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Metabolites Involved in Aerobic Degradation of the A and B Rings of Estrogen

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ABSTRACT Various bacteria, mainly actinobacteria and proteobacteria, are capable of aerobic estrogen degradation. In a previous study, we used the obligate aerobic alphaproteobacterium *Sphingomonas* sp. strain KC8 as a model microorganism to identify the initial metabolites involved in the oxygenolytic cleavage of the estrogen A ring: 4-hydroxyestrone, a *meta*-cleavage product, and a dead-end product pyridinestrone acid. In this study, we identified the downstream metabolites of this aerobic degradation pathway using ultraperformance liquid chromatography– high-resolution mass spectrometry (UPLC-HRMS). 4-Norestrogen-5(10)-en-3-oyl-coenzyme A and its closely related deconjugated (non-coenzyme A [non-CoA]) structure, 4-norestrogenic acid, were detected in the estrone-grown strain KC8 cultures. The structure of 4-norestrogenic acid was elucidated using nuclear magnetic resonance (NMR) spectroscopy. The extracellular distribution and the accumulation of 4-norestrogenic acid in the bacterial cultures indicate that the estrogen-degrading bacteria cannot degrade this deconjugated product. We also observed temporal accumulation and subsequent consumption of a common steroid metabolite, 3aα-H-4α(3′-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP), in the bacterial cultures. The metabolite profile and genomic analyses shed light on the biochemical mechanisms involved in the degradation of the A and B rings of natural estrogens. In this proposed aerobic pathway, C-4 of the meta-cleavage product is removed by a 2-oxoacid oxidoreductase through oxidative decarboxylation to produce the 4-norestrogen-5(10)-en-3-oyl-CoA. Subsequently, the B ring is cleaved by hydrolysis. The resulting A/B-ring-cleaved product is transformed into a common steroid metabolite HIP through β-oxidation reactions. Accordingly, the A and B rings of different steroids are degraded through at least three peripheral pathways, which converge at HIP, and HIP is then degraded through a common central pathway.

IMPORTANCE Estrogens, often detected in surface waters worldwide, have been classified as endocrine disrupting chemicals and carcinogens. Bacterial degradation is crucial for removing natural estrogens from natural and engineered ecosystems; however, current knowledge regarding the biochemical mechanisms and catabolic enzymes involved in estrogen biodegradation is very limited. Our estrogen metabolite profile and genomic analyses on estrone-degrading bacteria enabled us to characterize the aerobic estrogen degradation pathway. The results greatly expand our understanding of microbial steroid degradation. In addition, the characteristic metabolites, dead-end products, and degradation genes can be used as biomarkers to investigate the fate and biodegradation potential of estrogens in the environment.

The synthesis and secretion of estrogens occur in most animals, including invertebrates and vertebrates (1, 2). The biosynthetic pathways of estrogens include the elimination of the cholesterol side chain and hydroxylation of the steroid nucleus. In the liver, estrogens undergo structural modifications and are converted into inactive excretion products. The solubility of the resulting products is increased by conjugation with glucuronic acid or sulfate. These conjugates are excreted in urine and feces (3). Primary sources of environmental estrogens thus include human urine and livestock manure (4). In addition, literature and popular media have pointed to 17α-ethinylestradiol, a synthetic estrogen from oral contraceptives, as a major endocrine disrupting chemical (5). Because of the increase in the human population and increasing demands for livestock products, estrogen pollution has become a global concern and challenge (5–7).

Natural and synthetic estrogens have a serious impact on the environment. This has become an increasingly important issue, because exposure to estrogens has adverse physiological and reproductive effects on humans and aquatic animals (8–11). In addition to being endocrine-disrupting chemicals, estrogens have been classified by the World Health Organization as group 1 carcinogens (12). Wastewater treatment plants are crucial for removing estrogens produced by humans and livestock. However, municipal wastewater treatment plants are not able to remove estrogens completely (13, 14). Consequently, estrogens are often detected in surface waters of rivers and coasts near wastewater treatment plants (typically in the nanogram per liter range) (15–19).

Bacterial degradation plays a significant role in the removal of estrogens from the environment (20, 21). Bacteria capable of aerobic estrogen degradation have been isolated from various engineered and natural ecosystems, including activated sludge, seawater, compost, soils, and sandy aquifers; most of these isolates belong to the phyla *Actinobacteria* and *Proteobacteria* (22). For example, Fujii et al. (23) reported the isolation and characterization of the first estrogen-degrading sludge bacterium, *Novosphingobium tardaugens* strain ARI-1. Yoshimoto et al. (24) isolated several estrogen-degrading actinobacteria, identified as *Rhodococcus* spp.

The literature on estrogen degradation pathways is limited. In our recent study (20), we used *Sphingomonas* sp. strain KC8 (here referred to as strain KC8), a sludge isolate (25), as a model organism to study aerobic estrogen degradation. We identified three initial degradation metabolites: 4-hydroxyestrone (metabolite 2), the *meta*-cleavage product (metabolite 3), and a dead-end product pyridinestrone acid (see Fig. 1 for the structures of these molecules). We also identified strain KC8 gene clusters likely to be involved in the degradation of estrogen A and B rings (gene clusters I and II) and C and D rings (gene cluster III) (20). Pyridinestrone acid and the key catabolic gene *oecC* were detected in estrone (E1)-spiked samples collected from wastewater treatment plants and suburban rivers (20, 21), suggesting the prevalence of this aerobic degradation pathway (the 4,5-*seco* pathway) in the environment. However, the downstream metabolites and corresponding enzymes of this degradation pathway, especially those involved in the A/B ring degradation, remained to be unraveled. In the present study, two estrogen-degrading alphaproteobacteria, strain KC8 (25) and *Novosphingobium* sp. strain SLCC (21), were used as model microorganisms and incubated with E1. We used ultraperformance liquid chromatography–high-resolution mass spectrometry (UPLC-HRMS) and nuclear magnetic resonance (NMR) spectroscopy to identify the downstream metabolites of this aerobic pathway (Fig. 1). The metabolite profile analysis, together with the genomic analysis, enabled the prediction of biochemical mechanisms operating in the aerobic biodegradation of natural estrogens.
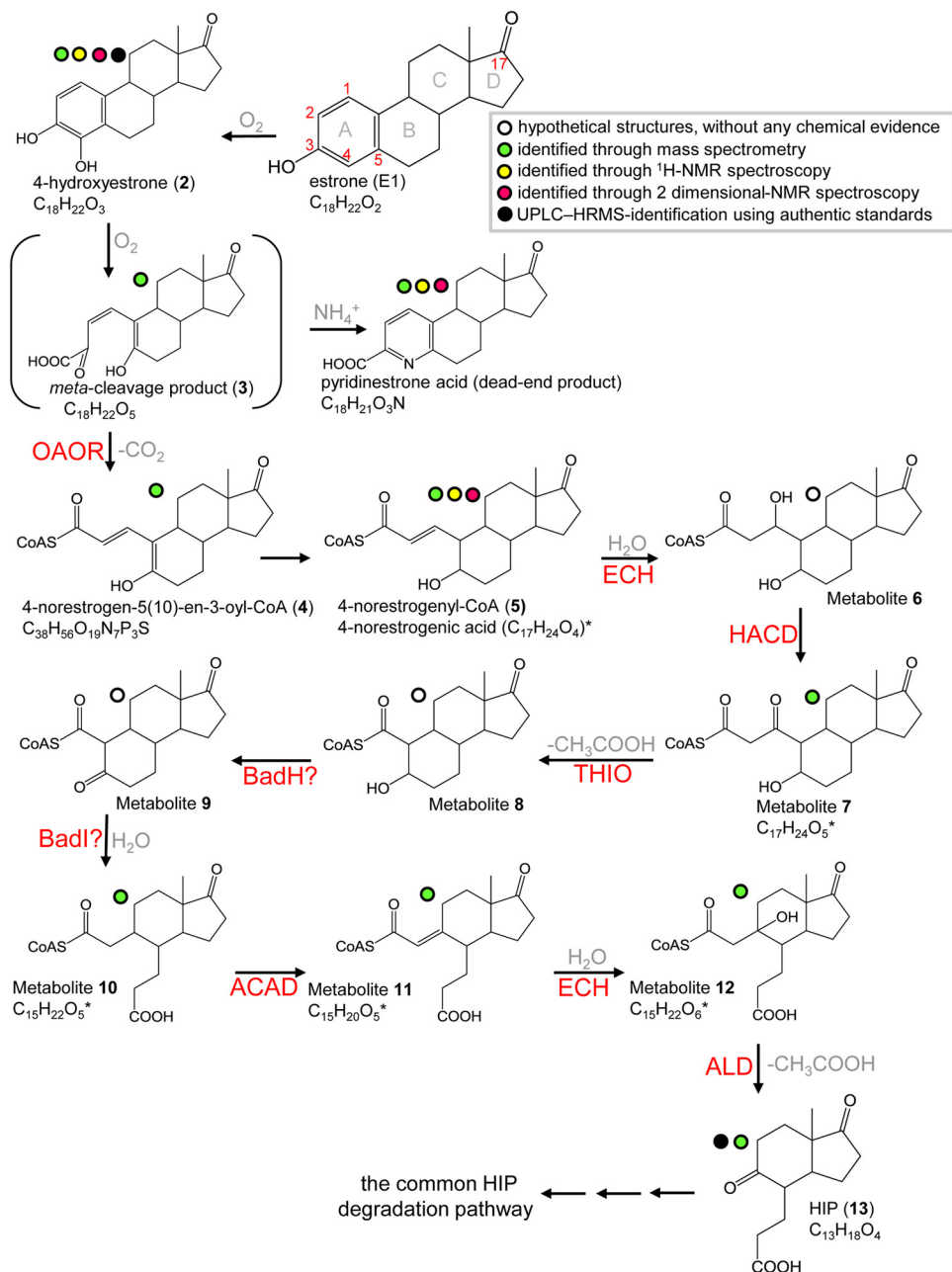
FIG 1 Proposed bacterial degradation pathway of natural estrogens. *, the deconjugated (non-CoA) metabolites were identified using UPLC-HRMS. The chemical structure of 4-norestrogenic acid, the deconjugated non-CoA structure of metabolite 5, was elucidated using NMR spectroscopy. Abbreviations: ACAD, acyl-CoA dehydrogenase; ALD, aldolase; BadH, 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase; BadI, 2-ketocyclohexanecarboxyl-CoA hydrolase; ECH, enoyl-CoA hydratase; HACD, β-hydroxyacyl-CoA dehydrogenase; OAOR, 2-oxoacid oxidoreductase; THIO, thiolase.

RESULTS AND DISCUSSION

UPLC-HRMS identification of ethyl acetate-extractable estrogen metabolites. In a previous study (20), we used [3,4C-^{13}C]E1 as a tracer to identify initial metabolites of the 4,5-seco pathway and concluded that bacterial estrogen degradation starts from the A ring. To identify the downstream intermediates of this aerobic pathway, the resting cells of strain KC8 were aerobically incubated with E1 ([2,4,16,16-D4]E1 and unlabeled E1 were mixed at a 1:1 molar ratio), and the hydrophobic estrogen metabolites were extracted using ethyl acetate. Therefore, pairs of adduct ions were observed
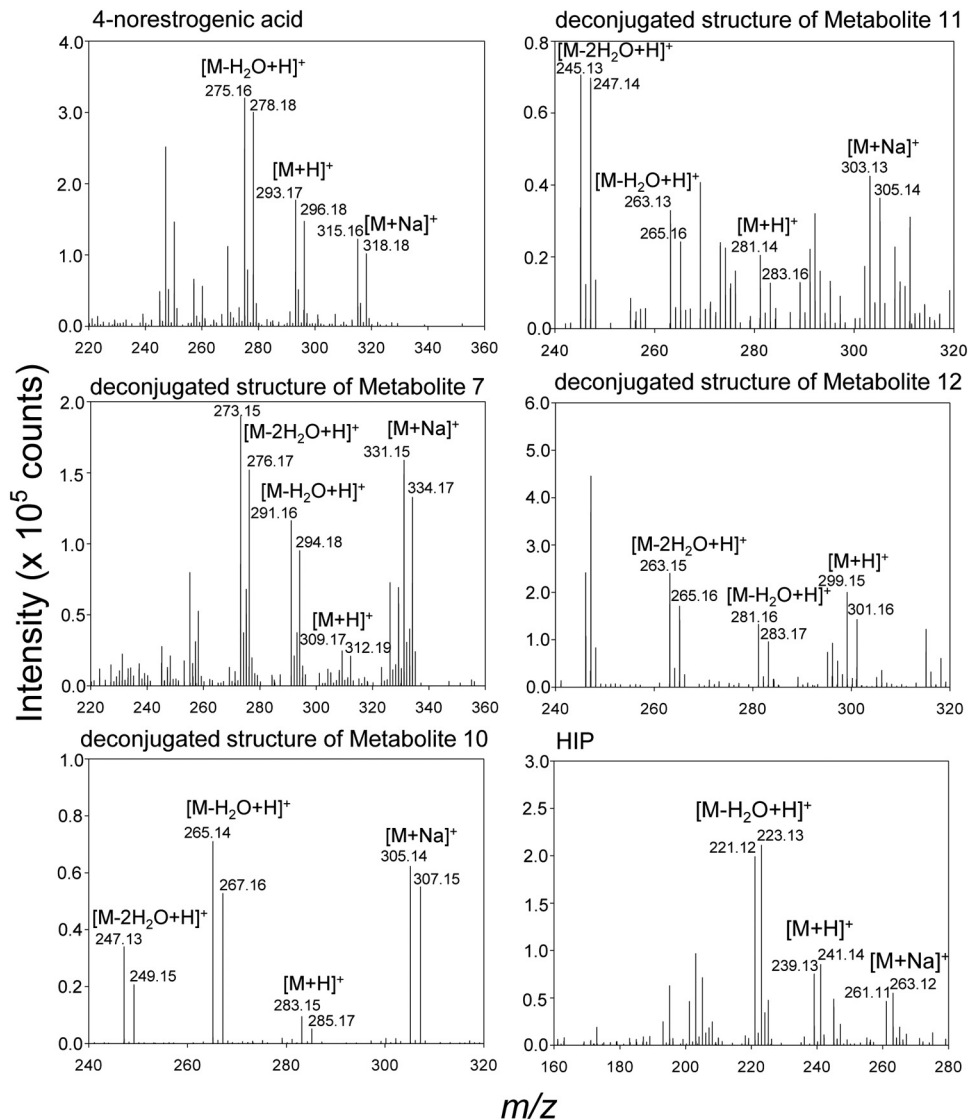
FIG 2 ESI-HRMS spectra of the estrogen metabolites of strain KC8 incubated with 1 mM E1. In the resting cell assay, the substrate was composed of [2,4,16-D4]E1 and unlabeled E1 (mixed at a 1:1 molar ratio). Only estrogen metabolites newly identified in this study are shown.

in the mass spectra of E1-derived metabolites (Fig. 2). We detected 10 E1-derived metabolites in total, including 6 undescribed intermediates (2 C_{17}, 3 C_{15}, and 1 C_{13} compound) of aerobic estrogen degradation (Table 1). 3aα-H-4α(3′-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP), the C_{13} metabolite, was identified by comparison with the authentic standard (purchased from Sigma-Aldrich) using UPLC-HRMS. The UPLC retention time and HRMS behavior of a detected metabolite were identical to those of the HIP standard (see Fig. S1 in the supplemental material). This result excludes the possibility of detecting structural isomers, which are often observed in chemical analyses relying only on mass spectrometry. A highly similar estrogen metabolite profile was also observed in the resting cell assay of strain SLCC, and the 6 novel estrogen metabolites were also detected (see Table S1).

Elucidating the structure of a novel estrogen metabolite through NMR analysis. 4-Norestrogenic acid, an unprecedented C_{17} estrogen metabolite, accumulated in the two E1-degrading strains (Tables 1 and S1). In addition, 4-norestrogenic acid was mostly (approximately 90% produced by bacterial cells) detected in extracellular fractions. To produce a sufficient amount of this compound for NMR analysis, strain SLCC

TABLE 1 UPLC-HRMS analysis of metabolites involved in aerobic E1 catabolism by strain KC8

Compound ID[a]	UPLC RT[b] (min)	Molecular formula (predicted mol wt)[c]	Dominant ion peaks	Identified product ions	Observation mode
E1	8.14	$C_{18}H_{22}O_2$ (270.16)	253.16	$[M-H_2O+H]^+$	ESI and APCI
			271.17	$[M+H]^+$	ESI and APCI
4-Hydroxyestrone	7.41	$C_{18}H_{22}O_3$ (286.16)	269.16	$[M-H_2O+H]^+$	APCI
			287.15	$[M+H]^+$	ESI and APCI
			309.15	$[M+Na]^+$	ESI
meta-Cleavage product	6.85	$C_{18}H_{22}O_5$ (318.15)	301.15	$[M-H_2O+H]^+$	ESI
			341.14	$[M+Na]^+$	ESI
Pyridinestrone acid	4.02	$C_{18}H_{21}O_3N$ (299.15)	282.17	$[M-H_2O+H]^+$	ESI
			300.16	$[M+H]^+$	ESI and APCI
			322.15	$[M+Na]^+$	ESI
Compound Ie	5.61	$C_{16}H_{24}O_4$ (280.17)	245.15	$[M-2H_2O+H]^+$	ESI
			263.16	$[M-H_2O+H]^+$	ESI and APCI
			281.17	$[M+H]^+$	ESI and APCI
			303.16	$[M+Na]^+$	ESI
4-Norestrogenic acid (metabolite 5)[d]	5.92	$C_{17}H_{24}O_4$ (292.17)	257.15	$[M-2H_2O+H]^+$	ESI
			275.16	$[M-H_2O+H]^+$	ESI and APCI
			293.17	$[M+H]^+$	ESI and APCI
			315.16	$[M+Na]^+$	ESI
Metabolite 7[d]	6.24	$C_{17}H_{24}O_5$ (308.16)	273.15	$[M-2H_2O+H]^+$	ESI and APCI
			291.16	$[M-H_2O+H]^+$	ESI and APCI
			309.17	$[M+H]^+$	ESI and APCI
			331.15	$[M+Na]^+$	ESI
Metabolite 10[d]	5.05	$C_{15}H_{22}O_5$ (282.15)	247.13	$[M-2H_2O+H]^+$	ESI and APCI
			265.14	$[M-H_2O+H]^+$	ESI and APCI
			283.15	$[M+H]^+$	ESI and APCI
			305.14	$[M+Na]^+$	ESI
Metabolite 11[d]	5.47	$C_{15}H_{20}O_5$ (280.13)	245.13	$[M-2H_2O+H]^+$	ESI and APCI
			263.13	$[M-H_2O+H]^+$	ESI and APCI
			281.14	$[M+H]^+$	ESI and APCI
			303.13	$[M+Na]^+$	ESI
Metabolite 12[d]	5.19	$C_{15}H_{22}O_6$ (298.14)	263.15	$[M-2H_2O+H]^+$	ESI and APCI
			281.16	$[M-H_2O+H]^+$	ESI and APCI
			299.15	$[M+H]^+$	ESI
HIP	3.78	$C_{13}H_{18}O_4$ (238.12)	221.12	$[M-H_2O+H]^+$	ESI and APCI
			239.13	$[M+H]^+$	ESI and APCI
			261.11	$[M+Na]^+$	ESI

[a]ID, identifier. Estrogen metabolites newly identified in this study are in boldface font.
[b]RT, retention time.
[c]The predicated molecular mass was calculated using the atom mass of ^{12}C (12.00), ^{16}O (15.99), and ^1H (1.01).
[d]The deconjugated non-CoA structures of the hypothetical CoA ester intermediates were identified using UPLC-HRMS.

(2 liters) was aerobically incubated with unlabeled E1 (1 mM) for 12 h. The extracellular distribution of 4-norestrogenic acid facilitates the separation of this compound from biomolecules in the cells. 4-Norestrogenic acid was sequentially purified using solid-phase extraction, HPLC, and thin-layer chromatography (TLC). The structure of 4-norestrogenic acid was elucidated using atmospheric pressure chemical ionization (APCI)-HRMS and 2-dimensional NMR spectroscopy.

The TLC-purified compound, obtained as a colorless oil, was assigned the molecular formula of $C_{17}H_{24}O_4$ based on a pseudomolecular ion peak $[M+H]^+$ at *m/z* 293.17 in the positive mode APCI-HRMS (Fig. 3A). The ^1H and ^{13}C NMR signals (Table 2; see also the original NMR spectra in Fig. S2) of this compound were compatible with those of 4-hydroxyestrone (a reference compound) except for the signals on the A and B rings, indicating distinct structural changes in the moiety. Considering the molecular formula, $C_{17}H_{24}O_4$, the double bond equivalence of 4-norestrogenic acid is six, including one ketone at δ_C 223.7 (C-17), one double bond at δ_C 145.3 (C-1)/131.7 (C-2), one acid carbonyl at δ_C 176.0 (C-3), and three rings (Fig. S2B). Comparing the ^{13}C NMR data of the two compounds (the TLC-purified compound and 4-hydroxyestrone), the major differences were the apparent downfield shifts of C-1, C-2, C-3, and C-6, upfield shifts of C-5 and C-10, and disappearance of C-4 (Table 2). These differences suggest that the A ring was cleaved. Mutual ^3J correlations of δ_H 6.26 (H-1)/δ_H 5.87 (H-2), δ_H 6.26 (H-1)/δ_H
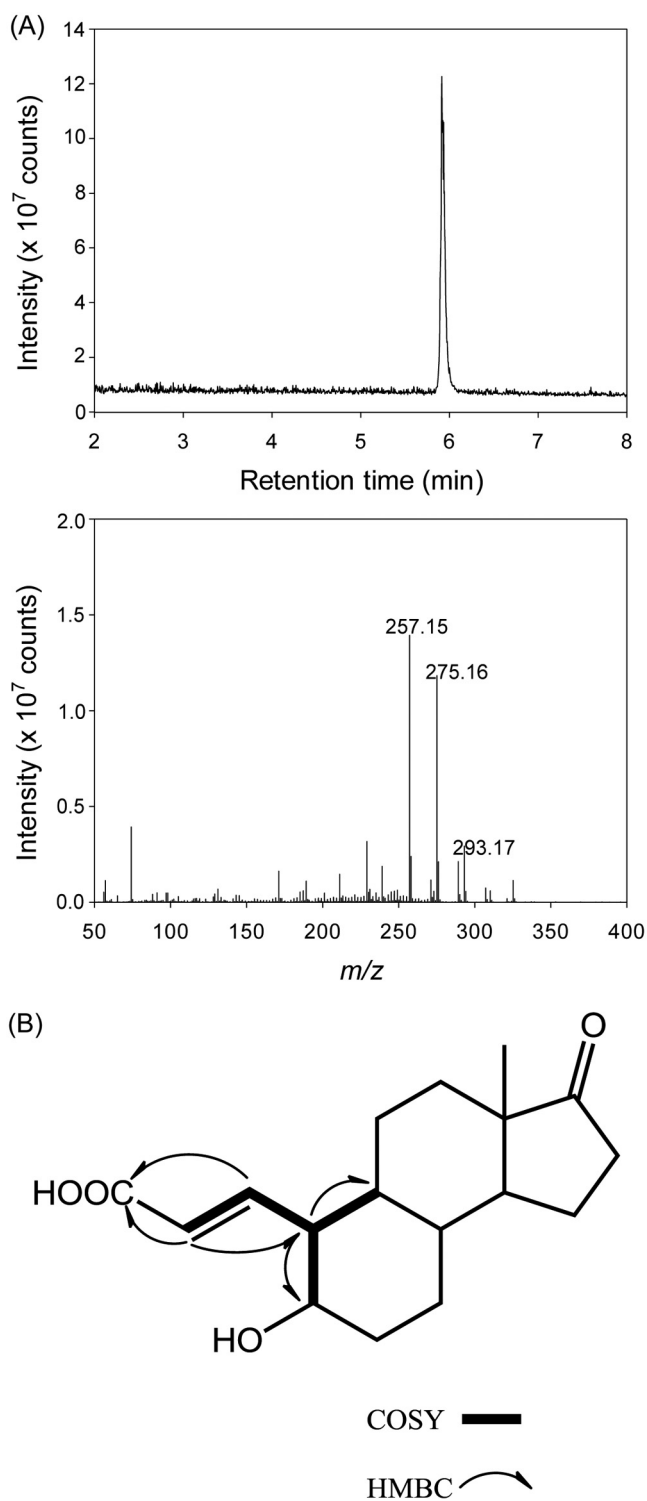
FIG 3 UPLC-APCI-HRMS (A) and key correlations in the 2-dimensional NMR data (B) of the TLC-purified 4-norestrogenic acid. Original NMR spectra are provided in Fig. S2 and S3 in the supplemental material.

1.82 (H-10), δ_H 1.82 (H-10)/δ_H 0.93 (H-9), and δ_H 1.82 (H-10)/δ_H 3.35 (H-5) were observed in the correlation spectroscopy (COSY) spectrum of the TLC-purified compound (Fig. 3B; see also the original COSY spectrum in Fig. S3A), which were further corroborated by the HMBC correlations of δ_H 6.26 (H-1)/δ_C 176.0 (C-3), δ_H 5.87 (H-2)/δ_C 56.3 (C-10) and 176.0 (C-3), and δ_H 1.82 (H-10)/δ_C 47.0 (C-9) and 73.8 (C-5) (Fig. 3B, see the original

TABLE 2 ^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of 4-norestrogenic acid and 4-hydroxyestrone (a reference)[a]

Position	4-Norestrogenic acid		4-Hydroxyestrone	
	^1H (δ [ppm]) (J [Hz])	^{13}C	^1H(δ [ppm]) (J [Hz])	^{13}C
1	6.26 dd (9.9, 15.5)	145.3	6.66 d (7.8)	117.1
2	5.87 d (15.5)	131.7	6.61 d (7.8)	113.3
3		176.0		143.5
4				143.5
5	3.35 m	73.8		125.0
6	2.03 m	35.1	2.94 dd (3.8, 12.3)	24.6
	1.36 m		2.63 m	
7	1.83 m	29.2	2.38 m	27.2
	1.10 m			
8	1.31 m	40.9	1.47 m	39.4
9	0.93 m	47.0	2.21 m	45.5
10	1.82 m	56.3		133.0
11	1.68 m	28.0	2.10 m	27.4
	1.22 m		1.37 m	
12	1.68 m	32.7	1.89 m	32.9
	1.21 m		1.44 m	
13		49.4		49.4
14	1.35 m	51.8	1.54 m	51.7
15	1.95 m	22.7	2.09 m	22.5
	1.59 m		1.68 m	
16	2.43 dd (8.8, 19.3)	36.8	2.51 dd (8.3, 18.4)	36.8
	2.07 dd (8.8, 19.3)		2.15 dd (8.3, 18.4)	
17		223.7		224.0
18	0.89 s	14.4	0.93 s	14.3

[a]Measured in methanol-d_4.

heteronuclear multiple bond correlation (HMBC) spectrum in Fig. S3B). The configuration of $\Delta^{1(2)}$ was determined to be E form based on the $J_{H-1/H-2}$ of 15.5 Hz. The TLC-purified compound is named 4-norestrogenic acid, and its structure is shown in Fig. 3B.

Role of 4-norestrogenic acid in the estrogen degradation pathway. At the beginning of the strain KC8 assay, only the substrate E1 was detected. After 4 h of aerobic incubation, two estrogen metabolites, HIP and 4-norestrogenic acid, apparently accumulated. After that, the amount of HIP gradually decreased over time (Fig. 4A). In contrast, 4-norestrogenic acid was not apparently consumed during the aerobic incubation. Similar temporal changes in estrogen metabolites were observed in the resting cell assay of strain SLCC (Fig. 4B). The cell extract of strain KC8 was incubated with 4-norestrogenic acid (0.1 mM), coenzyme A (CoASH; 1 mM), and ATP (5 mM). After 16 h of aerobic incubation, neither substrate consumption nor coenzyme A (CoA) ester production was observed (see Fig. S4). Moreover, strain KC8 cells were incubated with 0.1 mM 4-norestrogenic acid, and the bacterial cells did not import this compound within 6 days (see Fig. S5). These results suggest that transport protein, CoA ligase, or CoA transferase capable of channeling 4-norestrogenic acid back into the estrogen catabolic pathway is absent in strain KC8. It appears that 4-norestrogenic acid cannot be degraded by the estrogen-degrading bacteria. In contrast, the CoA ester of 4-norestrogenic acid, 4-norestrogenyl-CoA (metabolite 5 in Fig. 1), may be a crucial metabolite for aerobic estrogen degradation. The cellular CoA concentration fluctuates depending on the carbon source and growth state (26). CoA is an essential cofactor in numerous biosynthetic and energy-yielding metabolic pathways. When CoA is required in other metabolic pathways, the CoA esters in the estrogen degradation pathway (e.g., 4-norestrogenyl-CoA and metabolites 6 to 12) might be deconjugated by enzymatic activities (e.g., thioesterase or CoA transferase) (26). Alternatively, the abiotic hydrolysis of the CoA esters might occur due to the instability of the thioester structures (27). In this study, we incubated the bacteria with a very high concentration of E1 (1 mM), which may have led to the overproduction of 4-norestrogenic acid and other deconjugated (non-CoA) structures. CoA ester metabolites (28) and their deconjugated
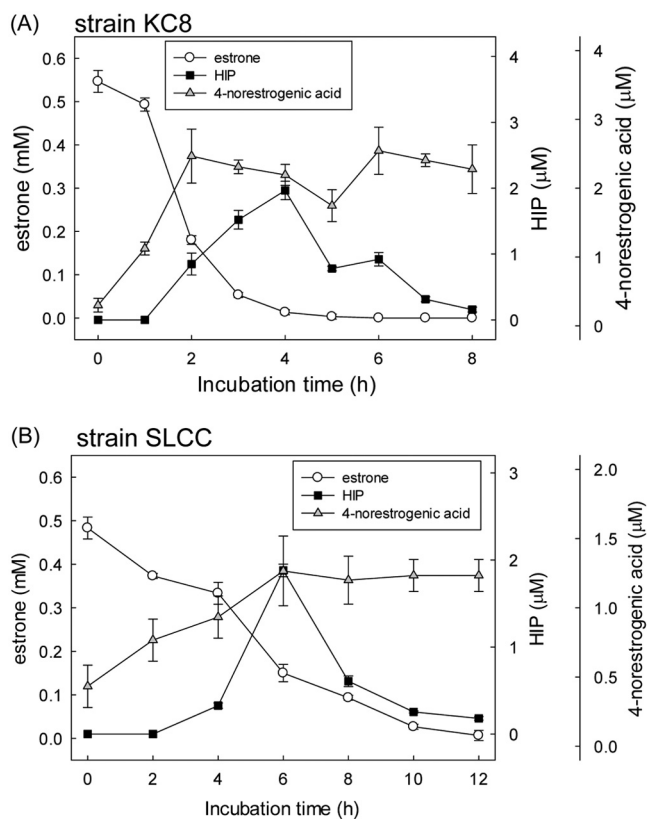
FIG 4 Time course of the E1 consumption as well as the production of 4-norestrogenic acid and HIP in strains KC8 (A) and SLCC (B). The resting cells were aerobically incubated with E1 (1 mM). The [2,4,16,16-D4]E1 and unlabeled E1 were mixed at a 1:1 molar ratio. Quantification of the estrogen metabolites was based on pesudomolecular ion counts corresponding to unlabeled compounds using UPLC-ESI-HRMS. Data shown are the means ± SD from three experimental measurements.

(non-CoA) structures (27) were also detected in *Sterolibacterium denitrificans* DSM 13999 anaerobically grown with a high concentration (millimolar scale) of cholesterol.

CoA ester intermediates are involved in the aerobic degradation of estrogen A and B rings. We then identified the hypothetical CoA ester intermediates. Strain KC8 cells in a resting cell assay were incubated with [2,4,16,16-D4]E1 and unlabeled E1 (mixed at a 1:1 molar ratio) for 4 h; therefore, pairs of adduct ion peaks should present in the MS spectra of the E1-derived CoA esters. After incubation, strain KC8 cells were lysed by sonication. The relatively hydrophilic CoA ester intermediates were separated from other bacterial metabolites by solid-phase extraction and subjected to UPLC-electrospray ionization (ESI)-HRMS analysis (Fig. 5). Similar procedures have been applied to extract and identify CoA ester intermediates derived from cholesterol (28) and bile acids (29), suggesting that the method combining solid-phase extraction and UPLC-ESI-HRMS is suitable for metabolite profile analyses of CoA esters. Two authentic standards, CoASH and benzoyl-CoA, were used to confirm the ionization efficiency and investigate the fragmentation patterns of the CoA esters under the UPLC-ESI-HRMS conditions. Pseudomolecular ion [M+H]$^+$ peaks were present in the HRMS spectra of CoASH (retention time, 2.92 min) (Fig. 5A) and benzoyl-CoA (retention time, 4.40 min) (Fig. 5B). Moreover, a fragment ion (*m/z* 768.12) peak corresponding to the CoA moiety was observed in the HRMS spectrum of benzoyl-CoA (Fig. 5B). Using the solid-phase extraction method, we observed an [M+H]$^+$ peak (*m/z* 1,040.26; retention time, 3.43 min) corresponding to a hypothetical CoA ester, 4-norestrogen-5(10)-en-3-oyl-CoA (metabolite 4) (Fig. 1), in the UPLC chromatogram of the bacterial extract (Fig. 5C). Moreover, the characteristic fragment ion peak of CoA (*m/z* 768.12) is also present in the MS spectrum of this compound. Furthermore, pairs of adduct ion peaks, with the
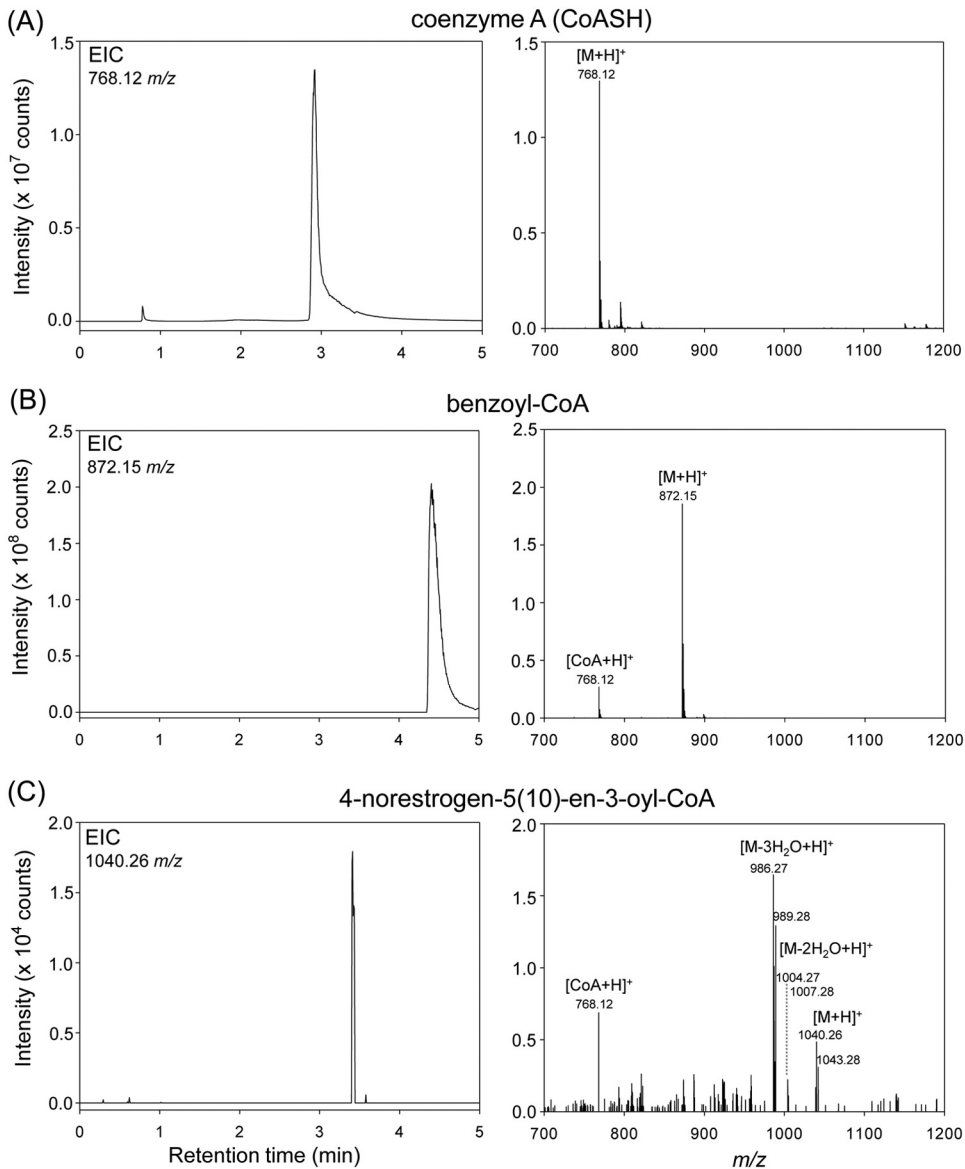
FIG 5 Extracted ion chromatograms (EIC) and MS spectra of two authentic standards, CoASH (A) and benzoyl-CoA (B), and the E1-derived 4-norestrogen-5(10)-en-3-oyl-CoA (metabolite 4) (C). In the resting cell assay of strain KC8, the substrate was composed of [2,4,16,16-D4]E1 and unlabeled E1 (mixed at a 1:1 molar ratio). The fragment ion (m/z 768.12) peak corresponding to the CoA moiety was observed in the MS spectra of benzoyl-CoA and 4-norestrogen-5(10)-en-3-oyl-CoA.

m/z difference of 3, were observed in the mass spectrum of 4-norestrogen-5(10)-en-3-oyl-CoA (Fig. 5C), consistent with the loss of an ^{2}H on C-4 of E1 after the decarboxylation step (Fig. 1). We did not detect other hypothetical CoA esters (metabolites 5 to 12). This might indicate that the following reactions leading to HIP are very fast and do not result in the accumulation of the CoA ester intermediates.

In the aerobic estrogen degradation by *Nocardia* sp. strain E110, the hydrolytic cleavage of the *meta*-cleavage product is followed by a decarboxylation reaction, producing a C_{17} metabolite with cleaved A and B rings (30). However, the enzymes involved were not identified. In the present study, the estrogen metabolite profile analyses of strains KC8 and SLCC also suggest that the B ring of the *meta*-cleavage product is opened via hydrolysis. The electron deficiency of the carbonyl carbon in the B ring facilitates the hydrolytic cleavage. Moreover, the identification of 4-norestrogen-5(10)-en-3-oyl-CoA, 4-norestrogenic acid, and the B-ring-cleaved metabolites suggests

(A)   Gene cluster II

Sphingomonas sp. strain KC8
Genome accession number: CP016306
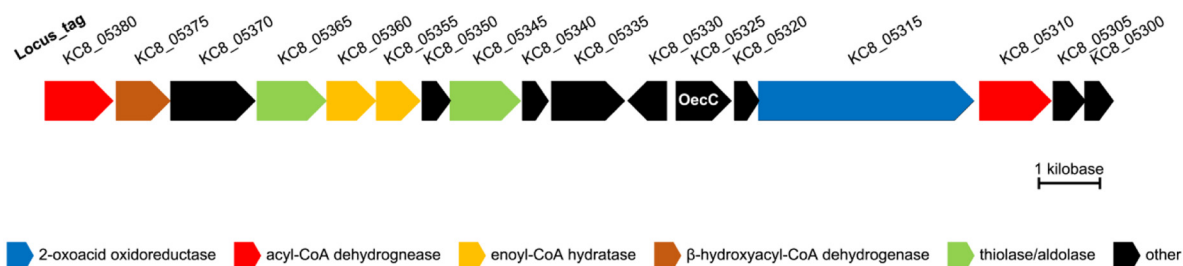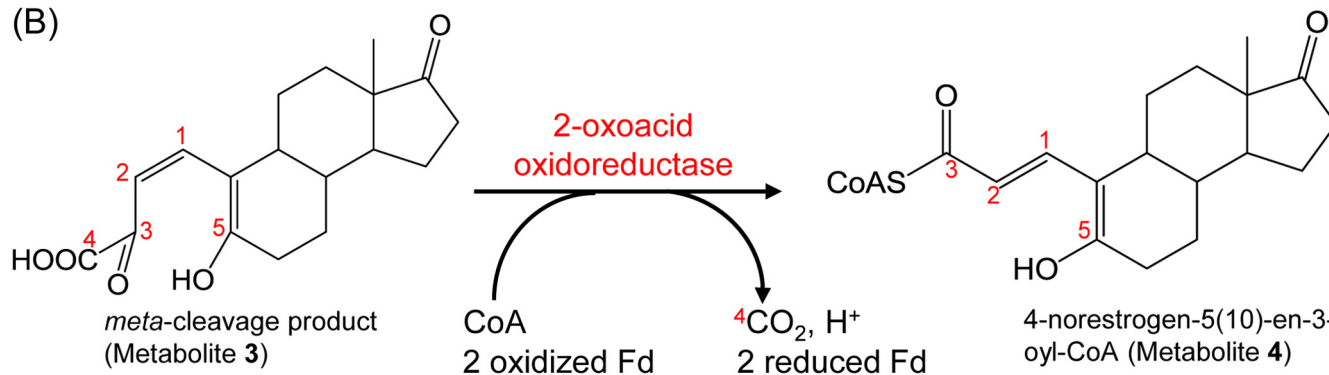
FIG 6 (A) Identification of genes encoding the β-oxidation enzymes and 2-oxoacid oxidoreductase in gene cluster II of strain KC8. (B) Oxidative decarboxylation of the *meta*-cleavage product by the 2-oxoacid oxidoreductase (the product of KC8_5315) of strain KC8. Fd, ferredoxin.

that in the aerobic estrogen degradation by proteobacteria, the C-4 removal through a decarboxylation reaction precedes the B ring cleavage. A similar hydrolytic ring cleavage mechanism was demonstrated in the degradation of cyclohexanecarboxylic acid by an alphaproteobacterium *Rhodopseudomonas palustris* (31, 32). Comparable estrogen metabolites (e.g., metabolite 10 corresponding to the hydrolytic product pimeloyl-CoA) have been detected in the two E1-degrading strains. However, genes similar (with a deduced amino acid sequence identity >40%) to 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase (BadH) or 2-ketocyclohexanecarboxyl-CoA hydrolase (BadI) were not present in gene cluster II of strain KC8. Nonetheless, further investigation is required to identify the corresponding genes and enzymes involved in the estrogen B ring cleavage.

Gene cluster II of strain KC8 is involved in the degradation of estrogen A and B rings. In a previous study (20), we identified a gene cluster (gene cluster II) in the genome of strain KC8, and the genes involved were specifically expressed during aerobic growth on E1. This gene cluster is also present in other estrogen-degrading bacteria. However, the functions of those gene products were unclear except for the 4-hydroxyestrone 4,5-dioxygenase (OecC, the product of KC8_05325). Here, we identified a gene (KC8_05315) encoding a member of the indolepyruvate ferredoxin oxidoreductase (IOR) family (Fig. 6A). Members of this enzyme family contain thiamine diphosphate and [4Fe-4S] clusters and catalyze the decarboxylation of 2-oxoacids [e.g., pyruvate, phenylpyruvate, (indol-3-yl)pyruvate, and 2-oxoglutarate] to form their CoA derivatives (33–35). The C-3 and C-4 of the *meta*-cleavage product represent a typical 2-oxo acid structure; thus, the *meta*-cleavage product and a CoA may serve as the substrates for the 2-oxoacid oxidoreductase of strain KC8, producing 4-norestrogen-5(10)-en-3-oyl-CoA through an oxidative decarboxylation (Fig. 6B).

In addition, we identified a gene (KC8_05370) encoding a 3-hydroxy-3-methylglutaryl-CoA synthase-like protein; this enzyme appears to use a CoA ester

metabolite derived from E1 as the substrate. Genes encoding the β-oxidation enzymes, including acyl-CoA dehydrogenase (KC8_05380 and KC8_05310), enoyl-CoA hydratase (KC8_05355 and KC8_05360), β-hydroxyacyl-CoA dehydrogenase (KC8_05375), and thiolase/aldolase (KC8_05345 and KC8_05365), are present in gene cluster II of strain KC8 (Fig. 6A). Strain KC8 cannot degrade sterols or cholic acid; thus, these β-oxidation genes are not involved in the side chain degradation. Moreover, gene clusters involved in the side chain degradation of steroids (36–40) are not present in the KC8 genome. The potential roles of these β-oxidation enzymes are shown in Fig. 1. Among the two thiolase/aldolase genes, the deduced amino acid sequence of KC8_05345 exhibited higher similarity to those of the aldolases of *S. denitrificans* (28) (gene product of SDENv1_10308; 45% sequence identity) and *Pseudomonas* sp. strain Chol1 (40) (gene product of C211_11377; 47% sequence identity), both of which are responsible for the transformation of steroid C_{22}-oyl-CoA to androsta-1,4-diene-3,17-dione; this implies that the product of KC8_05345 might catalyze the aldolytic side chain degradation of the CoA ester of metabolite 12 to produce HIP.

Gene cluster III of strain KC8 is involved in the HIP degradation. Two pieces of evidence imply that strain KC8 uses the same gene products to degrade the C and D rings of both androgens and estrogens: (i) a conserved gene cluster (gene cluster III) involved in the degradation of steroid C and D rings is present in the strain KC8 genome, and (ii) members of this gene cluster are commonly expressed in the E1- and testosterone-grown cells (20). The gene cluster for C/D ring degradation is widely distributed in testosterone- and cholesterol-degrading bacteria (41, 42), and HIP is a critical substrate for the gene products. The HIP catabolic pathway has been established in *Mycobacterium tuberculosis* (43); in this pathway, the hydrolytic cleavage of the steroid d ring through EchA20 is followed by the C ring cleavage through another hydrolase, IpdAB. We identified the corresponding strain KC8 genes (KC8_01060 [*echA20*], KC8_01080 [*ipdB*], and KC8_01085 [*ipdA*]). The detection of HIP (this study) and the expression of gene cluster III (20) in E1-grown strain KC8 suggest that the cleavage of the estrogen D ring also precedes the C ring degradation. Consequently, in the aerobic degradation pathway of estrogens, the A, B, D, and C rings of estrogens are sequentially opened (Fig. 1).

Conclusions. In contrast to the aerobic degradation of the A and B rings of cholesterol (36) and testosterone (44), this study suggests that CoA ester metabolites are largely involved in the aerobic degradation of estrogen A and B rings because of the following: (i) the gene cluster II of strain KC8, which is responsible for the aerobic degradation of estrogen A and B rings, includes at least 7 putative β-oxidation genes, one putative 2-oxoacid oxidoreductase gene, and one putative 3-hydroxy-3-methylglutaryl-CoA synthase gene; (ii) 4-norestrogen-5(10)-en-3-oyl-CoA, the product of the 2-oxoacid oxidoreductase, was detected in the E1-grown cells; and (iii) the biochemical mechanisms involved in estrogen B ring cleavage are highly similar to those involved in the bacterial degradation of cyclohexanecarboxylic acid, and this cyclohexanecarboxylic acid catabolic pathway involves a series of CoA ester intermediates (31, 32).

A highlight of this study was the detection of HIP in the E1-grown bacteria. HIP has been proposed to be a crucial metabolite for the aerobic (36, 44–46) as well as anaerobic (27, 28, 47) degradation of androgens, sterols, and bile acids. Moreover, genes involved in HIP degradation have been identified in the genomes of various aerobes (41, 42) and denitrifying bacteria (47). Accordingly, the A and B rings of different steroids are degraded through several peripheral pathways, including the 9,10-*seco* pathway (for aerobic degradation of androgens and sterols), the 4,5-*seco* pathway (for aerobic estrogen degradation), and the 2,3-*seco* pathway (for anaerobic degradation of androgens and sterols). Nevertheless, these peripheral steroid catabolic pathways finally converge at HIP, and HIP is further degraded through a common central pathway.

The aerobic estrogen degradation pathway was established in bacterial isolates cultivated under laboratory conditions with a very high substrate concentration (1 mM), but our previous studies indicated that the identified metabolites (e.g., the dead-end product pyridinestrone acid) and degradation genes (e.g., *oecC*) were detectable in the original sludge samples (21) or in various environmental samples treated with a low concentration (0.3 μM) of E1 (20). Moreover, an *oecC*-containing estrogen degrader, strain SLCC, isolated from the studied wastewater treatment plant was able to degrade E1 with a concentration (3.7 nM) close to environmental levels (21). These results suggest that bacteria may use a highly conserved pathway to degrade natural estrogens, regardless of the substrate concentrations. UPLC–HRMS-based metabolite identification has been successfully applied for monitoring the biodegradation of environmental estrogens (20–22). Compared with intermediates in the degradation pathway, dead-end products such as pyridinestrone acid and 4-norestrogenic acid may serve as more suitable biomarkers for environmental investigations of estrogen biodegradation, because dead-end products are less degradable and thus are more persistent in environmental samples.

MATERIALS AND METHODS

Chemicals and bacterial strains. Benzoyl-CoA, CoASH, [2,4,16,16-D4]E1 (95 atom % D), 4-hydroxyestrone, and 3α-H-4α(3′-propanoate)-7$\alpha\beta$-methylhexahydro-1,5-indanedione [HIP; also known as 3-(7a-methyl-1,5-dioxooctahydro-1H-inden-4-yl)propanoic acid] were purchased from Sigma-Aldrich. The other chemicals were of analytical grade and were purchased from Fluka, Mallinckrodt Baker, Merck, and Sigma-Aldrich. Strains KC8 and SLCC were isolated from the municipal wastewater treatment plants as described in our previous studies (21, 25).

Aerobic incubation of bacterial strains with [2,4,16,16-D4]E1. Strains KC8 and SLCC were used in the resting cell assays. Bacteria were first aerobically grown in R2A medium containing 0.1 mM unlabeled E1 (40 ml in a 250-ml Erlenmeyer flask) at 28°C with shaking (180 rpm). Cells were collected via centrifugation (8,000 $\times$ g, 20 min, 15°C) in the exponential growth phase at an optical density at 600 nm (OD$_{600}$) of 0.5 (optical path, 1 cm). The cell pellet was resuspended in a chemically defined medium described by Chen et al. (20). The cell suspension (OD$_{600}$ of 2; 10 ml) was fed with 1 mM E1 (unlabeled E1 and [2,4,16,16-D4]E1 mixed at a 1:1 molar ratio) and was aerobically incubated at 28°C with shaking (180 rpm). The cell suspensions of strains KC8 and SLCC were sampled (0.5 ml) every 1 and 2 h, respectively. To facilitate the extraction of carboxylic metabolites of E1, the samples were acidified with 30 μl of 6 N HCl. The resulting samples were extracted twice using equal volumes of ethyl acetate, and the estrogen metabolites were detected by UPLC-ESI-HRMS and UPLC-APCI-HRMS.

UPLC-ESI-HRMS. Ethyl acetate extractable samples were analyzed by UPLC-ESI-HRMS on a UPLC system coupled to an ESI-mass spectrometer. Separation was achieved on a reversed-phase C$_{18}$ column (Acquity UPLC BEH C$_{18}$, 1.7 μm; 100 mm by 2.1 mm; Waters) with a flow rate of 0.4 ml/min at 50°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). Separation was achieved using a linear gradient of solvent B from 5% to 99% over 12 min. Mass spectral data were collected in positive ESI mode in separate runs on a Thermo Fisher Scientific Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer (Waltham, MA, USA) operated in a scan mode of *m/z* 50 to 500. The source voltage was set at 3.2 kV, the capillary and source heater temperatures were 360°C and 350°C, respectively, and the sheath, auxiliary, and sweep gas flow rates were 30, 15, and 2 arbitrary units, respectively. The predicted elemental composition of individual intermediates was calculated using Xcalibur mass spectrometry software (Thermo Fisher Scientific).

UPLC-APCI-HRMS. The ethyl acetate extractable samples or TLC-purified 4-norestrogenic acid were also analyzed using UPLC-APCI-HRMS. The separation conditions for UPLC were the same as those for UPLC-ESI-HRMS. Mass spectral data were obtained using a Thermo Fisher Scientific Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer (Waltham, MA, USA) equipped with a standard APCI source operating in the positive ion mode. In APCI-HRMS analysis, the capillary and APCI vaporizer temperatures were 120°C and 400°C, respectively; the sheath, auxiliary, and sweep gas flow rates were 40, 5, and 2 arbitrary units, respectively. The source voltage was 6 kV and current was 15 μA. The parent scan was in the range of *m/z* 50 to 600.

Extraction and identification of CoA ester intermediates derived from E1. The preparation of the strain KC8 resting cells was the same as described above. The bacterial cells were incubated with unlabeled E1 (0.5 mM) and [2,4,16,16-D4]E1 (0.5 mM) for 4 h. After that, the cells were harvested by centrifugation and stored at −20°C. The frozen cells were gently resuspended in 0.6 ml of double distilled water, and then cells were disrupted by sonication (Bioruptor Pico Sonication System; Diagenode, Denville, NJ, USA) at 4°C for 20 min (20 cycles of 30 s on/30 s off). After sonicating, 30 μl of 6 N HCl was added to the cell lysate, which was vortexed for 5 min and then centrifuged at 13,500 $\times$ g for 1 min. The CoA esters were extracted by solid-phase extraction (Bakerbond SPE Octadecyl [C$_{18}$] disposable extraction column [1 ml] with 100 mg of sorbent) as described (28, 29) with minor modifications. The attached CoA esters were eluted with 1 ml of 30% aqueous methanol (vol/vol) and subjected to UPLC-ESI-HRMS analysis. Two reference compounds, CoASH and benzoyl-CoA, were used to confirm the extraction

efficiency of the solid-phase extraction method. The mobile phase for the UPLC separation comprised a mixture of two solvents: solvent A (2% [vol/vol] acetonitrile containing 0.1% [vol/vol] formic acid) and solvent B (acetonitrile containing 0.1% [vol/vol] formic acid); the separation was achieved with a linear gradient of solvent B from 0.1% to 90% within 10.5 min. The ESI-HRMS conditions were the same as described above, except that the parent scan was in the range of m/z 700 to 1,200.

Production of 4-norestrogenic acid by strain SLCC. Strain SLCC was first aerobically grown in R2A medium containing 0.1 mM unlabeled E1 (totally 4 liters in six 2-liter Erlenmeyer flasks) at 28°C with shaking (180 rpm). Cells were collected by centrifugation (8,000 × g, 20 min, 15°C) in the exponential growth phase at an OD_{600} of approximately 0.5. The cell pellet was resuspended in 2 liters of a chemically defined medium. The cell suspension was fed 1 mM unlabeled E1 and was aerobically incubated at 28°C with shaking (180 rpm) for 12 h. The supernatant was collected by centrifugation (8,000 × g, 20 min, 15°C). The estrogen metabolites were extracted via solid-phase extraction (Oasis HLB LP extraction cartridges, 6 ml; Waters] with 0.5 g of sorbent). The estrogen metabolites were eluted with 12 ml of methanol, concentrated to 1 ml with a centrifugal evaporator, and purified by HPLC and TLC sequentially. The chemical structure of the TLC-purified metabolite, 4-norestrogenic acid, was elucidated by NMR spectroscopy and UPLC-APCI-HRMS.

Extracellular-intracellular distribution of 4-norestrogenic acid. The resting cell assays of strains KC8 and SLCC were prepared as described above. The cell suspensions (OD_{600} of 2; 10 ml) were fed 1 mM unlabeled E1 and then aerobically incubated at 28°C with shaking. The cell suspensions were sampled (1 ml) after 3 h of aerobic incubation with E1. Cells were separated from the medium via centrifugation (13,500 × g, 10 min, 4°C), and the cell pellet was resuspended in 1 ml of double-distilled water. To facilitate the extraction of 4-norestrogenic acid, the samples were acidified using 30 μl of 6 N HCl. The resulting samples were extracted twice using ethyl acetate (1 ml), and the extracted 4-norestrogenic acid was quantified by UPLC-ESI-HRMS.

Incubation of the strain KC8 cell extract with 4-norestrogenic acid and CoASH. E1-grown strain KC8 cells were harvested by centrifugation. The cell pellet was resuspended in 100 mM HEPES (pH 7.5) containing 10% glycerol and then lysed with a French press (Thermo Fisher Scientific). Soluble proteins were collected by centrifugation (20,000 × g for 30 min, followed by 100,000 × g for 1.5 h) at 4°C. The reaction mixture (1 ml) contained 0.5 ml of soluble protein fraction (20 mg/ml), 0.1 mM 4-norestrogenic acid, 1 mM CoASH, 5 mM ATP, and 10 mM $MgSO_4$. The negative controls were reaction mixtures without soluble proteins or 4-norestrogenic acid. The reaction mixtures were incubated at 30°C for 16 h. The 4-norestrogenic acid and the CoA esters were extracted through liquid-liquid partition and solid-phase extraction, respectively, as described above.

Incubation of strain KC8 cells with 4-norestrogenic acid. E1-grown strain KC8 cells (OD_{600} of 0.2; 10 ml) were incubated in a chemically defined medium containing 0.1 mM 4-norestrogenic acid. 17α-Ethinylestradiol (final concentration, 0.05 mM), which cannot be utilized by strain KC8, was added to the bacterial culture to serve as an internal control. After different time intervals of incubation (0, 3, and 6 days), samples (0.5 ml) were withdrawn from the bacterial culture. The bacterial cells were removed via centrifugation (13,500 × g, 20°C, for 10 min). 4-Norestrogenic acid remaining in the supernatant was extracted using ethyl acetate, and the extracted 4-norestrogenic acid was quantified by UPLC-ESI-HRMS.

HPLC. A reversed-phase (RP) Hitachi HPLC system was used to separate the E1 metabolites. The separation was achieved on an analytical RP C_{18} column [Luna C_{18}(2), 5 μm, 250 mm by 4.6 mm; Phenomenex, Torrance, CA, USA] with a flow rate of 0.8 ml/min. The separation was performed isocratically at 35°C, with 40% (vol/vol) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid as an eluent. The estrogen metabolite 4-norestrogenic acid was detected at 210 nm using a photodiode array detector.

TLC. The estrogen metabolites were separated on silica gel aluminum TLC plates (silica gel 60 F_{254}, 0.2-mm thickness, 20 cm by 20 cm; Merck, Darmstadt, Germany). Ethyl acetate-water-acetic acid (50:1:1 [vol/vol/vol]) was used as the developing solvent system. The estrogen metabolites were visualized by spraying the TLC plates with 30% (vol/vol) H_2SO_4.

NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 27°C by using a Bruker AVIII 500 MHz FT-NMR. Chemical shifts (δ) were recorded and presented in parts per million with deuterated methanol (99.8%) as the solvent and internal reference.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .02223-18.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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