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Identification of 9α-Hydroxy-17-Oxo-1,2,3,4,10,19-Hexanorandrostan-5-Oic Acid in Steroid Degradation by *Comamonas testosteroni* TA441 and Its Conversion to the Corresponding 6-En-5-Oyl Coenzyme A (CoA) Involving Open Reading Frame 28 (ORF28)- and ORF30-Encoded Acyl-CoA Dehydrogenases

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***Comamonas testosteroni* TA441 degrades steroids via aromatization and meta-cleavage of the A ring, followed by hydrolysis, and produces 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid as an intermediate compound. Herein, we identify a new intermediate compound, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid. Open reading frame 28 (ORF28)- and ORF30-encoded acyl coenzyme A (acyl-CoA) dehydrogenase was shown to convert the CoA ester of 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid to the CoA ester of 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid. A homology search of the deduced amino acid sequences suggested that the ORF30-encoded protein is a member of the acyl-CoA dehydrogenase_fadE6_17_26 family, whereas the deduced amino acid sequence of ORF28 showed no significant similarity to specific acyl-CoA dehydrogenase family proteins. Possible steroid degradation gene clusters similar to the cluster of TA441 appear in bacterial genome analysis data. In these clusters, ORFs similar to ORFs 28 and 30 are often found side by side and ordered in the same manner as ORFs 28 and 30.**

Comamonas testosteroni and *Nocardia restrictus* have long been known to be representative steroid-degrading bacteria owing to their ability to assimilate the steroid carbons. The main intermediate compounds in bacterial steroid degradation were identified from the 1950s through the 1980s, and the mechanism of steroid degradation was predicted on the basis of the compounds identified for the purpose of obtaining medical materials (1–9). The degradation genes have been known since approximately 1980 (10–19), but the details of the degradation had remained unclarified, until we revealed the degradation mechanism of the A and B rings of the core steroidal structure (20–29). Our studies have suggested that an approximately 100-kb DNA region in *C. testosteroni* TA441 is a large steroid degradation gene cluster (Fig. 1, bottom) (28). Steroidal skeleton rings are degraded into 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound V) and 2-hydroxyhexa-2,4-dienoic acid (compound IV) via aromatization of the A ring and following hydrolysis (Fig. 1, top) by enzymes mainly encoded by a gene cluster consisting of open reading frame 18 (ORF18), ORF17, and *tesIHA2A1DEFG*. Coenzyme A (CoA) is added to compound V by an ORF18-encoded enzyme, and the compound is thought to be further degraded by β-oxidation by enzymes mainly encoded by a gene cluster consisting of *steAB*, ORF7, ORF6, *tesB*, ORF1, ORF2, ORF3, ORF4, ORF5, ORF21, ORF22, ORF23, ORF25, ORF26, ORF27, ORF28, ORF30, ORF31, ORF32, ORF33, and *tesR*. In a previous study, we identified 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid (compound VI) to be an intermediate compound in compound V degradation. ORF32-encoded enoyl-CoA hydratase was shown to be indispensable for the conversion of compound VI, likely adding a water molecule to a double bond at the C-6 position of this compound for further degradation by β-oxidation (29). Herein, we describe new findings related to the degradation of compound V to compound VI.

MATERIALS AND METHODS

General experimental procedures. Fast atom bombardment mass spectrometry (MS; negative-ion mode) was recorded on a JEOL JMS-700 mass spectrometer (JEOL Ltd., Tokyo, Japan) using a glycerin matrix. The electron ionization mass spectrum was recorded on a JEOL JMS-SX102 mass spectrometer (JEOL Ltd.). One- and two-dimensional nuclear magnetic resonance (NMR) spectra were recorded on a JNM-ECP500 or JNM-ECA600 spectrometer (JEOL Ltd.). Tetramethylsilane at 0 ppm in deuterated chloroform solution and the residual proton signal at 2.49 ppm in deuterated dimethyl sulfoxide (DMSO-d_6) solution or the residual proton signal at 3.30 ppm in deuterated methanol solution were used as internal references for ^{1}H chemical shifts. ^{13}C chemical shifts were obtained with reference to DMSO-d_6 (39.5 ppm) or deuterated chloroform (77.0 ppm) at 25°C.

Culture conditions. Mutant strains of *C. testosteroni* TA441 were grown at 30°C in a mixture of equal volumes of Luria-Bertani (LB) medium and C medium (a mineral medium for TA441) (21) with suitable carbon sources. Cholic acid and cholic acid analogs (chenodeoxycholic acid, deoxycholic acid, and lithocholic acid) were added as filter-sterilized DMSO solutions with a final concentration of 0.1% (wt/vol).

Construction of plasmids and gene-disrupted mutants. Each ORF was disrupted by inserting a kanamycin resistance gene without a terminator (the EcoT22I site in ORF28, the NruI site in ORF30, and between the EcoT22I and NruI sites in ORFs 28 and 30). The resultant plasmid was

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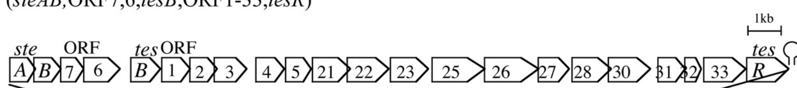

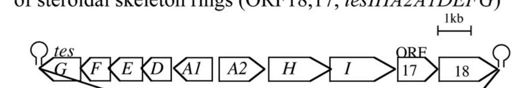

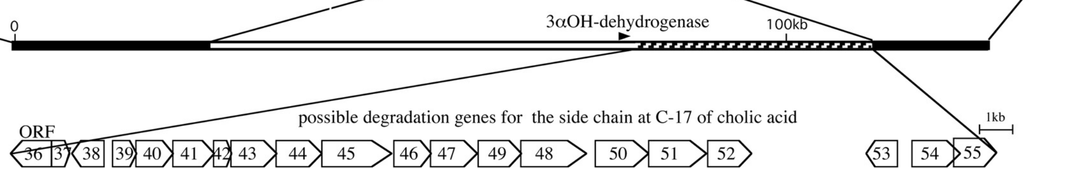

FIG 1 Proposed steroid degradation pathway in *Comamonas testosteroni* TA441 and degradation genes. The compounds are androsta-1,4-diene-3,17-dione (ADD; compound I), 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA; compound II), 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid (compound III), (2*Z*,4*Z*)-2-hydroxyhexa-2,4-dienoic acid (compound IV), 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound V), and 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid (compound VI). Compounds in brackets are presumed. Data for ORFs 36 to 52 are unpublished.

used to inactivate each ORF in TA441 via homologous recombination using electroporation. Kanamycin (final concentration, 400 mg/liter)-resistant mutants were picked up. The insertion of the kanamycin resistance gene was confirmed by Southern hybridization.

To construct mutants for complementation and conversion experiments, we transferred PCR-amplified ORF28, ORF30, or both ORF28 and ORF30 into pUC19 for transformation by *Escherichia coli*. The sequence of each DNA fragment was confirmed. The DNA fragments

were then transferred into the broad-host-range plasmid pMFY42 (30) to construct pMFYORF28, pMFYORF30, and pMFY28,30, respectively. Plasmid pMFY42 can be maintained in *Pseudomonas* and its relatives and provides tetracycline resistance. Each broad-host-range plasmid was introduced into the ORF28-, ORF30-, ORF28-ORF30-, and TesR-disrupted mutants (the $ORF28^-$, $ORF30^-$, $ORF28,30^-$, and $TesR^-$ mutants, respectively) via electroporation; a kanamycin- and tetracycline-resistant TA441 mutant was selected. Retention of the plasmids in the transformants was confirmed by Southern hybridization using suitable probes.

Isolation and identification of compound VII accumulated by the $ORF28^-$ mutant. The culture of the $ORF28^-$ mutant incubated with 0.1% (wt/vol) androsta-1,4-diene-3,17-dione (ADD) for 5 days (total volume, 500 ml) was extracted with 550 ml of ethyl acetate under neutral conditions. Then, the water layer was extracted twice with 550 ml of ethyl acetate under acidic conditions (pH 2.0 with HCl). The ethyl acetate layers under neutral and acidic conditions were treated individually with Na_2SO_4 and concentrated *in vacuo*. They were then dissolved in a small amount of methanol and subjected to high-performance liquid chromatography (HPLC), which revealed the presence of compound VII in the ethyl acetate layer extracted under acidic condition with a small amount of a new compound, compound VIII (retention time [RT] = 8.1 min).

HPLC analysis. After the addition of a double volume of methanol to the culture, the mixture was centrifuged and the supernatant was directly injected into a high-performance liquid chromatograph (Alliance 2695 with UV and 996 photodiode array detectors; Nihon Waters, Tokyo, Japan) equipped with an Inertsil ODS-3 column (4.6 by 250 mm; GL Sciences Inc., Tokyo, Japan). Elution was carried out using a linear gradient from 20% solution A (CH_3CN-CH_3OH-trifluoroacetic acid [TFA] [95:5:0.05]) and 80% solution B (H_2O-CH_3OH-TFA [95:5:0.05]) to 65% solution A and 35% solution B over 10 min; these conditions were maintained for 3 min and then changed to 20% solution A. The flow rate was 1.0 ml/min, and the temperature was 40°C. For the isolation of intermediate compounds, a Waters 600 HPLC with an Inertsil ODS-3 column (20 by 250 mm) was used, and elution was carried out using a linear gradient from 20% solution A and 80% solution B to 65% solution A and 35% solution B over 25 min, which was maintained for 10 min, followed by 20% solution A for 5 min. The accumulated compound was detected at 206 nm. The flow rate was 8 ml/min, and the temperature was 40°C.

UHPLC/MS analysis. Ultra-high-performance liquid chromatography (UHPLC)/MS analysis was performed only to confirm the peaks, as described below in "Complementation experiment with ORFs 28 and 30." The culture was extracted twice with a double volume of ethyl acetate under acidic conditions (pH 2, obtained with HCl). The ethyl acetate layer was dried and dissolved in methanol. UHPLC/MS was carried out using an Applied Biosystems Q Trap liquid chromatography tandem MS system with a reverse-phase column (2.1 by 150 mm; XTerra MSC_{18}; Waters) at a flow rate of 0.4 ml/min and a temperature of 40°C. Elution was carried out using a linear gradient from 20% solution C (CH_3CN) and 80% solution D (H_2O-HCOOH [100:0.05]) to 100% solution C over 4.5 min, which was maintained for 2 min. Electrospray ionization (negative) was used for detection. The conditions for MS were an ion spray voltage of 5.0 kV, a curtain gas pressure of 15 lb/in^2, a nebulizer gas pressure of 40 lb/in^2, an auxiliary gas pressure of 60 lb/in^2, and an ion source temperature of 400°C.

Transformation experiments. To prepare the substrates, we incubated the $ORF28^-$ mutant with a suitable steroid compound until the objective compound was accumulated, which was confirmed by HPLC analysis. The cells were removed via centrifugation and filter sterilization to prepare the substrate solution. The mutant cells for the experiments were precultured in LB medium with a small amount of cholic acid to induce steroid degradation genes. Cholic acid was used because the degradation intermediate compounds can be clearly distinguished from those obtained with ADD and deoxycholic acid. After incubation for approximately 20 h, the cells were washed twice, collected via centrifugation, and added to the prepared substrate solution. A small portion of the solution was analyzed every 24 h by HPLC.

RESULTS

Construction of $ORF28^-$ and $ORF30^-$ mutants and HPLC analysis of the intermediate compounds accumulated in the culture of the mutants incubated with cholic acid and the analogs. *Comamonas testosteroni* TA441 converts testosterone into 2-hydroxyhexa-2,4-dienoic acid and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound V) (Fig. 1). Compound V is thought to be degraded by β-oxidation via the CoA ester of 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid (compound VI), to which a water molecule is added by the ORF32-encoded enoyl-CoA hydratase. Because most of ORFs 1 to 33 are thought to be involved in this β-oxidation, the culture of each ORF (ORF 1 to 33)-disrupted mutant was incubated with testosterone and cholic acid analogs (cholic acid, chenodeoxycholic acid, deoxycholic acid, and lithocholic acid) and analyzed in detail. Compound VI was detected in the cultures of most of the mutants, but it was not detected in the culture of the ORF28-disrupted ($ORF28^-$) or ORF30-disrupted ($ORF30^-$) mutant incubated with testosterone, deoxycholic acid, or lithocholic acid. Instead, weak UV absorption was detected at an RT of 6.6 min (compound VII) with incubation with chenodeoxycholic acid and lithocholic acid (Fig. 2; note that only the HPLC charts for 7-day incubations with cholic acid analogs are shown). The UV absorption and RT of compound VII are characteristic, and they differ from those of the intermediate compounds identified in our previous studies (21, 22, 24–27, 29). Compound VII did not accumulate in the culture with cholic acid or chenodeoxycholic acid. Those compounds have a hydroxyl group at the C-7 position, but the other compounds do not, suggesting that ORFs 28 and 30 are involved in reactions related to the C-7 position of compound VII. A homology search indicated that the putative proteins encoded by ORFs 28 and 30 are acyl-CoA hydrogenases, which also supported that hypothesis.

Isolation and identification of putative intermediate compounds accumulated by the $ORF28^-$ mutant. Because the peak of compound VII was rather weak, we tested several steroid compounds to determine a suitable precursor for the production of compound VII in large quantities. The $ORF28^-$ and $ORF30^-$ mutants accumulated the largest amounts of compound VII when incubated with ADD, an intermediate compound in testosterone degradation. Therefore, ADD was used for the isolation of compound VII. Because the HPLC charts for the cultures of the $ORF28^-$ and $ORF30^-$ mutants were almost identical, we used only the $ORF28^-$ mutant for the isolation of compound VII.

The $ORF28^-$ mutant was incubated with ADD for 5 days, and the culture was extracted with ethyl acetate under neutral conditions and then under acidic conditions. Compound VII was purified from the ethyl acetate layer extracted under acidic conditions with a small amount of a new compound, compound VIII (RT = 8.1 min). In addition to the peaks for these compounds, several peaks were detected at an RT of approximately 6 to 8 min by HPLC. Compounds VII and VIII and a compound detected at an RT of 7.1 min were purified by HPLC (72.1 mg, 10.5 mg, and 32.8 mg, respectively, from 500 ml culture). The compound detected at an RT of 7.1 was identified as 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound V) through comparisons of the HPLC, MS, and NMR data to previously obtained data (26).

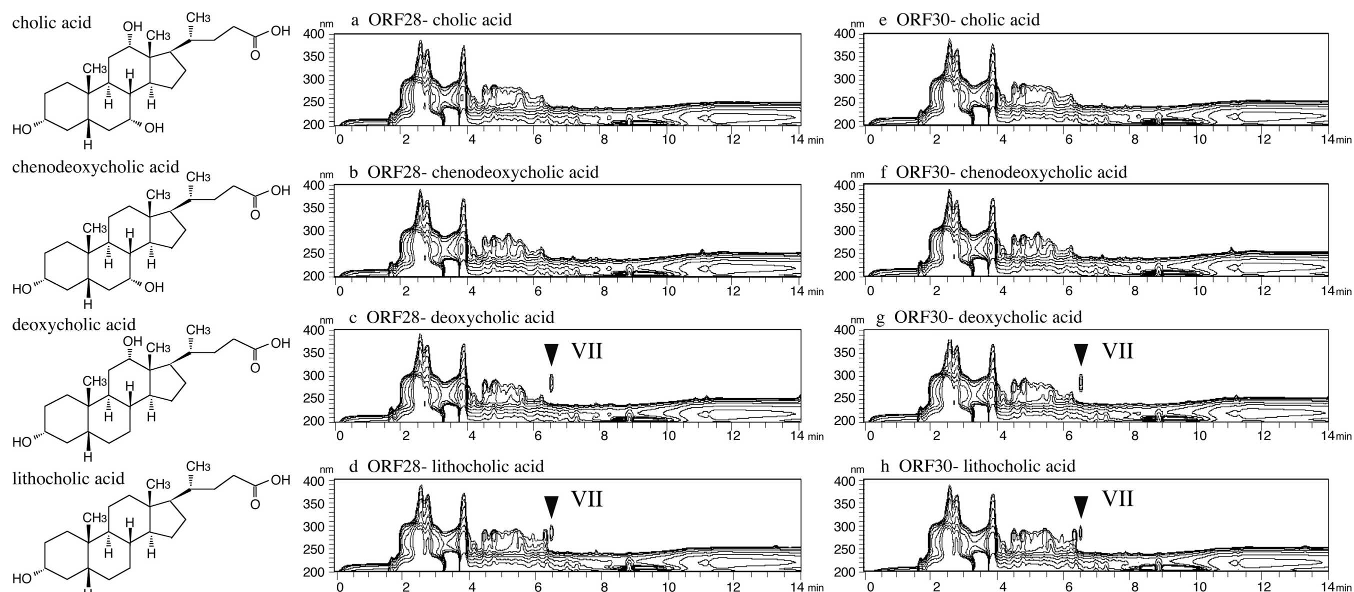

FIG 2 Three-dimensional HPLC analysis of the culture of the ORF28⁻ mutant (a to d) and ORF30⁻ mutant (e to h) incubated in 1/2 LB medium plus 1/2 C medium with 0.1% (wt/vol) cholic acid (a, e), chenodeoxycholic acid (b, f), deoxycholic acid (c, g), and lithocholic acid (d, h) for 5 days. The vertical axis indicates the wavelength (nm), and the horizontal axis indicates the RT (min); the UV absorbance of each compound is represented in contours. Arrowheads, a characteristic compound detected only in the culture of the ORF28⁻ and ORF30⁻ mutants (compound VII).

The high-resolution fast atom bombardment MS (negative) data for compound VII (found, *m/z* 239.1297 $[M-H]^-$; calculated, *m/z* 239.1283) indicated the molecular formula $C_{13}H_{20}O_4$. Compound VII was identified to be 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid on the basis of the NMR analysis (Table 1; Fig. 3), including double-quantum filtered correlation spectroscopy, heteronuclear single-quantum correlation spectroscopy, heteronuclear multiple bond correlation, and one-dimensional nuclear Overhauser effect spectroscopy data (31). Compound VIII was identified to be the lactonized form of compound VII, on the basis of NMR data (Table 1; Fig. 3) and high-resolution electron ionization MS data (found, *m/z* 222.1254 $[M]^+$; calculated, *m/z* 222.1256). Compound VIII was not detected in the culture but was produced in the stored fraction containing purified compound VII. Compound VIII was likely produced nonenzymatically from compound VII via dehydration under acidic conditions due to the TFA in the HPLC solvent.

Complementation experiment with ORFs 28 and 30. Compound VII was identified to be 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid. Both ORF28- and ORF30-encoded proteins showed significant homology to acyl-CoA dehydrogenases (ACADs); therefore, the actual substrate in the degradation is considered to be the CoA ester of this compound. Both the ORF28⁻ and ORF30⁻ mutants accumulated compound VII when incubated with a steroid compound lacking the hydroxyl group at the C-7 position, implying that both proteins are involved in the conversion of the CoA ester of compound VII. To confirm this involvement, we constructed a mutant with disruptions in both ORF28 and ORF30 (the ORF28,30⁻ mutant) for use in a complementation experiment. We used deoxycholic acid as a starting steroid compound in this experiment. When ADD was used as the starting steroid compound, compound V appeared at an RT of 7.1 min on the HPLC analysis. The putative product, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid (compound

TABLE 1 NMR data for compounds accumulated by the ORF28⁻ mutant incubated with ADD

	δ (ppm)[a]			
	Compound VII		Compound VIII	
No.	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
5	174.84	(OH) 11.94 br s	171.76	
6	37.06	2.33 ddd (15.6, 7.5, 7.5)	26.72	2.49 ddd (17.2, 7.7, 6.5)
		2.17 ddd (15.6, 8.4, 7.2)		2.36 ddd (17.2, 8.1, 7.2)
7	23.56	1.50–1.56 m	20.38	2.01 m
		1.50–1.56 m		1.64 m
8	39.20	1.49 m	31.41	2.08 m
9	64.74	(OH) 4.28 br s	77.12	4.53 ddd (2.8, 2.8, 2.8)
		3.73 ddd (3.4, 3.4, 3.4)		
11	29.04	1.61–1.66 m	25.93	1.79–1.83 m
		1.56–1.61 m		1.79–1.83 m
12	26.17	1.31 ddd (12.8, 4.2, 2.7)	26.37	1.47 m
		1.43 ddd (13.0, 12.8, 4.6)		1.29 ddd (12.9, 12.9, 6.0)
13	47.32		46.82	
14	42.27	1.77 ddd (12.4, 11.2, 5.8)	40.94	1.62 ddd (12.6, 12.6, 5.8)
15	21.48	1.45 m	20.78	1.90 ddd (12.1, 8.3, 5.8)
		1.81 m		1.47 m
16	34.98	2.35 dd (19.2, 8.2)	34.79	2.38 dd (19.0, 8.0)
		1.99 ddd (19.2, 8.9, 8.9)		2.04 m
17	219.89		218.92	
19	12.48	0.76 s	12.25	0.83 s

[a] In DMSO-d_6. Abbreviations for NMR signals are as follows s, singlet; d, doublet; m, multiplet; br, broad. Values in parentheses are *J* (in Hz).

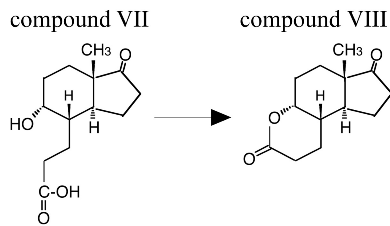

FIG 3 Structures of compound VII (9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid) and compound VIII (lactonized form of compound VII).

VI), appeared at an RT of 6.9 min. When deoxycholic acid was used, the compound accumulated by the ORF28$^-$, ORF30$^-$, and ORF28,30$^-$ mutants was 12α-hydroxy-9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid, which appeared at an RT of 4.8 min, which can be clearly distinguished from compound VI.

The mutants constructed for the complementation experiment were the following: the ORF28$^-$ mutant with a broad-host-range plasmid, pMFY42 (as a negative control), and with pMFY42 carrying ORF28 (pMFYORF28); the ORF30$^-$ mutant with pMFY42 (as a negative control) and with pMFY42 carrying ORF30 (pMFYORF30); and the ORF28,30$^-$ mutant with pMFY42 (as a negative control), with pMFYORF28 (pMFYORF28), with pMFYORF30 (pMFYORF30), and with pMFY42 carrying ORF28 and ORF30 (pMFYORF28,30). The culture was analyzed by HPLC every 24 h after the start of incubation.

After 5 days of incubation, a small amount of compound VII was detected in the cultures of the complemented mutants: the ORF28$^-$ mutant with pMFYORF28, the ORF30$^-$ mutant with pMFYORF30, and the ORF28,30$^-$ mutant with pMFYORF28,30 (Fig. 4a, c, and e). A considerable amount of compound VII was detected in the cultures of the other mutants (Fig. 4b, d, and f to h), indicating that both ORF28 and ORF30 are indispensable for the conversion of compound VII. Moreover, a clear peak was detected at an RT of 6.9 min in the cultures of the complemented mutants. The RTs and the UV absorbance curves of this compound were almost identical to those of compound VI. To identify this compound, we performed the same experiment and analyzed the culture with a UHPLC/MS system. The data for the culture of the ORF28,30$^-$ mutant with pMFY42 are presented as representative data for the noncomplemented mutants, and the data for the culture of the ORF28,30$^-$ mutant with pMFYORF28,30 are presented as representative data for the noncomplemented mutants. All UHPLC analyses were performed under the conditions described in Materials and Method, and MS data were obtained in negative-ion mode.

A peak in the MS spectrum appears with an approximately

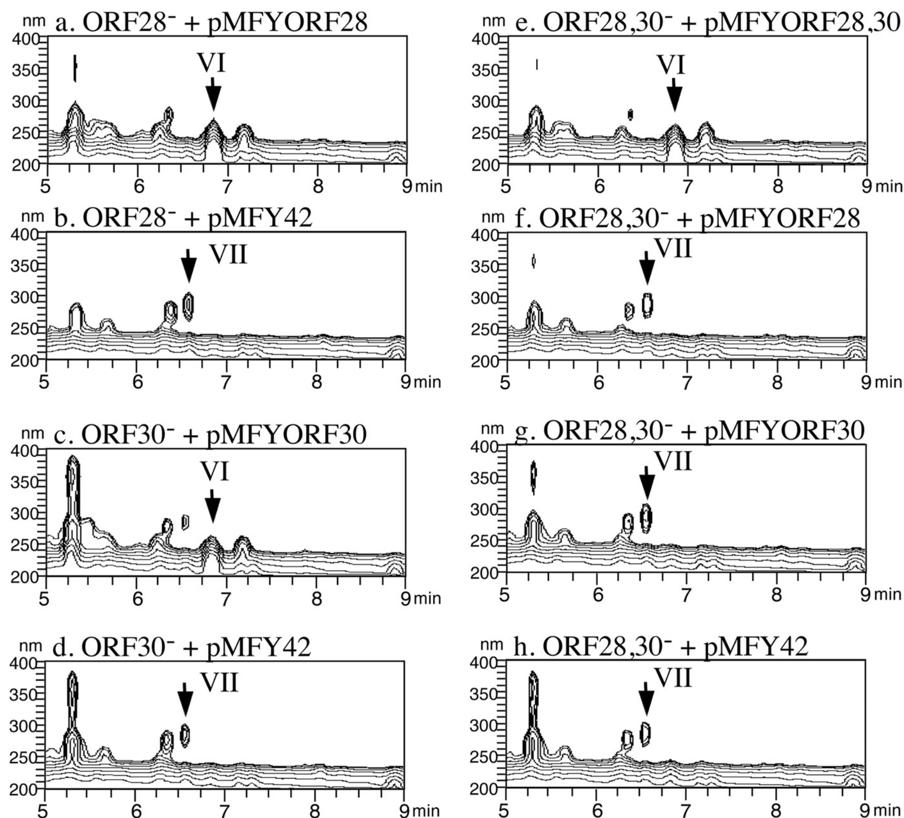

FIG 4 Complementation experiments with ORF28 and ORF30. HPLC charts of the cultures of the ORF28$^-$ mutant with pMFYORF28 (broad-host-range plasmid pMFY42 carrying ORF28) (a), the ORF28$^-$ mutant with pMFY42 (a negative control) (b), the ORF30$^-$ mutant with pMFYORF30 (pMFY42 carrying ORF30) (c), the ORF30$^-$ mutant with pMFY42 (a negative control) (d), a mutant doubly disrupted in both ORF28 and 30 (the ORF28,30$^-$ mutant) with pMFYORF28,30 (pMFY42 carrying both ORF28 and ORF30) (e), the ORF28,30$^-$ mutant with pMFYORF28 (f), the ORF28,30$^-$ mutant with pMFYORF30 (g), and the ORF28,30$^-$ mutant with pMFY42 (a negative control) (h) incubated with deoxycholic acid for 7 days are shown. Compound VII, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid; compound VI, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid.

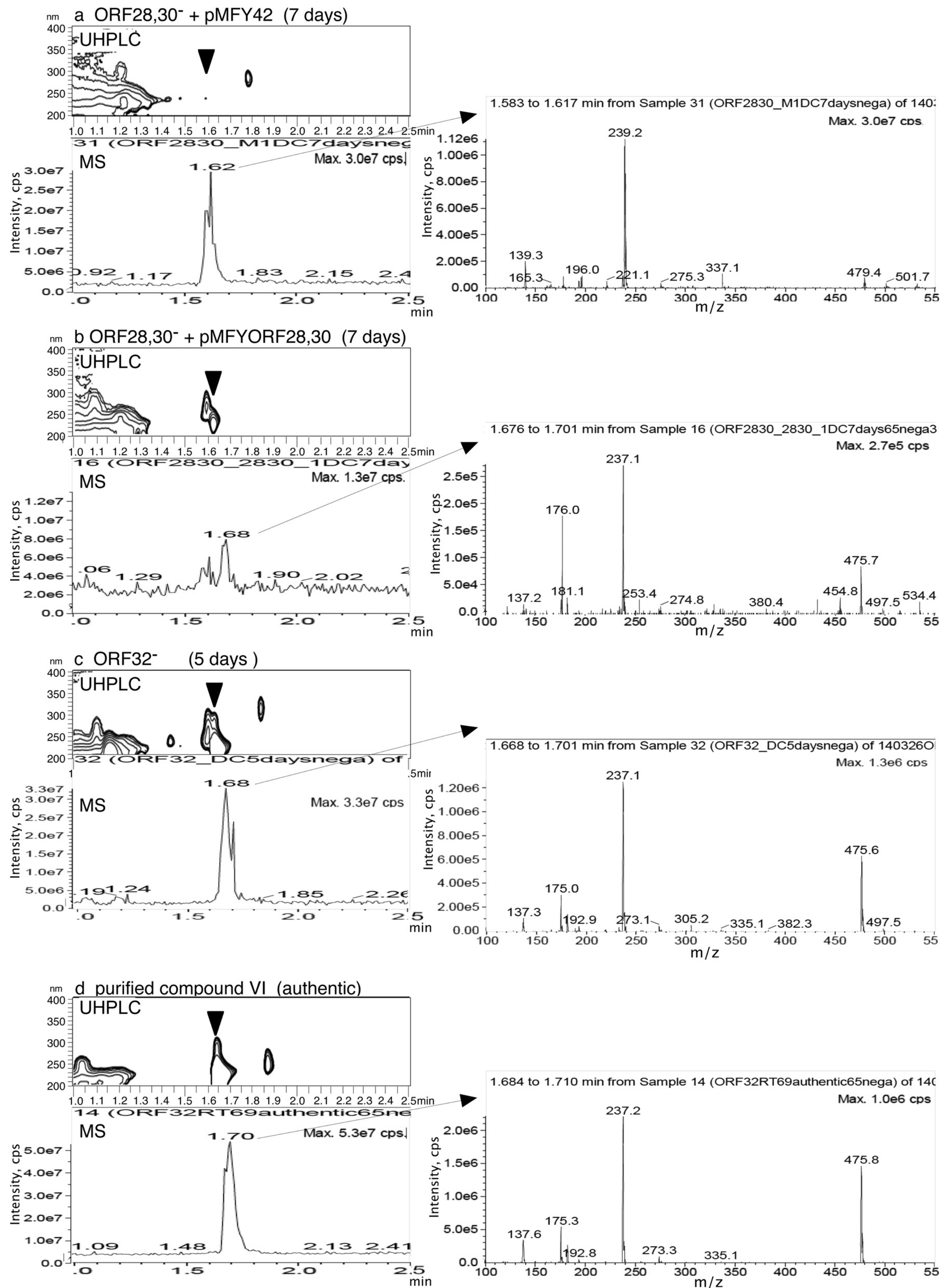

FIG 5 UHPLC/MS analysis of the culture of the complementation experiments with ORF28 and ORF30. (a) The ORF28,30$^-$ mutant with pMFY42 incubated for 7 days (a negative control); (b) the ORF28,30$^-$ mutant with pMFYORF28,30 incubated for 7 days; (c) the ORF32$^-$ mutant incubated for 5 days; (d) purified compound VI (authentic). Electrospray ionization (ESI; negative) was used for detection. Compound VII, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid; compound VI, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid.

0.5-min delay compared with the time to the corresponding peak in the UHPLC chromatogram. In the culture of the ORF28,30$^-$ mutant with pMFY42, a peak is barely detected at an RT of 1.59 min with UHPLC (Fig. 5a). This peak was identified as compound VII through comparison of its UHPLC RT with that of purified compound VII and the molecular weight (MW) of 240 from the MS analysis. The compound appeared as a small peak in the UHPLC chromatogram, but it was detected in large quantity by MS. The culture of the ORF28,30$^-$ mutant with pMFYORF28,30 yielded a small but clear peak at an RT of 1.64 min in the UHPLC chromatogram (Fig. 5b), and the results of MS analysis revealed an MW of 238. A peak with the same UHPLC RT and MW was detected in the culture of the ORF32$^-$ mutant incubated with deoxycholic acid (Fig. 5c). Then, compound VI, purified in the previous study, was analyzed in the same way to provide authentic data (Fig. 5d). On the basis of a comparison of the RT and the MW of 238 from MS analysis, the compound detected at an RT of 1.64 min in the culture of the ORF28,30$^-$ mutant with pMFY-ORF28,30 was identified to be compound VI.

Conversion of 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound VII) by cloned ORF28- and ORF30-encoded enzymes. Conversion of the identified intermediate compound by the cell extracts of the recombinant *E. coli* strain expressing the objective gene was successful in most of the reactions involved in the aromatization and cleavage of the steroidal A ring (20–22, 25, 28). In these experiments, the gene-disrupted mutant was incubated until most of the added steroid compound was converted to the intermediate compound. The culture was centrifuged and filter sterilized to remove the mutant cells completely, and the supernatant was used as the reaction solution. The cell extract of the recombinant *E. coli* strain expressing the objective gene was added and analyzed after an hour of incubation at 30°C. For the reactions involved in the β-oxidation in steroid degradation, the transformation experiments were carried out in the same way, but a decrease of the free acid of the substrate CoA ester and an increase of the free acid of the product CoA ester were not detected (the actual substrate and the products are considered to be the CoA esters, but a quantitative change of the compound without CoA was thought to occur) (26, 29). Conversion by the cell extracts of recombinant *E. coli* strains expressing ORFs 28 and 30 was also unsuccessful; we were not able to detect any change in the content of compound VII or compound VI. Then, we performed the transformation experiments with the TesR$^-$ mutant as the host. TesR is a positive regulator of steroid degradation genes in TA441; without TesR, steroid degradation genes are not induced (23, 32). Transformation using the TesR$^-$ mutant is sometimes successful when transformation by the cell extract of recombinant *E. coli* is not successful (27).

To prepare the compound VII solution, we incubated the ORF28$^-$ mutant with deoxycholic acid for 5 days until only compound VII was detected as the main accumulated intermediate using HPLC (Fig. 6a). After the solution was centrifuged and filter sterilized to remove ORF28$^-$ mutant cells completely, it was used as a substrate solution. The TesR$^-$ mutant with pMFYORF28,30 and the TesR$^-$ mutant with pMFY42 (negative control) were precultured in LB medium with kanamycin and tetracycline until adequate growth for the experiment was obtained (30 to 40 h), and the cells were collected via centrifugation. The mutant cells were incubated in the substrate solution at 30°C, which was sampled daily for HPLC analysis.

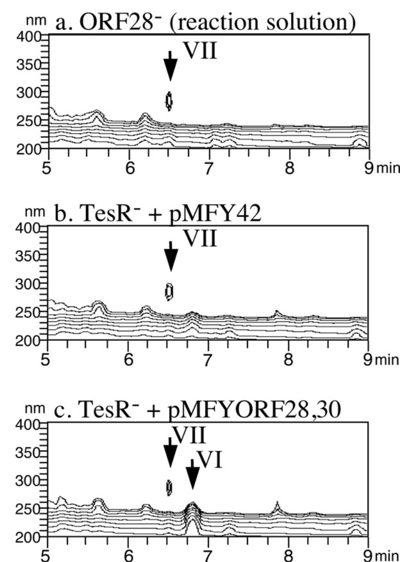

FIG 6 Transformation of α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound VII) by the TesR$^-$ mutant expressing ORF28 and ORF30. (a) A culture of the ORF28-disrupted mutant incubated until intermediate compounds other than 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid were almost undetectable was used as a reaction solution after filter sterilization. (b and c) The reaction solution was treated with a precultured TesR$^-$ mutant carrying pMFY42 (a negative control) (b) and the TesR$^-$ mutant carrying pMFYORF28,30 (c). HPLC charts of the reaction solution, analyzed 2 days after the start of the incubation, are shown. The TesR$^-$ mutant is a mutant strain of TA441 with a mutation in TesR, a positive regulator of steroid degradation genes. Compound VI, 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid.

Figure 6b and c show HPLC charts of the reaction solution 5 days after the start of incubation. In the reaction solution of the TesR$^-$ mutant with pMFYORF28,30, compound VII was converted to compound VI. This result shows that only ORF28- and ORF30-encoded enzymes are indispensable for conversion. Because these enzymes are considered acyl-CoA dehydrogenases, the substrate is a CoA of compound VII that coexists with compound VII in the substrate solution. The CoA ester of the intermediate compounds is likely contained in a small quantity in the reaction solution. The success in detecting the product in this study was probably due to the strong UV absorbance of compound VI and the long incubation period (5 days). A small amount of compound VI was thought to be produced from its CoA ester during the long incubation, and the strong UV absorbance of compound VI led to detection by HPLC.

DISCUSSION

Several steroid compounds, such as testosterone, are converted into 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound V) and 2-hydroxyhexa-2,4-dienoic acid by steroid-degrading bacteria to be completely degraded and used as carbon sources. Most of the steroid degradation genes of *C. testosteroni* TA441 identified to date are in the two main steroid degradation clusters, both of which are located in an approximately 100-kb DNA region that is considered a hot spot of steroid degradation genes (Fig. 1) (28, 33). ORFs 1 to 33 in a steroid degradation gene cluster consist of *steA*, *steB*, ORF7, ORF6, *tesB*, ORFs 1 to 33, and *tesR* and have been implicated in the β-oxidation of compound V. A previous study determined that 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound VI) is

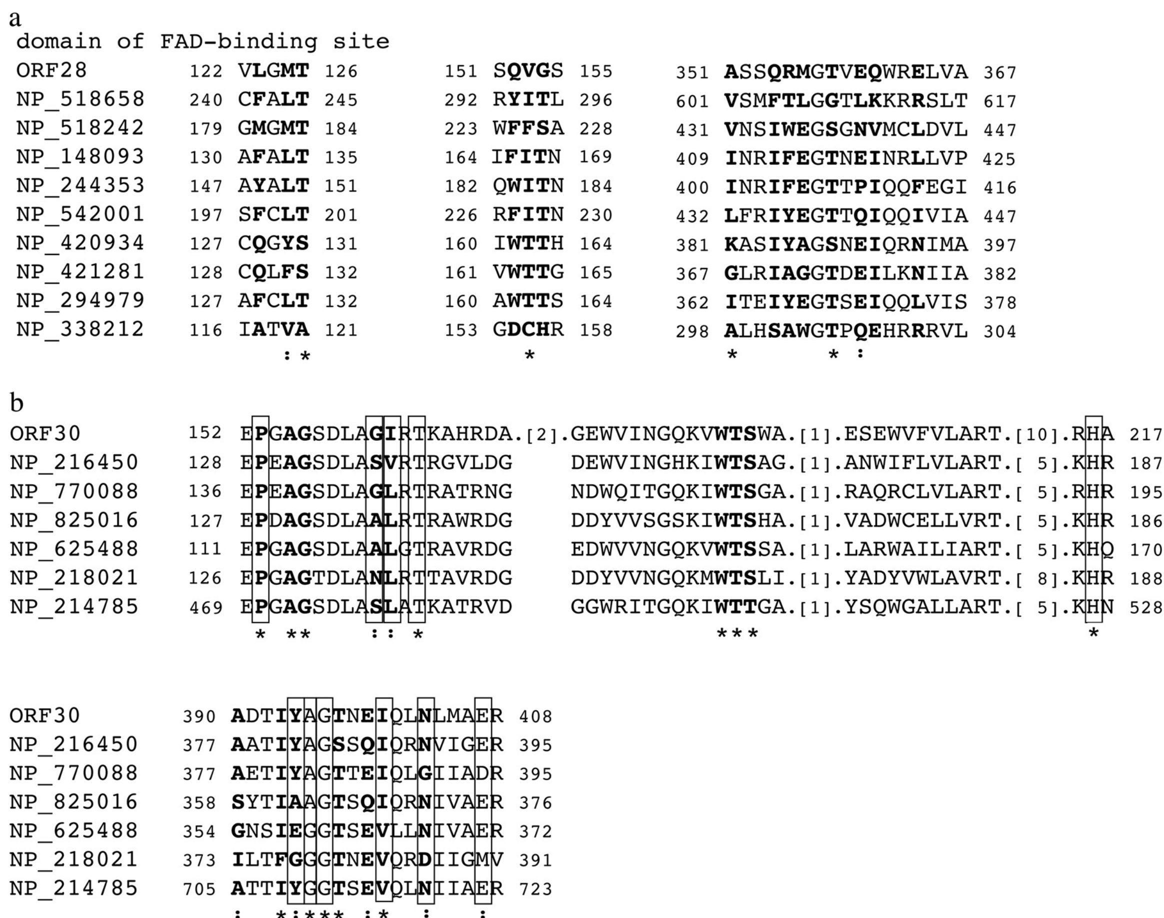

FIG 7 (a) Alignment of the deduced amino acid sequence of ORF28 with acyl-CoA dehydrogenases (lipid metabolism; COG1960). Only highly conserved regions are shown. Boldface, amino acids for the putative FAD-binding site, asterisks, mostly identical amino acids; colons, mostly similar amino acids. GenBank accession numbers are given on the left. NP_518658 and NP_518242, *Ralstonia solanacearum* GMI1000 (40); NP_148093, *Aeropyrum pernix* K1 (41); NP_244353, *Bacillus halodurans* C-125 (42); NP_542001, *Brucella melitensis* bv. 1 strain 16M (43); NP_420934 and NP_421281, *Caulobacter crescentus* CB15 (44); NP_294979, *Deinococcus radiodurans* R1 (45); NP_338212, *Mycobacterium tuberculosis* CDC1551 (46). (b) Alignment of the deduced amino acid sequence of ORF30 with putative acyl-CoA dehydrogenases similar to FadE6, FadE17, and FadE26. Only highly conserved regions are shown. Boldface, amino acids for the putative FAD-binding site; boxed regions, amino acids for the putative substrate-binding site; asterisks, identical amino acids; colons, similar amino acids. GenBank accession numbers are given on the left. NP_216450, NP_218021, and NP_214785, *Mycobacterium tuberculosis* H37Rv (47); NP_770088, *Bradyrhizobium diazoefficiens* (*Bradyrhizobium japonicum*) USDA 110 (48, 49); NP_825016, *Streptomyces avermitilis* MA-4680 (50); NP_625488, *Streptomyces coelicolor* A3(2) (51).

one of the intermediates in the degradation of compound V, and ORF32 encodes an enoyl CoA hydratase that adds a water molecule to the CoA ester of compound VI (29).

9α-Hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound VII) was identified in this study to be another intermediate compound in the degradation of compound V. The experiments showed that both ORF28 and ORF30 are involved in the conversion of compound VII. A homology search indicated that both of these ORFs encode acyl-CoA dehydrogenases, suggesting that ORF28- and ORF30-encoded proteins convert the CoA ester of compound VII to that of compound VI. The best-known structure of all acyl-CoA dehydrogenases is the medium-chain acyl-CoA dehydrogenase. The structure is a homotetramer, which is classified as a dimer of dimers. Because our experiments showed that ORFs 28 and 30 are indispensable for the conversion, the deduced amino acid sequences of these ORFs were examined in detail using COACH, a ligand-binding site prediction program (34, 35).

The identity of the deduced amino acid sequence of ORF28 to the corresponding protein of *C. testosteroni* (strains CNB-2, S44, ATCC 11996, and KF-1) is more than 90%. The identity to the corresponding proteins of proteobacteria is approximately 40%. All the proteins whose amino acid sequences showed approximately 40% identity to the deduced amino acid sequence of ORF28 were classified as CaiA, acyl-CoA dehydrogenases (lipid metabolism; cluster of orthologous group 1960 [COG1960]). The homology is not significantly high, but the overall amino acid sequence of the ORF28-encoded protein is similar to that of COG1960 proteins, whereas only a partial sequence is similar to that of other acyl-CoA dehydrogenases. Therefore, the ORF28-encoded protein was assigned to COG1960 acyl-CoA dehydrogenases. The COG1960 classification contains models that may span more than one domain and are not assigned to any domain superfamily (description from the National Center for Biotechnology Information). The putative flavin adenine dinucleotide (FAD)-binding site, corresponding to amino acids 123, 125, 126, 131, 152,

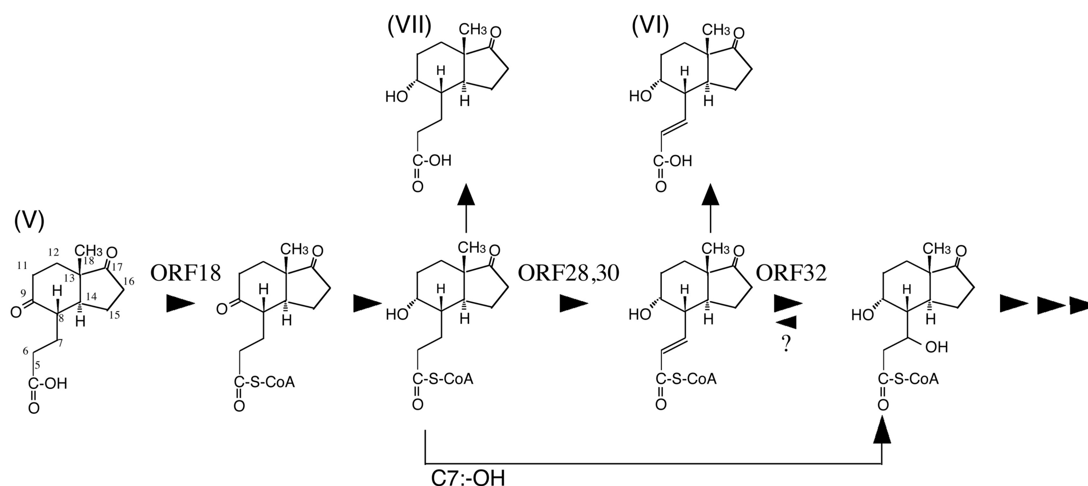

FIG 8 Proposed degradation pathway of steroidal B, C, and D rings in *Comamonas testosteroni* TA441. The compounds are 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound V), 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (compound VII), and 9α-hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-en-5-oic acid (compound VI).

153, 154, 199, 351, 354, 355, 356, 358, 360, 361, and 364 in the deduced amino acid sequence of ORF28, is the best-conserved domain in COG1960 proteins. The alignment of the partial amino acid sequences of COG1960 proteins (selected by the mode possibly wide variety of species) with the deduced amino acid sequence of the corresponding region in ORF28 is shown in Fig. 7a (amino acids composing the putative FAD-binding site are in bold). Amino acids in the putative FAD-binding site, except for amino acids 131 and 199, are contained in the presented sequences. The FAD-binding region is indispensable for acyl-CoA dehydrogenase activity. However, less than half of the domain amino acids are conserved in ORF28. Moreover, all of the COG1960 proteins presented in Fig. 7a are putative proteins that have been found through genome analysis. Their activities have not been experimentally confirmed, and their substrates are unclear.

The deduced amino acid sequence of ORF30 shows more than 90% identity to the corresponding protein of *C. testosteroni* (strains ATCC 11996, CNB-2, KF-1, and S44) and more than 50% identity (maximum, approximately 65% identity) to putative acyl-CoA dehydrogenases (ACAD_fadE6_17_26 family) of a number of species of bacteria. The ACAD_fadE6_17_26 family includes the eukaryotic acyl-CoA dehydrogenases involved in β-oxidation as well as amino acid catabolism enzymes. These enzymes share high sequence similarity but differ in their substrate specificities (description from the National Center for Biotechnology Information). The putative FAD-binding site, which is at amino acids corresponding to amino acids 153, 155, 156, 161, 162, 188, 189, 190, 239, 245, 390, 393, 394, 397, 399, 400, and 403 in the deduced amino acid sequence of ORF30, is well conserved. The putative substrate binding site is also well conserved and shows the highest hit with the substrate binding site of 3-thiaoctanoyl-CoA. In the deduced amino acid sequence of ORF30, the conserved amino acids are 115, 118, 119, 123, 153, 161, 162, 164, 216, 268, 272, 275, 276, 279, 338, 340, 394, 395, 396, 400, 404, and 407. The alignment of the partial deduced amino acid sequence of ORF30 with the corresponding region of the amino acid sequences of ACAD_fadE6_17_26 (selected by the mode possibly wide variety of species) is shown in Fig. 7b (amino acids of the putative FAD-binding site are in bold; amino acids of the putative substrate binding site are boxed). In contrast to the amino acids in ORF28, the amino acids in the FAD-binding and putative substrate binding sites are well conserved in ORF30. From this information, the ORF30-encoded protein can be classified with the ACAD_fadE6_17_26 family.

The ORF30-encoded protein seems to be the main component of CoA dehydrogenase and should have activity on its own. However, the conversion did not occur without ORF28 in this study. Possible steroid degradation gene clusters similar to that of TA441, ORF28 and ORF30, are often found in bacterial genome analysis data (33). To the best of our knowledge, ORFs similar to ORF28 and ORF30 lie side by side in the same order as ORFs 28 and 30 in all of these putative steroid degradation gene clusters in the database (found in *Cupriavidus necator* [*Ralstonia eutropha*] JMP134 [GenBank accession number NC_007347], *C. necator* [*R. eutropha*] H16 [GenBank accession number NC_008314], *Burkholderia cenocepacia* J2315 [GenBank accession number NC_011001], *Burkholderia* sp. strain 383 [GenBank accession number NC_007511], *Cupriavidus taiwanensis* LMG 19424 [GenBank accession number NC_010530], *Shewanella pealeana* ATCC 700345 [GenBank accession number NC_009901], *Shewanella halifaxensis* HAW-EB4 [GenBank accession number NC_010334], and *Pseudoalteromonas haloplanktis* TAC125 [GenBank accession number NC_007481]). These results may indicate that the ORF28- and ORF30-encoded proteins form an acyl-CoA dehydrogenase of a new family that is indispensable in bacterial steroid degradation.

Compound V was converted to compound VII via the reduction of the ketone group at C-9 to a hydroxyl group. The gene involved in this reaction remains unclear. The ORF28,30^{-} mutant incubated with cholic acid and chenodeoxycholic acid, which have a hydroxyl group at C-7, did not accumulate compound VII, implying that the C-7 hydroxyl group is directly dehydrogenated to a ketone group in the degradation of steroid compounds that contain it. Conversion of the CoA ester of 7α,9α-dihydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid to the CoA ester

of compound VI may occur, because a small amount of compound VI was detected in the culture of the ORF32$^-$ mutant incubated with steroid compounds with a hydroxyl group at the C-7 position. Based on the present information, we propose that the conversion of compound V to compound VII in TA441 occurs as shown in Fig. 8.

The degradation begins with the addition of CoA to compound V by ORF18-encoded CoA transferase, followed by conversion of a ketone group to a hydroxyl group at the C-9 position. Then, a double bond is introduced at the C-6 position of the CoA ester of compound VII by the ORF28- and ORF30-encoded CoA dehydrogenases. The resultant CoA ester of compound VI likely has a water molecule added to a double bond at C-6 by ORF32-encoded hydratase to produce the CoA ester of 7α,9α-dihydroxy-17-oxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid. On the basis of the β-oxidation process, the subsequent reaction would be dehydrogenation of the hydroxyl group to produce a ketone group at the C-7 position, and then two carbons, one at the C-5 position and one at C-6, would be removed by a thiolase.

Compound V and its derivatives have been isolated as intermediate compounds in steroid degradation in the Gram-positive bacteria *Arthrobacter simplex* (36) and *Corynebacterium equi* (*Rhodococcus equi*) (37), and the lactone derivative of compound V has been isolated from Gram-negative *Pseudomonas* species (38). Compound V has also been predicted to be an intermediate compound in cholic acid degradation by the Gram-positive bacterium *Streptomyces rubescens* on the basis of a compound, 2,3,4,6,6aβ,7,8,9,9aα,9bβ-decahydro-6aβ-methyl-1*H*-cyclopenta[*f*]quinoline-3,7-dione, isolated from the culture of this bacterium incubated with cholic acid (39). A steroid degradation pathway similar to that of *C. testosteroni* might be widespread among bacteria. Because steroid compounds are known for their wide variety of functions, further development of the study on bacterial steroid degradation is expected.

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