Steroid degradation genes in *Comamonas testosteroni* TA441: isolation of genes encoding a Δ4(5)-isomerase and 3α- and 3β-dehydrogenases and evidence for a 100kb steroid degradation gene hot spot

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Running title: steroid degradation by *C. testosteroni* TA441

Abstract

In previous studies, we identified two major *Comamonas testosteroni* TA441 gene clusters involved in steroid degradation. Because most of the genes included in these clusters were revealed to be involved in degradation of basic steroidal structures and a few were suggested to be involved in the degradation of modified steroid compounds, we investigated the spectrum of steroid compounds degradable for TA441 to better identify the genes involved in steroid degradation. TA441 degraded testosterone, progesterone, epiandrosterone, dehydroepiandrosterone, cholic acid, deoxycholic acid, chenodeoxycholic acid, and lithocholic acid. The results suggested TA441 having 3α-dehydrogenase and Δ4(5)-isomerase, and 3β-,17β-dehydrogenase gene, we isolated these genes, all of which had high homology to the corresponding genes of *C. testosteroni* ATCC11996. Results of gene-disruption experiments indicated that 3β,17β-dehydrogenase is a unique 3β-dehydrogenase which also acts as a 17β-dehydrogenase in TA441, and there will be at least one more enzyme with 17β-dehydrogenating activity. The 3α-dehydrogenase and Δ4(5)-isomerase genes were found adjacent in the DNA region between the two main steroid degradation gene clusters together with a number of other genes that may be involved in steroid degradation, suggesting the presence of a steroid degradation gene hot spot over 100 kb in size in TA441.

1. Introduction

Steroid compounds have various pharmaceutical and hormonal effects. Degradation of steroid compounds by bacteria was intensively studied during the 1950s-1970s for the purpose of obtaining materials for steroid drug synthesis, especially from two representative steroid degrading bacteria, Gram-positive *Rhodococcus* (formerly *Nocardia*) *restrictus* and Gram-negative *Comamonas* (formerly *Pseudomonas*) *testosteroni* [1-23]. The major intermediate compounds in bacterial steroid degradation were identified in these studies and a degradation pathway was proposed based on these compounds. As new functions have been discovered for several steroid compounds[24-26], bacterial steroid degradation is re-emerging as an area of research interest. However, before our study on steroid degradation in *C. testosteroni* TA441, the bacterial steroid degradation pathway remained incomplete and the identification of degradation genes for only a few early-steps had been reported.

In our previous studies on strain TA441, we clarified the detailed degradation pathway in strain TA441 and identified two major steroid degradation gene clusters probably containing most of the genes necessary for degradation of the basic steroid skeleton using gene-disrupted mutants. One cluster contains a dehydrogenase gene for converting a β-oriented hydroxyl group at the C-12 position to a ketone group (*steA*), a hydrogenase gene for converting the ketone group at the C-12 position to an α-oriented hydroxyl group (*steB*), ORF7 and ORF6 of unknown function, a *meta*-cleavage enzyme gene *tesB*, 16 other ORFs (ORF1-33), and a positive regulator gene *tesR*. The other major steroid degradation cluster consists of ORF18, ORF17, and *tesIHA2A1DEFG*, located at least 20kb from *steA* and *tesR*.

C. testosteroni TA441 degrades testosterone into 2-hydroxyhexa-2,4-dienoic acid and

9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid via aromatization of the A-ring followed by ring cleavage and hydrolysis using enzymes encoded by the genes in the second cluster as well as TesB. The degradation product 2-hydroxyhexa-2,4-dienoic acid is further degraded by TesEGF in a process that is similar to bacterial biphenyl degradation [27-34]. CoenzymeA (CoA) is added to 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid by the ORF18-encoded CoA-transferase. Most of ORFs1 to 33 are considered to be involved in β-oxidation of the resultant 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid CoA-ester.

To the best of our knowledge, only a limited number of steroid degradation genes have been isolated from other strains of *C. testosteroni* (Table 1); genes for Δ1- and Δ4(5)-dehydrogenases from ATCC17410 [35, 36], a *meta*-cleavage enzyme gene [37], a positive regulator gene [38-40], a 3β,17β-dehydrogenase gene [41-43], a 3α-dehydrogenase gene [44-46], and a 3-ketosteroid-Δ4(5)-isomerase gene [21, 47] from ATCC11996. The 3-ketosteroid-Δ4(5)-isomerase gene is located just downstream of the 3α-dehydrogenase gene, and genes encoding repressors of 3α-dehydrogenase were recently found on the complementary strand of the 3α-dehydrogenase gene [48]. *C. testosteroni* TA441 was thought to have genes encoding a 3β,17β-dehydrogenase, a 3α-dehydrogenase, and a 3-ketosteroid-Δ4(5)-isomerase, but to date these genes have not been isolated from this strain.

In previous studies, we primarily used testosterone for the analysis of steroid degradation by *C. testosteroni* TA441 because testosterone has a simple structure and has long been used as a representative steroid in studies of bacterial steroid degradation. Here, we report on an investigation of the spectrum of steroid compounds degradable for *C. testosteroni* TA441 and the identification of genes for a 3β-dehydrogenase, a

3α-dehydrogenase, and a 3-ketosteroid-Δ4(5)-isomerase in *C. testosteroni* TA441.

2. Materials and Methods

2.1. Culture conditions

Comamonas testosteroni TA441 and the gene-disrupted mutant were grown at 30 °C in Luria-Bertani broth (LB-medium), C-medium (a mineral medium used for growth of TA441 [27]), or 1/2LB+1/2C-medium (mixture of equal volume of LB and C-media) with suitable carbon sources when it's necessary. Structure of steroid compounds used in this study (testosterone, progesterone, dehydroepiandrosterone, epiandrosterone, 17α-hydroxyprogesterone, cortisone, cortizole, cholesterol, cholic acid, cheno deoxycholic acid, and deoxycholic acid) are shown in Fig. 1. These compounds except for cholesterol were added as a filtered dimethyl sulfoxide (DMSO) solution at a final concentration of 0.1% (w/v). Cholesterol was added without filtration at a final concentration of approximately 0.1% (w/v), because of its low solubility in DMSO. Gene-disruption was performed in the same manner as previous experiments by inserting a kanamycin-resistance gene without terminator into the objective gene [28].

2.2. Growth of TA441 on steroid compounds

Growth was monitored as colony forming units (CFU) because the culture with a steroid compound was usually turbid, which prevented measurement of cell absorbance. TA441 was pre-cultured in LB-medium, washed twice, resuspended in C-medium. The same amount of the cells was inoculated to C-medium with 0.1% (w/v) each steroid and the growth was monitored by counting colonies that appeared on LB plates on which

appropriate dilutions of the culture had been spread and incubated at 30 °C.

2.3. HPLC analysis of the culture of gene-disrupted mutants

Gene-disrupted mutants were pre-cultured in LB-medium with kanamycin (400 μg/mL), inoculated to 10 ml 1/2LB +1/2C medium with 0.1 % (w/v) of each steroid, and incubated at 30°C. Twice the volume of methanol was added to a small amount of the culture 24 h, 32 h, and 48 h after the start of the incubation, which was then centrifuged and the supernatant was directly injected into the HPLC. For the three-dimensional HPLC data, a Waters 2690 HPLC (Waters Corporation, MA) was used with Waters 996 Photodiode Array Detector (Waters Corporation, MA) and an Inertsil ODS-3 column (4.6 x 250 mm; GL Science, Tokyo, Japan), and elution 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 minutes, maintained for 13 minutes, and then changed to 20% solution A for 5 minutes. The flow rate was 1 mL/min.

2.4. Accession number

The accession number for a DNA fragment containing 3β-dehydrogenese gene is ***AB474240***, and for 3α-dehydrogenese and 3-ketosteroid-Δ4(5)-isomerase genes is ***AB489116***.

3. Results

3.1. Growth of *C. testosteroni* TA441 on steroid compounds

Previously, we elucidated the pathway and genes involved in testosterone degradation by *C. testosteroni* TA441. We utilized testosterone in those studies to reveal details about degradation of the basic skeleton of steroid compounds, and did not focus upon the degradability of modified steroid compounds. As modified steroid compounds such as the cholic acid analogs are attracting attention due to newly discovered functions (e.g. reduction of internal cholesterol level in animals and induction of defensive reactions in plants [24-26]), in this study we examined degradability of steroid compounds containing different side chains and double bonds (Fig. 1) by *C. testosteroni* TA441 in order to identify the genes involved in the degradation of modified steroid compounds**.** Pre-cultured *C. testosteroni* TA441 cells were inoculated into C-medium (a mineral medium used for growth of TA441 [27]) containing 0.1% (w/v) of each steroid compound and cell growth was monitored at 0, 24, 48, and 72 h by determining the number of colony forming units per unit volume (CFU/ml) (Fig. 1). The CFU reached ca. 10^8/ml in control cultures without a steroid compound carbon source. This amount of growth was probably due to pre-culture in LB media; therefore a CFU of ca. 10^8/ml was regarded as no growth in cultures with steroid compounds.

C. testosteroni TA441 showed significant growth on testosterone, progesterone, dehydroepiandrosterone, cholic acid and its analogs (deoxycholic acid, chenodeoxycholic acid, lithocholic acid), and epiandrosterone. *C. testosteroni* TA441 grew faster on cholic acid and its analogs (with the exception of lithocholic acid) than on the other steroid compounds tested, but these compounds seemed to cause cell lysis after about 48 h. We do not know why this occurred, but similar lysis was observed when *C. testosteroni* TA441 was grown on succinate as the sole carbon source. Growth on lithocholic acid was quite similar to growth on epiandrosterone so that the data is not

shown in Fig. 1. The start of growth on epiandrosterone was slow. We consider this was due to the low water solubility of this steroid, because small flocks, probably consisting of bacterial cells and small particles of epiandrosterone, appeared a few hours after the start of incubation.

Growth of *C. testosteroni* TA441 on 17α-hydroxyprogesterone was so unstable that further study will be required to confirm that the organism can grow on this compound. *C. testosteroni* TA441 showed negligible growth on cortisone and cholesterol, and also on estrone, 17β-estradiol, ergosterol, and several other steroid compounds with a large side chain at the C-17 position, such as plant sterols (data not shown). In subsequent experiments, we used only cholesterol as a representative of these undegradable steroid compounds.

3.2. HPLC analysis of intermediate compounds in culture media of gene-disrupted mutants of *C. testosteroni* TA441 incubated with steroid compounds

Because *C. testosteroni* TA441 degrades steroid compounds so rapidly that intermediates can be detected only transiently and in small amounts, HPLC analysis of intermediates in the culture of *C. testosteroni* TA441 is of limited value. Intermediate compounds are usually detected with greater success in gene-disrupted mutants, that gene-disrupted mutants are more suitable to confirm the ability of *C. testosteroni* TA441 to degrade steroid compounds by HPLC. *tesD* encodes the hydrolase for 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid (4,9-DSHA), an intermediate compound in bacterial steroid degradation, and disruption of *tesD* causes cells to accumulate 4,9-DSHA or its analogs. These compounds have a characteristic intense yellow color under neutral condition and absorb UV strongly with

a maximum at about 320 nm under acidic condition. In addition, although 4,9-DSHA and its analogs are potentially unstable, 4,9-DSHA and its analogs are nonenzymatically converted to 4-aza-17-oxoandrosta-1,3,5(10)-triene-3-carboxylic acid (4,9-DSHA-N) and its analogs with ammonium ion in the culture, which are quite stable and behave as dead-end compounds for *C. testosteroni* TA441.

Pre-cultured TesD-disrupted mutant cells were inoculated and incubated in 1/2LB+1/2C-medium (mixture of equal volume of LB and C-media) with 0.1% (w/v) steroid compound. Chromatograms of culture broth analyzed by HPLC at 24 h are shown in Fig. 1. Compounds with strong absorbance at 320 nm were 4,9-DSHA (retention time (RT) = 10.5 min) and its analogs, and a compound detected at RT = 3.5 min with a maximum absorbance at about 280 nm is 4-aza-9,17-dioxo-9,10-secoandrosta-1,3,5(10)-triene-3-oic acid (4,9-DSHA-N) (closed arrowheads).

Among the steroid compounds tested, significant amounts of 4,9-DSHA and/or its analogs were detected in cultures of the TesD-disrupted mutant incubated with testosterone, progesterone, dehydroepiandrosterone, and cholic acid analogs. In the culture with epiandrosterone and 17α-hydroxyprogesterone, a minimal amount of 4,9-DSHA-N was detected. In the cultures incubated with cortisone and cholesterol, 4,9-DSHA and its analogs, and 4,9-DSHA-N were undetectable**.**

To confirm whether *C. testosteroni* TA441 is capable of degrading epiandrosterone and 17α-hydroxyprogesterone, we performed the same experiment with a TesH-disrupted mutant. TesH is a Δ1-dehydrogenase that introduces a double bond at the C-1 position of the steroid A-ring. TesH-disrupted mutants incubated with steroid compounds accumulate androsta-4-ene-3,17-dione (4-AD) and/or its analogs with

compounds of the same structure as 4-AD and/or its analogs except for the presence of a hydroxyl group at the C-9 position (9-OH-4-AD and/or its analogs) when the subjected steroid compounds are degradable for *C. testosteroni* TA441. In this experiment, dehydroepiandrosterone and progesterone were used as positive controls for epiandrosterone and 17α-hydroxyprogesterone, respectively. 4-AD and 9-OH-4-AD were supposed to accumulate when *C. testosteroni* TA441 degrades these four compounds. Significant amounts of 4-AD and 9-OH-4-AD were detected in the culture incubated with progesterone and epiandrosterone as well as dehydroepiandrosterone, indicating that *C. testosteroni* TA441 degrades epiandrosterone (Fig. 2). On the other hand, only a negligible amount of 9-OH-4-AD was detected in the culture incubated with 17α-hydroxyprogesterone (Fig. 2), suggesting that TA441 is not interpreted to degrade this steroid.

Because 4,9-DSHA has a quite characteristic absorption curve under both acidic and neutral conditions, compounds with absorption curves similar to 4,9-DSHA are considered to be 4,9-DSHA analogs. When we isolated 4,9-DSHA as an intermediate compound of bacterial steroid degradation in a previous study [28], we purified the compound by HPLC under acidic conditions with trifluoroacetic acid (TFA) to obtain sharp peaks because 4,9-DSHA analogs produce broad peaks when isolated by HPLC under neutral conditions. Since the strong acidic condition caused by residual TFA led to conversion of 4,9-DSHA to several compounds during evaporative concentration, we interrupted evaporation just before samples were completely desiccated and added a solvent suitable for further analysis by NMR and MS**.** We attempted to identify the 4,9-DSHA analog at RT = 9.2 min when we isolated and identified 4,9-DSHA, but NMR analysis was unsuccessful because only small amount of the compound was

isolated and a part of it was converted automatically to other compounds, which resulted in isolation of an insufficient amount and purity of 4,9-DSHA analog at RT = 9.2. High-resolution FAB-MS (positive) analysis of the ion of this compound at *m/z* 351.1835 $(M-H)^+$ (Calculated *m/z*: 351.1808) indicated that the molecular formula is $C_{19}H_{27}O_6$, suggesting 4,9-DSHA analog at RT = 9.2 is a compound produced by addition of 2 H to 4,9-DSHA. It is not possible to add 2 H to 4,9-DSHA without changing the absorption curve unless the addition is to a ketone group at the C-17 position. Conversion of a ketone group to a hydroxyl group at the C-17 position and vice versa is a constantly occurring reaction before the cleavage of the A-ring because steroid degradation intermediate compounds usually have a ketone group at the C-17 position and are accompanied by a small amount of the compound with a hydroxyl group at the C-17 position [34]. Based on these results, we speculate that the 4,9-DSHA analog with a RT = 9.2 min in HPLC analyses is 4,5-9,10-diseco-3,17-dihydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid.

Structure of 4,9-DSHA analogs with a RT = 3.2 min and RT = 7.3 were speculated from the absorption curves, RT (under the present HPLC condition, more hydrophilic compounds are detected earlier), together with other intermediate compounds in degradation of cholic acid and chenodeoxycholic acid. We identified 7,12-dihydroxy-9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid and 7-hydroxy-9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid as the major intermediates produced by hydrolysis in cultures of ORF18-disrupted mutants incubated with cholic acid and chenodeoxycholic acid, respectively (33). This result indicates that the hydroxyl groups at positions C-7 and C-12 are present before hydrolysis, and that therefore the major 4,9-DSHA analog observed at RT = 3.2 min in cultures incubated

with cholic acid is 4,5-9,10-diseco-3,7,12-trihydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid. The major 4,9-DSHA analog observed at RT = 7.3 min in cultures incubated with chenodeoxycholic acid is 4,5-9,10-diseco-3,7-dihydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid, while the major 4,9-DSHA analog with a RT = 7.8 min in cultures incubated with deoxycholic acid is 4,5-9,10-diseco-3,12-dihydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid.

Several peaks were detected by HPLC in the culture of TesD-disrupted mutant incubated with chenodeoxycholic acid. The peak at RT = 10.5 min is considered to be 4,9-DSHA itself because the RT is in complete agreement with the RT of 4,9-DSHA run as a standard. A small amount of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid identified in the culture of ORF18-disrupted mutants incubated with chenodeoxycholic acid (33) will support that 4,9-DSHA was produced from chenodeoxycholic acid. These results imply that during degradation of chenodeoxycholic acid in *C. testosteroni* TA441, a double bond would be produced at Δ6(7) by dehydration, followed by conversion to a single bond by addition of 2 H. Therefore, the 4,9-DSHA analog with a RT = 10.6 is likely to be 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2,6-trien-4-oic acid.

To further confirm our results and investigate which steroid or intermediate compound(s) induce steroid degradation genes in *C. testosteroni* TA441, we performed Northern hybridization analyses. Unfortunately, we were unable to obtain unequivocal results, probably because some steroid compounds tested were less soluble in water and phenol, which was used in RNA isolation, and residual steroid compounds may have prevented hybridization. With regard to the steroid compounds that were soluble, results were consistent with those of growth and HPLC analysis: steroid degradation genes

were induced when the subjected steroid is degradable for *C. testosteroni* TA441.

3.3. Isolation of a putative 3β,17β-dehydrogenase, a 3α-dehydrogenase, and 3-ketosteroid-Δ4(5)-isomerase genes from *C. testosteroni* TA441

Our results obtained in section 3.1 and 3.2 indicated that in addition to genes already identified as being involved in degradation of the basic steroid skeleton, *C. testosteroni* TA441 produces 3β-, 17β- and 3α-dehydrogenases, a 3-ketosteroid-Δ4(5)-isomerase, and enzymes for degradation of the side chain at the C-17 position of cholic acid and progesterone. Genes encoding 3β,17β- and 3α-dehydrogenases and 3-ketosteroid-Δ4(5)-isomerase have been isolated from *C. testosteroni* ATCC11996 [21, 41, 44]. Because the DNA sequences of *C. testosteroni* ATCC11996 and TA441 revealed to date are quite similar, we constructed PCR primers based on DNA sequences of *C. testosteroni* ATCC11996 genes to isolate corresponding genes from strain TA441. PCR amplification was performed with these primers and total TA441 DNA as the template, and probes were generated based on the obtained fragments after confirming the sequence. Using these probes, DNA fragments containing both the putative 3α-dehydrogenase and 3-ketosteroid-Δ4(5)-isomerase genes, as well as a fragment containing the putative 3β,17β-dehydrogenase gene were isolated. DNA sequencing confirmed that these fragments contained the genes of interest, and the deduced amino acid sequences were highly homologous (approximately 95 % identity) (Fig. 3) [21, 41, 44].

Negative regulators of 3α-dehydrogenase, RepA and RepB, which are encoded on the complementary strand of the 3α-dehydrogenase and 3-ketosteroid-Δ4(5)-isomerase genes was reported for *C. testosteroni* ATCC11996 (Fig. 3A) [39], and corresponding

putative genes were found in *C. testosteroni* TA441. However, the deduced amino acid sequence of the product of the *repA* gene had only approximately 85% identity with *C. testosteroni* ATCC11996 RepA. While 85% identity is not low, it is conspicuously lower than the identity between the other corresponding proteins involved in steroid degradation from these two strains. The deduced amino acid sequence of the product of the DNA region correspond to *repB* showed high homology (around 96% identity) to *C. testosteroni* ATCC11996 RepB, but we were unable to find a stop codon, which resulted in a larger ORF (ORF64) containing the gene region correspond to *repB*. The entire deduced amino acid sequence of ORF64 showed the highest homology (92% identity) to the putative enzyme-regulator fusion protein of *C. testosteroni* CNB-2 [49], as well as several other putative enzyme-regulator fusion proteins belong to the GreA/GreB family of elongation factors. Other than *C. testosteroni*, *Bordetella parapertussis* showed the highest amino acid identity (73%) [50]. *C. testosteroni* ATCC11996 3α-dehydrogenase and 3-ketosteroid-Δ4(5)-isomerase are comprehensively studied by other research groups (3α-dehydrogenase, [44-46]; 3-ketosteroid-Δ4(5)-isomerase, [21, 47]), and we consider that further examination of these enzymes in *C. testosteroni* TA441 is of less value.

The isolated DNA fragment containing a putative 3β□17β-dehydrogenase gene was composed of three putative ORFs (ORFs61-63) with a partial ORF in the upstream DNA region of ORF61, and a possible terminator immediately downstream of ORF62 (Fig. 3B). The deduced amino acid sequence of the product of ORF62 showed high homology (96% identity) to 3β□17β-dehydrogenase (3β□17β-DH) of *C. testosteroni* ATCC11996. The partial ORF corresponds to *stdC* of *C. testosteroni* ATCC11996, which was reported to be induced when ATCC11996 is incubated with steroid

compounds [51], suggesting the possibility that the partial ORF, ORF61, and the putative 3β□17β-DH gene are involved in steroid degradation. The putative amino acid sequence of ORF61 shows high homology to putative proteins of the DUF1329 family. DUF1329 is a hypothetical protein but is found in wide range of bacteria, especially in *Pseudomonas* species. The deduced amino acid sequence of ORF63 showed significant homology to Sel1 domain-containing proteins and is not considered to be involved in steroid degradation.

3.4. Analysis of ORF61 and ORF62 (a putative 3β□17β-dehydrogenase gene) using gene-disrupted mutants

Because ORF61 might be involved in steroid degradation, and its function in steroid degradation is unclear, we constructed gene-disrupted mutants of ORF61 and ORF62 in *C. testosteroni* TA441. ORF61- and the ORF62-disrupted mutants were cultured and their growth was monitored in the presence of various steroid compounds as described in section 3.1. Both mutants grew on testosterone, but the ORF62-disrupted mutant grew slower than the *C. testosteroni* TA441 (positive control) and the ORF61-disrupted mutant (Table 2).

The 3β□17β-dehydrogenase of *C. testosteroni* ATCC11996 was first reported as a 3β-dehydrogenase. We considered the main role of the enzyme encoded by ORF62 of *C. testosteroni* TA441 to be 3β-dehydrogenation and conducted the same growth experiment with epiandrosterone, which has a ketone group at the C-17 position and a β-oriented hydroxyl group at the C-3 position. Growth of both *C. testosteroni* TA441 and the ORF61-disrupted mutant on epiandrosterone was slower than on testosterone, but cell density reached more than 10^9 CFU/ml in approximately 48 h. However, the

ORF62-disrupted mutant did not show significant growth on epiandrosterone. These results suggested that the ORF62-encoded enzyme is a unique 3β-dehydrogenase in *C. testosteroni* TA441, and that *C. testosteroni* TA441 might have another dehydrogenase more suitable for the 17β-hydroxyl group.

To confirm results of the growth experiment, gene-disrupted mutants were incubated with testosterone, epiandrosterone, and dehydroepiandrosterone (we added this compound to support the data), and the cultures were analyzed by HPLC as described in section 3.2. In this experiment, the *C. testosteroni* TA441 mutant without 17β-dehydrogenase activity and incubated with testosterone should accumulate 17-hydroxy-androsta-1,4-diene-3-one. The *C. testosteroni* TA441 mutant without 3β-dehydrogenase activity should not accumulate particular intermediate compounds because the 3β-hydroxyl group has to be converted to a ketone group before dehydrogenation on the A-ring by 3-ketosteroid-Δ1-dehydrogenase, 3-ketosteroid-Δ4-dehydrogenase, and 3-ketosteroid isomerase.

The results are shown in Fig. 4, with a higher detector sensitivity since epiandrosterone has a weaker absorbance. We were unable to detect degradation of epiandrosterone and dehydroepiandrosterone with the ORF62-disrupted mutant, while all three steroid compounds decreased to undetectable levels with the ORF61-disrupted mutant. These results are consistent with growth results, indicating the enzyme encoded by ORF62 is a unique 3β-dehydrogenase in *C. testosteroni* TA441. Conversion of testosterone to androsta-1,4-diene 3,17-dione (ADD) was slow in the culture of the ORF62-disrupted mutant, and testosterone was still detected 10 days after the start of the incubation. indicating that the ORF62-disrupted mutant still converted a hydroxyl group at the C-17 position to a ketone group, though at a slower rate than the *C.*

testosteroni TA441 control. This enzyme is considerd to have 17β-dehydrogenase activity, but *C. testosteroni* TA441 probably has at least one more dehydrogenase that works on a hydroxyl group at the C-17 position.

4. Discussion

We previously elucidate a bacterial degradation pathway for the basic skeleton of steroid compounds and to determine the genes involved therein in *Comamonas testosteroni* TA441 using testosterone and ADD as representative steroid compounds due to their simple structures [27-29, 30 , 31-34]. Here we investigated the spectrum of degradable steroid compounds in the purpose of understanding the whole genes involved in steroid degradation in *C. testosteroni* TA441. For the production of useful steroidal compounds, more familiar steroid compounds with modification, which includes cholic acid, cholesterol, and plant sterols, are preferable as starting materials for developing new chemicals with useful effects. These compounds are also attractive from a research perspective because a number of useful effects of these steroid compounds are known and are still under intensive investigation in searching for novel useful effects [24-26].

Among the steroid compounds we tested, *C. testosteroni* TA441 degraded testosterone, progesterone, epiandrosterone, dehydroepiandrosterone, cholic acid, deoxycholic acid, chenodeoxycholic acid, and lithocholic acid (Fig. 5). Cholesterol and some plant sterols were also tested, but *C. testosteroni* TA441 did not degrade them, indicating that the structure of the side chain at position C-17 is one of the most important factors determining the degradability of steroid compounds by *C. testosteroni*

TA441. Regarding cholic acid degradation and modification, *C. testosteroni* TA441 may be an attractive candidate for applications because *C. testosteroni* TA441 degrades cholic acid effectively without interruption even in nutrient rich broth and it will be possible to accumulate excess amount of an objective intermediate compound using gene-disrupted mutants.

The presence of a substituent group at the C-11 position also considerably affects steroid degradability of *C. testosteroni* TA441, as evidenced by the inability of *C. testosteroni* TA441 to degrade cortisone and cortisol. A substituent group at the C-11 position probably prevents hydroxylation at the C-9 position, which is indispensable for aromatization of the A-ring required for bacterial steroid degradation. In contrast, hydroxyl groups at the C-7 and C-12 positions did not affect steroid degradation by *C. testosteroni* TA441 and remained after cleavage of the A- and B-rings, except for a β-oriented hydroxyl group at C-12 should be converted to α-oriented hydroxyl group before cleavage of B-ring [34]. Genes indispensable for this configrational change were found in one of the two major steroid degradation gene clusters in *C. testosteroni* TA441 [34].

For the complete degradation of testosterone, progesterone, epiandrosterone, dehydroepiandrosterone, and cholic acid and its analogs, *C. testosteroni* TA441 requires 3α-dehydrogenase, 3-ketosteroid-Δ4(5)-isomerase, 3β-dehydrogenase, and 17β-dehydrogenase genes, a set of genes for degradation of the side chain at the C-17 position of cholic acid and progesterone, as well as already identified steroid degradation genes. Among the predicted genes, we isolated a putative 3α-dehydrogenase gene, a putative 3-ketosteroid-Δ4(5)-isomerase gene, and a putative 3β,17β-dehydrogenase gene, whose deduced amino acid sequences showed about 95%

identity with those of the corresponding genes of *C. testosteroni* ATCC11996. Because 3α-dehydrogenase and 3-ketosteroid-Δ4(5)-isomerase are well studied in *C. testosteroni* ATCC11996 while 3β,17β-dehydrogenase was less investigated, we constructed a putative 3β,17β-dehydrogenase gene-disrupted mutant of *C. testosteroni* TA441 and found that it did not degrade epiandrosterone, which has a β-oriented hydroxyl group at the C-3 position and a ketone group at the C-17 position. The putative 3β,17β-dehydrogenase gene-disrupted mutant did degrade testosterone, which has a β-oriented hydroxyl group at the C-17 position and a ketone group at the C-3 position, but the growth was noticeably slower than that of the *C. testosteroni* TA441 control. Both of these results were supported by HPLC analysis of the intermediate compounds. From these results, this enzyme in *C. testosteroni* TA441 is considered to be a unique 3β-dehydrogenase possessing a degree of 17β-dehydrogenase activity, but *C. testosteroni* TA441 is likely to have at least one more enzyme with 17β-dehydrogenation activity. Dehydrogenation and hydrogenation at the C-17 position is thought to be a reversible reaction in *C. testosteroni* TA441 because almost all the major intermediate compounds isolated so far have a ketone group at the C-17 position and are accompanied by a small amount of a compound of the same structure with the exception of a hydroxyl group at the C-17 position. *C. testosteroni* TA441 may have more than one enzyme with 17β-dehydrogenating/hydrogenating activity to deal with intermediate compounds having considerable structural differences.

3α-dehydrogenase [44-46, 48, 52] and 3-ketosteroid-Δ4(5)-isomerase [9, 21, 47, 53-71] of *C. testosteroni* ATCC11996 were studied in detail, that we did little experimental examination of corresponding genes in *C. testosteroni* TA441. Cholic acid and its analogs have an α-oriented hydroxyl group at the C-3 position. These

compounds are well-utilized by *C. testosteroni* TA441 upon induction of the putative 3α-dehydrogenase gene, suggesting that *C. testosteroni* TA441 uses 3α-dehydrogenase to produce a ketone group at the C-3 position. A TesI (Δ4-dehydrogenase)-disrupted mutant of *C. testosteroni* TA441 can grow on dehydroepiandrosterone, which has a double bond at Δ5(6), but cannot grow on epiandrosterone, which lacks a double bond at Δ5(6), indicating that *C. testosteroni* TA441 transfers the double bond at Δ5(6) to Δ4(5) using 3-ketosteroid-Δ4(5)-isomerase. Two genes encoding negative regulators of 3α-dehydrogenase, *repA* and *repB*, are found on the complementary chain of the 3α-dehydrogenase gene in *C. testosteroni* ATCC11996. The putative genes were also found in the corresponding positions in *C. testosteroni* TA441 (Fig. 3), but these putative proteins showed some differences to those of *C. testosteroni* ATCC11996. Identity between the RepA proteins of each strain was around 85%, which is obviously lower than homology between other corresponding steroid degradation enzymes, and RepB of *C. testosteroni* TA441 (303 aa) is suggested to be a fusion protein by homology search, that is longer than RepB of *C. testosteroni* ATCC11996 (78 aa).

The entire genome sequence of *C. testosteroni* CNB-2 was recently reported [49], and it has approximately 95% identity with the sequenced DNA region of *C. testosteroni* TA441. We compared the genes/ORFs of *C. testosteroni* TA441 that are involved in steroid degradation, the two main steroid degradation gene clusters (the region from *steA* to *tesR* and from *tesG* to ORF18) and the two DNA fragments isolated in this study, with the corresponding DNA region of *C. testosteroni* CNB-2. Our comparison revealed that the *steA* to *tesR* region, the 3α-dehydrogenase and 3-ketosteroid-Δ4(5)-isomerase genes, and the *tesG* to ORF18 region are on the same DNA strand several dozen kilobases apart. Nearly 90% of these interspatial DNA regions in *C. testosteroni* TA441

have been sequenced to date. Most of the corresponding putative ORFs in *C. testosteroni* TA441 and CNB-2 are in the same order and the deduced amino acid sequences show more than 95% identity. A homology search suggested the possibility that a number of genes in this region are involved in steroid degradation, which implies that this DNA region of more than 100 kb is a hot spot of steroid degradation genes. Particularly, DNA region about 15kb away from *tesG* is likely to be involved in the degradation of the cholic acid side chain at the C-17 position, because the initial step in the degradation of the cholic acid C-17 side chain is suggested to be a β-oxidation reaction and a number of putative β-oxidation genes are included in this region. Although none of the genes involved in degradation of the side chain at position C-17 in cholic acid have been isolated from *C. testosteroni* strains, the acyl-CoA-dehydrogenase for 7α,12α-dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylate CoA-ester was isolated from *Pseudomonas* sp. Chol1 [72].

7α,12α-Dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylate CoA-ester is an intermediate compounds in the degradation of the side chain at C-17 position in colic acid, which is produced by removal of two carbons at C-23 and C-24 positions of cholic acid. A putative acyl-CoA-dehydrogenase gene showing about 70% amino acid identity to the acyl-CoA-dehydrogenase for 7α,12α-dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylate CoA-ester of *Pseudomonas* sp. Chol1 was found in the DNA region about 15kb away from *tesG* in *C. testosteroni* TA441. In addition, one of the Tn5-mutants of *C. testosteroni* TA441 constructed in our previous study accumulates 7α,12α-dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylic acid when incubated with cholic acid (manuscript in preparation). Tn-5 was inserted into the putative acyl-CoA-dehydrogenase gene for

7α,12α-dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylate CoA-ester in this mutant, strongly supporting speculation that this putative gene is the acyl-CoA-dehydrogenase for 7α,12α-dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylate CoA-ester. These information suggest the hypothesis that putative genes in the neighboring region are also involved in degradation of the a side chain at position C-17 of cholic acid. Further analyses of the possible steroid degradation gene hot spot as well as studies on degradation of the side chain at position C-17 of cholic acid as well as steroid C- and D-rings are now underway.

Although *C. testosteroni* TA441 degraded the side chain at C-17 position of cholic acid and progesterone, it did not degrade cholesterol and other steroid compounds that have a more complicated side chain at the C-17 position, including plant sterols and estrogens. To the best of our knowledge, Gram-positive bacteria, such as *Rhodococcus* sp., are capable of degrading a wider range of steroid compounds [73]. Gram-positive bacteria have isozymes for steroid degradation [74, 75] and will have more than one steroid degradation pathway [76], which may be responsible for the ability of these bacteria to degrade such a wide range of steroid compounds. *C. testosteroni* TA441 basically has only one steroid degradation pathway because gene-disruption usually lead to accumulation of intermediate compounds. It also indicates that there is only one enzyme for a single reaction, which resulted in successful investigation on the degradation pathway and the genes involved there in. Conceivably, for pharmaceutical or biomedical applications, combined use of Gram-positive bacteria and gene-disrupted mutants of *C. testosteroni* TA441 might be effective for producing useful steroidal compounds, though studies on bacterial steroid degradation in more genera will be necessary to fully elucidate the genes and pathways involved.

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Figure legends and table captions

Figure 1. Structure of steroid compounds examined in this study, growth curves of *C. testosteroni* TA441 with each steroid compound serving as carbon source, and HPLC analysis of cultures of TesD-disrupted mutants incubated with each steroid compound. Compounds with strong absorbance at 320 nm were 4,9-DSHA (retention time (RT) = 10.5 min) and its analogs, and a compound detected at RT = 3.5 min with a maximum absorbance at about 280 nm is 4-aza-9,17-dioxo-9,10-secoandrosta-1,3,5(10)-triene-3-oic acid (4,9-DSHA-N) non-enzymatically converted from 4,9-DSHA (closed arrowheads).

Figure 2. HPLC analysis of the culture of the TesH (Δ1-dehydrogemase)-disrupted mutant incubated with epiandrosterone, 17α-hydroxyprogesterone, dehydroepiandrosterone (control), and progesterone (control). The TesH-disrupted mutant accumulated androsta-4-ene-3,17-dion (4-AD) and 9-hydroxyandrosta-4-ene-3,17-dion (9-OH-4-AD) when subjected compounds are degradable for *C. testosteroni* TA441.

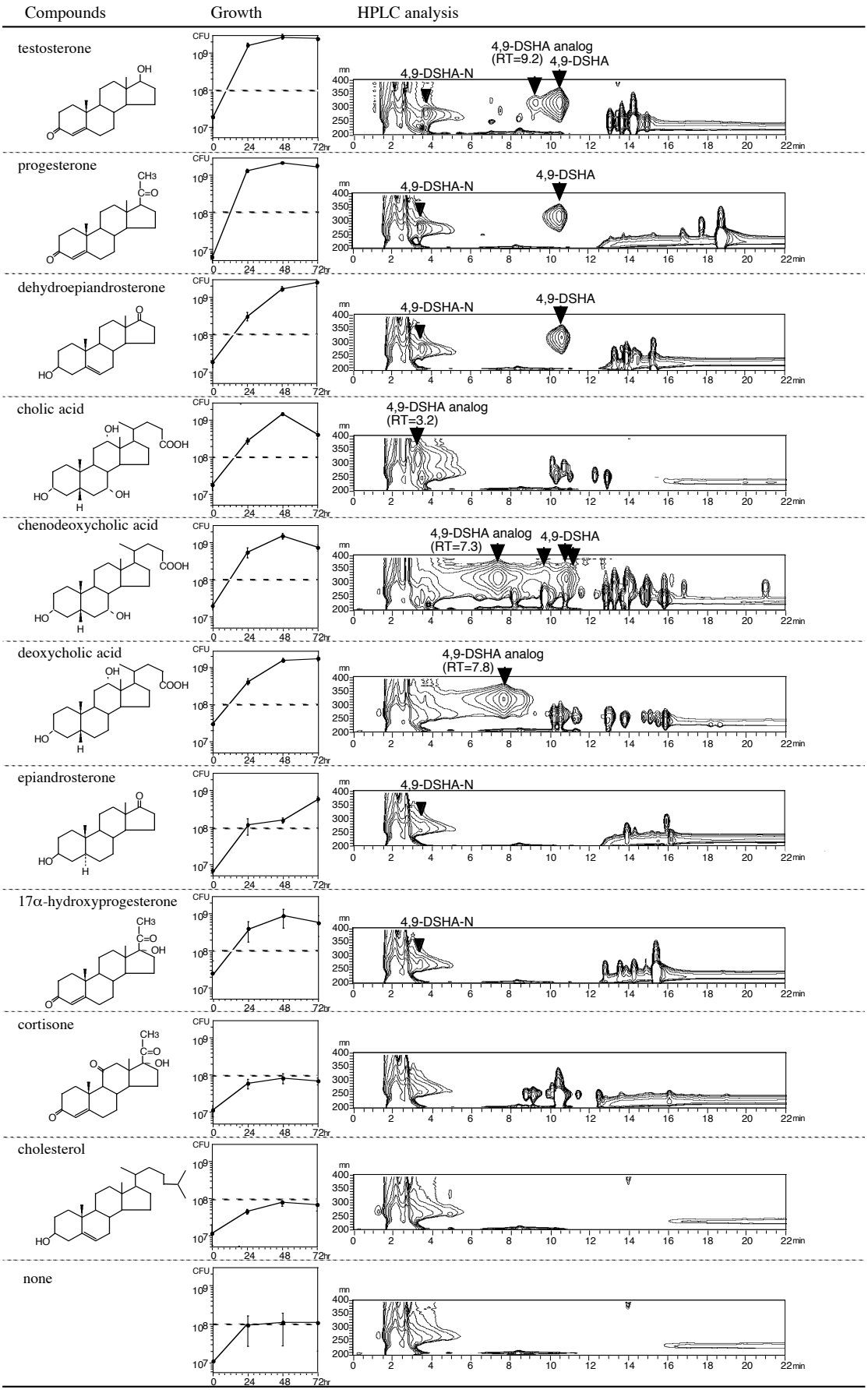
Figure 3. The isolated DNA fragment of *C. testosteroni* TA441 containing putative 3α-dehydrogenase and 3-ketosteroid-Δ4(5)-isomerase genes (above) and the DNA fragment containing a putative 3β,17β-dehydrogenase gene (below) with corresponding genes of *C. testosteroni* ATCC11996. Numbers (%) indicate amino acid identity between the corresponding proteins.

Figure 4. HPLC analysis of the culture of the ORF62 (a putative 3β,17β-dehydrogenase gene)-disrupted mutant and ORF61-disrupted mutant incubated with testosterone (RT = 13.6), epiandrosterone (RT = 15.4), or dehydroepiandrosterone (RT = 14.3) (indicated with open arrowheads) for 1 day and 10 days.

Figure 5. Proposed *Comamonas testosteroni* TA441 steroid degradation pathway. Compounds (R1, R2=H) are: 3-oxo-5β-cholan-24-oic acid (I); androsta-4-ene-3,17-dione (II); androsta-1,4-diene-3,17-dione (ADD) (III); 9-hydroxy-1,4-androstadiene-3,17-dione (IV); 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (V); 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (VI); 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) (VII); 2-hydroxyhexa-2,4-dienoic acid (VIII); 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (IX); 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid CoA ester (X).

Table 1. Steroid degradation genes identified in *Comamomas testosteroni* TA441, ATCC11996, and ATCC17410.

Table 2. Growth (measured as CFU/ml) of *C. testosteroni* TA441, the ORF61-disrupted mutant, and the ORF62 (putative 3β,17β-dehydrogenase)-disrupted mutant incubated for 24 h with 0.1 % (w/v) testosterone or epiandrosterone as the sole carbon source.



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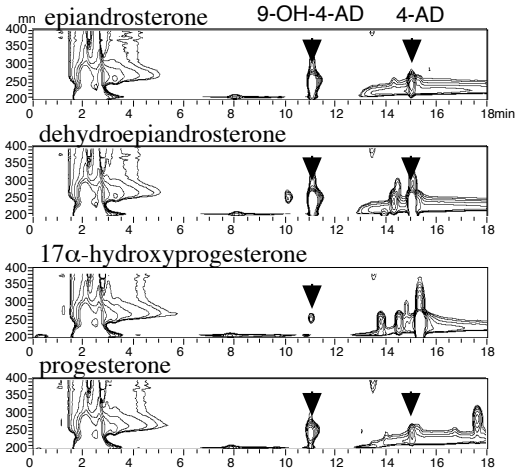

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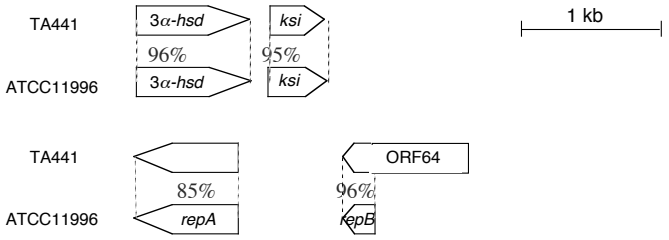
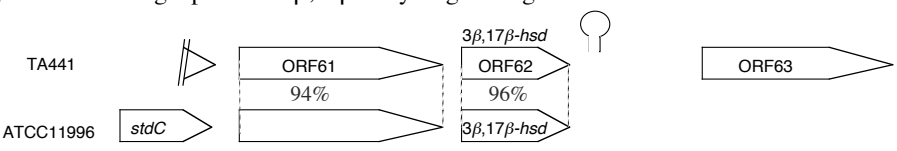
DNA fragment containing a putative 3α-dehydrogenase gene and a putative 3-ketosteroid-Δ5-isomerase ge



DNA fragment containing a putative 3β,17β-dehydrogenase gene



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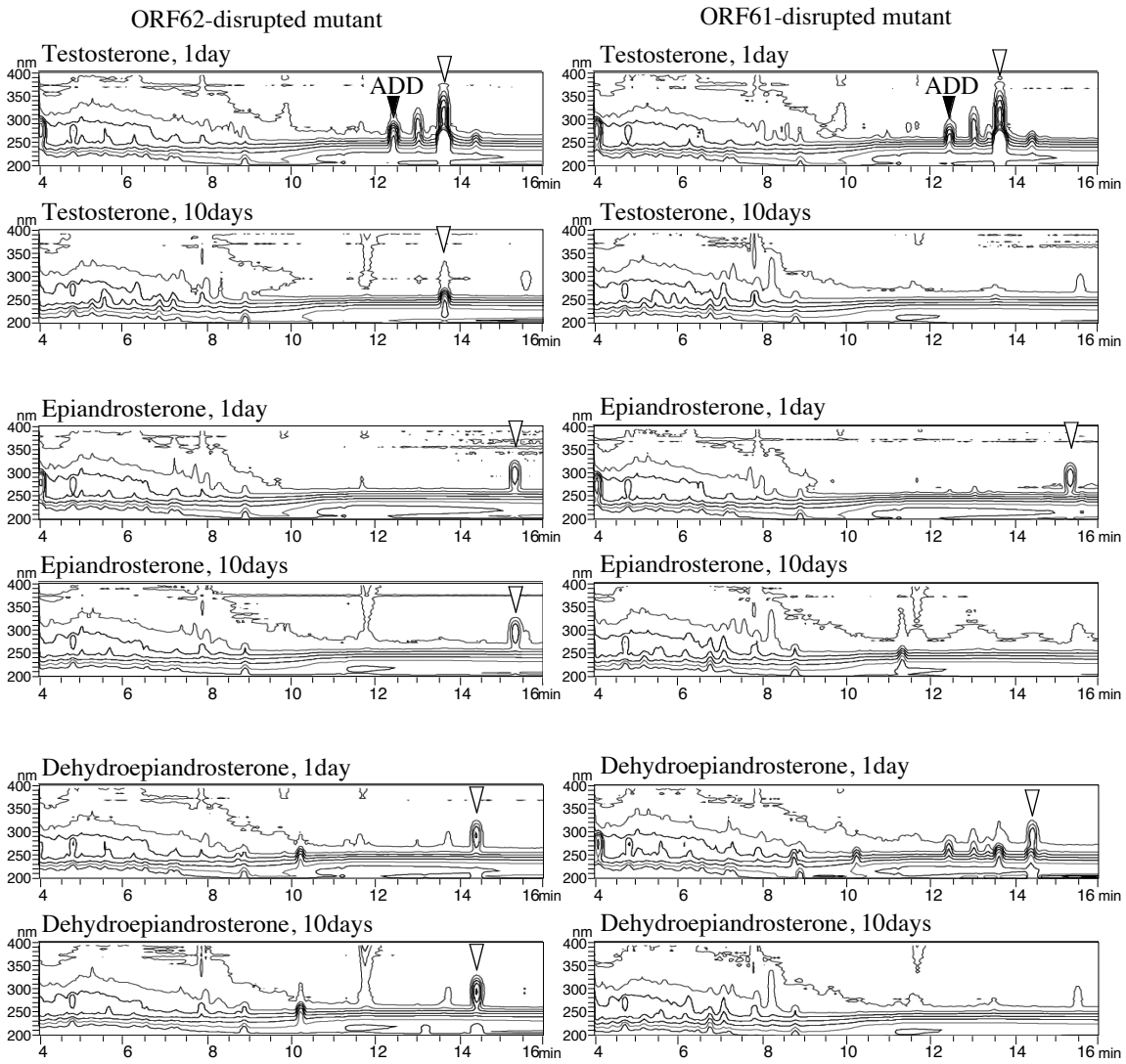

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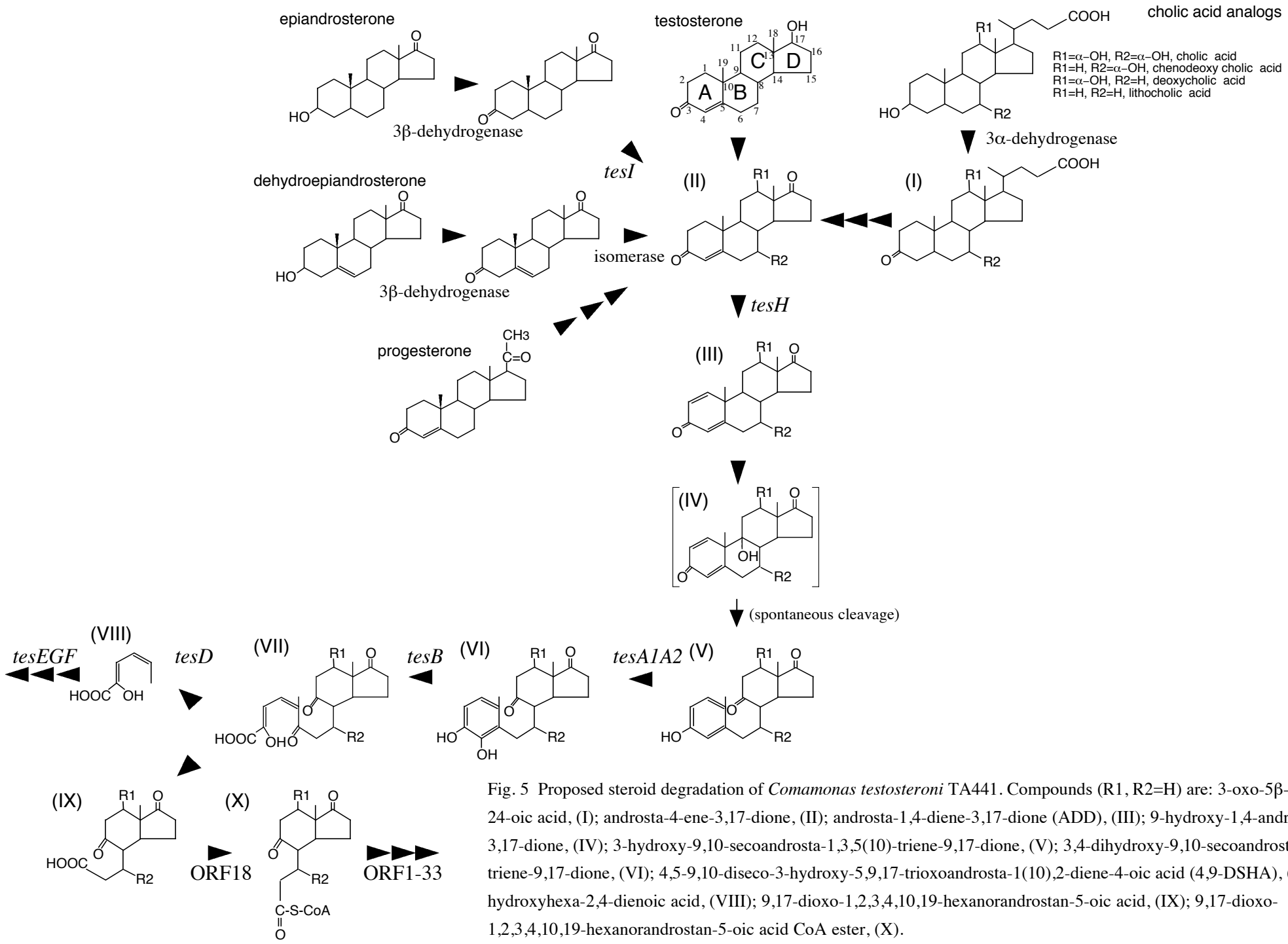

Fig. 5 Proposed steroid degradation of *Comamonas testosteroni* TA441. Compounds (R1, R2=H) are: 3-oxo-5β-cholan-24-oic acid, (I); androsta-4-ene-3,17-dione, (II); androsta-1,4-diene-3,17-dione (ADD), (III); 9-hydroxy-1,4-androstadien 3,17-dione, (IV); 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, (V); 3,4-dihydroxy-9,10-secoandrosta-1,3,5(triene-9,17-dione, (VI); 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA), (VII); 2-hydroxyhexa-2,4-dienoic acid, (VIII); 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid, (IX); 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid CoA ester, (X).

Table 1. Steroid degradation genes identified in *Comamomas testosteroni* TA441, ATCC11996, and ATCC17410.

	Gene / ORF (accession number) [reference]			Protein / (putative protein)
strain	TA441	ATCC11996	ATCC17410	
	ORF18	-	-	CoA-transferase at C-5 of cleaved B-ring
	ORF17	-	-	reductase component of C-9 hydroxylase
	tesI	-	(PRK07121) [35]	3-ketosteroid Δ4-dehydrogenase
	tesH	-	(PRK12835) [36]	3-ketosteroid Δ1-dehydrogenase
	tesA2	-	-	hydroxylase at C-4 of aromatized A-ring
	tesA1	-	-	hydroxylase at C-4 of aromatized A-ring
	tesE	-	-	2-hydroxyhexa-2,4-dienoic acid hydratase
	tesF	-	-	propionaldehyde dehydrogenase
	tesG	-	-	4-hydroxy-2-oxo-hexanoate aldolase
	steA	-	-	dehydrogenase at C-12 from α-OH to ketone
	steB	-	-	hydrogenase at C-12 from ketone to β-OH
	ORF7	-	-	(hypothetical protein)
	ORF6	-	-	(NADH:flavin oxidoreductase)
	tesB	(AAM77244) [37]	-	*meta*-cleavage enzyme for aromatized A-ring
	ORF1	-	-	(CoA-transferase alpha subunit)
	ORF2	-	-	(CoA-transferase beta subunit)
	ORF3	-	-	(enoyl-CoA hydratase/isomerase family)
	ORF4	-	-	(enoyl-ACP reductase)
	ORF5	-	-	(enoyl-CoA hydratase/isomerase family)
	ORF21	-	-	(acyl-CoA dehydrogenase)
	ORF22	-	-	(acyl-CoA dehydrogenase)
	ORF23	-	-	(thiolase)
	ORF25	-	-	(6-aminohexanoate-cyclic-dimer hydrolase)
	ORF26	-	-	(6-aminohexanoate-cyclic-dimer hydrolase)
	ORF27	-	-	(short-chain dehydrogenase/reductase)
	ORF28	-	-	(acyl-CoA dehydrogenase)
	ORF30	-	-	(acyl-CoA dehydrogenase)
	ORF31	(ACI39936) **	-	(short-chain dehydrogenase/reductase)
	ORF32	(AAV40816) **	-	(MaoC domain protein)
	ORF33	(AAV40815) **	-	(acetyl-CoA acetyltransferase)
	tesR	*teiR* [38]	-	LuxR-type transcription factor
	*	*stdC* [41]	-	steroid binding protein
	ORF61*	ORF [41]	-	unknown
	ORF62*	3*β*,17*β*-*hsd* [41-43]	-	steroid 3β,17β-dehydrogenase
	3*α*-*hsd* *	3*α*-*hsd* [44, 45] *hsdA* [46]	-	3-hydroxysteroid 3α-dehydrogenase
	ksi *	*ksi* [21, 47]	-	3-ketosteroid Δ4(5)-isomerase
	*	*repA* [48]	-	repressor of 3-hydroxysteroid 3α-dehydrogenase
	ORF64*	*repB* [48]	-	repressor of 3-hydroxysteroid 3α-dehydrogenase

Broken lines: the genes/ORFs above and below are in separated DNA region.
*: the gene/ORF isolated in this study. **: available only on online database.

Table 2. Growth (measured as CFU/ml) of *C. testosteroni* TA441, the ORF61-disrupted mutant, and the ORF62 (putative 3β,17β-dehydrogenase)-disrupted mutant incubated for 24 h with 0.1 % (w/v) testosterone or epiandrosterone as the sole carbon source.

	TA441 (standard error)	ORF62$^-$ (standard error)	ORF61$^-$ (standard error)
(0 hr)	3.27×10^7 (3.43×10^6)	1.75×10^7 (3.19×10^6)	1.69×10^7 (5.72×10^6)
testosterone	1.74×10^9 (4.82×10^7)	1.21×10^9 (1.76×10^8)	1.85×10^9 (1.23×10^8)
epiandrosterone	7.52×10^8 (1.99×10^8)	2.41×10^7 (4.80×10^6)	6.6×10^8 (6.00×10^7)