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The genes encoding the hydroxylase of 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione in steroid degradation in *Comamonas testosteroni* TA441

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Abstract

Steroid degradation genes of *Comamonas testosteroni* TA441 are encoded in at least two gene clusters: one containing the *meta*-cleavage enzyme gene *tesB*; and another consisting of ORF18, 17, *tesI*, *H*, ORF11, 12, and *tesDEFG*. TesH and I are, respectively, the Δ^1- and $\Delta^4(5\alpha)$-dehydrogenase of the 3-ketosteroid, TesD is the hydrolase for the product of *meta*-cleavage reaction, and TesEFG degrade one of the product of TesD. In this report, we describe the identification of the function of ORF11 (*tesA2*) and 12 (*tesA1*). The TesA1- and TesA2-disrupted mutant accumulated two characteristic intermediate compounds, which were identified as 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA) and its hydroxylated derivative, 3,17-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione by MS and NMR analysis. A complementation experiment using a broad-host range plasmid showed that both TesA1 and A2 are necessary for hydroxylation of 3-HSA to 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA).

Keywords: *Comamonas testosteroni*; Steroid; Testosterone; *Meta*-cleavage; Hydroxylase

1. Introduction

Comamonas testosteroni (formerly *Pseudomonas testosteroni*) is known for its ability to utilize testosterone and various steroids as sole carbon and energy sources. The mechanism by which testosterone is degraded in *C. testosteroni* was eagerly studied and the main intermediate compounds in the degradation pathway were determined in 1960s [1–9]. The main pathway of testosterone degradation in *C. testosteroni* was predicted by these results, even though most of the genes involved in the degradation have not been determined, the exception being for genes for initial 17β-dehydrogenation [10–12] and subsequent Δ^1-dehydrogenation [13], which were reported in 1990s. *C. testosteroni* strain TA441 utilizes certain steroids as well as a number of aromatic compounds. For the purpose of isolating the steroid degradation genes in TA441, we simultaneously identified genes and intermediate compounds that are accumulated by the gene-disrupted mutants and revealed the testosterone degradation pathway in TA441 as presented in Fig. 1 [14–16]. The process is initiated by dehydrogenation of the 17β-hydroxyl group on testosterone to 4-androstene-3,17-dione (4-AD) (reaction (1) in Fig. 1), which then undergoes Δ^1-dehydrogenation to 1,4-androstadiene-3,17-dione (ADD) by TesH (reaction (2)) [16], followed by 9α-hydroxylation to produce 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA) (reaction (3) and the following spontaneous cleavage). ORF17, which is located in the region downstream of *tesH*, is probably the reductase component of 9α-hydroxylase [16]. C-4 of 3-HSA is hydroxylated to yield 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA) (reaction (4)), which is cleaved between C-4 and C-5 via a *meta*-cleavage reaction by

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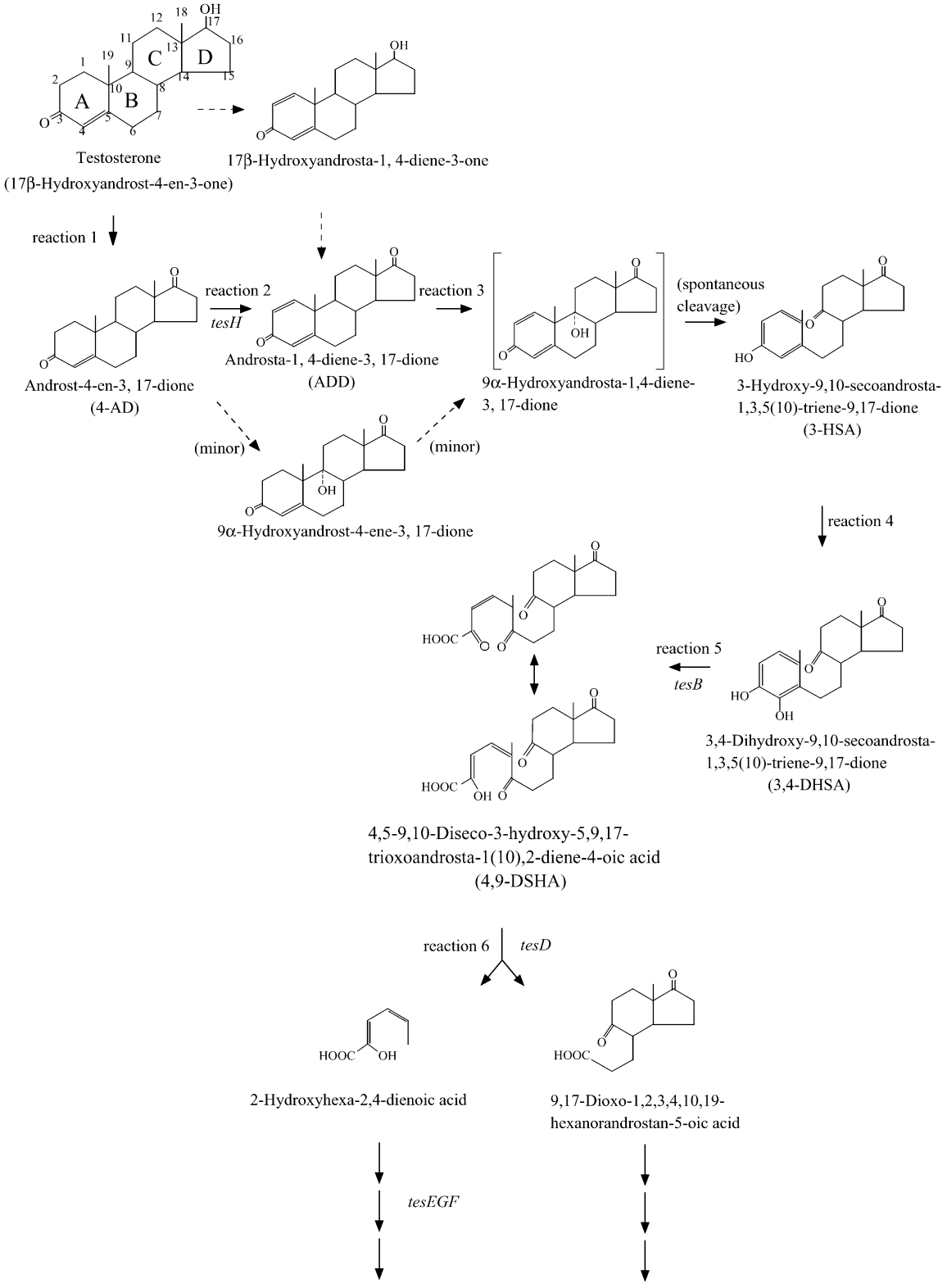

Fig. 1. Proposed testosterone degradation pathway in *C. testosteroni* TA441. Closed arrows indicate the main pathway and broken lines indicate minor reactions.

TesB (reaction (5)) [14]. The product of the *meta*-cleavage reaction of 3,4-DHSA, 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandorosta-1(10),2-dien-4-oic acid (4,9-DSHA), is degraded into 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid and 2-hydroxyhexa-2,4-dienoic acid by TesD (reaction (6)) [15]. One of the products, probably 2-hydroxyhexa-2,4-dienoic acid, is degraded by TesEFG [15]. TesB and TesDEFG show homology to corresponding enzymes in bacterial aromatic compound degradation, such as BphC and BphDHIJ, respectively. Identities are about 40%, between TesB and BphC, and between TesD and BphD, and about 60, 80, 80% between TesEFG and BphHIJ, respectively. Although they showed significant homology to the corresponding aromatic compound degradation enzymes, northern hybridization analysis showed that these enzymes were induced in steroid-grown TA441 cells (testosterone and cholic acid) but not in aromatic compound-grown TA441 cells (phenol, biphenyl, and 3-(3-hydroxyphenyl) propionic acid) [16]. Steroid degradation genes of TA441 are encoded in at least two gene clusters: one containing the *meta*-cleavage enzyme gene *tesB* and the following ORF1, 2, and 3, all of which are necessary in steroid degradation; and another consisting of ORF18, 17, *tesI*, *H*, ORF11, 12, and *tesDEFG* (Fig. 2). TesI, is the 3-ketosteroid $\Delta^4(5\alpha)$-dehydrogenase, which is not necessary for degradation of testosterone ($\Delta^4(5\alpha)$-bond is double), but is indispensable for degradation of androsterone ($\Delta^4(5\alpha)$-bond is single) [16]. Putative terminators are found just downstream of ORF18 and *tesG*, and the genes in the downstream region of these terminators are not necessary for steroid degradation. In the present study, we describe the function of ORF11 and ORF12.

2. Materials and methods

2.1. Culture conditions

C. testosteroni TA441 and the mutant strains were grown at 30 °C in LB medium, C medium, or mixed LB + C medium (mixture of equal volume of LB and C media) [14,15]. Testosterone was added as a filtered DMSO solution at a final concentration of 0.1% (w/v).

2.2. Construction of plasmids and mutant strains

Bacterial strains and plasmids used in this study are listed in Table 1. ORF11, ORF12, and *tesD* were disrupted by deletion and insertion of a Km-resistance gene into the two *Nru*I sites of p12-26HB1 (Fig. 2 and Table 1). About 1.8 kb gene region between the two *Nru*I sites was deleted in the resultant plasmid, p1112D-Km^r. Plasmid p1112D-Km^r was used for inactivation of the ORF11, ORF12, and *tesD* gene in

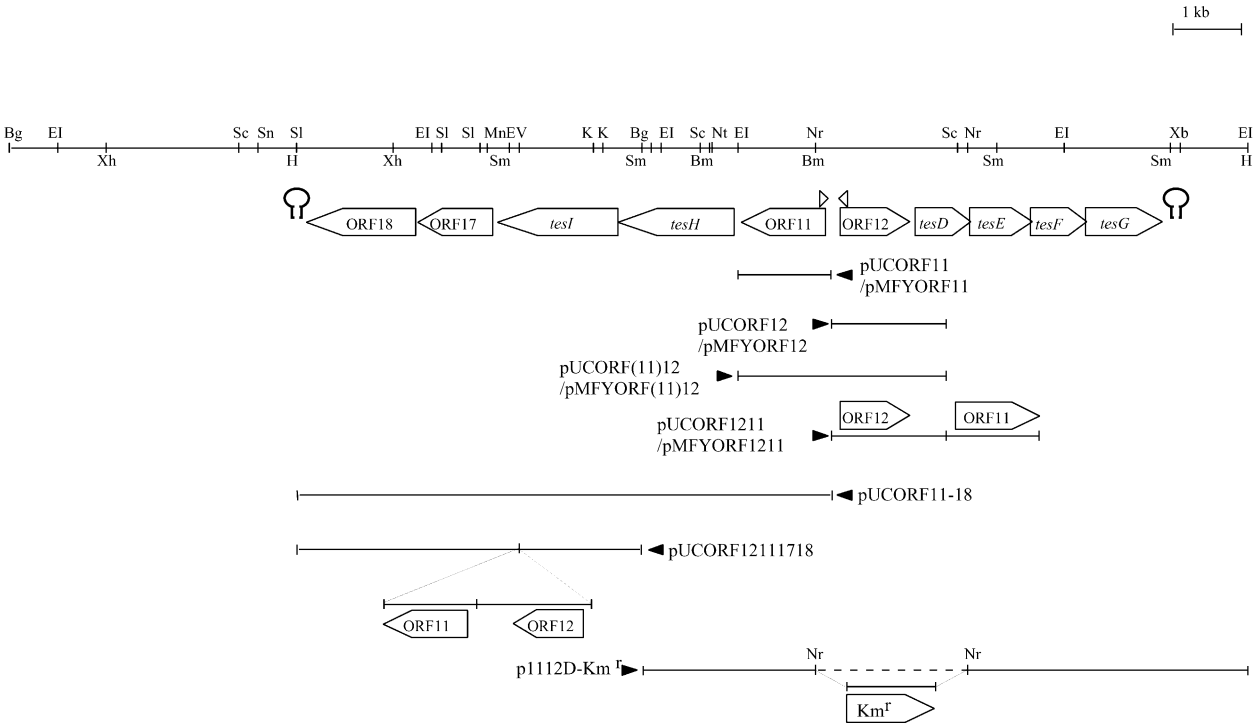

Fig. 2. Partial restriction map of a steroid degradation gene cluster of *C. testosteroni* TA441. The genes and putative ORFs are indicated by large open arrows. Open arrowheads below these ORFs indicate putative promoters, and the putative terminators locate just downstream of ORF18 and *tesG*. Deletion plasmids are indicated below the restriction map; the remaining gene segments (lines) are also shown. The closed arrowheads indicate the direction of transcription regulated by the *lac* promoter of the pUC19 vector. Abbreviations are as follows: Bb, *Bgl*II; Bm, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; Hc, *Hin*cII; Hd, *Hin*dIII; Mn, *Mun*I; Ms, *Mst*I; Nr, *Nru*I; Nt, *Not*I; Ps, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; Sn, *Sna*BI; Sp, *Sph*I; Xb, *Xba*I; Xh, *Xho*I.

Table 1
Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Bacterial strains		
C. testosteroni TA441	Wild-type, Tes^{+*}	[27]
$ORF11^{-}$	ORF11::Km^r mutant of TA441	[15]
$ORF12^{-}$	ORF12::Km^r mutant of TA441	[15]
$ORF1112D^{-}$	ORF11, ORF12, and *tesD*::Km^r mutant of TA441	This work
Plasmids		
pUC19	Ap^r, *lac*Z	[28]
pUC118	Ap^r, *lac*Z	[28]
pSuperCosI	Ap^r, Cosmid vector	[29]
pMFY42	Tc^r, Km^r, RSF1010-based broad-host range plasmid	[21]
pC26	Ap^r, pSuperCosI with *Bam*HI insert of TA441	[15]
p12-26HB1	Ap^r, pUC19 with 8.8 kb *Hin*dIII-*Bgl*II insert of pC26	This work
pUCORF11	Ap^r, pUC19 with 1.2 kb *Stu*I-*Eco*RI insert of pC26	This work
pUCORF12	Ap^r, pUC19 with 1.4 kb *Sph*I-*Stu*I insert of pC26	This work
pUCORF(11)12	Ap^r, pUC19 with 2.7 kb *Sph*I-*Eco*RI insert of pC26	This work
pUCORF1211	Ap^r, pUCORF(11)12 based plasmid (1.4 kb *Nae*I-*Stu*I fragment of the insert of pUCORF(11)12 is inverted)	This work
pUCORF1718	Ap^r, pUC118 with 5.2 kb *Bgl*II-*Hin*dIII insert of pC26	This work
pUCORF12111718	Ap^r, pUCORF1718 with 2.7 kb *Sph*I-*Eco*RI insert of pUCORF1211	This work
pUCORF11-18	Ap^r, pUC19 with 8.8kb *Avr*II-*Hin*dIII insert of pC26	This work
pMFYORF11	Ap^r, pMFY42 with the same insert as pUCORF11	This work
pMFYORF12	Ap^r, pMFY42 with the same insert as pUCORF12	This work
pMFYORF (11)12	Ap^r, pMFY42 with the same insert as pUCORF(11)12	This work
pMFYORF1211	Ap^r, pMFY42 with the same insert as pUCORF1211	This work

Tes^{+*}: grow on testosterone as the sole carbon source, Ap: ampicillin, Km: kanamycin, Tc: tetracycline.

TA441 by homologous recombination. The plasmid was introduced into TA441 by electroporation, and a Km-resistant and carbenicillin-sensitive TA441 mutant was selected. Insertion of the Km-resistance gene was confirmed by southern hybridization using the Km-resistance gene and ORF12 as probes. The ORF11-disrupted mutant and ORF12-disrupted mutant were constructed in the same manner in the previous study [15].

2.3. Three-dimensional HPLC

Twice the volume of methanol was added to the culture, which was then centrifuged and the supernatant was directly injected into the HPLC. A Waters 2690 HPLC (Nihon Waters, Tokyo, Japan) was utilized with an Inertsil ODS-3 column (2.1 mm × 150 mm), and elution carried out using a linear gradient from 20% solution A (CH_3CN:CH_3OH: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, then maintained for 3 min, and then changed to 20% solution A for 5 min. The flow rate was 0.21 mL/min.

2.4. Isolation of the intermediate products X1 and X2 accumulated by ORF12-disrupted mutant

For the isolation of the intermediate products accumulated by ORF12-disrupted mutant (compounds X1 and X2), a 300 ml culture of ORF12-disrupted mutant incubated in mixed LB + C medium with 0.1% testosterone was twice extracted with the same volume of ethyl acetate. The ethyl acetate layer was concentrated in vacuo, and the residue was dissolved in a small amount of methanol and loaded onto the Waters 650 HPLC (Nihon Waters, Tokyo, Japan) with an Inertsil ODS-3 column (20 mm × 250 mm, GL Science, Tokyo, Japan) and a solvent with the composition CH_3CN:MeOH:H_2O:TFA of 50:40:10:0.05, flow rate 8 mL/min, at room temperature. X1 and X2 were detected at 210 nm. The fractions containing X1 and X2 were collected and dried. X1 and X2 were dissolved in a small amount of methanol and purified by HPLC by elution with the composition CH_3CN:H_2O of 1:1 at 40 °C. Other conditions are the same as described above.

2.5. General experimental procedures

For gas chromatography–mass spectrometry (GC–MS), a GCMS-QP5050A (Shimadzu Corporation, Kyoto, Japan) fitted with a capillary column HP-5MS (0.25 mm internal diameter by 30 m, 0.25 μm film thickness, Agilent Technologies, CA, USA) was used. UV spectra were recorded with an Ultrospec 3300 (Amersham Biosciences Corp., NJ, USA). NMR spectra (^1D, ^1H and ^{13}C, distortionless enhancement by polarization transfer [DEPT], pulsed field gradients-correlated spectroscopy [PFG-DQFCOSY], pulsed field gradients-heteronuclear multiple quantum coherence [PFG-HMQC], pulsed field gradients-heteronuclear multiple bond coherence [PFG-HMBC]) were taken on a JNM-

ECP500 spectrometer (JEOL, Tokyo, Japan) in $CDCl_3$ solution with tetramethylsilane (TMS) at 0 ppm as the internal standard for ^1H and ^{13}C chemical shifts.

3. Results

3.1. HPLC analysis of the culture media of ORF11-disrupted mutant and ORF12-disrupted mutant incubated with testosterone

C. testosteroni TA441 utilizes certain steroids, such as testosterone, using enzymes that are induced when TA441 is incubated with steroids. In previous studies, we identified two steroid degradation gene clusters in TA441. One contains *meta*-cleavage enzyme gene *tesB* and three following ORFs [14], all of which were necessary for steroid degradation in TA441, and the other consists of ORF18, 17, *tesI*, *H*, ORF11, 12, and *tesDEFG* (Fig. 2) [15,16]. TesD degrades 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid (4,9-DSHA) into 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid and 2-hydroxyhexa-2,4-dienoic acid. The latter compound is probably degraded by TesEFG [15]. TesH and I are, respectively, the Δ^1- and $\Delta^4(5\alpha)$-dehydrogenase of the 3-ketosteroid. The putative amino acid sequences of ORF11 and 12, encoded in the center of this gene cluster, showed homology to some oxygenases, such as the phenol hydroxylase of *Bacillus stearothermophilus* BR219 [17] (ORF11) and component B of nitrilotriacetate monooxygenase of *Chelatobacter* strain ATCC 29600 [18] (ORF12), though the identity was about 30% at maximum. The ORF11-disrupted mutant and ORF12-disrupted mutant showed limited growth on testosterone, indicating that both are necessary for steroid degradation in TA441 [15]. To analyze the function of these ORFs, the mutants were incubated in the mixed LB and C medium with testosterone for 2 days. The mixed LB and C medium is a mixture of equal volumes of both media, and gene-disrupted mutants cultured in the mixed medium accumulate a larger amount of intermediate compounds than in normal C medium. Fig. 3 shows the results of three-dimensional HPLC analysis of the culture of both mutants. The ORF12-disrupted mutant converted most of the testosterone into the intermediate compound X1, showing a characteristic UV-spectrum, and a smaller amount of compound X2, which showed a similar UV-spectrum to that of X1. The ORF11-disrupted mutant accumulated a smaller amount of X1 and X2 than the ORF12-disrupted mutant together with androst-4-en-3,17-dione (4-AD), androsta-1,4-diene-3,17-dione (ADD), and 17-hydroxy-1,4-androstadiene-3-one, which are intermediates in the early steps in testosterone degradation (Fig. 1). This is probably because the disruption of ORF11 affected the expression of genes in the region downstream of ORF11 (*tesH*, *tesI*, ORF17, and ORF18: TesH is the Δ^1-dehydrogenase of 4-AD to produce ADD and ORF17 is probably the reductase component of 9α-hydroxylase of ADD). The estimated

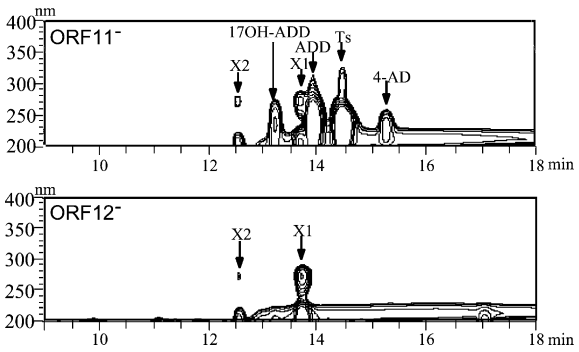

Fig. 3. Three-dimensional HPLC analysis of the culture of the ORF11-disrupted mutant and the ORF12-disrupted mutant incubated with testosterone. The vertical axis indicates wavelength, the horizontal axis indicates the RT, and the UV absorbance of each compound is represented in contour. For the analysis, a Waters 2690 HPLC was used with an Inertsil ODS-3 column (2.1 mm × 150 mm; GL Science, Tokyo, Japan), and elution was carried out using a linear gradient from 20% solution A (CH_3CN:CH_3OH: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, maintained for 3 min, and then changed to 20% solution A for 5 min. The flow rate was 0.21 ml/min. Abbreviations are as follows: Ts, testosterone; 4-AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; and 17OH-ADD, 17β-hydroxy-1,4-androstadiene-3-one.

amount of X1 in the culture of the ORF12-disrupted mutant presented in Fig. 3 was around 90% of the predicted amount, and that of X2 was only a few percent. In the case of ORF11-disrupted mutant, the rates were about 15% and a few percent. Usually, ORF12-disrupted mutant accumulates around 70–90% of X1 and ORF11-disrupted mutant accumulates around 20%.

3.2. Isolation and purification of the intermediate compounds X1 and X2

The ORF12-disrupted mutant was incubated with 0.1% testosterone at 30 °C for about 2 days, and the resultant culture was centrifuged to remove cells and other precipitation. The supernatant of the culture was extracted twice with the same volume of ethyl acetate, and the ethyl acetate fraction was dried and concentrated in vacuo. The remnant was dissolved in a small amount of methanol and separated by HPLC (Waters 600 with ODS-3 column) eluted with acetonitrile:methanol:water:TFA (50:10:40:0.05). The fractions containing X1 and X2 were collected from the eluent. Since these fractions were not sufficiently pure, the fractions were concentrated, dissolved in a small amount of methanol, and separated by HPLC again with acetonitrile:water (1:1) as the mobile phase. The purity of the isolated X1 and X2 was confirmed by HPLC and analyzed further.

3.3. Characterization of compounds X1 and X2

Maximum UV absorbance of X1 and X2 are 280.6 (2325) and 280.0 (1918). The molecular formula and weight of X1 are $C_{19}H_{24}O_3$ and 300, and of X2 are $C_{19}H_{26}O_3$ and 302. The

Table 2
NMR data for X1 and X2[a,b]

Number	X1		X2	
	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)
1	131.2	6.99 d (8.2)	131.1	6.98 d (8.2)
2	112.8	6.60 dd (8.2, 2.7)	112.6	6.58 dd (8.2, 2.8)
3	153.8		153.6	
4	115.7	6.67 d (2.7)	115.7	6.65 d (2.8)
5	141.8		142.3	
6	31.0	2.72 ddd (13.3, 11.5, 4.6)	31.0	2.67 ddd (13.3, 11.9, 5.0)
		2.46 ddd (13.3, 11.9, 5.5)		2.43 m
7	26.7	1.88 m, 1.69 m	27.2	1.76 m, 1.57 m
8	49.7	2.52 m	50.4	2.41 m
9	211.3		212.4	
10	127.9		128.1	
11	37.4	2.55–2.45 m	37.9	2.52 ddd (15.6, 14.2, 7.3), 2.38 m
12	30.4	2.02 m, 1.70 m	35.5	2.02 ddd (12.8, 6.4, 1.8), 1.53 m
13	47.6		43.3	
14	49.2	1.89 m	49.7	1.62 m
15	22.5	2.06 m	24.1	1.72 m
		1.73 m		1.50 m
16	36.1	2.58 br dd (19.0, 7.8), 2.26 ddd (19.0, 9.6, 9.6)	31.1	2.19 m, 1.64 m
17	218.8		80.5	3.77 dd (8.7, 8.7)
18	13.5	1.17 s	10.7	1.08 s
19	18.4	2.26 s	18.3	2.25 s

Abbreviations for NMR signals are as follows: s, singlet; d, doublet; m, multiplet; br, broad.
[a] The molecular formula and weight of X1 and X2 are $C_{19}H_{24}O_3$ and 300, and $C_{19}H_{26}O_3$ and 302, respectively.
[b] Maximum UV absorbance of X1 and X2 are 280.6 (2325) and 280.0 (1918).

UV-spectra and MS data of X1 and X2 corresponded to those of 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA) and 3,17-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9-one (3,17-DHSA) presented by Dodson et al. in 1958 and 1961 [1–3]. Table 2 shows the ^{1}H NMR, ^{13}C NMR, and 2D-NMR (FGDQCOSY, FGHMQC, HMBC, and DEPT) data of compounds X1 and X2. NMR data of X1 agreed, on the whole, with the partial ^{13}C NMR data of 3-HSA presented by Furukawa et al. in 1979 [19], and with the partial ^{13}C NMR data of Hamato et al. in 1988 [20], the exceptions being for C1 (131.2 ppm in our data and 127.3 ppm in theirs), C10 (127.9 ppm and 130.9 ppm, respectively), C8 (49.7 ppm and 49.0 ppm, respectively), and C14 (49.2 ppm and 49.6 ppm, respectively). From these results, X1 and X2 were identified as 3-HSA and 3,17-DHSA, respectively (Fig. 4). Our data presented in Table 2 is the first complete ^{13}C and ^{1}H NMR data of 3-HSA, together with complete NMR data of 3,17-DHSA. NMR data of 3,17-DHSA has not been reported before.

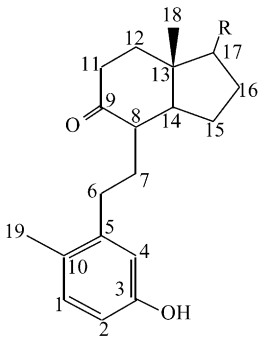

Fig. 4. Structure of compounds X1 and X2. R—O: 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione and R—OH: 3,17-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9-one.

3.4. Conversion of 3-HSA by ORF11 and ORF12

As the ORF11- and ORF12-disrupted mutants accumulated 3-HSA and both of the ORFs showed about 30% identity to some oxygenases, ORF11 and 12 were expected to encode the hydroxylase for converting 3-HSA to 3,4-dihydroxy-9, 10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA). We constructed pUC19-derivative plasmids encoding ORF11 (pUCORF11 in Fig. 2) and ORF12 (pUCORF12) and introduced the plasmids into *E. coli*. The *E. coli* cells harboring each of the plasmids were incubated with induction by IPTG, and cell free extract was prepared as described in our previous study of TesD [15]. To prepare 3-HSA, the ORF12-disrupted mutant was incubated with testosterone until all the added testosterone was converted to 3-HSA and 3,17-DHSA. The cells were removed by centrifugation and the supernatant was used as reaction solution. The reaction solution was treated with *E. coli* harboring each of the plasmids encoding ORF11 and ORF12, and the cell free extract. As 3,4-DHSA was not detected by HPLC, the remainder of the 3-HSA was analyzed by HPLC at suitable intervals. However, neither the *E. coli* cells nor their cell free extracts showed conversion of 3-HSA (data not shown). Then we constructed pUC19-derivative plasmids encoding ORF11 and 12 in the opposite direction (pUCORF(11)12), ORF12 and 11 in the same direction (pUCORF1211), ORF11–18 (pUCORF11–18), and

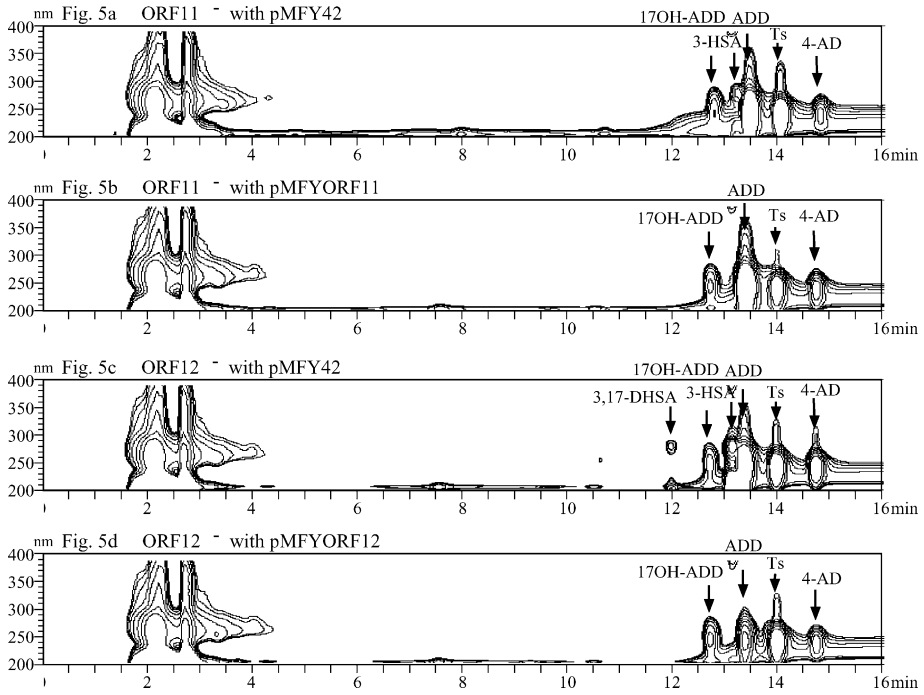

Fig. 5. HPLC charts of the culture of ORF11-disrupted mutant carrying a broad-host range plasmid pMFY42 incubated with testosterone (a), ORF11-disrupted mutant carrying pMFYORF11 (pMFY42 based plasmid expressing ORF11) (b), ORF12-disrupted mutant carrying pMFY42 incubated with testosterone (c), and ORF12-disrupted mutant carrying pMFYORF12 (pMFY42 based plasmid expressing ORF12) (d). The analysis was carried out under conditions identical to those described in the legend of Fig. 3. Abbreviations are as follows: Ts, testosterone; 4-AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; 17OH-ADD, 17β-hydroxy-1,4-androstadiene-3-one; 3-HAS, 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione-1,4-androstadiene-3,17-dione; and 3,17-DHSA, 3,17-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione-1,4-androstadiene-3,17-dione. Identification of these compounds except for 3-HSA and 3,17-DHSA was performed in the previous study [15].

ORF11–12, 17, and 18 (pUCORF12111718) (Fig. 2), and introduced each of them into *E. coli*. The conversion experiment was performed in the same manner as above using *E. coli* cells and the cell free extract with these plasmids, but reduction of 3-HSA was not observed. As this might be due to proteins encoded by ORF11 and ORF12 not having activity when expressed in *E. coli* cells, the gene region encoding each ORF was transferred into the broad-host range plasmid, pMFY42 [21], which can be maintained in *Pseudomonas* and its relatives (pMFYORF11 and pMFYORF12). Plasmids pMFYORF11 and pMFY42 (negative control) were individually introduced into the ORF11-disrupted mutant of TA441, and pMFYORF12 and pMFY42 (negative control) were individually introduced into the ORF12-disrupted mutant. These mutants were incubated with testosterone and the remaining 3-HSA was analyzed by HPLC at suitable intervals. HPLC chart of the culture about 38 h after start of the incubation is presented in Fig. 5. 3-HSA accumulated in the culture of the mutants with pMFY42 (negative control), while 3-HSA was not detected in the culture of the ORF11-disrupted mutant with pMFYORF11 or the ORF12-disrupted mutant with pMFYORF12. This indicates that both ORFs are involved in testosterone degradation in TA441. Then we considered a similar complementation experiment using a mutant gene-disrupted for both ORF11 and 12 to confirm that both ORFs are necessary for conversion of 3-HSA. ORF activity may be determined by measuring the amount of unreacted 3-HSA, but detection of the produced 3,4-DHSA would be a more direct means of determining ORF activity. The method for confirming presence of 3,4-DSHA in culture is described in the next section.

3.5. Detection of 3,4-DSHA

The complementation experiment described in the previous section indicated that both ORF11 and ORF12 are involved in the conversion of 3-HSA in testosterone degradation by TA441. The product of the hydroxylation of 3-HSA is thought to be 3,4-DHSA, but we were not successful in detecting it in our previous study. 3,4-DHSA was reported to be one of the intermediate compounds in steroid degradation by *Nocardia restrictis* [6], which degrades steroids by a degradation pathway as almost identical to that of *C. testosteroni* [1–9]. In testosterone degradation in TA441, a *meta*-cleavage enzyme TesB, encoded in another steroid degradation cluster of TA441, converts 3,4-DHSA to 4,9-DSHA [14]. Fig. 6a shows the HPLC chart of the culture of a TesB-disrupted mutant incubated with testosterone in the mixed LB + C media. This mutant is assumed to accumulate 3,4-DHSA; however, only 4-AD and ADD, the first and the second intermediates in the testosterone degradation of TA441, were detected as intermediate compounds. As we could not detect 3,4-DHSA

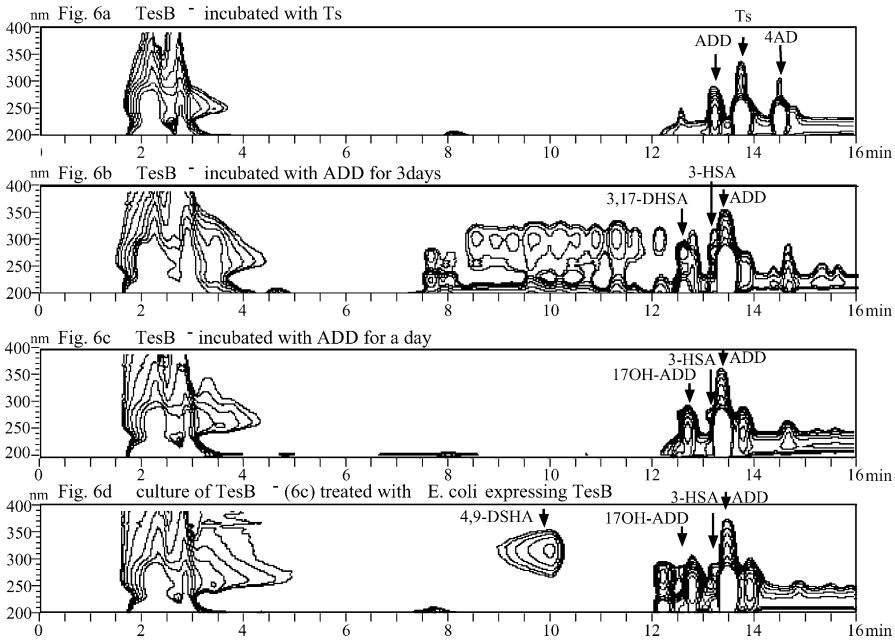

Fig. 6. HPLC charts of the culture of TesB-disrupted mutant incubated with testosterone (a), with ADD for three days (b), for one day (c), and the culture of (c) treated with the cell free extract of *E. coli* expressing TesB (d). The analysis was carried out under conditions identical to those described in the legend of Figs. 3 and 4, 9-DSHA: 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid. Other abbreviations are the same as those described in the legend of Fig. 5.

in the culture of the TesB-disrupted mutant incubated with testosterone, we incubated the TesB-disrupted mutant with several steroids, with the expectation of detecting the substrate of the *meta*-cleavage reaction. To our surprise, cultures of TesB-disrupted mutant incubated with cholic acid and those incubated with ADD became dark red within a few days, while the same color change was not observed following incubation with testosterone. A dark red color is often observed when catechol derivatives accumulate during bacterial degradation of aromatic compounds. Catechol is a compound comprising a benzene ring with two adjacent hydroxyl moieties and automatically oxidizes to produce quinone derivatives, which gives dark reddish color. As ADD is an intermediate compound in testosterone degradation by TA441, the accumulated compound should be 3,4-DHSA. HPLC analysis of this dark red culture showed a number of peaks between RT 8 and 12, which were not detected in the culture of any other gene-disrupted mutant incubated with testosterone (Fig. 6b) [15,16]. These compounds would be produced by automatic oxidation of 3,4-DHSA and would color the culture medium dark red. The isolation and characterization of these compounds was difficult, and therefore we confirmed the accumulation of 3,4-DHSA by conversion of 3,4-DHSA to 4,9-DSHA by TesB, followed by detection of 4,9-DSHA by HPLC. As a preliminary experiment showed that accumulation of red compounds prevented the TesB reaction from proceeding, we interrupted the incubation of the TesB-disrupted mutant with ADD before the culture showed pigmentation. Following incubation for about 1 day, fewer peaks were detected between RT 8 and 12 than in the 3-day culture, and a small amount of 3-HSA was detected (Fig. 6c). The cells of the TesB-disrupted mutant were separated from the culture by centrifugation, and the supernatant was transferred to a new test tube. About 2 h after the addition of the *E. coli*$^{-}$ cells expressing TesB, the reaction solution was yellow (4,9-DSHA is brilliant yellow under neutral and basic conditions [15]). HPLC analysis of this yellow solution showed the accumulation of 4,9-DSHA (Fig. 6d), indicating that we could detect 3,4-DHSA by conversion to 4,9-DSHA. When we treated the culture of TesB-disrupted mutant incubated with testosterone in the same way with *E. coli*$^{-}$ cells expressing TesB, smaller amount of 4,9-DSHA was detected.

3.6. Conversion of 3-HSA by ORF11 and ORF12 in ORF11, 12, and tesD-disrupted mutant

For the complementation experiment for both ORF11 and 12, we constructed the gene-disrupted mutant 1112D^{-} (an ORF11, ORF12, and *tesD*-disrupted mutant). As TesB is encoded in a different gene cluster from that of ORF11 and 12, when 3,4-DHSA is produced in this mutant, it is converted to 4,9-DSHA by TesB, and the resultant 4,9-DSHA accumulates because TesD, the hydrolase for 4,9-DSHA, is disrupted (Fig. 7). We constructed pMFY42-based broad-host range plasmids encoding ORF11 and 12 in the opposite direction (pMFYORF(11)12) and ORF12 and 11 in the same direction (pMFYORF1211). Plasmids pMFYORF11, pMFYORF12, pMFYORF(11)12, pMFYORF1211, and pMFY42 were individually introduced into the gene-disrupted mutant 1112D^{-}.

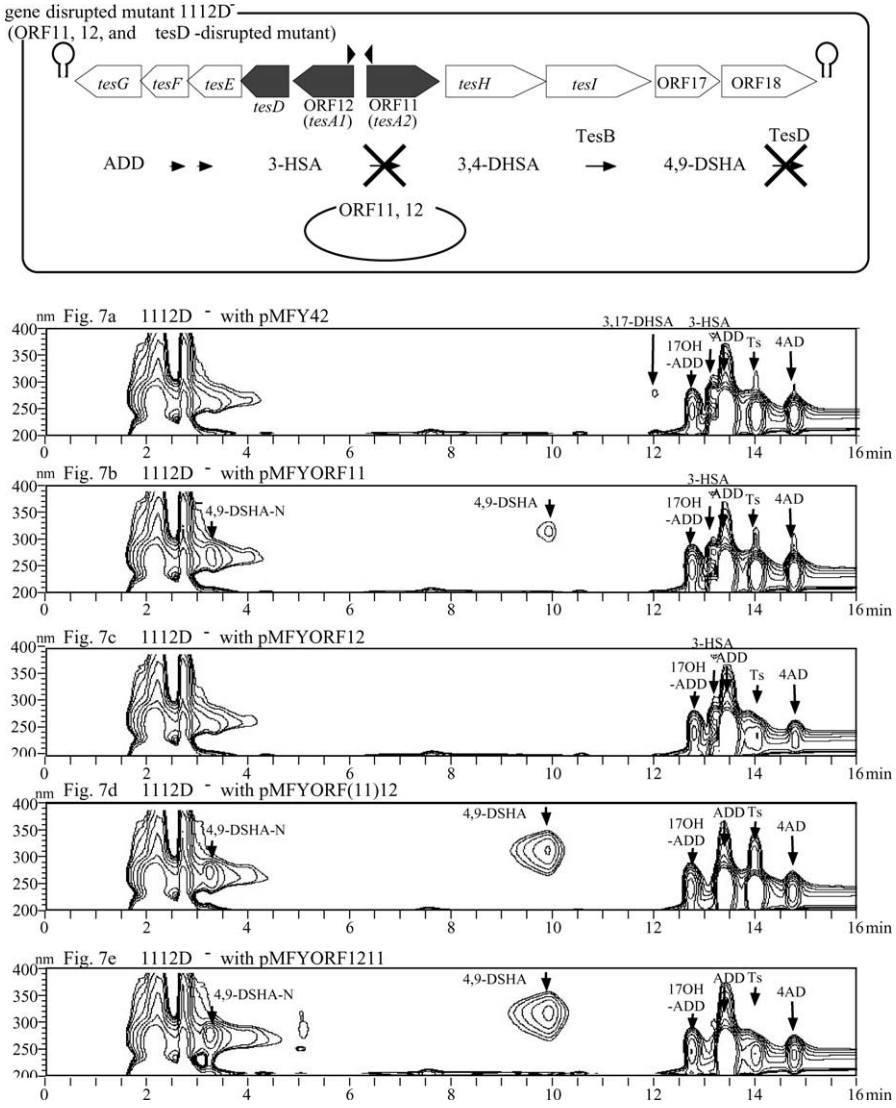

Fig. 7. Complementation experiment of ORF11 and ORF12. HPLC chart of the culture of the 1112D⁻ (ORF11, 12 and TesD-disrupted mutant) carrying pMFY42 incubated with testosterone (a), 1112D⁻ carrying pMFYORF11 (b), 1112D⁻ carrying pMFYORF12 (c) 1112D⁻ carrying pMFYORF(11)12 (d), and 1112D⁻ carrying pMFYORF1211. pMFYORF(11)12 is a broad-host range plasmid encoding ORF11 and ORF12 in the opposite direction and pMFYORF1211 is a broad-host range plasmid encoding ORF12 and ORF11 in the same direction. The analysis was carried out under the same conditions stated in the legend of Fig. 3. 4,9-DSHA-N: 4-aza-9,17-dioxo-9,10-secoandrosta-1,3,5(10)-triene-3-carboxylic acid. 4,9-DSHA-N was produced from 4,9-DSHA non-enzymatically by ammonium ion in the culture media added as a nitrogen source [15]. Other abbreviations are the same as those described in the legend of Figs. 5 and 6.

The mutants were incubated with ADD, and the culture was analyzed by HPLC at suitable intervals. The amount of 4,9-DSHA detected by HPLC peaked at 38 h after the start of the incubation (Fig. 7). In the culture of 1112D⁻ with pMFY42 (negative control), 1112D⁻ with pMFYORF11, and 1112D⁻ with pMFYORF12, 3-HSA accumulated and 4,9-DSHA was only detected in a very small amount in the culture of 1112D⁻ with pMFYORF11 (Fig. 7a–c). In the culture of 1112D⁻ with pMFYORF(11)12 and 1112D⁻ with pMFYORF1211, 4,9-DSHA was detected with little amount of 3-HSA. The amount of 4,9-DSHA was larger in the culture of 1112D⁻ with pMFYORF1211 than in that of 1112D⁻ with pMFYORF(11)12. These results indicate that both ORF11 and 12 are necessary for effective hydroxylation of 3-HSA at the C4-position. We therefore named ORF11 and ORF12 *tesA2* and *tesA1*, respectively.

4. Discussion

In *C. testosteroni* TA441, steroids are degraded via aromatization of the A-ring, then *meta*-cleaved and further degraded by hydrolysis. In our previous study, we reported two steroid degradation gene clusters in TA441; one containing the *meta*-cleavage enzyme gene *tesB*, which converts 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA) to 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid (4,9-DSHA), and the

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TesA1           ----------------MQSTDATFDPKDFRRALGMFGTGVTIVTTRAENGEPVGITANSF  44
U39411          MADQIRS------ATEGGDPTS--DPKGFRRALGTFPTGVTIVTAPGVDG-PAGVTANSF  51
AE008260        ------------------MTVATLDPRALRDAFGAFVTGVTIVTTRDDAGKPVGFTANSF  42
AF380367        ----------------------MEIKKFRNALGCFVTGVTIVTTRDLYGKPVGVTANSF  37
AP005957        MNDLPKQPAVPDPANELASDSSPIDPRDFRSALGTYGTGVTIITAVAPDGRPYGITCNSF  60
BX640449        ---------------MSAAFPPAFDAAFFRTALGRFATGVTVVTTAGPDGQPVGLTVSSF  45
BX640420        ---------------MSAAFPPAFDAAFFRTALGRFATGVTVATTAGPDGQPVGLTVSSF  45
BX640434        ---------------MSAAFPPAFDAAFFRTALGRFATGVTVVTTAGPDGQPVGLTVSSF  45
                                          :   :* *:* : ****: *:    * * *.* .**

TesA1           NSVSLEPPMVLWSLAKNARSLPVFQSADTWNVHILSNEQEALSNRFAR-AGEDKFSGLPL 103
U39411          ASVSLDPPLVLWSIGHTSRSHSKFQQSATFAINILADDQVGVSQVFAG-GSADKFSLVDW 110
AE008260        TSVSLDPPLLLICLARTSRNFATMTGAKNFAVNILSESQKDLSNTFAR-PVEDRFAAAEW 101
AF380367        SSVSLDPPLVLWSLAKASYSFPAFSSCDAFCVHVLAEGQEAIATQFAK-GSNDKFANVNI  96
AP005957        ASVSLNPPLVLWSLGVYSSSLTVFQNASHFAVHVLGASQQALANIFAK-SSDDKFAGVDW 119
BX640449        NSVSLNPPLILWSLARTSSSLAAFERCQRYVVNVLSASQIALARRFATGKTPERFAGLTL 105
BX640420        NSVSLNPPLILWSLARTSSSLAAFERCQRYVVNVLSASQIALARRFATGKTPERFAGLTL 105
BX640434        NSVSLNPPLILWSLARTSSSLAAFERCQRYVVNVLSASQIALARRFATGKTPERFAGLTL 105
                 ****:**::* .:.  : . . :  .  : :::*.  *  ::  **     ::*:

TesA1           DSEAAHAPLLQD-CSARFRCKTAFQYDGGDHIIFVGEVTDYDANPHPPLLYVTGGYALAS 162
U39411          HTGRTGAPLIDN-ALAYFDCVCEARHEGGDHTIMIGRVVDFGRAEGSPLAFSQGRYGVTL 169
AE008260        SDAPQGSPVFAE-VAAWFECTMQEVIEAGDHVILLGRIGAFDNSGLNGLGYARGGYFTPM 160
AF380367        KDGFGSVPVIDN-YMVRFDCSIYNRYDGGDHIIIVGEVIDFVVDNNKNPLAFYKGSFKNP 155
AP005957        TPGLGNAPVLAE-SVANFQCRSVNRYYGGDHVIFLGAVEAYAYNAKDTLLFARGAYGRFV 178
BX640449        AQAPAGTPMLGEGCAAWFECRNRSRYEEGDHIIMVGQVEHCGHSGVPPLVFHAGGFDLTP 165
BX640420        AQAPAGTPMLGEGCAAWFECRNRSRYEEGDHIIMVGQVEHCGHSGVPPLVFHAGGFDLTP 165
BX640434        AQAPAGTPMLGEGCAAWFECRNRSRYEEGDHIIMVGQVEHCGHSGVPPLVFHAGSFDLTP 165
                       *:: :   . * *        *** *::* :

TesA1           RKANAVASEPAADTNTVYSENLIGYLMGRAHFQFLSGLRKPMAAYGMTDADFYVLSLLSI 222
U39411          DHPEAAKARDHKSEEYGLDDLPFLSLIAKAHYKEDADLEEQRSAAGCTPVGSKILAGLYG 229
AE008260        LAAKAVSAAAEG-------EIVVGAVLERRGEIYLVGDDVLSLPGCVVAGGDPVAALTSR 213
AF380367        AYNCVGAILPASDGGVTENNAV---------------------------------------- 177
AP005957        ANDERKKTP----------------------------------------------------- 187
BX640449        PHGGASS------------------------------------------------------- 172
BX640420        PHGGASS------------------------------------------------------- 172
BX640434        PHGGASS------------------------------------------------------- 172

TesA1           QQPQSPAEIASHMAYTGTDIGAVALQSLISKGWVEESRERAESLQLTPLGNEAILHVLAA 282
U39411          SAPLTADELARRMYLDRREVVDSLNEFVADG---HVESCDSGRFALTESGKQRRRRMIEY 286
AE008260        LQDLTGLSVRTGFLYSVYENKADGRQHIVYHALAEGDDAPRQGRFLSPGALGSAKFDSGA 273

TesA1           AKAVETDLIASLGEMEAATLRNLLKKAIAATDPGLPKLWQARA------ 325
U39411          VSRYQDEQLASISRSDLGVLRGCCKLFLRARGAGLAKALALALSSRFEQ 335
AE008260        TADIVNRFALESSIGNFGVYVGNETAGKVHPISMKAVNP---------- 312
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Fig. 8. Alignment of the TesA1 and proteins showing similarity to TesA1: (*) amino acids identical among all the proteins; (:) and (.) amino acids of high and low similarity, respectively. Abbreviations are U39411: nitrilotriacetate monooxygenase component B of *Chelatobacter heintzii* (*Aminobacter aminovorans*) ATCC 29600 [18,23], AE008260: flavoprotein oxidoreductase Atu4239 of *Agrobacterium tumefaciens* strain C58 [24], AF380367: putative NADH:FMN oxidoreductase of *Burkholderia* sp. DBT1 (available only on database under accession number AF380367), AP005957: nitrilotriacetate monooxygenase of *Bradyrhizobium japonicum* USDA110 [25], BX640449: putative monooxygenase component of *Bordetella bronchiseptica* RB50 [26], BX640420: putative monooxygenase component of *Bordetella pertussis* Tohama I [26], and BX640434: putative monooxygenase component of *Bordetella parapertussis* 12822 [26].

other containing *tesD*, a gene encoding the hydrolase for 4,9-DSHA, and consisting of ORF18, 17, *tesI*, *H*, *A2*, *A1*, and *tesDEFG*. Of these, TesA1 and TesA2 were considered to be oxygenases because they showed homology (maximum about 30%) to some oxygenases, such as the phenol hydroxylase of *B. stearothermophilus* BR219 [17] (TesA2) and component B of nitrilotriacetate monooxygenase of *Chelatobacter* strain ATCC 29600 [18] (TesA1), but the functions were not clarified in the previous study. In the present study, two characteristic intermediate compounds accumulated by the gene-disrupted mutants of TesA1 and TesA2 grown on testosterone were isolated, purified, and identified by MS and NMR analysis as 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA) and its hydroxylated derivative, 3,17-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,17-DHSA). Several sets of NMR data were available for 3-HSA, but they were not complete [19,20]. Our data in the present study are the first complete set of NMR data for 3-HSA and 3,17-DHSA, as well as the first report identifying the enzyme genes for conversion of 3-HSA in bacterial steroid degradation. Hydroxylation and dehydroxylation at the C17-position is probably a reversible reaction, as both 3-HSA and 3,17-DHSA accumulated when either testosterone, which has a hydroxy group at position C17, or ADD, which has a ketone group at position C17, was used as the substrate. 3-HSA is

the major intermediate compound, and 3,17-DHSA is minor. Although we could not detect hydroxylation of 3-HSA in *E. coli* transformants expressing TesA1 and/or TesA2, or in the cell free extract, complementation experiments using broad-host plasmids carrying the same gene region showed that both TesA1 and TesA2 are necessary for the effective hydroxylation of 3-HSA at the C4-position. One possible reason for the absence of the activity in *E. coli* expressed TesA1 and TesA2 is overexpression. TesA2 may have low hydroxylation activity without TesA1, as a weak conversion of 3-HSA was detected from TesA2 in the complementation experiment.

For more information, we analyzed the putative amino acid sequences of *tesA1* and *tesA2* using the sequence motif search of GenomeNet service (Kyoto University Bioinformatics Center, http://motif.genome.ad.jp/). GenomeNet service search provides motif searches against PROSITE Pattern (Swiss Institute of Bioinformatics), PROSITE Profile (Swiss Institute of Bioinformatics), BLOCKS (Fred Hutchinson Cancer Research Center), ProDom (Insitut National de la Recherche Agronomique), PRINTS (University of Manchester), and Pfam (Washington University, St. Louis and Sanger Centre) at a time. Although TesA2 showed weak hydroxylation activity, no putative functional domains were found in TesA2. Prodom presented a group of enzymes with ID 008747 as similar to TesA2. Most of the enzymes included in this group are thought to be hydroxylases from their amino acid sequences, but most of them were only putative hydroxylases. Of these, an oxygenase of *Rhodococcus erythropolis* TA421 [22] showed the maximum identity, 33%, with TesA2. *E. coli* expressed TA421 oxygenase showed oxidization of indole, which resulted in pigmentation on the *E. coli* colony, but *E. coli* with the plasmid expressing TesA2 did not show the pigmentation. The search results for TesA1 by BLOCKS, ProDom, and Pfam indicated a flavin reductase-like domain in TesA1. The region containing the flavin reductase-like domain in TesA1 proposed by the search was not identical, but all the amino acids proposed as the flavin reductase-like domain are contained in the region from the aa number 10–160. Fig. 8 shows the alignment of TesA1 and proteins showing homology to it. Most of the proteins which have homology to TesA1 consist of 170–180 aa, and a few consist of about 320 aa, which is almost the same length as TesA1. Though they have the similar length, they showed little homology with each other after about 170 aa. NtaB, component B of nitrilotriacetate monooxygenase of *Chelatobacter* strain ATCC 29600 [18], showed the highest homology — about 33% — to TesA1. The nitrilotriacetate monooxygenase of ATCC 29600 is a two-component monooxygenase encoded by *ntaA* and *ntaB*, which are divergently oriented. NtaB is a flavin mononucleotide (FMN)-containing protein with nitrilotriacetate-stimulated NADH-oxidizing activity. The role of NtaA remains unclear, but its presence was absolutely necessary for oxygenation of nitrilotriacetate. NtaA shows about 40% homology to dibenzothiophene monooxygenase SoxA. The mixture of purified NtaA and NtaB showed oxygenation of nitrilotriacetate, while addition of purified NtaA to NtaB overexpressed by *E. coli* did not show oxygenation. As some of these characteristics of NtaA and NtaB are similar to those of TesA2 and TesA1, TesA1 and TesA2 are thought to be a two-component oxygenase.

Our study clarified the functions of all genes, except for ORF17 and ORF18, in the gene cluster consisting of ORF18, 17, *tesI*, *H*, *A2*, *A1*, and *tesDEFG*. However, the function of most of the genes in the other cluster containing *tesB* is unclear. Further studies are required to confirm the nature of the substrates and products of the enzymes derived from these genes. Another steroid degradation gene cluster is likely present in TA441 because some of the predicted enzyme genes such as an oxygenase component of the hydroxylase of ADD at the C9-position were not found in the analyzed gene region. Characterization of the predicted but not yet isolated steroid degradation genes will also be important.

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