017) and androgens (Wang et al., 2014), with nitrate as the terminal electron acceptor. A denitrifying betaproteobacterium, \textit{Denitratisoma oestradiolicum} strain AcBE2-1 (= DSM 16959), is able to degrade natural oestrogens (E1 and E2) but not cholesterol or androgens (Fahrbach et al., 2006). However, a closely related strain, \textit{Denitratisoma} sp. DHT3, isolated from denitrifying sludge in a wastewater treatment plant, had the capacity to degrade E1, E2 and testosterone (Wang et al., 2019). Based on the stoichiometric analysis, the above-mentioned bacterial isolates were shown to completely degrade specific steroids. Interestingly, these steroid hormone-degrading anaerobes share common physiological traits. First, colony growth on an agar plate was very marginal or not observed; thus, their isolation and purification were conducted via repeating serial dilution. Second, all strains utilized an extremely narrow spectrum of substrates. Third, they are able to use either oxygen or nitrate as the electron acceptor, but \textit{D. oestradiolicum} AcBE2-1 cannot degrade E2 with oxygen as the electron acceptor (Fahrbach et al., 2006).

\begin{table}[h!]
\centering
\begin{tabular}{llllll}
\hline
Phylum/class & Strain & Origin & G + C content (mol\%) & Accession number & Steroid substrates & Growth on agar plate \\
\hline
\textbf{Actinobacteria} & \textit{Rhodococcus ruber} M1, N361 (DSM 43338) & Activated sludge & 70.5 & GCF_001646835.1 & AD, cholesterol, testosterone & Yes \\
& \textit{Rhodococcus aetherivorans} 10BC-312 (DSM 44752) & Activated sludge & NA & NA & AD, ADD, testosterone & Yes \\
& \textit{Rhodococcus ruber} Chol-4 & Activated sludge & 70.6 & GCF_000347955.2 & AD, ADD, cholesterol, 17β-Estradiol, testosterone, progesterone & Yes \\
& \textit{Amycolatopsis sp.} 75iv2 (ATCC 39116) & Soil & 69.1 & AFWY00000000 & Cholesterol, testosterone & Yes \\
& \textit{Rhodococcus equi} ATCC 13557 & NA & NA & NA & 17α-Ethynylestradiol (partial degradation) & Yes \\
& \textit{Rhodococcus erythropolis} ATCC 4277 & Soil & 67.0 & NA & 17α-Ethynylestradiol (partial degradation) & Yes \\
\textbf{Alphaproteobacteria} & \textit{Novosphingobium tardaugens} ARI-1 (NBRC 16725) & Activated sludge & 61.2 & CP034719 & 17β-Estradiol, oestrone, estriol & Yes \\
& \textit{Sphingomonas sp.} KC8 & Activated sludge & 63.7 & CP016306 & 17β-Estradiol, oestrone, testosterone & Yes \\
& \textit{Sphingomonas wittichii} RW1 & River & 68.4 & CP00699 & Testosterone & Yes \\
\textbf{Betaproteobacteria} & \textit{Comamonas testosterone} (ATCC 11996, DSM 50244) & Soil & 61.5 & AHIL00000000.1 & Testosterone & Yes \\
& \textit{Cupriavidus necator} H16 (ATCC17699) & Activated sludge & 66.5 & NC_008313 & Testosterone & No \\
& \textit{Endozoicomonas montiporae} CL-33 (BCRC 17933) & Coral & 48.4 & NC_008314 & Testosterone & Yes \\
\textbf{Gammaproteobacteria} & \textit{Pseudomonas resinovorans} CA10 (NBRC 106553) & Activated sludge & 65.6 & AP013068 & Testosterone & NA \\
\hline
\end{tabular}
\caption{Selection of the bacterial strains capable of aerobic degradation of steroid hormones.}
\end{table}

AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; NA, not available.

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1998), is able to degrade testosterone, but the presence of the co-substrate (acetate) in denitrifying medium is essential (Yang et al., 2016). Recently, Azoarcus sp. strain Aa7 capable of degrading ADD under denitrifying conditions has been isolated from soil (Yuce et al., 2019). The bacterial strains capable of anaerobic steroid degradation are shown in Table 2. Notably, all these denitrifiers are facultative anaerobes and can be handled easily under aerobic conditions. Moreover, the genomes of most of these strains are available.

**Bacterial degradation pathways of steroid sex hormones**

**Aerobic biodegradation of androgens through the 9,10-seco pathway**

The aerobic catabolic pathways for major classes of steroids – including sterols, androgens, oestrogens, progestogens and bile acids – have been elucidated in various bacteria. The cholesterol degradation pathway in actinobacteria has been studied in some detail, partially resulting from the biotechnological applications of actinobacteria in steroid drug production (Fernandes et al., 2003; Donova and Egorova, 2012). Moreover, cholesterol catabolism plays a critical role in mycobacterial pathogenicity (Pandey and Sassetti, 2008; VanderVen et al., 2015; Crowe et al., 2017; 2018). Kieslich (1985) was the first to propose a general scheme of aerobic cholesterol degradation, with some common androgens – including AD and androsta-1,4-diene-3,17-dione (ADD) – as key intermediates in this pathway. The degradation involves a series of enzymes that have been mainly investigated by Dr. Lindsay D. Eltis’ team. The genomic analysis revealed that, in *Mycobacterium tuberculosis* strain H37Rv, a gene cluster with over 80 catabolic genes is responsible for the cholesterol catabolic pathway (van der Geize et al., 2007; Crowe et al., 2015). Moreover, the key enzymes involved in this pathway, including the oxygenases for the degradation of cholesterol side chain (Rosloniec et al., 2009; Ouellet et al., 2010) and oxygenolytic cleavage of the A/B-rings (Yam et al., 2009; Capyk et al., 2011), as well as the hydrolases for the C/D-rings degradation (Crowe et al., 2017), have been characterized. This aerobic pathway is widely distributed in actinobacteria (Bergstrand et al., 2016), suggesting that aerobic steroid degradation is crucial for their survival in environments. Some actinobacteria can also utilize androgens; one may thus envisage that actinobacteria use homologous enzymes to aerobically degrade androgens through a highly similar pathway (Donova, 2007; Bergstrand et al., 2016).

Aerobic androgen degradation has been mainly studied using *Comamonas testosterone* (a betaproteobacterium) as the model organism. The studies on aerobic testosterone catabolism were initiated in the 1960s by

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**Table 2.** Characterized bacterial strains that are capable of degrading steroid hormones under anaerobic conditions.

| Class                  | Strain                                      | Origin      | G + C content (mol%) | Accession number | Steroid substrates                  | Electron acceptors (growth with steroids) | Growth on agar plate |
|------------------------|---------------------------------------------|-------------|----------------------|------------------|-------------------------------------|------------------------------------------|----------------------|
| Betaproteobacteria     | Azoarcus sp. Aa7 (DSM 16959)                | Soil        | 66.1                 | QVR00000000       | ADD, cholate, deoxycholate           | Nitrate                                  | Yes                  |
|                       | Denitratisoma oestradiolicum AcBE2-1 (DSM   | Activated sludge | 61.4             | NCXS00000000     | E1, E2                              | Nitrate                                  | Marginal             |
|                       | 16959)                                       |             |                      |                  |                                     |                                          |                      |
|                       | Denitratisoma sp. DHT3                      | Activated sludge | 64.9             | CP020914         | E1, E2, testosterone                | Nitrate                                  | No                   |
|                       | Sterolibacterium dentifrices Chol-1S (DSM 13999) | Activated sludge | 65.3             | LT837804         | AD, cholesterol, testosterone        | Nitrate, oxygen                          | No                   |
|                       | Sterolibacterium sp. 72Chol (DSM 12783)     | Ditch       | NA                   | NA               | Cholesterol                         | Nitrate, oxygen                          | No                   |
|                       | Thauera terpenica 58E6 (DSM 12139)          | Ditch       | 64.2                 | ATJV01000070     | Testosterone                       | Nitrate                                  | Yes                  |
|                       | Thauera terpenica GDN1                       | Estuarine   |                      |                  |                                     |                                          |                      |
| Testosterone^a         | Nitrate                                     | Activated sludge | 61.7             | CP011971         | AD, E1, E2, testosterone            | Nitrate, oxygen                          | No                   |

AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; NA, not available.

^a^A co-substrate such as acetate is essential (Yang et al., 2016; Shih et al., 2017).

^b^The strain formed tiny colony on nutrient agar plate but unable to grow after 2–6 times of sub-culturing.

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Sih et al. (1966), and 30 years later, Horinouchi et al. (2001, 2003 Horinouchi et al., 2004) used a gene disruption technique to identify the degradation genes as well as catabolic intermediates accumulated in C. testosteroni mutants. The aerobic degradation pathway of androgens was established based on these molecular studies (Horinouchi et al., 2012; Horinouchi et al., 2018). Under aerobic conditions, C. testosteroni tends to oxidize the 17-hydroxyl group of testosterone into a carbonyl group. However, this dehydrogenation reaction is not a prerequisite for core ring cleavage. In contrast, oxidation of the A-ring is thought to initiate the core ring degradation. The process includes two reactions: oxidation of the 3-hydroxyl moiety and oxidation of C-1/C-2 of androgens (Fig. 2). 3α- or 3β-hydroxysteroid dehydrogenases, members of short-chain dehydrogenase/reductase family, are involved in the oxidation of 3-hydroxyandrogens such as epiandrosterone, whereas 3-ketosteroid-Δ1-dehydrogenase (TesH) is responsible for the introduction of a double bond between C-1 and C-2 of AD. The formation of the 3-oxo-1,4-diene structure at an early stage is critical since it enables cleavage of the core ring. The subsequent step is the hydroxylation at C-9 in the B-ring by a monooxygenase, 3-ketosteroid 9α-hydroxylase (encoding by orf17). The resulting structure, 9α-hydroxy-androsta-1,4-diene-3,17-dione, is very unstable and undergoes an abiotic cleavage between C-9 and C-10 in the B-ring and the simultaneous aromatization of the A-ring, producing a seco steroid, 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA). This 9,10-seco steroid is the key intermediate in this aerobic pathway, named the 9,10-seco pathway by Philipp (2011). After the production of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA) through the 4-hydroxylation reaction by another monooxygenase – TesA1/A2 – the catecholic A-ring is subject to metabolism by an extradiol dioxygenase, TesB. Subsequently, the hydrolyase TesD mediates the hydrolytic cleavage between the C-5 and C-10 of 4,5,9,10-diseco-3-hydroxy-5,9,17-triioxandrosta-1(10),2-diene-4-oic acid (4,9-DSHA), producing 3α-H-4α(3′-propanoate)-7αβ-methylhexahydro-1,5-indanediol (HIL). Overall, the aerobic degradation of the A/B-rings of androgens requires at least two monooxygenases and an extradiol dioxygenase, with molecular oxygen as a co-substrate (Fig. 2).

One the other hand, the regulation of the androgen degradation genes has been reported. In C. testosteroni, the gene product of teiR (a testosterone-inducible regulator) positively regulates the transcription of genes involved in the initial steps of steroid degradation (Horinouchi et al., 2004). Pruneda-Paz et al (2004) further demonstrated that the teiR-disrupted mutant strain lost the ability to use testosterone as its sole carbon source. By contrast, the repressor protein TetR may be specifically responsible for the expression of the 3β,17β-hydroxysteroid dehydrogenase gene (Pan et al., 2015; Wu et al., 2015).

Thus far, bacterial steroid uptake is poorly understood. Actinobacteria transport cholesterol via the ATP-dependent MCE4 protein, a member of the Mammalian Cell Entry (MCE) superfamily that locates in cytoplasmic membrane (Casali and Riley, 2007; Mohn et al., 2008). In Gram-negative proteobacteria, the outer membrane and periplasmic space complicate steroid uptake and catabolism. Furthermore, the lipopolysaccharide leaflet on the outer surface of the outer membrane impedes steroids from passive diffusion through the membrane bilayer (Plišiat and Nikaido, 1992). The ATP-dependent MCE proteins are ubiquitous among proteobacteria (Casali and Riley, 2007). These proteins form a conserved hexameric ring module spanning the periplasmic space to transport phospholipids and other hydrophobic molecules (Malinverni and Silhavy, 2009; Ekiert et al., 2017). Thus, the possibility that MCE-like transporter might play a role in steroid uptake in proteobacteria cannot be excluded.

Literature on aerobic biodegradation of progestogen is relatively limited. Liu et al. (2013) identified some androgens (e.g. AD and ADD) as intermediates of aerobic progestosterone degradation. Moreover, Horinouchi et al. (2012) suggested that C. testosteroni degrades progestosterone through the 9,10-seco pathway. The 9,10-seco pathway is also responsible for the bile acid degradation in Pseudomonas sp. strain Cho1 (Philipp, 2011). Interestingly, the strain Cho1 accumulated extracellular androgenic intermediates such as ADD during the degradation of bile acids (Holert et al., 2014). The unusual release of the androgens by the bile acid-degrading bacteria into the environment may have hormonal effects on the coexisting fauna (Mendelski et al., 2019). In summary, the aerobic catabolic pathways of sterols, bile acid, androgens and progestogens proceed through the oxygen-dependent 9,10-seco pathway, with 9,10-seco steroids (e.g. 3-HSA and its 17-hydroxyl structure) as characteristic intermediates.

**Aerobic degradation of oestrogens through the 4,5-seco pathway**

Oestrogens are the most concerning endocrine disruptors (Ghayee and Auchus, 2007) and are also potential carcinogens (Yager and Davidson, 2006). A complete aerobic pathway of oestrogen degradation was recently proposed in the alphaproteobacterium Sphingomonas sp. strain KC8 (Wu et al., 2019), and several gene clusters involved in this aerobic pathway have also been identified (Chen et al., 2017; Wu et al., 2019). Compared to the aerobic 9,10-seco degradation pathway, the oestrogen degradation pathway is different in several aspects: (i) the core ring cleavage occurs between C-4 and C-5 in the A-ring; thus, the pathway is named the...
4,5-seco pathway; (ii) the A/B-rings degradation contains a series of coenzyme A (CoA)-esters; and (iii) this pathway produces oestrogen-derived dead-end-products: pyridinestrone acid (PEA) and 4-norestenic acid (Fig. 3).

Under aerobic conditions, E2 is first oxidized to E1 by 17β-oestradiol dehydrogenase (OecA). The C-4 of E1 is then hydroxylated by a monoxygenase oestrone 4-hydroxylase (OecB), and the resulting catecholic A-ring is opened through meta-cleavage by an extradiol dioxygenase, 4-hydroxyestrone 4,5-dioxygenase (OecC) (Fig. 3).

The meta-cleavage product of 4-hydroxyestrone is unstable, and in the presence of ammonium may undergo an abiotic recylization to produce a nitrogen-containing compound pyridinestrone acid. It is known that meta-cleavage metabolites produced in bacterial cultures are often abiotically recylized with ammonium to generate pyridine 2-carboxylic acid products (Dagley et al., 1960; Mycroft et al., 2015). The production of steroid metabolites through non-enzymatic reactions has also been demonstrated in the 9,10-seco pathway (Kieslich, 1985). The addition of a hydroxyl...
group at C-9 of ADD results in the formation of an unstable 9α-hydroxylated intermediate, which undergoes a spontaneous split of the B-ring and then generates the 3-HSA for further A-ring degradation. By contrast, once pyridinestrone acid is produced in bacterial cells, it is not able to be further degraded and is excreted into the extracellular environment. Pyridinestrone acid is thus a dead-end-product of the 4,5-seco pathway.

Only a minor part (approximately 2% in the oestrogen-grown strain KC8 cultures) of the meta-cleavage product is abiotically transformed into pyridinestrone acid, and the majority (> 95%) of the meta-cleavage molecules is
further degraded by the strain KC8. A member of the indolepyruvate ferredoxin oxidoreductase family, 2-oxoacid oxidoreductase (OAOR), removes the C-4 and adds a CoA to the carboxyl C-3 of the meta-cleavage product, producing 4-norestrogen-5(10)-en-3-oyl-CoA through oxidative decarboxylation (Fig. 3). This CoA-ester has been identified using mass spectrometry and its non-CoA moiety – 4-norestrogenic acid – has been structurally determined through NMR spectroscopic analyses (Wu et al., 2019). The deconjugated structure, 4-norestrogenic acid, is often detected in the extracellular environment. Subsequently, the C-2 and C-3 are removed through a cycle of thiolytic β-oxidation, and the B-ring is opened through hydrolysis (Fig. 3). A similar hydrolytic ring cleavage mechanism has been demonstrated in the degradation of cyclohexanecarboxylic acid by the alphaproteobacterium Rhodopseudomonas palustris (Pelletier and Harwood, 1998; 2000). Subsequently, the removal of C-1 and C-10 through aldolytic cleavage results in the production of HIP. Except for 4-norestrogen-5(10)-en-3-oyl-CoA, no other CoA esters proposed in this aerobic pathway have been detected; however, at least five deconjugated (non-CoA) metabolites corresponding to these hypothetical CoA esters have been detected in the bacterial cultures (Wu et al., 2019). The dead-end-products pyridinestrone acid and 4-norestrogenic acid are less biodegradable and tend to accumulate in bacterial cultures (Wu et al., 2019) or environmental samples (Chen et al., 2017, 2018); thus, these compounds may serve as biomarkers for investigating environmental aerobic oestrogen biodegradation.

In addition to strain KC8, most metabolites involved in the 4,5-seco pathway were also identified in another oestrogen degrader, Novosphingobium sp. strain SLCC (Chen et al., 2018; Wu et al., 2019). Initial metabolites, such as 4-hydroxyestrone and the meta-cleavage product, were also identified in Sphingomonas sp. strain ED8 (Kurisu et al., 2010) and an actinobacterium Nocardi a sp. strain E110 (Coombe et al., 1966). Accordingly, it is speculated that these aerobes may adopt the 4,5-seco pathway for oestrogen degradation. Although the gene cluster for the oestrogen A/B-rings degradation has been identified, only three catabolic genes – oecA, oecB and oecC – have been functionally characterized (Chen et al., 2017). The actual role of other genes, 2-oxoacid oxidoreductase (OAOR), for example, remains to be validated.

Anaerobic degradation of steroids through the 2,3-seco pathway

Compared to the extensive study (more than 50 years) of the aerobic 9,10-seco pathway, investigations of anaerobic androgen degradation are relatively recent and limited. The anaerobic 2,3-seco pathway was first proposed in the testosterone-degrading gammaproteobacterium S. denitrificans DSM 18526 after the discovery of the ring-cleavage product 17-hydroxy-1-oxo-2,3-secoandrost-3-onic acid (2,3-SAOA) (Wang et al., 2013), although some metabolites (1-dehydrotestosterone, 1-testosterone, AD, ADD and 1-hydroxysteroids) involved in the initial steps of testosterone transformation were identified prior to this study (Chiang et al., 2010; Leu et al., 2011). This 2,3-secosteroid, along with other initial metabolites, was also identified in the denitrifying betaproteobacterium S. denitrificans DSM 13999 cultivated with testosterone (Wang et al., 2014).

Thus far, only a few enzymes involved in the 2,3-seco pathway have been characterized from the strains DSM 13999 and DSM 18526. Some redox enzymes, such as 17β-hydroxysteroid dehydrogenase and 3-ketosteroid Δ^1^-dehydrogenase (AcmB), catalyse the transformation of testosterone into 1-dehydrotestosterone, AD and ADD (Chiang et al., 2008a; Chiang et al., 2008b; Lin et al., 2015). The same sets of enzymes are also responsible for the redox reactions of androgens under aerobic conditions (Yang et al., 2016). The molybdoenzyme 1-testosterone hydratase/dehydrogenase (AtcABC) mediates the hydration reaction at the C-1 of 3-oxo-1-en structures – which includes 1-testosterone – as well as the subsequent oxidation of the 1-hydroxyl group (Yang et al., 2016). A phylogenetic analysis of AtcABC sequences suggested that this heterotrimeric protein belongs to the xanthine oxidase family containing molybdopterin, FAD and iron–sulphur clusters. The formation of the 1,3-dioxo structure in the A-ring is critical since it enables cleavage of the steroidal core ring (Fig. 4). The hydratase responsible for the A-ring cleavage has not been characterized, partially due to the lack of a commercially available substrate (17β-hydroxy-androstan-1,3-dione or androstan-1,3,17-trione). Subsequently, the C-3 and C-4 of the cleaved A-ring are then removed from the secosteroids through a putative aldolytic cleavage (Wang et al., 2014), producing 17β-hydroxy-2,5-seco-3,4-dinorandrost-1,5-dione (2,5-SAD) (Fig. 4). The following B-ring degradation remains completely unclear; however, the downstream metabolites HIP and HIP-CoA have been identified as intermediates in the anaerobic androgen degradation pathway (Warnke et al., 2017).

The mechanisms and enzymes involved in anaerobic oestrogen degradation remain poorly studied, although some anaerobic proteobacterial degraders have been isolated. It is very likely that anaerobic bacteria must adopt an oxygen-independent pathway different from the 4,5-seco pathway to degrade phenolic A-ring of oestrogen. Recently, a comparative genomic analysis indicated that 2 certain gene homologues shared in the genomes of S. denitrificans DSM 18526 and two Denitratisoma strains might play a role in anaerobic oestrogen degradation.
Wang et al. (2019) used the Denitratisoma sp. DHT3 as a model organism to identify initial steps of the anaerobic degradation pathway for E2. Their study suggested that denitrifying degraders utilize a convergent catabolic pathway – the 2,3-seco pathway – to catabolize different steroid structures.

The HIP degradation pathway, a common central pathway for bacterial degradation of the steroid C,D-rings. It is interesting that HIP – a C13 metabolite with the remaining steroid C/D-rings – is a common intermediate identified in all bacterial steroid catabolic pathways (the aerobic 9,10-seco and the 4,5-seco pathways, as well as the anaerobic 2,3-seco pathway). The HIP degradation pathway has been mainly established in aerobic actinobacteria, activated by a specific acyl-CoA synthetase FadD3 (Casabon et al., 2013), although this activation enzyme was also

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characterized in anaerobic S. denitrificans DSM 13999 (Warnke et al., 2018). The remaining carbons of the steroid B-ring are then removed through a cycle of thiolytic β-oxidation. Subsequently, the hydrolytic cleavage of the steroid D-ring is mediated by an enoyl-CoA hydratase (EchA20), whereas the C-ring cleavage is mediated by another hydratase IdpAB (Crowe et al., 2017; 2018) (Fig. 5A). Surprisingly, the homologues of these ring-cleavage enzymes were identified in the genomes of aerobic C. testosteronei (Horinouchi et al., 2012; Crowe et al., 2017) and Sphingomonas sp. KC8 (Chen et al., 2017), as well as anaerobic S. denitrificans DSM 13999 (Warnke et al., 2017), Sdo denitrificans, and T. terpenica (Yang et al., 2016) (Table 3). Further gene mining showed that most experimentally verified steroid-degrading bacteria contain gene clusters involved in the HIP degradation pathway (Fig. 5B). These data thus indicate that bacteria adopt divergent pathways to degrade the steroidal A/B-rings, depending on oxygen conditions and steroid structures. However, all these steroid catabolic pathways then converge at the HIP, and bacteria use the same set of enzymes to degrade the remaining steroid C/D-rings.

Culture-independent approaches expand insight into the diversity of sex hormone degraders and ecological significance

Early microcosm and mesocosm studies suggested that oestrogens and testosterone can be biodegraded to CO2 in river sediments (Jürgens et al., 2002), marine sediments (Ying et al., 2003), agricultural soils (Fan et al., 2007) and activated sludges (Andersen et al., 2003) under aerobic or anaerobic conditions. Czajka and Londry (2006) investigated the anaerobic degradation of natural and synthetic oestrogens in lake sediments under methanogenic as well as sulphate-, iron- and nitrate-reducing conditions, showing that natural oestrogens were degraded under all tested conditions, whereas synthetic EE2 was not apparently degraded by microorganisms. In addition, anaerobic biotransformation of testosterone into oestrogens, including E1 and E2, in testosterone-spiked estuarine sediment samples under fermentative condition was observed (Shih et al., 2017). These reports suggested that various anaerobes might play a role in steroid hormone degradation in oxygen-limited environments.

However, microbial profiles and functional genes involved in these bioprocesses were lacking. Due to the certain extent of genes and metabolites involved in microbial catabolism of testosterone and oestrogens (Tables 3–5), recent culture-independent studies have focused on using this information to discover the signatures of microbial degraders and their degradation activities regarding these sex hormones in different environments.

The microautoradiography–fluorescence in situ hybridization (MAR-FISH) technique was applied to identify active oestrone-assimilating bacteria in activated sludge using [2,4,6,7-3H(N)]oestrone as a tracer. Some studies have revealed that several active proteobacterial taxa incorporated trace oestrone (submicrogram per liter concentrations) in activated sludges, indicating that the main degraders of oestrogen in wastewater treatment plants are different from those reported in culture-dependent studies (Zang et al., 2008; Thayanukul et al., 2010; Kurisu et al., 2015). However, this technique has several disadvantages. First, oestrogens are highly hydrophobic; thus, these steroid compounds may easily attach to cell membranes (Lin et al., 2015) or passively transport into cell via the outer membrane transporter (Wiener and Horanyi, 2011; Lin et al., 2015; Wei et al., 2018). Therefore, distinguishing between types of metabolic activities – passive diffusion or active uptake, and redox transformation or complete degradation – of radiolabelled bacterial cells is difficult. Taxonomic identification of labelled cells also relies on oligo probes targeting specific bacterial taxa used in each study, resulting in an incomplete profile of oestrogen-incorporated bacteria.

Integrated multi-omics approaches, including (meta) genomic analysis and metabolite profiling, have been applied to identify steroid hormone degraders and their catabolic pathways in environments. Chen et al. (2016) revealed that C. testosteronei spp. play a major role in aerobic androgen degradation in activated sludge based on the detection of the signature metabolite and key gene in the 9,10-seco pathway, 3-HSA and tesB. A similar strategy was used to interrogate anaerobic androgen degraders in denitrifying sludge and anoxic estuary sediments. In both ecosystems, the signature metabolite 2,3-SAOA and degradation key gene alCA were identified, indicating that these microbial communities degrade androgen through the 2,3-seco pathway. However, the main degraders were Thauera spp. (phylogenetically...
close to *T. terpenica* 58Eu) instead of the model organism *S. denitrificans* DSM 18526 (Yang et al., 2016; Shih et al., 2017). Another example of interrogating environmental degradation is using the $^{13}$C-metabolomic approach to identify major oestrogen degradation pathways in river waters. The occurrence of $^{13}$C-labelled pyridinestrone acid – the dead-end-product of the 4,5-seco pathway – in $[3,4^{13}$C]$^{13}$C-oestrone-treated water samples indicated that river microorganisms degrade natural oestrogens via the 4,5-seco pathway. These characteristic metabolites or dead-end-products were identified using ultra-performance liquid chromatography–high-resolution mass spectrometry (UPLC—HRMS). The characteristic metabolites and dead-end-products of each pathway and their UPLC—HRMS behaviours are shown in Fig. 6 and Table 5 respectively.

Some genome-based studies have also broadened the diversity of aerobic androgen degraders. Horinouchi et al. (2012) used homology to search for gene clusters and their actual roles of these degraders in natural ecosystems – soils, deep sea, eukaryotic hosts, and even in the Antarctica Dry Valleys – indicating the ecological significance of steroid degraders (Holert et al., 2018). For example, the fact that steroid-degrading gammaproteobacteria isolated from sponges and corals suggests that microbial steroid metabolism plays a role in symbiosis relationships (mutualism) with their animal hosts (Ding et al., 2016; Holert et al., 2018). Despite this, the actual roles of these degraders in nature ecosystems remain elusive because the hormone degradation activities are mostly identified in chemically defined media or mesocosms supplied with large amount of hormones (micro- to milli-molar), which is much higher (1000- to 10 000-fold) than those detected in environments (Yang et al., 2016; Chen et al., 2016; Shih et al., 2017; Chen et al., 2018). It has been speculated that steroid hormone degraders in environments might be members of rare biosphere due to the low content of

### Table 3. Homologues involved in HIP degradation in steroid-degrading bacteria.

| Steroid hormone degraders | Enoyl-CoA hydratase (Ech A20) | Hydrolase α subunit (IdpA) | Hydrolase β subunit (IdpB) |
|---------------------------|-------------------------------|---------------------------|---------------------------|
| Aerobic actinobacteria    |                               |                           |                           |
| *R. jostii* RHA1           | WP_007300903.1                | WP_011597005.1            | WP_011597004.1            |
| *M. tuberculosis* H37Rv    | NP_218067.1                   | NP_218068.1               | NP_218069.1               |
| *M. smegmatis* Mc2-155     | YP_890227.1                   | YP_890228.1               | YP_890229.1               |
| Aerobic alphaproteobacteria|                               |                           |                           |
| *Sphingomonas* sp. KC8     | ARS25865.1                    | ARS25890.1                | ARS25889.1                |
| *N. taraugensis* NBR1 1752 | WP_021688816.1                | WP_021688820.1            | WP_021688819.1            |
| *Altererythrobothacterium* sp. MH-BS| WP_067540119.1 | WP_067540155.1            | WP_067540130.1            |
| Aerobic betaproteobacteria |                               |                           |                           |
| *C. testosteroni* ATCC 11996| WP_003078394.1                | WP_003078407.1            | WP_003078404.1            |
| *C. testosteroni* CNB-2    | ACY32026.1                    | ACY32022.1                | ACY32023.1                |
| Denitrifying betaproteobacteria |                               |                           |                           |
| *S. denitrificans* DSM 13999 | SMB21421.1                    | SMB21413.1                | SMB21414.1                |
| *D. oestradiolicum* DSM 16959 | TWO82302.1                    | TWO81338.1                | TWO80907.1                |
| *Denitratisoma* sp. DHT3   | QDX80568.1                    | QDX82838.1                | QDX80569.1                |
| *T. trepenica* 58Eu        | WP_021250107.1                | WP_021250110.1            | WP_021250109.1            |
| Denitrifying gammaproteobacteria |                               |                           |                           |
| *S. denitrificans* DSM18526| WP_066917844.1                | WP_016491273.1            | WP_066917840.1            |

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Table 4. Selected catabolic genes as biomarkers for identification of the three major steroid degradation pathways.

| Enzyme | Gene name & protein ID | Substrate | Product |
|--------|------------------------|-----------|---------|
| The 9,10-seco pathway | | | |
| 3-Ketosteroid 9x-hydroxylase (reductase subunit) | ADD | 3-HSA | ORF17 WP_003078230.1 ORF17 ACY32105.1 KshA2 KshA2 KshA |
| 3,4-Dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione-4,5-dioxygenase | TesB | TesB | WP_003078410.1 ACY32021.1 ATcA SMB21166.1 AtcA AtcA |
| 4,5-9,10-Deseco-3-hydroxy-5,9,17-trioxoandrost-1(10)-2-diene-4-oate hydrolase | TesD | TesD | WP_003075542.1 ACY32100.1 AtcA AtcA |
| The 2,3-seco pathway | | | |
| 1-Testosterone hydratase/ dehydrogenase (subunit A) | 1-Testosterone | 1-7-Hydroxy-androstan-1,3-dione | AtcA SMB21166.1 AtcA TWO78727.1 AtcA QDX80120.1 AtcA |
| The 4,5-seco pathway | | | |
| 4-Hydroxyestrone 4,5-Dioxygenase | 4-Hydroxyestrone | Meta-cleavage product<sup>a</sup> | OecC WP_010123492.1 OecC WP_021690447.1 OecC WP_006753109.1 |
| 2-Oxoacid oxidoreductase | Meta-cleavage product<sup>a</sup> | 4-Noresterone-5(10)-en-3-oyl-CoA | OecC WP_003075542.1 OecC WP_010953414.1 |

ADD, androsta-1,4-diene-3,17-dione; 3-HSA, 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione; 4,9-DSHA, 4,5,9,10-deseco-3-hydroxy-5,9,17-trioxoandrost-1(10)-2-diene-4-oic acid; HIP, 3α-H-4α-(3-propanoate)-7β-methylhexahydro-1,5-indandione.<br><sup>a</sup>See Figure 3 for the chemical structure of the meta-cleavage product.
hormone substrates (Wei et al., 2018). Under a substrate concentration (3.7 nM of oestrone as the sole carbon source) close to environmental levels, pyridinestrone acid – the dead-end-product of the 4,5-seco pathway – was detected in bacterial cultures, indicating that the oestrogen degradation ability of Novosphingobium sp. SLCC remained active (Chen et al., 2018). Moreover, the FISH-MAR study (Thayanukul et al., 2010) indicated that bacteria are able to assimilate trace oestrogens (submicrogram per litter concentrations). Accordingly, in situ studies of mesocosms periodically amended with a low concentration (nM) of steroid substrate may be essential to elucidate the ecological roles of these rare biospheres in their habitats.

### Table 5. UPLC-HRMS information of characteristic metabolites involved in bacterial degradation of steroid hormones.

| Compound ID | Chemical structure | UPLC behaviour (RT, min) | Molecular formula/ (predicted molecular mass)* | Dominant ion peaks | Identification of product ions | Mode observed |
|-------------|--------------------|--------------------------|-----------------------------------------------|-------------------|--------------------------------|---------------|
| Aerobic 9,10-seco pathway | | | | | | |
| 3,17-Dihydroxy-9,10-seconandrosta-1,3,5(10)-triene-9-one (3,17-DHSA) | <image> | 5.53a | C20H26O3 | 302.1881 | 267.1740 | [M-2H2O + H]+ | ESI and APCI |
| 3-Hydroxy-9,10-seconandrosta-1,3,5(10)-triene-9,17-dione (3-HSA) | <image> | 5.35a | C19H24O3 | 300.1725 | 283.1667 | [M-H2O + H]+ | ESI and APCI |
| Aerobic 4,5-seco pathway | | | | | | |
| Pyridinestrone acid (PEA) | <image> | 4.02b | C18H21O3N | 299.1521 | 282.17 | [M-H2O + H]+ | ESI |
| 4-Norestrogenic acid | <image> | 5.92b | C17H24O4 | 292.1675 | 307.1740 | [M-H2O + H]+ | ESI and APCI |
| Anaerobic 2,3-seco pathway | | | | | | |
| 17-Hydroxy-1-oxo-2,3-secoandrost-3-ene-17-oic acid (2,3-SAOA) | <image> | 5.08a | C19H26O4 | 322.2144 | 305.21 | [M-H2O + H]+ | ESI and APCI |
| 1,17-Dioxo-2,3-secoandrostan-3-one (DSAO) | <image> | 5.00a | C19H28O4 | 320.1988 | 303.20 | [M-H2O + H]+ | ESI and APCI |
| The central HIP degradation pathway | | | | | | |
| 3α-H-4α-H-3β-propanoate)-7αβ-methylhexahydro-1,5-indanedione (HIP) | <image> | 2.39a | C17H26O4 | 320.1988 | 294.1831 | [M-2H2O + H]+ | ESI and APCI |
| | | | | | | |
| RT, retention time. The UPLC separation was achieved on a reversed-phase C18 column (Acquity UPLC® BEH C18; 1.7 µm; 100 x 2.1 mm; Waters) with a flow rate of 0.4 ml min⁻¹ at 35°C (column oven temperature). The mobile phase comprised a mixture of two solvents: solvent A [2% (vol/vol) acetonitrile containing 0.1% (vol/vol) formic acid] and solvent B [methanol containing 0.1% (vol/vol) formic acid]. Condition 1: separation was achieved using a linear gradient of solvent B from 10% to 99% across 8 min. Condition 2: separation was achieved using a linear gradient of solvent B from 5% to 99% across 12 min. aCondition 1 for the UPLC separation. bCondition 2 for the UPLC separation. cThe predicated molecular mass was calculated using the atom mass of 12C (12.0000), 16O (15.9949) and 1H (1.0078).
Challenges and future perspectives

Steroid hormone contamination appears to be widespread in various ecosystems, and its long-term impact on wildlife has been studied in some detail. Elucidating the physiology of microbial degraders and biochemical mechanisms involved in steroid hormone degradation may offer a solution to improve biodegradation efficiency in engineered ecosystems. Although conventional culture-dependent and molecular approaches have provided insights into each biodegradation step, investigation of steroid hormone biodegradation remains challenging. For example, the fact that most steroid-degrading anaerobes cannot grow on solid media (e.g. agar plate) makes many molecular biological approaches difficult. Although the oestrogen-degrading alphaproteobacteria (e.g. *Sphingomonas* spp. and *Novosphingobium* spp.) are able to form colonies on

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agar plates, the presence of glycosphingolipids on their cell wall and the lack of suitable gene transfer vectors increase the difficulties in genetic manipulation (Saito et al., 2006). Fortunately, the combination of transcriptional analysis and metabolite profiling provides an alternative to determining the steps in the 2,3-seco and 4,5-seco pathways. Nevertheless, the information regarding the steroid B-ring degradation in anaerobic proteobacterial degraders, steroid hormone chemosensory and steroid transport systems remains unclear. Thus, the isolation of suitable bacterial strains for molecular biological approaches is crucial for future studies on steroid biodegradation.

The ecological role of steroid hormone degraders in environments remains uncertain. Metagenomics is a conventional approach to address ecological relevance of microbial hormone degradation, but challenges remain because sequences of degradation genes usually comprise low coverage within metagenome data set (Holert et al., 2018). This might be due to the low abundance of microbial degraders in ecosystems where the hormone input is low (Wei et al., 2018). As a result, interrogation on ecosystems with long-term hormone contamination may expand new insights into diversity of hormone degraders and catabolic genes. Moreover, recent discoveries of steroid degraders in eukaryotic hosts (Ding et al., 2016; Holert et al., 2018) suggest a bidirectional interaction between steroid degraders and their eukaryotic hosts. The discovery of cobalamin auxotrophy in many steroid-degrading anaerobes (Wei et al., 2018; Wang et al., 2019) indicates cobalamin cross-feedings within microbial communities. These findings may also offer another avenue for future studies.

Acknowledgements
The authors acknowledge the generous financial support by the Ministry of Science and Technology of Taiwan (MOST 107-2311-B-001-021-MY3).

Conflict of interest
None declared.

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