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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 O_2 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.

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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-ethynyloestradiol (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 progestogens). 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, progestogens (such as protesterone) 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

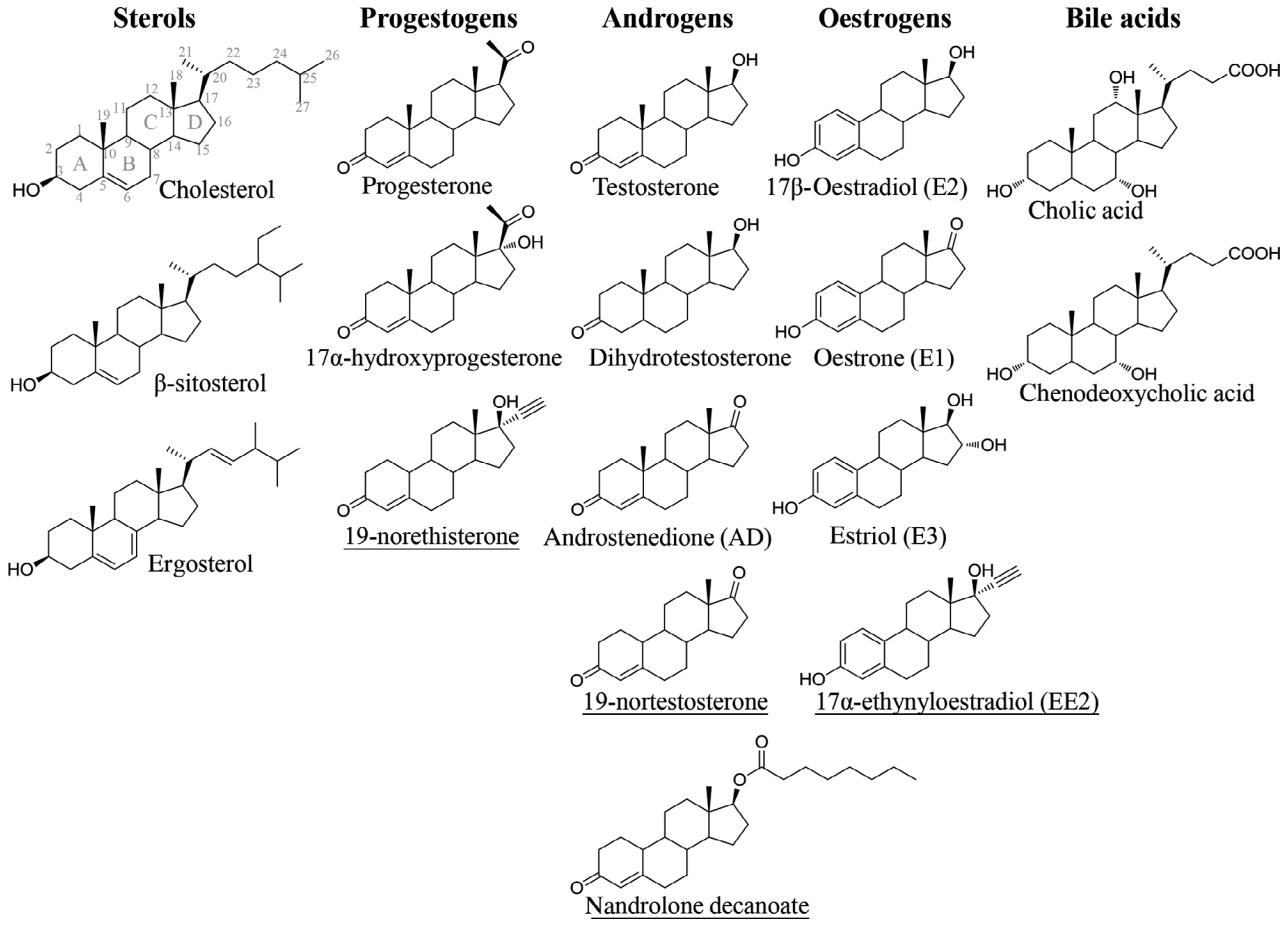


Fig. 1. The chemical structures of prevalent natural and synthetic steroid hormones. The ring identification (A-D) and carbon numbering (1-27) systems for steroids are shown on cholesterol. Underlined compounds are synthetic steroids.

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´es and Schmachtenberg, 2012). Sulphated steroids (e.g. 17β-oestradiol disulphate) are potent olfactory chemosignals

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*., 2014). 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 Hellou, 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 Sedlak, 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 Ellestad, 2014; Gorga *et al*., 2015; Shih *et al*., 2017; Šauer *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; Šauer *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 $\mu g\ l^{-1}$) 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 $\mu g\ l^{-1}$ 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

a high removal efficiency (91–100%) for androgens and progestogens; however, the removal efficiency for oestrogens was relatively lower (67–80%).

The removal of steroid hormones through microbial activities is also observed in natural ecosystems such as soils (Fan *et al*., 2007; Mashtare *et al*., 2013), river water (Jürgens *et al*., 2002), sandy aquifers (Ying *et al*., 2003), as well as seawater and marine sediments (Homklin *et al*., 2011; Gorga *et al*., 2015; Liu *et al*., 2015a). In general, microorganisms degrade steroids slowly under oxygen-limited or oxygen-fluctuating conditions. Thus, anaerobic environments such as river and marine sediments are reservoirs for steroids (Mackenzie *et al*., 1982; Hanselman *et al*., 2003; Czajka and Londry, 2006).

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*., 2012; Fernández de Las Heras *et al*., 2013; Guevara *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. zopfii*) were capable of degrading oestrogens completely (Yoshimoto *et al*., 2004; Kurisu *et al*., 2010). On the other hand, the oestrogen-degrading alphaproteobacterial *Novosphingobium tardaugens* NBRC 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 *R. equi* ATCC 13557 and *R. erythropolis* ATCC 4277 displayed partial EE2 degradation activity when co-incubated with glucose and adipic acid, respectively (O'Grady *et al*., 2009).

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). *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, *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, *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 *D. oestradiolicum* AcBE2-1 cannot degrade E2 with oxygen as the electron acceptor (Fahrbach *et al*., 2006). The betaproteobacterium *Thauera terpenica* strain 58Eu (= DSM 12139), isolated from a ditch (Foss and Harder,

Table 1. Selection of the bacterial strains capable of aerobic degradation of steroid hormones.

			Genome information			
Phylum/class	Strain	Origin	G + C content (mol%)	Accession number	Steroid substrates	Growth on agar plate
Actinobacteria	*Rhodococcus ruber* M1, N361 (DSM 43338)	Activated sludge	70.5	GCF_001646835.1	AD, cholesterol, testosterone	Yes
	Rhodococcus aetherivorans 10BC-312 (DSM 44752)	Activated sludge	NA	NA	AD, ADD, testosterone	Yes
	Rhodococcus ruber Chol-4	Activated sludge	70.6	GCF_000347955.2	AD, ADD, cholesterol, 17β-Oestradiol, testosterone, progesterone	Yes
	Amycolatopsis sp. 75iv2 (ATCC 39116)	Soil	69.1	AFWY00000000	Cholesterol, testosterone	Yes
	Rhodococcus equi ATCC 13557	NA	NA	NA	17α-Ethinyloestradiol (partial degradation)	Yes
	Rhodococcus erythropolis ATCC 4277	Soil	67.0	NA	17α-Ethinyloestradiol (partial degradation)	Yes
Alphaproteobacteria	*Novosphingobium tardaugens* ARI-1 (NBRC 16725)	Activated sludge	61.2	CP034719	17β-Oestradiol, oestrone, estriol	Yes
	Sphingomonas sp. KC8	Activated sludge	63.7	CP016306	17β-Oestradiol, oestrone, testosterone	Yes
	Sphingomonas wittchii RW1	River	68.4	CP000699	Testosterone	Yes
Betaproteobacteria	*Comamonas testosterone* (ATCC 11996, DSM 50244)	Soil	61.5	AHIL00000000.1	Testosterone	Yes
	Cupriavidus necator H16 (ATCC17699)	Activated sludge	66.5 66.7	NC_008313 NC_008314	Testosterone	No
Gammaproteobacteria	*Endozoicomona montiporae* CL-33 (BCRC 17933)	Coral	48.4	CP013251	Testosterone	Yes
	Pseudomonas resinovorans CA10 (NBRC 106553)	Activated sludge	65.6	AP013068	Testosterone	NA

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

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 (Yücel *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 details, 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 involved 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 (Rosłoniec *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 testosteroni* (a betaproteobacterium) as the model organism. The studies on aerobic testosterone catabolism were initiated in the 1960s by

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

			Genome information				
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	QVLR00000000	ADD, cholate, deoxycholate	Nitrate	Yes
	Denitratisoma oestradiolicum AcBE2-1 (DSM 16959)	Activated sludge	61.4	NCXS00000000	E1, E2	Nitrate	Marginal
	Denitratisoma sp. DHT3	Activated sludge	64.9	CP020914	E1, E2, testosterone	Nitrate	No
	Sterolibacterium denitrificans 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[b]
	Thauera terpenica 58Eu (DSM 12139)	Ditch	64.2	ATJV01000070	Testosterone[a]	Nitrate	Yes
	Thauera terpenica GDN1	Estuarine		sediment	NA	NA	
Testosterone[a]	Nitrate	Yes					
Gammaproteobacteria	*Steroidobacter denitrificans* FS (DSM 18526)	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.

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-Δ^1-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 secosteroid, 3-hydroxy-9,10-seconandrosta-1,3,5(10)-triene-9,17-dione (3-HSA). This 9,10-secosteroid 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-seconandrost-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 *meta*-cleavage by an extradiol dioxygenase, TesB. Subsequently, the hydrolase TesD mediates the hydrolytic cleavage between the C-5 and C-10 of 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA), producing 3aα-H-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP). 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 (Plésiat 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 progesterone degradation. Moreover, Horinouchi *et al.* (2012) suggested that *C. testosteroni* degrades progesterone through the 9,10-*seco* pathway. The 9,10-*seco* pathway is also responsible for the bile acid degradation in *Pseudomonas* sp. strain Chol1 (Philipp, 2011). Interestingly, the strain Chol1 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-secosteroids (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

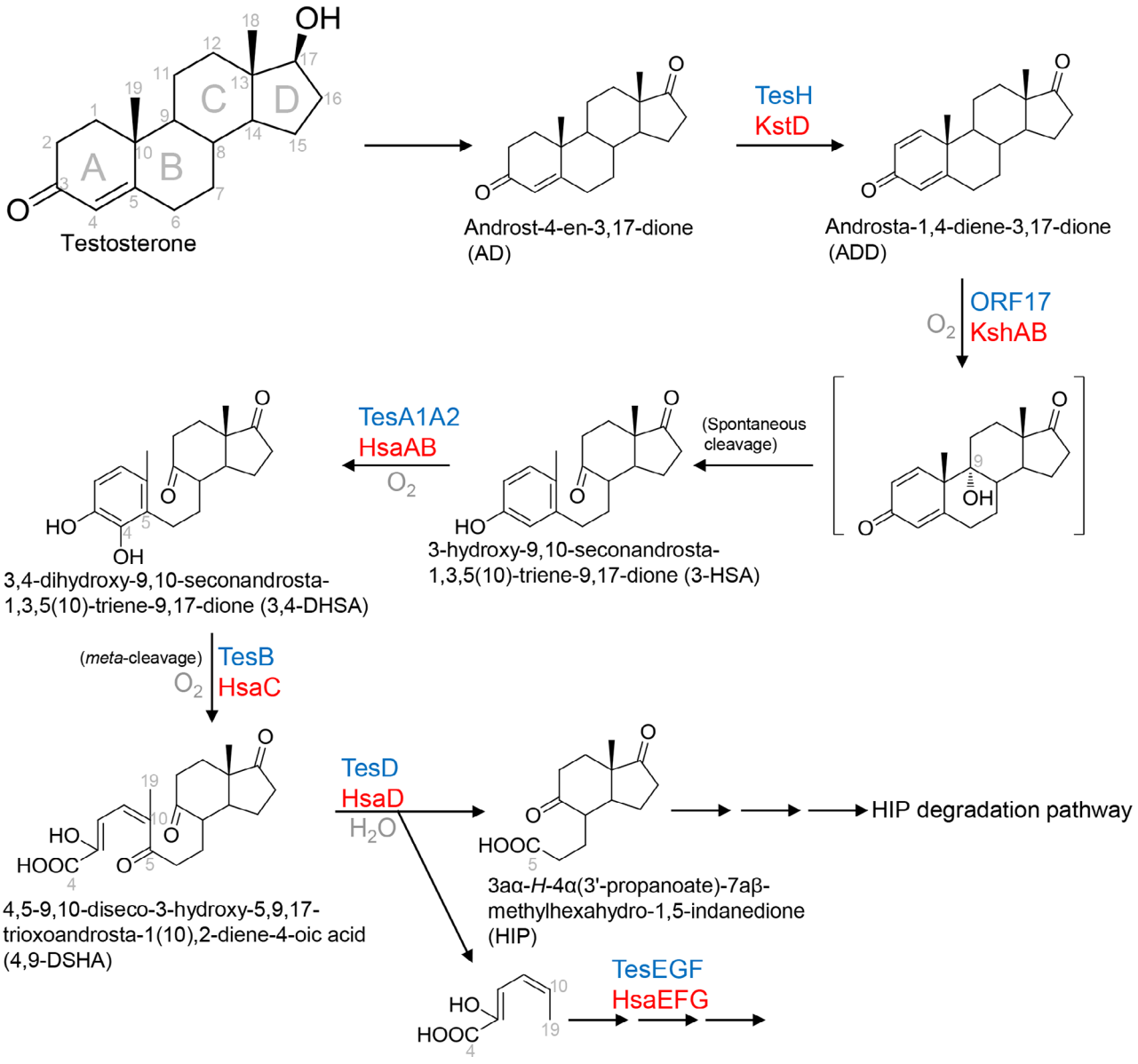


Fig. 2. The aerobic 9,10-*seco* pathway for bacterial degradation of androgens. Characterized or annotated enzymes from proteobacteria are marked in blue, and those from actinobacteria are marked in red. Protein nomenclature is based on that of *Comamonas testosteroni* strain TA441, *Mycobacterium tuberculosis* strain H37Rv, and *Rhodococcus jostii* strain RHA1. The structure in the bracket (9α-hydroxy-androsta-1,4-diene-3,17-dione) is very unstable and has never been detected. Highly similar pathways were proposed for the aerobic degradation of cholic acid (Holert *et al.*, 2014) and cholesterol (Bergstrand et al., 2016).

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-norestrogenic 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 monooxygenase 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 recyclization to produce a nitrogen-containing compound pyridinestrone acid. It is known that *meta*- cleavage metabolites produced in bacterial cultures are often abiotically recyclized with ammonium to generate picolinic acid (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

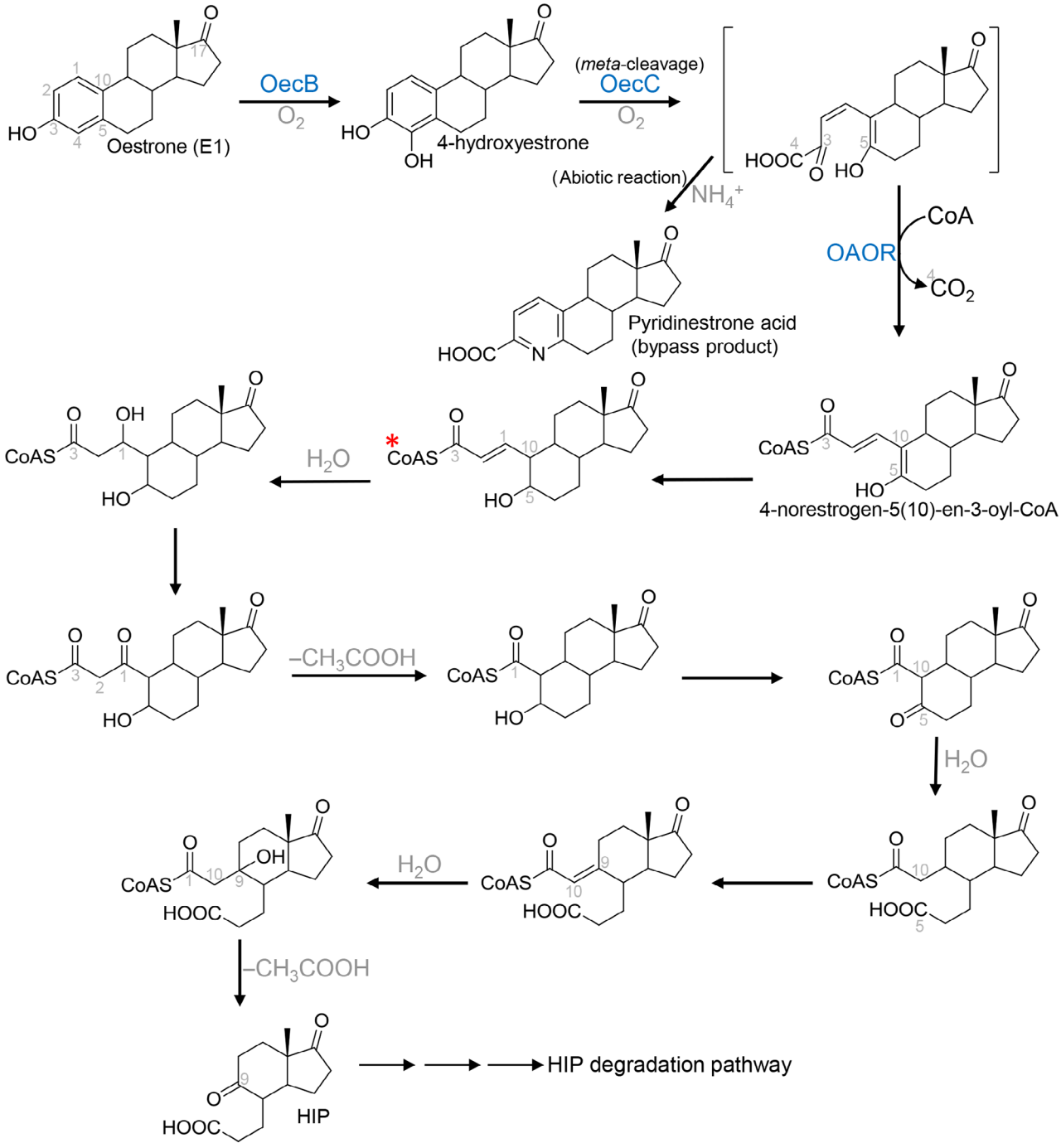


Fig. 3. The aerobic 4,5-*seco* pathway for bacterial degradation of natural oestrogens. Characterized or annotated enzymes from proteobacteria are marked in blue. Protein nomenclature is based on that of *Sphingomonas* sp. strain KC8. *, the deconjugated structure, 4-norestrogenic acid, is often detected in extracellular environments.

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 carboxylic 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 *Nocardia* 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-secoandrostan-3-oic 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 hydrolase 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-SDAD) (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 certain gene homologues shared in the genomes of *S. denitrificans* DSM 18526 and two *Denitratisoma* strains might play a role in anaerobic oestrogen degradation

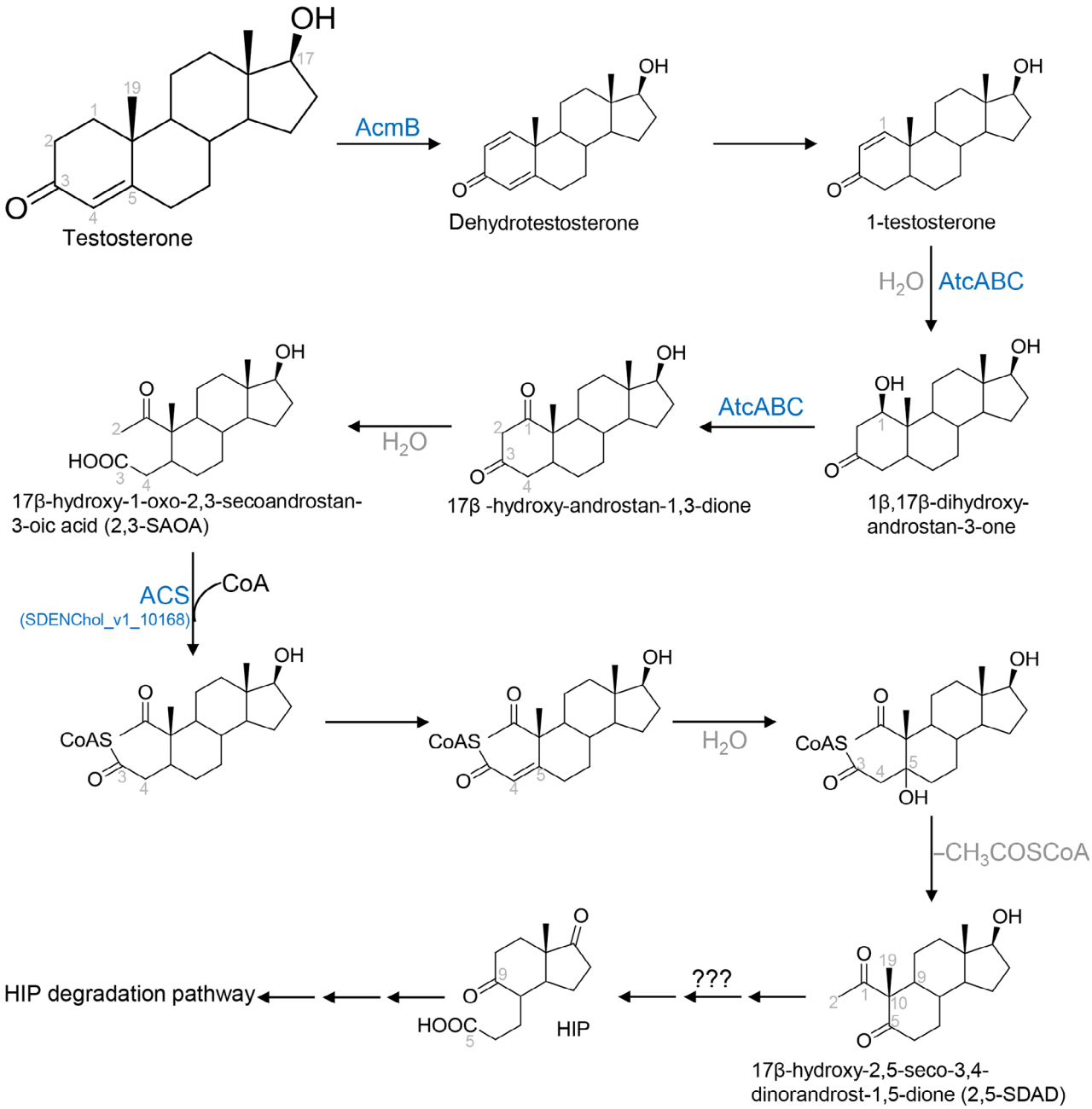


Fig. 4. The anaerobic pathways for bacterial degradation of androgens and oestrogens. Protein nomenclature is based on that of *Sterolibacterium denitrficans* DSM 13999, *Steroidobacter denitrificans* DSM 18526 and *Denitratisoma* sp. strain DHT3. AdoHcy, *S*-adenosylhomocysteine; SAM, *S*-Adenosylmethionine.

(Chen *et al.*, 2019). 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 C_{13} 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

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 hydrolase IpdAB (Crowe *et al*., 2017; 2018) (Fig. 5A). Surprisingly, the homologues of these ring-cleavage enzymes were identified in the genomes of aerobic *C. testosteroni* (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 denitficians*, 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.

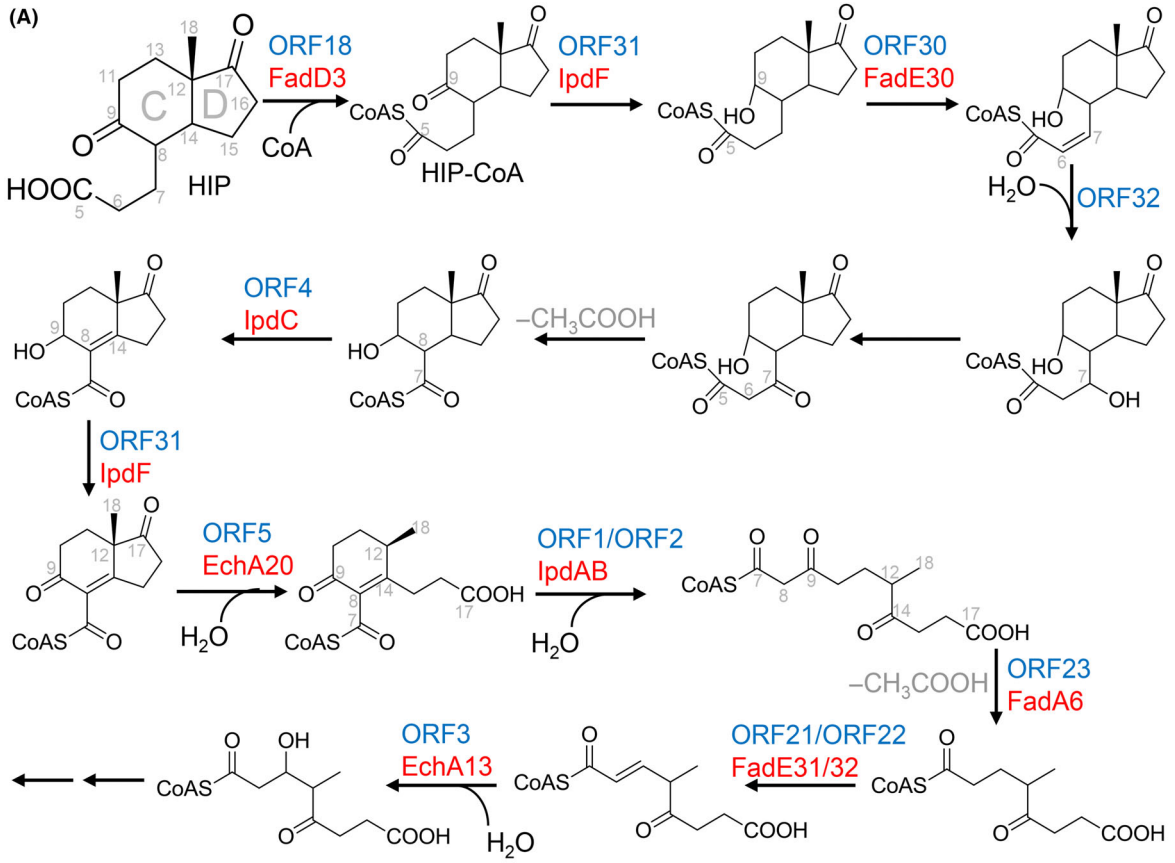
Culture-independent approaches expand insight into the diversity of sex hormone degraders and ecological significance

Early microcosm and mesocosm studies suggested that oestrogens and testosterone can be biodegraded to CO_2 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-^{3}H(*N*)]oestrone as a tracer. Some studies have revealed that several active proteobacterial taxa incorporated trace oestrone (submicrogram per litter 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. testosteroni* 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 *atcA* were identified, indicating that these microbial communities degrade androgen through the 2,3-*seco* pathway. However, the main degraders were *Thauera* spp. (phylogenetically

Fig. 5. The HIP degradation pathway shared by all of the studied steroid-degrading bacteria. (A) The proposed HIP degradation pathway. Characterized or annotated enzymes from proteobacteria are marked in blue, and those from actinobacteria are marked in red. (B) The gene cluster for HIP degradation is widely present in steroid-degrading actinobacteria and proteobacteria. The evolutionary history was inferred from 16S rRNA gene sequences using the maximum-likelihood method in MEGA 7 (Kumar *et al*., 2016). Bootstrap values were calculated from 1,000 resamplings. Bacterial strains marked in black indicate that the aerobic steroid degradation capabilities were physiologically confirmed. Bacterial strains in red are steroid-degrading denitrifiers. The steroid degradation capabilities of bacteria marked in grey remain to be experimentally confirmed. Gene nomenclature is based on that of *Comamonas testosteroni* strain TA441 (ORF1 ~ 33) and *Mycobacterium tuberculosis* strain H37Rv. Orthologous genes are marked with the same colours.



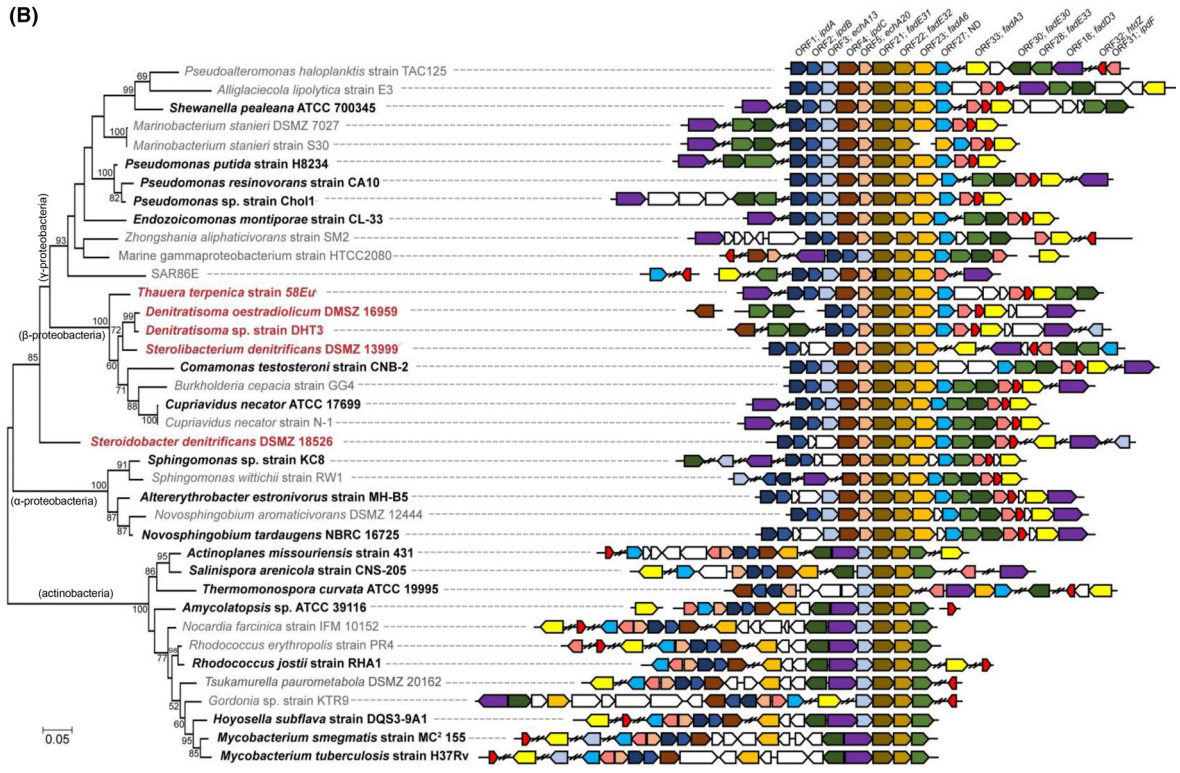


Table 3. Homologues involved in HIP degradation in steroid-degrading bacteria.

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	ARS25885.1	ARS25890.1	ARS25889.1
N. tardaugens NBRC 16725	WP_021688816.1	WP_021688820.1	WP_021688819.1
Altererythrobacter sp. MH-B5	WP_067540119.1	WP_067540155.1	WP_067540130.1
Aerobic betaproteobacteria			
C. testosterone 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

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 hormone 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 [3,4C-^{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 related to testosterone degradation in bacterial genomes. The result showed that *C. testosteroni* KF1, *Cupriavidu necator* JMP134, *Cup. taiwanensis* LMG 19424, *Ralstonia eutropha* H16, *Burkholderia cenocepacia* J2315l, *Burkholderia* sp. 383, *Shewanella pealeana* ATCC700345, *S. halifaxensis* HAW-EB4 and *Pseudoalteromonas haloplanktis* TAC125 are putative androgen degraders due to the high amino acid sequence identity of enzymes involved in the degradation of the testosterone core ring. Recently, a large scale of genomic study using a hidden Markov models (HMMs) search revealed that of the over 8,000 published bacterial genomes, only 256 actinobacterial and proteobacterial genomes harbour the genes involved in the 9,10-*seco* pathway. For further validation, nine predicted steroid-degrading strains were selected for growth experiments and metabolite identification. Among them, only three proteobacterial strains – *Pseudomonas resinovorans* NBRC106553, *Cupriavidus necator* ATCC17699 and *Sphingomonas wittchii* RW1 – and one actinobacterial strain – *Amycolatopsis* sp. ATCC39166 – are able to degrade testosterone completely (Bergstrand *et al*., 2016).

Although most steroid hormone degraders were isolated from sludge communities in wastewater treatment plants, it is interesting that some degraders and catabolic activities were identified in soils, coral, or river and marine sediments, as mentioned above. A metagenome analysis further revealed that the genes involved in the aerobic 9,10-*seco* pathway (degradation pathway for androgens, bile acids and sterols) are ubiquitous in different 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 4. Selected catabolic genes as biomarkers for identification of the three major steroid degradation pathways.

Enzyme The 9,10-*seco* pathway	Substrate	Product	Gene name & protein ID *C. testosteroni* DSM 50244	*C. testosteroni* CNB-2	*R. ruber* Chol-4	*R. rhodochrous* DSM 43269	*R. jostii* RHA1	*M. tuberculosis* H37Rv
3-Ketosteroid 9α-hydoxylase (reductase subunit)	ADD	3-HSA	ORF17 WP_003078230.1	ORF17 ACY32105.1	KshA2 KXF84594.1	KshA2 HQ425874.1	KshA WP_050787406.1 WP_016884082.1	KshA NP_218043.1
3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione-4,5-dioxygenase	3,4-DHSA	4,9-DSHA	TesB WP_003078410.1	TesB ACY32021.1	HsaC WP_010593244.1 WP_017679866.1	NA	HsaC WP_011597910.1 WP_011596919.1	HsaC NP_218085.1
4,5-9,10-Deseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oate hydrolase	4,9-DSHA	HIP	TesD WP_003075542.1	TesD ACY32100.1	HsaD WP_010593414.1	NA	HsaD WP_009479043.1 WP_011596918.1	HsaD ACY32100.1

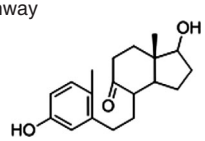
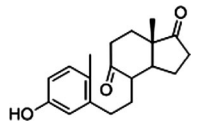
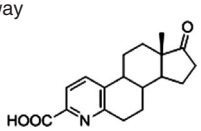
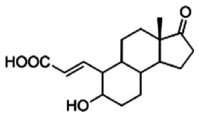
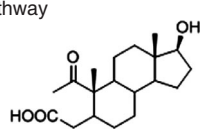
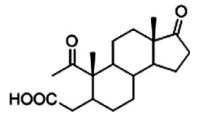
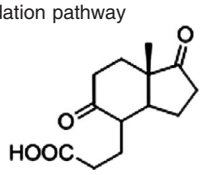
The 2,3-*seco* pathway			*S. denitrificans* DSM 13999	*D. oestradiolicum* DSM 16959	*Denitratisoma* sp. DHT3	*T. terpenica* 58Eu	*S. denitrificans* DSM 18526
1-Testosterone hydratase/dehydrogenase (subunit A)	1-Testosterone	17-Hydroxy-and-rostan-1,3-dione	AtcA SMB21166.1	AtcA TWO78727.1	AtcA QDX80120.1	AtcA WP_021248954.1	AtcA WP_066918684.1

The 4,5-*seco* pathway			*Sphingomonas* sp. KC8	*N. tardaugens* NBRC 16725	*Altererythrobacter* sp. MH-B5
4-Hydroxyestrone 4,5-Dioxygenase	4-Hydroxyesterone	*Meta*-cleavage product[a]	OecC WP_010123492.1	OecC WP_021690447.1	OecC WP_067531096.1
2-Oxoacid oxidoreductase	*Meta*-cleavage product[a]	4-Norestrogen-5(10)-en-3-oyl-CoA	OAOR WP_003075542.1	OAOR ACY32100.1	OAOR WP_010593414.1

ADD, androsra-1,4-diene-3,17-dione; 3-HSA, 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 4,9-DSHA: 4,5-9, 10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid; HIP: 3aα-*H*-4α (3'-propanoate)-7αβ-methylhexhydro-1,5-indanedione.

[a]See Figure 3 for the chemical structure of the *meta*-cleavage product.

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)[c]	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)		5.53[a]	$C_{19}H_{26}O_3$ 302.1881	267.1740 285.1844 303.1946 325.1766	$[M\text{-}2H_2O + H]^+$ $[M\text{-}H_2O + H]^+$ $[M + H]^+$ $[M + Na]^+$	ESI and APCI ESI and APCI ESI and APCI ESI
3-Hydroxy-9,10-seconandrosta-1,3,5(10)-triene-9,17-dione (3-HSA)		5.35[a]	$C_{19}H_{24}O_3$ 300.1725	283.1667 301.1801 323.1625	$[M\text{-}H_2O + H]^+$ $[M + H]^+$ $[M + Na]^+$	ESI and APCI ESI and APCI ESI
Aerobic 4,5-seco pathway						
Pyridinestrone acid (PEA)		4.02[b]	$C_{18}H_{21}O_3N$ 299.1521	282.17 300.16 322.15	$[M\text{-}H_2O + H]^+$ $[M + H]^+$ $[M + Na]^+$	ESI ESI and APCI ESI
4-Norestrogenic acid		5.92[b]	$C_{17}H_{24}O_4$ 292.1675	257.15 275.16 293.17 315.16	$[M\text{-}2H_2O + H]^+$ $[M\text{-}H_2O + H]^+$ $[M + H]^+$ $[M + Na]^+$	ESI ESI and APCI ESI and APCI ESI
Anaerobic 2,3-seco pathway						
17-Hydroxy-1-oxo-2,3-secoandrostan-3-oic acid (2,3-SAOA)		5.08[a]	$C_{19}H_{30}O_4$ 322.2144	305.21 323.22 345.20	$[M\text{-}H_2O + H]^+$ $[M + H]^+$ $[M + Na]^+$	ESI and APCI ESI and APCI ESI
1,17-Dioxo-2,3-secoandrostan-3-oic acid (DSAO)		5.00[a]	$C_{19}H_{28}O_4$ 320.1988	303.20 321.21 343.19	$[M\text{-}H_2O + H]^+$ $[M + H]^+$ $[M + Na]^+$	ESI and APCI ESI and APCI ESI
The central HIP degradation pathway						
3aα-*H*-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP)		2.39[a] 3.78[b]	$C_{17}H_{26}O_4$ 294.1831	259.17 277.18 317.17	$[M\text{-}2H_2O + H]^+$ $[M\text{-}H_2O + H]^+$ $[M + Na]^+$	ESI and APCI ESI and APCI ESI

RT, retention time.
The UPLC separation was achieved on a reversed-phase C_{18} column (Acquity UPLC® BEH C18; 1.7 μm; 100 × 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.
[a]Condition 1 for the UPLC separation.
[b]Condition 2 for the UPLC separation.
[c]The predicated molecular mass was calculated using the atom mass of ^{12}C (12.0000), ^{16}O (15.9949) and ^{1}H (1.0078).

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.

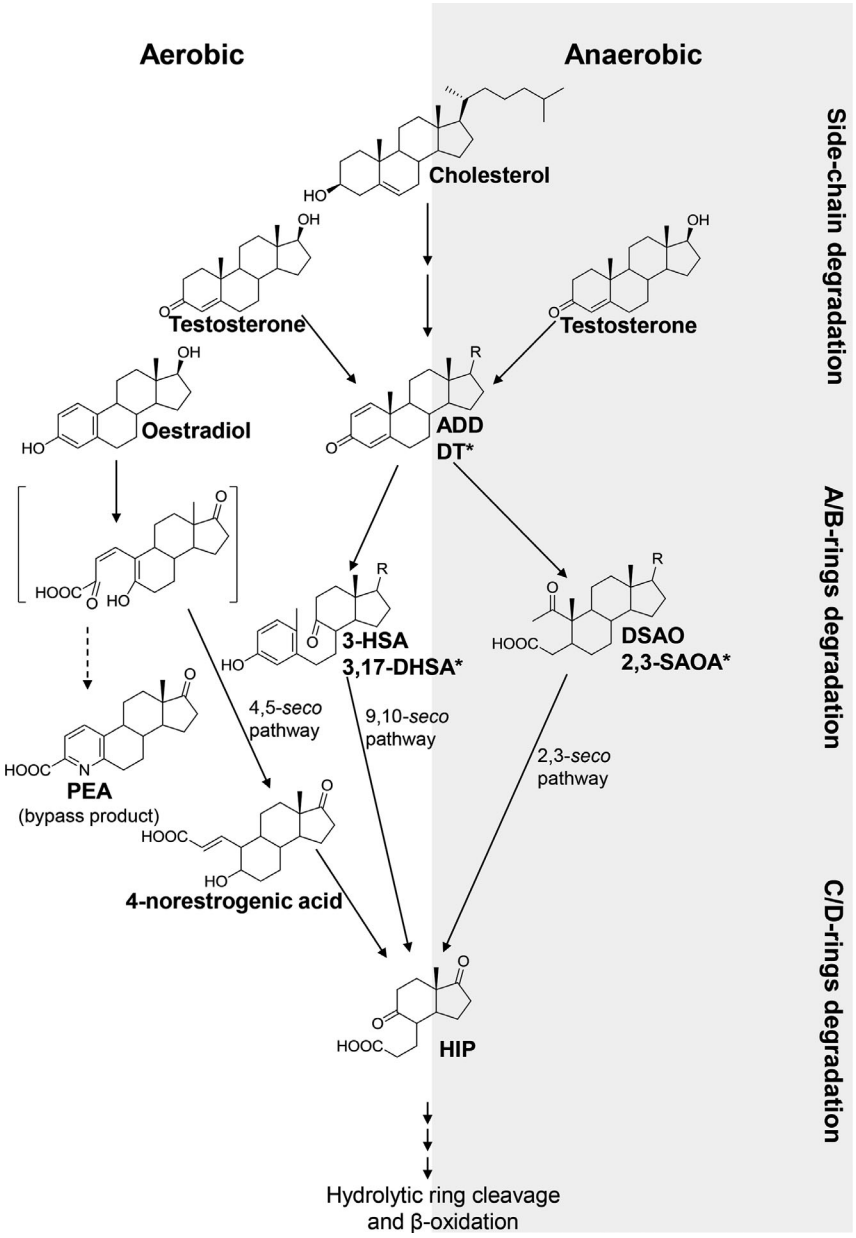


Fig. 6. Characteristic metabolites involved in bacterial steroid degradation pathways. 4-Norestrogenic acid is the deconjugated structure of a critical CoA-ester intermediate in the aerobic 4,5-*seco* pathway. R at the C17 position represents a keto or hydroxyl or group. *, the corresponding 17-hydroxyl structures. Abbreviations: ADD, androsta-1,4-diene-3,17-dione; DSAO, 1,17-dioxo-2,3-secoandrostan-3-oic acid; DT, 1-dehydrotestosterone; HIP, 3aα-H-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione; 3-HSA, 3-hydroxy-9,10-seconandrosta-1,3,5(10)-triene-9,17-dione; 3,17-DHSA, 3,17-dihydroxy-9,10-seconandrosta-1,3,5(10)-triene-9-one; PEA, pyridinestrone acid; 2,3-SAOA, 17-hydroxy-1-oxo-2,3-secoandrostan-3-oic acid.

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

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 transcriptomic 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.

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Conflict of interest

None declared.

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