

A New Bacterial Steroid Degradation Gene Cluster in *Comamonas testosteroni* TA441 Which Consists of Aromatic-Compound Degradation Genes for Seco-Steroids and 3-Ketosteroid Dehydrogenase Genes

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Received 2 December 2002/Accepted 27 May 2003

In *Comamonas testosteroni* TA441, testosterone is degraded via aromatization of the A ring, which is cleaved by the *meta*-cleavage enzyme TesB, and further degraded by TesD, the hydrolase for the product of TesB. TesEFG, encoded downstream of TesD, are probably hydratase, aldolase, and dehydrogenase for degradation of 2-oxohex-4-enoic acid, one of the products of TesD. Here we present a new and unique steroid degradation gene cluster in TA441, which consists of ORF18, ORF17, *tesI*, *tesH*, ORF11, ORF12, and *tesDEFG*. TesH and TesI are 3-ketosteroid- Δ^1 -dehydrogenase and 3-ketosteroid- $\Delta^4(5\alpha)$ -dehydrogenase, respectively, which work in the early steps of steroid degradation. ORF17 probably encodes the reductase component of 9α -hydroxylase for 1,4-androstadiene-3,17-dione, which is the product of TesH in testosterone degradation. Gene disruption experiments showed that these genes are necessary for steroid degradation and do not have any isozymes in TA441. By Northern blot analysis, these genes were shown to be induced when TA441 was incubated with steroids (testosterone and cholic acid) but not with aromatic compounds [phenol, biphenyl, and 3-(3-hydroxyphenyl)propionic acid], indicating that these genes function exclusively in steroid degradation.

Comamonas testosteroni is able to utilize certain C₁₉ and C₂₁ steroids, as well as a number of aromatic compounds, as sole carbon and energy sources via a complex metabolic pathway involving many steroid-inducible enzymes. The mechanism by which testosterone is degraded in *Nocardia restrictus* and *C. testosteroni* was proposed in the 1960s (4, 8, 14). However, the genes involved in this degradation pathway in *C. testosteroni* have yet to be identified, except for the initial 17 β -dehydrogenation (1, 3, 7) and the following Δ^1 -dehydrogenation (13). In previous studies, we identified the testosterone degradation genes *tesB* and *tesDEFG* among some open reading frames (ORFs) which are involved in testosterone degradation in *C. testosteroni* TA441 (9, 10). *tesB* encodes the *meta*-cleavage enzyme for 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA) (10), and *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) (9). TesEFG are probably the enzymes for degradation of 2-oxohex-4-enoic acid, which is one of the two products of the hydrolysis of 4,9-DSHA by TesD.

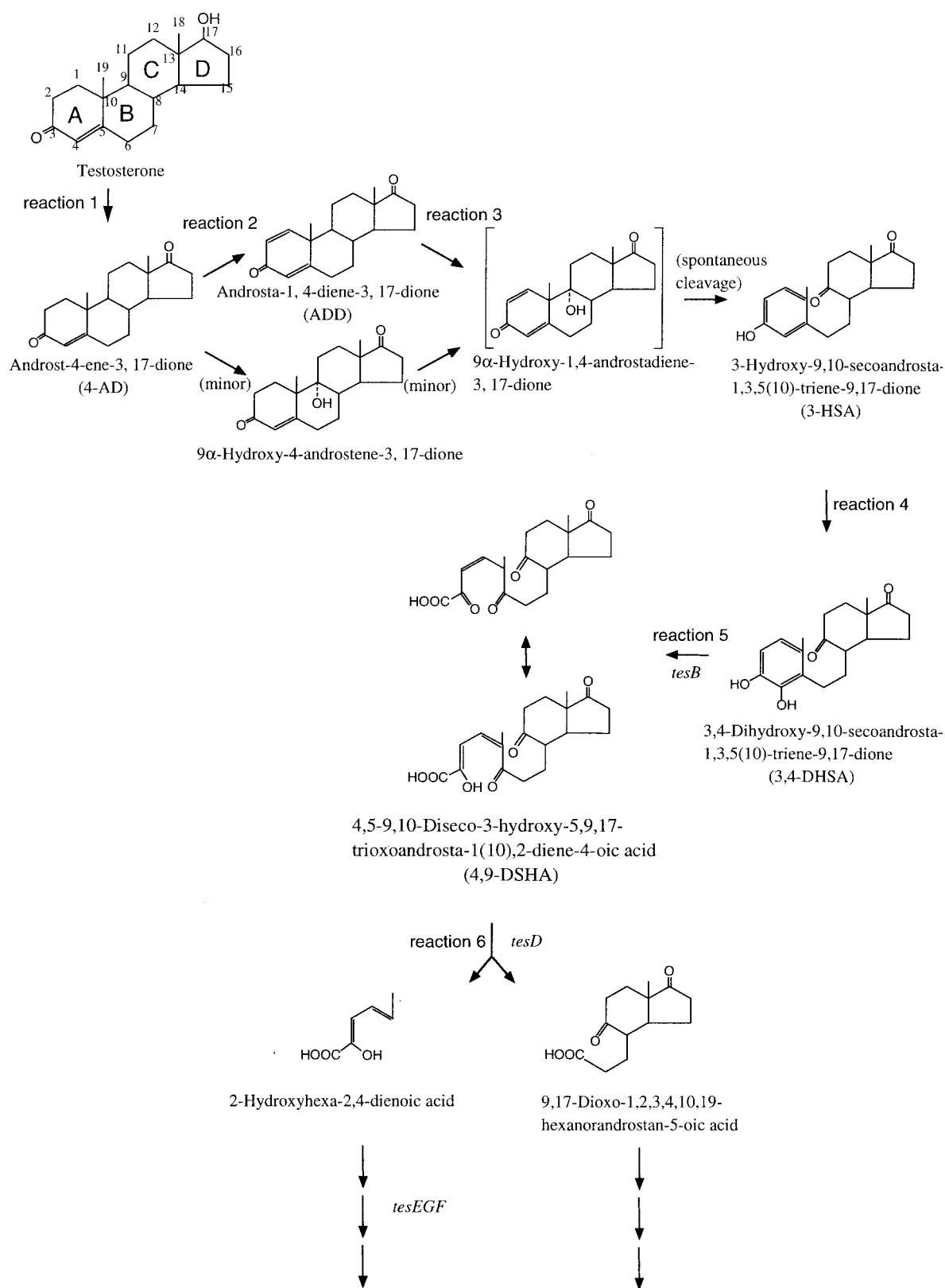
Studies on these genes have shown the probable testosterone degradation pathway of *C. testosteroni* TA441 to be that presented in Fig. 1. The process is initiated by dehydrogenation of the 17 β -hydroxyl group on testosterone to 4-androstene-3,17-dione (4-AD) (Fig. 1, reaction 1), which then undergoes Δ^1 -dehydrogenation to 1,4-androstadiene-3,17-dione (ADD) (reaction 2), 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). The C-4 of 3-HSA is hydroxy-

lated to yield 3,4-DHSA (Fig. 1, reaction 4), which is cleaved between C-4 and C-5 via a *meta*-cleavage reaction by TesB (reaction 5) (10). The product of the *meta*-cleavage reaction of 3,4-DHSA, 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) (9). One of the products, probably 2-hydroxyhexa-2,4-dienoic acid, is degraded by TesEFG.

TesB, -D, -E, -F, and -G show homology to the corresponding enzymes in bacterial aromatic compound degradation, such as BphC, -D, -H, -I, and -J, respectively, in biphenyl degradation. However, the homologies between TesB and BphC and between TesD and BphD are about 40% at most. In the upstream gene region of TesD, ORF11 and ORF12 were identified. ORF11 is oriented in the direction opposite that of ORF12 in relation to *tesG*. Both of the ORFs are indispensable, are induced in TA441 grown on testosterone, and are suggested to encode oxygenases. The region from ORF11 to *tesG* was not included in the gene region of approximately 20 kb containing *tesB*.

In the present study, we report a new steroid degradation gene cluster consisting of ORF18, ORF17, *tesI*, *tesH*, ORF11, ORF12, and *tesDEFG*. 3-Ketosteroid- Δ^1 -dehydrogenase (TesH) and 3-ketosteroid- $\Delta^4(5\alpha)$ -dehydrogenase (TesI) are encoded in sequence, followed by two ORFs, ORF17 and ORF18, which are indispensable and are induced in TA441 cells grown on steroids. ORF17 probably encodes the reductase component of 9α -hydroxylase for ADD, which is the product of the reaction catalyzed by TesH in testosterone degradation. In contrast to the 3-ketosteroid- Δ^1 -dehydrogenases in *Rhodococcus erythropolis* SQ1 (KstD1 and KstD2) (17, 18), TesH is a unique Δ^1 -dehydrogenase for 4-AD in TA441. The enzymes encoded by ORF11 to ORF18 and by ORF12 to *tesG* are induced when TA441 is grown with steroids

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FIG. 1. Proposed pathway of testosterone degradation in *C. testosteroni* TA441.

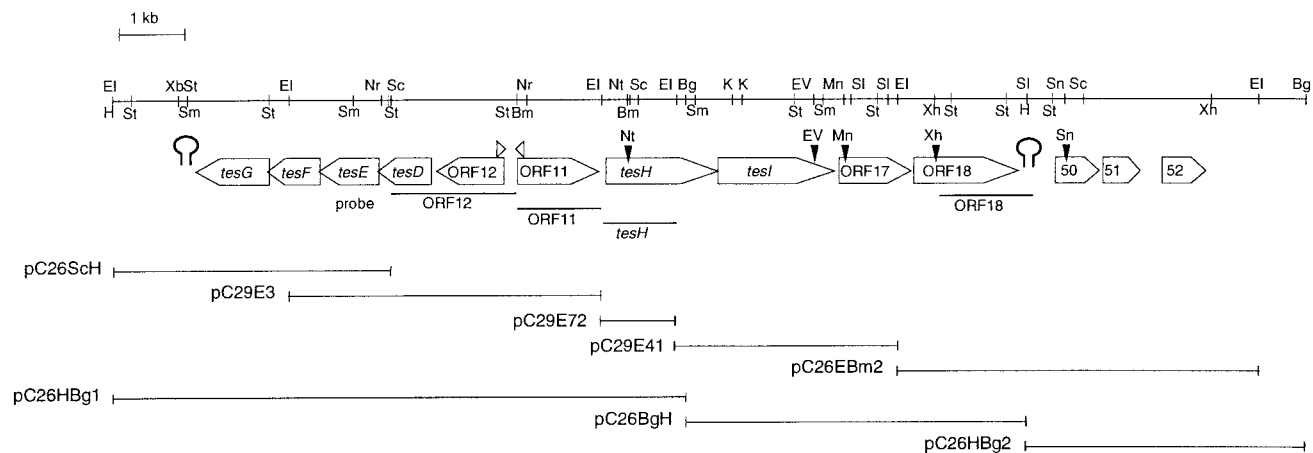


FIG. 2. Partial restriction map of the DNA fragment containing the steroid degradation genes of *C. testosterone* TA441. Large open arrows, genes and putative ORFs; small open arrowheads above large arrows, putative promoters. Putative terminators are located just downstream of *tesG* and ORF18. Small solid arrowheads above genes and ORFs indicate restriction sites where the Km^r gene was inserted. DNA segments used as probes are shown just below the ORFs. Deletion plasmids and remaining gene segments are also shown below the restriction map. Abbreviations: Bm, *Bam*HI; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; H, *Hind*III; K, *Kpn*I; Mn, *Mun*I; Nr, *Nru*I; Nt, *Not*I; Ps, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; Sn, *Sna*BI; Xb, *Xba*I; Xh, *Xho*I.

but not when it is grown with aromatic compounds [phenol, biphenyl, and 3-(3-hydroxyphenyl)propionic acid], indicating that the enzymes encoded by this gene cluster are used exclusively for steroid degradation in TA441.

MATERIALS AND METHODS

Culture conditions. *C. testosterone* TA441 and mutant strains were grown at 30°C in either Luria-Bertani (LB) medium, C medium (10), or a mixed LB-plus-C medium (a mixture of equal volumes of LB and C media) with suitable carbon sources when necessary. Steroids and aromatic compounds, except for 3-(3-hydroxyphenyl)propionic acid, were added in a filtered dimethyl sulfoxide solution at a final concentration of 0.1% (wt/vol). 3-(3-Hydroxyphenyl)propionic acid was added as a CH_3CN solution at a final concentration of 0.1% (wt/vol).

Construction of plasmids and mutant strains for gene disruption experiments. The *tesH* gene was disrupted by insertion of a kanamycin resistance gene into the *Not*I site in *tesH*. The resultant plasmid, pTesH- Km^r , was used for inactivation of the *tesH* gene in TA441 by homologous recombination. The plasmid was introduced into TA441 by electroporation, and a Km^r and carbenicillin-sensitive TA441 mutant was selected. Insertion of the Km^r gene in *tesH* was confirmed by Southern hybridization using the Km^r gene and *tesH* as probes. Gene disruption of *tesI*, ORF17, and ORF18 was performed in the same manner. The restriction site at which the Km^r gene was inserted is indicated in Fig. 2.

Growth of TA441 and mutant strains on testosterone. Growth was monitored by CFU counting, because the culture to which testosterone was added in a dimethyl sulfoxide solution was turbid, which prevented measurement of cell absorbance. Mutants were grown with 0.1% (wt/vol) testosterone or androsterone, and 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, as previously described (10).

Three-dimensional HPLC. A volume of methanol equivalent to twice the volume of culture was added to the culture, which was then centrifuged. The supernatant was directly injected into a high-pressure liquid chromatography (HPLC) system. A Waters 2690 HPLC was utilized with an Inertsil ODS-3 column (2.1 by 150 mm), and elution was carried out using a linear gradient from 20% solution A (CH_3CN-CH_3OH -trifluoroacetic acid [95:5:0.05]) and 80% solution B (CH_3CN-H_2O -trifluoroacetic acid [95:5:0.05]) to 65% solution A and 35% solution B over 10 min; the latter proportion was maintained for 3 min, and then the solvent was adjusted to 20% solution A for 5 min. The flow rate was 0.21 ml/min.

Isolation of the intermediate product X4 accumulated by the TesH disruption mutant. For isolation of the intermediate product accumulated by the TesH disruption mutant (the compound X4), a 1,200-ml culture of the *tesH* disruption mutant incubated in 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 a Waters 600 HPLC (Nihon Waters, Tokyo, Japan) with an Inertsil ODS-3

column (20 by 250 mm; GL Science) and a solvent composed of CH_3CN and H_2O (1:1), with a flow rate of 1 ml/min, at 40°C. X4 was detected at 245 nm. The fraction containing X4 was collected and studied further.

General experimental procedures. For gas chromatography-mass spectrometry (GC-MS), an API 2000 LC/GC/MS spectrometer (Perkin-Elmer, Inc., Boston, Mass.) was used. UV spectra were recorded with an Ultrospec 3300 (Amersham Pharmacia Biotech, Piscataway, N.J.). Nuclear magnetic resonance (NMR) (1D , 1H , and ^{13}C , DEPT, PFG-DQFQSY, PFG-HMQC, PFG-HMBC) spectra were taken on a JNM-ECP500 spectrometer (JEOL, Tokyo, Japan) in a $CDCl_3$ solution with tetramethylsilane at 0 ppm as an internal standard for 1H and ^{13}C chemical shifts.

Northern blot analysis. For the data presented in Fig. 8, TA441 cells incubated in LB medium for about 12 h were collected and added to newly prepared LB medium containing 0.1% testosterone and succinate. Total RNA was purified every 2 h after the start of the incubation and analyzed by Northern hybridization with suitable probes. Succinate was used as a negative control. For the data presented in Fig. 9, TA441 cells incubated in LB medium for about 12 h were collected and added to newly prepared LB medium containing 0.1% of each substrate [testosterone, cholic acid, phenol, biphenyl, 3-(3-hydroxyphenyl) propionic acid, and succinate]. Total RNA was purified at 4 h after the start of the incubation and analyzed by Northern hybridization with suitable probes.

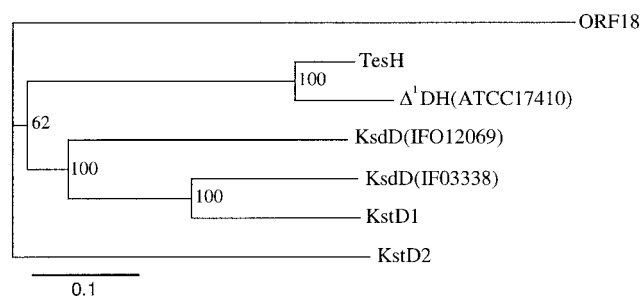


FIG. 3. Phylogenetic tree for TesH and Δ^1DH s. The branching pattern was generated by the neighbor-joining method (CLUSTAL X). The putative amino acid sequence of ORF18 was used as an outgroup. KsdD(IFO12069), Δ^1DH of *A. simplex* IFO12069 (accession number S61889); KsdD(IFO3338), Δ^1DH of *R. rhodochrous* IFO3338 (accession number AB007847); KstD1 and KstD2, Δ^1DH s of *R. erythropolis* SQ1 (accession numbers AF096929 and AY078169, respectively); Δ^1DH (ATCC17410), Δ^1DH of *C. testosterone* ATCC 17410 (accession number M68488).

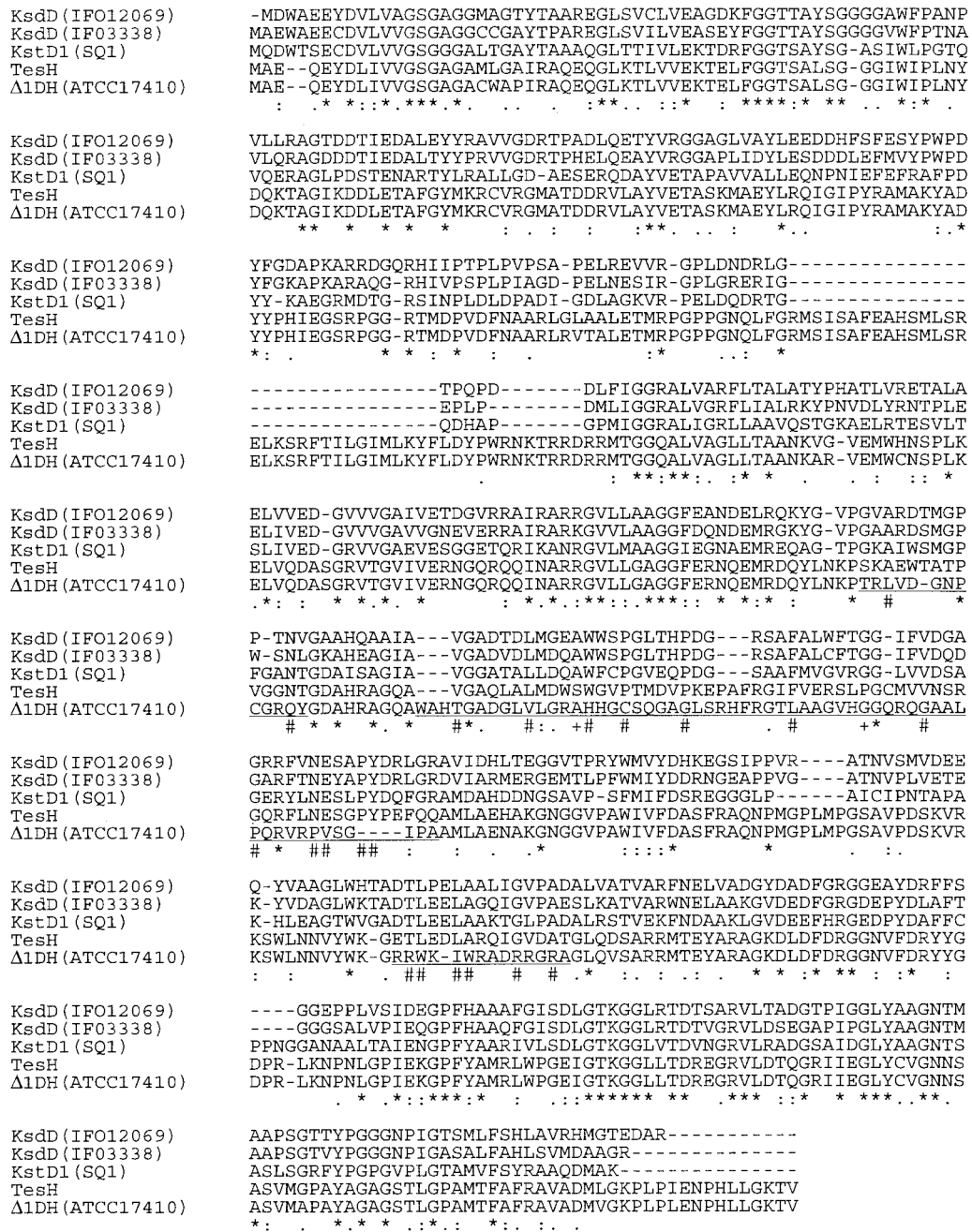


FIG. 4. Alignment of TesH and the Δ^1 DHs. Asterisks indicate amino acids identical among all the proteins; colons and periods indicate amino acids of high and low similarity, respectively. Regions in which the amino acid sequences of TesH and the Δ^1 DH of ATCC 17410 differ greatly are underlined. In these regions, number signs indicate amino acids identical among all the proteins except the Δ^1 DH of ATCC 17410, and plus signs indicate amino acids identical among all the proteins except TesH. Abbreviations are the same as for Fig. 3.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB076368.

RESULTS

Isolation and sequencing of the gene segment downstream of ORF11. The two DNA segments of *C. testosteroni* TA441 containing genes necessary for testosterone degradation have been reported previously. One contains *tesB*, the gene encod-

ing the *meta*-cleavage enzyme which converts 3,4-DHSA to 4,9-DSHA (10). In another gene segment, *tesD*, a gene encoding the hydrolase for 4,9-DSHA, and *tesEFG*, genes encoding the enzymes for the following reaction (Fig. 2) (9), occur in sequence. ORF11 and ORF12, showing homology to some oxygenases, are located upstream of *tesD*, and ORF11 is oriented in the direction opposite that of ORF12 in relation to *tesG*. Downstream of *tesG*, a putative terminator was found, which indicates that this is one end of this gene cluster.

ORF17	MSSEATTAS RYHPLRVRAV IDETHDKSI VFEVPEALAE QFSYRPGQFL TLRLPIEGR-	59
KshB	MTTVEVP HSS RSAVLTVSGV IEETS DARSL VFEIPAELKD KFDYKPGQFL TLRTPSDQTG	60
	:: .. : * * * * *::: :***: * : :*::***** :***: *	
	RxYS G-rich region	
ORF17	YVPRCYS MSS APMLDDALRV TVKRVDKGRG SNWVCDRVQV GDSIELMPPS GLFSRNLSQ	119
KshB	SVARCYS LAS SPFTDDAPKV TVKRTVDGYG SNWLCDLQV GDTIEVLPPS GVFTPKSLD H	120
	*::*****: : * : * * : * * * * : * * * * *::: * * * * *::: * * * * * : * * * * : * : * : * * : * :	
ORF17	NFLLLAGGSG ITPVFSILRT VLKQHGGNVV LFYANRDERS VIFKKDLQQL AAEYPDRLQV	179
KshB	DFLLFGAGSG ITPVISILKS ALTQSGSNVV LIYANRDEKS VIFGAELREL AAQHPGRLTV	180
	*****: * * * * *::: *	
ORF17	IHWLDSVQGA PSQKQLAAWA TPWVADAGQA FICGPGPFMD AAQTAMIEAG MPAEQVHVER	239
KshB	VHWIETVQGL PAVSQLATLA KPFVAYE--A FMCGPGPFMD AVHKALAEAG MPRTQVHAEV	238
	*****: * : * * : *	
ORF17	FVSLPDEETL LLMQEATAPV EAVDQALVQ LRLDGEEYEF NCSGTETILE AGLRAGINVP	299
KshB	FNSLAG-DPF RDVEVAEVS D EEAADAATVE VELDGETHTL VWPRKQTLVD IMLAKGLDVP	297
	* * * : * : *	
	CxxxxCxx Cxxxxxxxx xxxxxxxxxxx xxxxxxxxxxx C	
ORF17	FSCQAGMCAS CMCQVQDGSV HLRHNEVLDA KDLSKKWTLA CQSVPSTSEKL RVKFP E	355
KshB	YSCKEGEGSG CACTVTEGEV QMDNSEILDA EDVANGYILG CKRSRSP I A- - - - -	346
	* * * : *	

FIG. 5. Alignment of the putative amino acid encoded by ORF17 with *R. erythropolis* SQ1 KshB. KshB is the ferredoxin reductase component of the two-component class IA monoxygenase KSH. Asterisks indicate amino acids identical in the two proteins; colons and periods indicate amino acids of high and low similarity, respectively. Three conserved motifs of ferredoxin reductase are boxed.

To obtain the gene segment downstream of ORF11, several pUC19-derived plasmids were constructed from pC26 and pC29, SuperCosI-derived plasmids carrying the DNA of TA441 described previously (9) (Fig. 2). Analysis of a 17-kb region downstream of ORF11 revealed nine ORFs (*tesH*, *tesI*, and ORFs 17, 18, 50, 51, 52, 53, and 54). Of the 17-kb region sequenced, about 10 kb comprising the region from *tesH* to ORF52 is shown in Fig. 2, because the following experiments showed that ORFs 50 to 54 are not involved in testosterone degradation in TA441. Two ORFs just downstream of ORF11 are named *tesH* and *tesI*, because they encode enzymes with high homologies to 3-ketosteroid- Δ^1 -dehydrogenase (Δ^1 DH) (83.0% identity) and 3-ketosteroid- $\Delta^4(5\alpha)$ -dehydrogenase [$\Delta^4(5\alpha)$ DH] (75.8% identity), respectively, in *C. testosteroni* ATCC 17410 (6, 13). TesH also showed identities of about 33, 34, 38, and 33% to the following Δ^1 DHs, respectively: *Arthrobacter simplex* IFO12069 KsdD (11), *Rhodococcus rhodochrous* IFO3338 KsdD (12), and *R. erythropolis* SQ1 KstD1 and KstD2 (identity between KstD1 and KstD2, 35%) (17, 18). The phylogenetic tree and the alignment of these Δ^1 DHs are shown in Fig. 3 and 4, respectively (KstD2 is not included in the alignment).

The alignment revealed three regions in which the amino acid sequences of TesH and the Δ^1 DH of ATCC 17410 differ greatly (Fig. 4). These differences are the results of a frameshift caused by the lack of several nucleotides in the gene sequence of the Δ^1 DH of ATCC 17410 relative to that of *tesH*. A search of TesI homology revealed significant homology only with the $\Delta^4(5\alpha)$ DH of ATCC 17410 (75.0% identity). Alignment of TesI and the $\Delta^4(5\alpha)$ DH of ATCC 17410 also revealed three significantly different regions as a result of a frameshift.

Downstream of *tesI*, two ORFs, ORF17 and ORF18, followed by a putative terminator, were found. The putative amino acid sequence of ORF17 showed homology with several kinds of ferredoxin reductases and possessed three conserved motifs of ferredoxin reductase: (i) the consensus sequence

(RXFS), which is considered to be the binding region of a flavin adenine dinucleotide-isoalloxazine ring, (ii) the G-rich region, which is considered to be the binding region of an NAD(P)-ribose, and (iii) the consensus sequence (CXXXX CXXC-any 29 amino acids-C) of the chloroplast type Fe-S cluster (Fig. 5). The putative amino acid sequence of ORF17 showed the highest homology, about 46.0%, with KshB, the ferredoxin reductase component of *R. erythropolis* SQ1 3-ke-tosteroid-9 α -hydroxylase (KSH) (16). All of this evidence indicates that ORF17 encodes the ferredoxin reductase component of 9 α -hydroxylase for ADD in testosterone degradation in *C. testosteroni* TA441. However, none of the putative proteins that we cloned showed significant homology to KshA, the terminal oxygenase component of *R. erythropolis* SQ1 KSH (16). ORF18 showed the maximum homology, about 45%, to several putative acid-coenzyme A (CoA) ligases, about 30% homology to long-chain fatty acid-CoA ligases, and lower homologies to other CoA ligases.

Downstream of ORF18, several putative ORFs (ORF50 to ORF54) were found. Because the growth of the gene disruption mutants of these ORFs on testosterone was almost equal to that of TA441 (data not shown), these ORFs are not considered to be involved in testosterone degradation.

Growth of *tesH*, *tesI*, ORF17, and ORF18 disruption mutants on testosterone. *tesH*, *tesI*, ORF17, and ORF18 in TA441 were individually disrupted with a Km^r gene by homologous recombination, and the resultant mutant strains were designated TesH⁻, TesI⁻, ORF17⁻, and ORF18⁻, respectively. Figure 6a shows a comparison of the growth of each mutant strain and TA441 on testosterone as the sole carbon source. The growth of each strain until around 6 h after the start of the incubation was probably caused by the nutrients carried by the cells from preculture on LB medium. After that, growth of the TesH⁻, ORF17⁻, and ORF18⁻ mutants on testosterone as the sole carbon source was limited, indicating that these genes

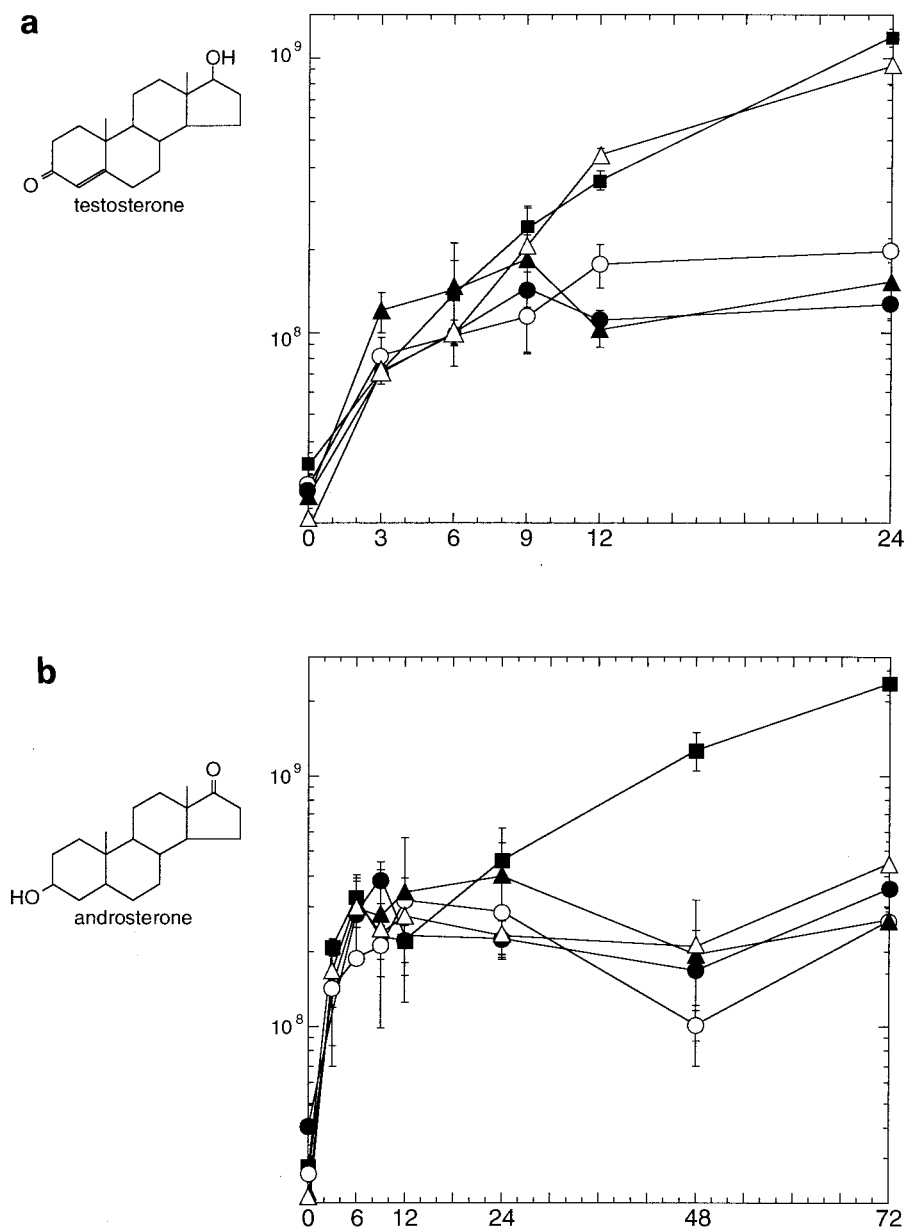


FIG. 6. Growth of TA441 and gene disruption mutants on testosterone (a) or androsterone (b) as the sole carbon source. The genes were disrupted by insertion of a Km^r gene. Each strain was grown in LB medium, washed twice, and inoculated into 10 ml of C medium supplemented with 0.1% (wt/vol) testosterone or androsterone. Symbols: ■, TA441; ▲, TesH disruption mutant; △, TesI disruption mutant; ●, ORF17 disruption mutant; ○, ORF18 disruption mutant. Growth is expressed in CFU. Data are averages of more than three experimental determinations.

are necessary for testosterone degradation in TA441. The growth of the TesI⁻ mutant was almost the same as that of TA441. This result is consistent with the function of TesI as $\Delta^4(5\alpha)$ DH; because testosterone has a double bond at the $\Delta^4(5\alpha)$ position, $\Delta^4(5\alpha)$ DH is not necessary for testosterone degradation. To confirm the function of TesI as $\Delta^4(5\alpha)$ DH, we also examined the growth of these mutants and TA441 on androsterone, whose $\Delta^4(5\alpha)$ bond is single (Fig. 6b). Only TA441 showed significant growth on androsterone; the TesI⁻ mutant, as well as the other mutants, showed limited growth.

HPLC analysis of the culture media of the mutants. The TesH⁻, TesI⁻, ORF17⁻, and ORF18⁻ gene disruption mutants were incubated in a mixed LB-plus-C medium with tes-

tosterone, and after about 40 h the culture media were analyzed by HPLC. The mixed LB-plus-C medium is a mixture of equal volumes of LB and C medium and was used because TA441 mutants accumulated larger amounts of intermediate compounds in this medium than in C medium (9). Figure 7 shows a three-dimensional HPLC chart of the culture of each mutant. The chart of the culture of the TesD disruption mutant, characterized in our previous study (9), is presented as authentic (Fig. 7a). In the culture of the TesH⁻ mutant, 4-AD and an intermediate compound named X4 accumulated (Fig. 7b). Compound X4 was not detected in the culture of any other mutants. The identification of X4 is described in the next section. The TesI⁻ mutant did not accumulate a significant

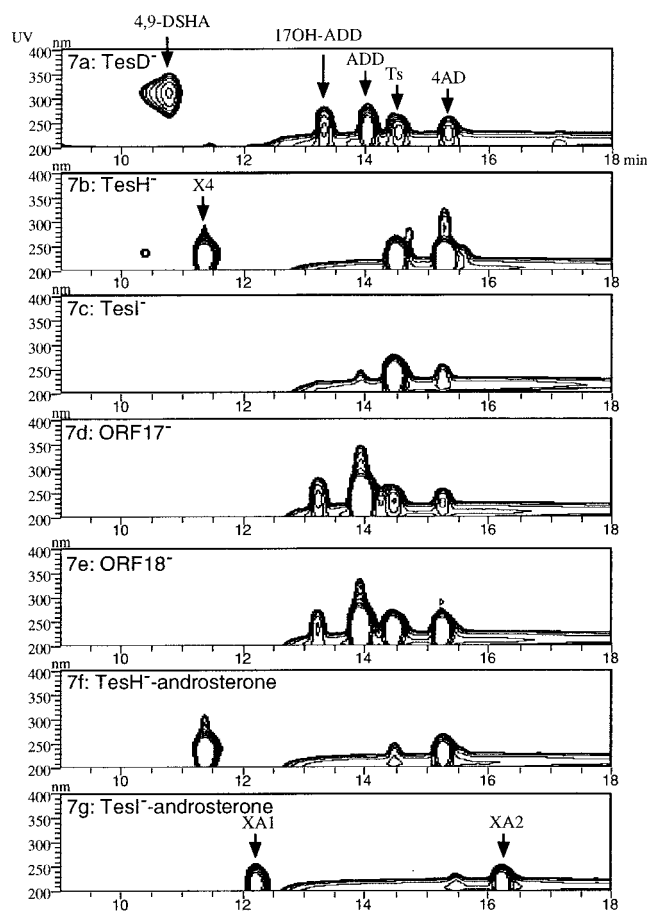


FIG. 7. Intermediate compounds accumulating in the cultures of the gene disruption mutants of TA441 incubated with testosterone (a through e) or androsterone (f and g). Data are represented in three-dimensional HPLC charts (the vertical axis indicates wavelength, the horizontal axis indicates reaction time, and the UV absorbance of each compound is represented in contour). (a) HPLC chart of the culture of the *TesD*⁻ disruption mutant grown on the mixed LB-plus-C medium with testosterone (presented as authentic). (b through g) HPLC charts showing cultures of the gene disruption mutants of *tesH*⁻ (b), *tesI*⁻ (c), ORF17⁻ (d), and ORF18⁻ (e) incubated with testosterone and of the gene disruption mutants of *tesH*⁻ (f) and *tesI*⁻ (g) incubated with androsterone. Ts, testosterone; 17OH-ADD, 17-hydroxy-1,4-androstadiene-3-one.

amount of any intermediate (Fig. 7c), a result usually observed in the culture of TA441 or a well-growing mutant strain. The ORF17⁻ and ORF18⁻ mutants accumulated 4-AD, ADD, and 17 β -hydroxylated ADD (Fig. 7d and e). These intermediate compounds are detected with most gene disruption mutants of steroid degradation genes (9, 10; also unpublished data). Figure 7f and g show the culture of the *TesH*⁻ and *TesI*⁻ mutants, respectively, incubated with androsterone. In the culture of the *TesH*⁻ mutant incubated with androsterone, 4-AD and X4 accumulated, the same result as for incubation with testosterone. In the culture of the *TesI*⁻ mutant, two intermediate compounds (XA1 and XA2) accumulated; these were not detected in the culture of any other mutant.

Purification and identification of compound X4. For isolation of X4, the *TesH*⁻ mutant was incubated with testosterone, and the culture was extracted with ethyl acetate. The ethyl

acetate layer was concentrated, and the residue was dissolved in a small amount of methanol. The solution was loaded onto the HPLC column, and the fraction containing X4 was collected from the eluent. The purified X4 gave a molecular ion at m/z 325 ($M + Na^+$) and m/z 303 ($M + H^+$) by direct MS analysis, indicating its molecular formula to be $C_{19}H_{26}O_3$. Compound X4 showed maximum absorbance at 241.5 nm ($\epsilon = 16,000$) in methanol solution, which agreed well with the value obtained by Dodson and Muir (5) for 9 α -hydroxyandrost-4-ene-3,17-dione. Because complete NMR data were not available from previous studies of 9 α -hydroxyandrost-4-ene-3,17-dione, complete NMR assignments were performed (Table 1). The ^{13}C NMR spectrum confirmed the presence of 19 carbons including 2 methyls at 12.7 and 19.9 ppm and 2 carbonyl carbons at 198.8 and 219.9 ppm. One hydroxy signal was observed at 76.7 ppm. The 1H NMR spectrum confirmed two methyl protons at 0.93(s) and 1.35(s) ppm and one olefinic methine proton at 5.90(d) ppm. These characteristic signals of 1H NMR spectra are consistent with the partial 1H NMR spectral data of 9 α -hydroxyandrost-4-ene-3,17-dione reported by Bell et al. (2), in which 9 α -hydroxyandrost-4-ene-3,17-dione was prepared by the conversion of 4-AD by the fungus *Diaporthe celastrina*. Because the measured value of compound X4 correlated well with the MS, UV spectrum, and partial NMR analysis data reported in previous studies, X4 was identified as 9 α -hydroxyandrost-4-ene-3,17-dione.

Induction of the steroid degradation genes in TA441. Total RNA of TA441 incubated in LB medium with testosterone or succinate (negative control) was purified every 2 h for 8 h and subjected to Northern blot analysis. LB medium, which has been shown not to inhibit the induction of testosterone degradation genes in TA441 (9), was used to obtain almost the same volume of cells from cultures of all the compounds tested. Northern blot analysis showed that *tesH* and ORF18 were induced in testosterone-grown cells (Fig. 8). Induction of

TABLE 1. NMR data for X4

Carbon no.	^{13}C (ppm)	1H NMR (δ [ppm]) ^a
1	28.6	2.43 m, 1.75 m
2	33.9	2.42 m
3	198.8	
4	127.2	5.90 d (1.8)
5	167.7	
6	31.5	2.51 ddd (15.1, 6.4, 1.8), 2.36 ddd (15.1, 5.1, 1.8)
7	24.2	1.75 m, 1.54 m
8	37.2	2.11 ddd (12.0, 11.5, 3.7)
9	76.7	
10	44.5	
11	26.2	1.78 ddd (14.2, 13.3, 4.1), 1.65 ddd (14.2, 4.1, 2.3)
12	27.0	1.69 ddd (13.3, 4.1, 2.3), 1.56 m
13	47.2	
14	44.3	1.86 ddd (12.0, 6.0, 6.0)
15	21.6	1.91 m, 1.56 m
16	35.7	2.48 br.dd (18.8, 8.7), 2.13 ddd (18.8, 8.7, 8.7)
17	219.9	
18	12.7	0.93 s
19	19.9	1.35 s

^a Coupling constants (J values in hertz) are given in parentheses.

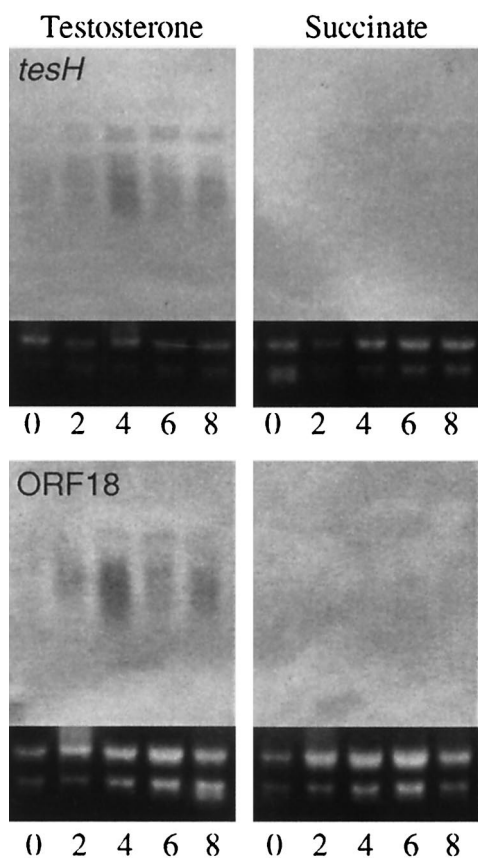


FIG. 8. Induction of *tesH* and ORF18 in TA441 incubated with testosterone. Total RNA was purified every 2 h and analyzed by Northern hybridization with suitable probes (indicated in Fig. 2). Succinate was used as a negative control.

ORF11, ORF12, and *tesB* in TA441 cells incubated with steroids (testosterone and cholic acid) or with aromatic compounds [phenol, biphenyl, and 3-(3-hydroxyphenyl)propionic acid] at 4 h after the start of the incubation is shown in Fig. 9. Cholic acid was tested because TA441 shows good growth on it. Induction of ORF11, ORF12, and *tesB* was examined because these genes are located at the very beginning in each sequence of genes (putative operon). ORF11, ORF12, and *tesB* were induced during growth on steroids but not during growth on aromatic compounds. The *mhpB* gene, which encodes the *meta*-cleavage enzyme for 3-(3-hydroxyphenyl)propionic acid degradation, was used as a control. In contrast to ORF11, ORF12, and *tesB*, *mhpB* was induced in cells incubated with 3-(3-hydroxyphenyl)propionic acid but not with steroids.

DISCUSSION

In this paper, we reported a bacterial steroid degradation gene cluster with a unique combination of genes that has not been reported before. It consists of ORF18, ORF17, *tesI*, *tesH*, ORF11, ORF12, and *tesDEFG*. *TesDEFG*, reported in our previous study (9), are the hydrolase for 4,9-DSHA (*TesD*) and enzymes for the subsequent reaction (*TesEFG*). The present study revealed the presence of a Δ^1 DH gene and a $\Delta^4(5\alpha)$ DH gene, *tesH* and *tesI*, followed by two ORFs, ORF17

and ORF18, in the region upstream of *tesD*. All these genes are involved and induced in testosterone degradation in TA441. Involvement of *TesH* and *TesI* in androsterone degradation, as well as induction of isolated testosterone degradation genes in TA441 cells incubated with cholic acid, indicates that these genes are targeted not only to testosterone but also to various kinds of steroids. Putative terminator sequences were found just next to the end of ORF18 and just next to the end of *tesG*, and ORFs located downstream of ORF18 were not necessary for steroid degradation in TA441, indicating that this cluster consists of ORF18 through *tesG*. Genes *tesH*, *tesI*, and probably ORF17 encode enzymes for steroids with an androstane structure, and ORF11, ORF12, and *tesDEFG* encode enzymes for the aromatized A ring of seco-steroids. (The roles of ORF11 and ORF12 will be described in a subsequent paper.) Gene disruption experiments and analysis of the culture media of the mutants showed that all these enzymes are involved in steroid degradation. The induction of these genes by steroids (testosterone and cholic acid) but not by aromatic compounds [phenol, biphenyl, and 3-(3-hydroxyphenyl)propionic acid] was confirmed by Northern blot analysis. These results clearly show

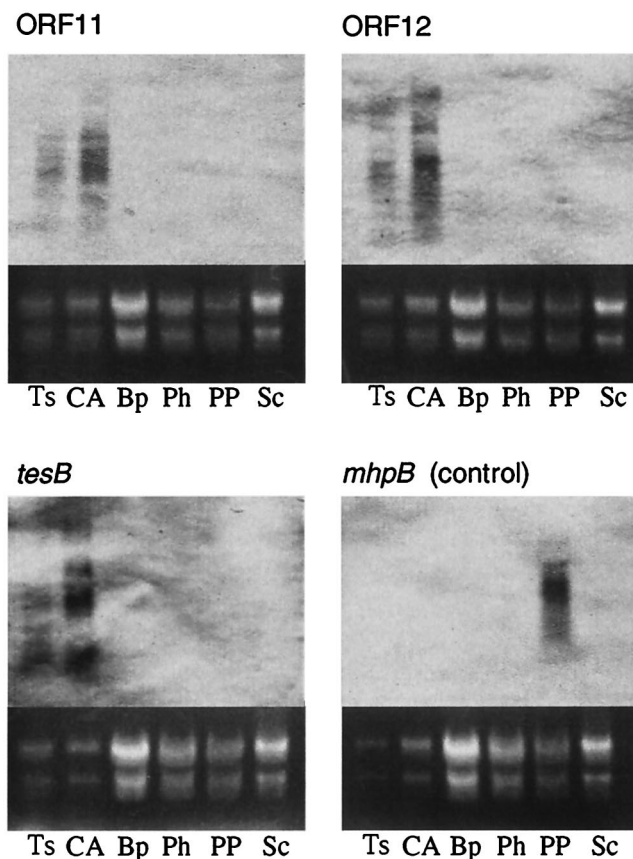


FIG. 9. Induction of ORF11, ORF12, and *tesB* in TA441 cells incubated with steroids or aromatic compounds. Cells were collected 4 h after the start of incubation and were subjected to Northern blot analysis with suitable probes (ORF11, ORF12, *tesB*, and *mhpB*). *mhpB* is the gene encoding the *meta*-cleavage enzyme of TA441 in 3-(3-hydroxyphenyl)propionic acid degradation and was used as a control. Substrates tested were testosterone (Ts), cholic acid (CA), biphenyl (Bp), phenol (Ph), 3-(3-hydroxyphenyl)propionic acid (PP), and succinate (Sc).

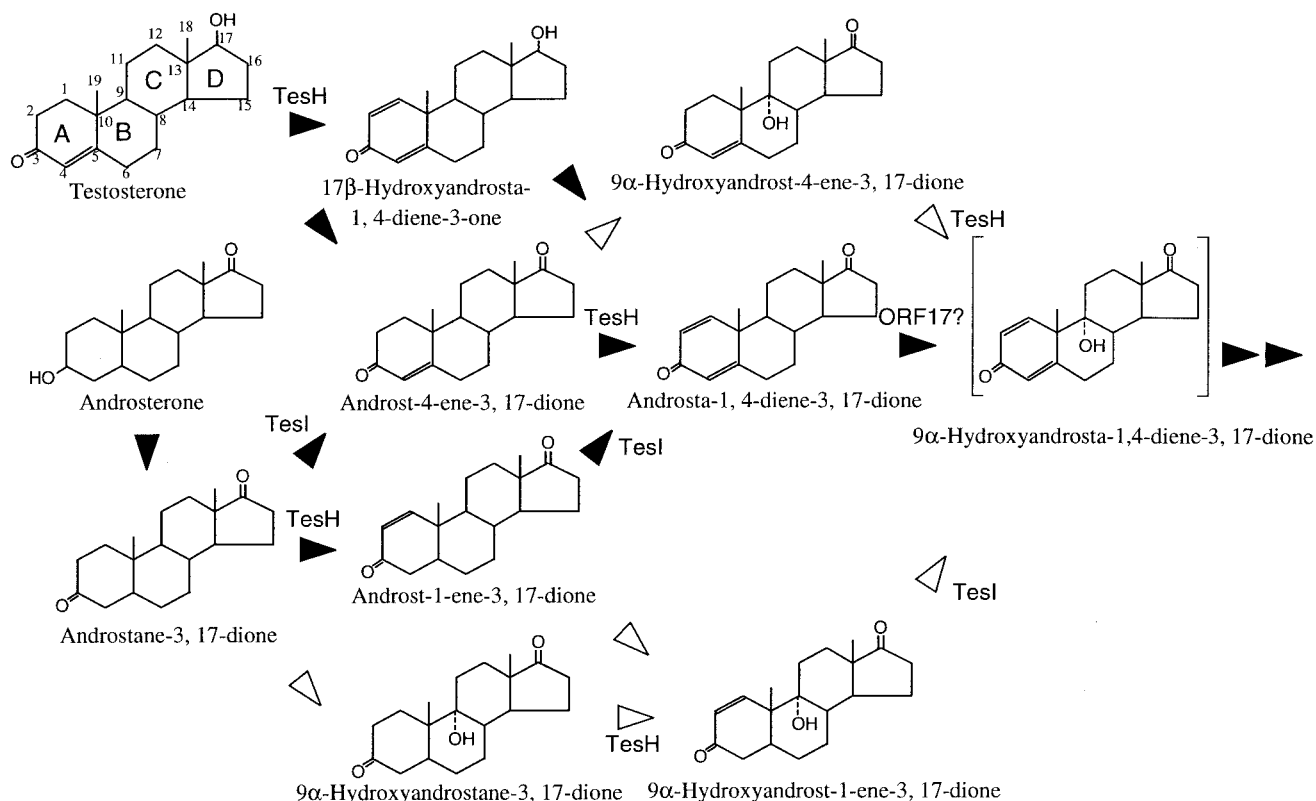


FIG. 10. Conversion of testosterone and androsterone in *C. testosteroni* TA441. Solid arrowheads indicate the probable main degradation pathway. Open arrowheads indicate minor reactions.

that *tesDEFG* developed exclusively for steroid degradation in TA441 in spite of their similarity to aromatic compound degradation genes. From the evolutionary viewpoint, the relationship between the *tes* genes and aromatic compound degradation genes is particularly interesting.

TesH showed about 83 and 38% identity with *C. testosteroni* ATCC 17410 Δ^1 DH (13) and *R. erythropolis* SQ1 KstD1 (17, 18), respectively. *R. erythropolis* SQ1 has two Δ^1 DHs, KstD1 and KstD2, and the *kstD1* disruption mutant of SQ1 still possessed the ability to grow on testosterone. The *tesH* disruption mutant of TA441 did not show significant growth on testosterone and accumulated 4-AD and 9 α -hydroxyandrost-4-ene-3,17-dione, indicating that TesH is a unique Δ^1 DH in TA441. 4-AD and ADD were detected in the cultures of most of the mutants incubated with testosterone and in the culture of TA441 in the early phase of growth on testosterone, while 9 α -hydroxyandrost-4-ene-3,17-dione was detected only in the culture of the *tesH* disruption mutant. These results indicate that in TA441, Δ^1 -dehydrogenation of 4-AD to ADD is faster and more significant than 9 α -hydroxylation of 4-AD. Figure 10 shows proposed testosterone and androsterone conversion in TA441.

TesI, encoded just next to TesH, showed 75.8% identity with *C. testosteroni* ATCC 17410 $\Delta^4(5\alpha)$ DH, and the *tesI* disruption mutant grew quite well on testosterone [in which the $\Delta^4(5\alpha)$ bond is double] but failed to show significant growth on androsterone [in which the $\Delta^4(5\alpha)$ bond is single], indicating that TesI is a unique $\Delta^4(5\alpha)$ DH for steroid degradation in TA441. In the culture of the *tesI* disruption mutant incubated with

androsterone, two intermediate compounds (XA1 and XA2), which were not detected in the cultures of other mutants, were detected by HPLC. By comparison with peaks detected in the *tesH* disruption mutant incubated with androsterone, these compounds are believed to be 9 α -hydroxyandrost-1-ene-3,17-dione and androst-1-ene-3,17-dione.

The putative amino acid sequence of ORF17 possessed conserved motifs of a ferredoxin reductase component of hydroxylases and showed similarity to ferredoxin reductases; the highest identity, about 48%, was to *R. erythropolis* SQ1 KshB (16). Since KshB is the ferredoxin reductase component of SQ1 KSH, there is a high possibility that ORF17 encodes a ferredoxin reductase component of KSH. This is supported by the result of HPLC analysis of the culture of the ORF17 disruption mutant incubated with testosterone: most of the testosterone added was converted to ADD. KshA is the terminal oxygenase of SQ1 KSH, though none of the putative proteins that were identified in TA441 as involved in steroid degradation showed significant homology to KshA. Examination of the function of ORF17 and a search for the probable terminal oxygenase component are now under way. ORF18 is also necessary and induced in testosterone degradation and may encode an acid-CoA ligase. CoA-ligase adds CoA to a carboxyl group, but its role in steroid degradation is unclear.

Another gene cluster isolated from TA441 contains *tesB*, encoding a *meta*-cleavage enzyme for 3,4-DHSA, followed by three putative ORFs, ORF1 to ORF3, all of which are necessary for testosterone degradation in TA441. Recently, a testosterone-inducible biphenyl-2,3-diol-1,2-dioxygenase of *C.*

testosteroni ATCC 11996, TIP1, which is almost identical to TesB (only two amino acids are different), was reported (15). The *tip1* gene is followed by ORF1, *tip6*, ORF2, and ORF3, which are almost identical to ORFs 1 to 3 in TA441. (The ORF2 of ATCC 11996 presented in the report of Skowasch et al. is shorter than ORF2 in TA441. This is probably the result of an error in DNA sequence determination, which resulted in the division of an ORF, which corresponds to ORF2 in TA441, into *tip6* and ORF2, because the estimated molecular weight of TIP6 in their sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiment was not consistent with the molecular weight of the putative protein encoded by *tip6* but was consistent with ORF2 of TA441.) Extradiol dioxygenase, another name of the *meta*-cleavage enzyme, is well known for its wide substrate specificity, so the activity on 2,3-dihydroxy biphenyl and the approximately 40% homology to BphC's are not considered to be enough evidence to conclude that it is biphenyl-2,3-diol-1,2-dioxygenase. To identify it as biphenyl-2,3-diol-1,2-dioxygenase, it is necessary to examine the growth of the gene disruption mutant on biphenyl and its induction in biphenyl-grown cells. TA441 does not grow on biphenyl. Northern blot analysis showed that *tesB* is induced in TA441 incubated with steroids (testosterone and cholic acid) but not with aromatic compounds [phenol, biphenyl, and 3-(3-hydroxyphenyl)propionic acid]. These results showed that TesB is an enzyme only for steroid degradation. The gene cluster containing *tesB* must be another steroid degradation gene cluster in TA441. Analysis of genes in this cluster is also necessary in order to clarify the entire degradation pathway of steroids in *C. testosteroni*.

ACKNOWLEDGMENTS

We thank Hiroyuki Koshino for practical advice on identification of purified intermediate compounds. We thank Y. Ichikawa and R. Nakazawa (Biodesign DNA Sequence Facility, RIKEN) for determination of the nucleotide sequence.

This work was partly supported by a grant from the Eco Molecular Sciences Research Program of RIKEN. M.H. was supported by a grant from the Special Postdoctoral Researchers Program of RIKEN.

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