Steroid degradation in *Comamonas testosteroni*

Masae Horinouchi*, Toshiaki Hayashi, and Toshiaki Kudo[a]

Chemical Biology Core Facility, RIKEN Advanced Science Institute, 2-1 Hirosawa,

Wako-shi, Saitama 351-0198, Japan

[a] Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki-shi, Nagasaki

852-8521, Japan

*Correspondence to:

Chemical Biology Core Facility, RIKEN Advanced Science Institute, RIKEN, 2-1

Hirosawa, Wako-shi, Saitama 351-0198, Japan

Tel: +81 48 467 5354

Fax: +81 48 462 1357

E-mail: masae@riken.jp

Abstract

Steroid degradation by *Comamonas testosteroni* and *Nocardia restrictus* have been intensively studied for the purpose of obtaining materials for steroid drug synthesis. *C. testosteroni* degrades side chains and converts single/double bonds of certain steroid compounds to produce androsta-1,4-diene 3,17-dione or the derivative. Following 9α-hydroxylation leads to aromatization of the A-ring accompanied by cleavage of the B-ring, and aromatized A-ring is hydroxylated at C-4 position, cleaved at Δ4 by *meta*-cleavage, and divided into 2-hydroxyhexa-2,4-dienoic acid (A-ring) and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (B,C,D-ring) by hydrolysis. Reactions and the genes involved in the cleavage and the following degradation of the A-ring are similar to those for bacterial biphenyl degradation, and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid degradation is suggested to be mainly β-oxidation. Genes involved in A-ring aromatization and degradation form a gene cluster, and the genes involved in β-oxidation of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid also comprise a large cluster of more than 10 genes. The DNA region between these two main steroid degradation gene clusters contain 3α-hydroxysteroid dehydrogenase gene, Δ5,3-ketosteroid isomerase gene, genes for inversion of an α-oriented-hydroxyl group to a β-oriented-hydroxyl group at C-12 position of cholic acid, and genes possibly involved in the degradation of a side chain at C-17 position of cholic acid, indicating this DNA region of more than 100kb to be a steroid degradation gene hot spot of *C. testosteroni.*

1. Brief overview of steroid degradation by *Comamonas testosteroni*

1-1. Introduction

Steroid compounds are known for the wide variety and have long been used as medicinal chemicals for their hormonal effects and various other functions on animals. Several species of bacteria degrade certain steroid compounds to utilize as the sole carbon and energy sources, and the intermediate compounds of steroid degradation by these bacteria have been intensively studied for the purpose of obtaining materials for steroid drug synthesis. For these studies, testosterone was often used as representative steroid compound and Gram-negative *Comamonas* (formerly *Pseudomonas*) *testosteroni* and Gram-positive *Rhodococcus* (formerly *Nocardia*) *restrictus* were used as representative steroid degrading bacteria. Among *C. testosteroni,* strain ATCC 1196 has been traditionally used and some other steroid-degrading *C. testosteroni* strains such as ATCC 17410 and TA441 were reported a few decades later when genetic engineering showed explosive progress.

1-2. Degradation pathway of steroid compound in *C. testosteroni*

In 1958, it was already revealed that testosterone degradation by *C. testosteroni* starts with conversion of a hydroxyl group at C-17 position to a ketone group and introduction of a double bond at C-1 position of the A-ring, being followed by hydroxylation at C-9 position of the B-ring which leads to aromatization of the A-ring (Figure 1.) [1, 2]. Then an intermediate compound with the aromatized A-ring, 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA), and an intermediate compound with the C,D-rings with cleaved B-ring,

9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid, were identified in 1965 [3]. Then

aromatized A-ring of 3-HSA is cleaved and the compound is divided into two

compounds, A-ring and B,C,D-rings, by hydrolysis. [1, 2, 4-12]. The degradation

pathway was confirmed with detail using gene-disrupted mutants in early 2000s, with

further studies revealing that A-ring is degraded with the pathway similar to a common

bacterial aromatic compound degradation pathway and B,C,D-rings is degraded mainly

by β-oxidation [13-18]. Investigation of the detail of degradation pathway of B,C,D-ring

and other side chains of steroid compounds are still in progress.

1-3. Steroid degradation enzymes in *C. testosteroni*.

Although testosterone has been used as the representative steroid compound to

reveal steroid degradation in *C. testosteroni*, *C. testosteroni* also degrades several other

steroids (e.c. cholic acid and its derivatives, testosterone, progesterone, epiandrosterone,

dehydroepiandrosterone) and converts them to androsta-1,4-diene 3,17-dione (ADD) or

the derivative corresponding to the initial steroid compound before breaking down

steroidal four rings. This suggests *C. testosteroni* has all the enzymes required for

degradation of these compounds to ADD or the derivative, and among the predicted

enzymes, Δ5,3-ketosteroid isomerase, 3α-dehydrogenase, and 3β-dehydrogenase have

long been studied since 1950s [19-21]. These three enzymes are indispensable for

degradation of steroid compounds with a double bond at Δ5 (e.c.

dehydroepiandrosterone), with an α-oriente-hydroxyl group at C-3 position (e.c. cholic

acid, androsterone), and with a β-oriente-hydroxyl group at C-3 position (e.c.

epiandrosterone), respectively. Crystal structure analysis and genetic analysis started

after around 1990.

1-4. Steroid degradation genes and the gene clusters in *C. testosteroni*

Investigation of steroid degradation genes of in *C. testosteroni* launched fully in 1990s when 3β-dehydrogenase gene, 3α-dehydrogenase gene, and Δ5,3-ketosteroid isomerase gene were isolated consistently, then degradation genes for basic steroidal structure were revealed with detailed degradation pathway in the next decade (Figure 1., table 1) [13-18, 22-28]. The aromatized A-ring is cleaved and completely degraded by *meta*-cleavage pathway, a common bacterial degradation pathway for aromatic compounds, and the genes involved in the A-ring degradation show significant homology to the corresponding genes in bacterial aromatic compound degradation. Degradation pathway of intermediate compound of B,C,D-rings, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid, is still under investigation, but the main part of the degradation is suggested to be β-oxidation. The degradation is like an aggregation that irrelevant reactions just gathered up, but the genes for the reactions are exclusively induced only when *C. testosteroni* is incubated with steroid compounds.

  Genes of *C. testosteroni* for degradation of basic steroidal structure constitute two main steroid degradation gene clusters; one mainly consists of genes involved in aromatization and cleavage of the A-ring (named *tesG*-ORF18 in strain TA441) and the other consists of genes involved in β-oxidation of the B,C,D-rings (named *steA* to *tesR* in strain TA441) (Table 1). Recent study indicated these two steroid degradation gene clusters are on the same DNA strand several dozens of kilo bases apart and the 3α-dehydrogenase gene and the adjacent Δ5,3-ketosteroid isomerase gene are located in

the DNA region between these clusters (Table 1, identified steroid degradation genes of

C. testosteroni strains are shown with putative genes of *C. testosteroni* CNB-2, whose

whole genome determination was completed in 2009 [29]). Quite a few possible steroid

degradation genes are found in this DNA region, suggesting that this DNA region of

more than 100kb to be the steroid degradation gene hot spot of *C. testosteroni*.

2. Detail of each reaction of steroid degradation by *Comamonas testosteroni*

2-1. 3β-,17β-Dehydrogenation

In mammals, 3β-hydroxy-Δ5-steroid dehydrogenase catalyzes both dehydrogenation from 3β-hydroxyl group to ketone group as well as transposition of a double bond at Δ5 to Δ4, but in bacterial steroid degradation, the hydroxyl group at C-3 position must be converted to a ketone group before introduction of a double bond into A-ring.

3β,17β-Dehydrogenase (3β,17β-hsd) of *C. testosteroni* was purified in 1970s from strain ATCC11996. The enzyme was initially isolated as 3β-hsd and the cloned enzyme was revealed to act on both 3β-hydroxyl group and 17β-hydroxyl group of steroid compounds (Figure 2.) [22, 30-32]. In 2002, crystallographic analysis at 1.2 A resolution revealed the enzyme to have nearly identical subunits that form a tetramer with the active site containing a Ser-Tyr-Lys triad, typical for short-chain dehydrogenases/reductases (SDR) [33].

The gene is not in the putative steroid degradation gene hot spot of *C. testosteroni*, but putative genes in the surrounding DNA region, *stdC* and *sip48*, were induced when strain ATCC11996 was incubated with steroid compounds degradable for the strain (Figure 3.) [22, 34-36]. The deduced amino acid sequence of *stdC* showed the maximum identity (ca. 80%) to PhaR, polyhydroxyalkonate synthesis repressor and Sip48 showed significant homology to DUF1329 family proteins. DUF1329 family proteins are hypothetical proteins, but can be found in wide genera of Gram-negative bacteria, especially in *Pseudomonas* sp.

Genes encoding Sip48 and 3β,17β-hsd were also isolated from strain TA441 with several kb DNA region downstream of 3β,17β-hsd gene. A possible terminator was

found just downstream of the putative 3β,17β-hsd gene and the homology search indicated that a protein encoded by a putative ORF in the downstream DNA region (ORF63, Figure 3.) to be a secretion lowering protein 1, suggesting that the DNA region downstream of the 3β,17β-hsd are not involved in steroid degradation [37].

A gene-disrupted mutant of 3β,17β-hsd of strain TA441 did not show significant growth on epiandrosterone, which has a 3β-hydroxyl group and 17-ketone group, while the gene-disrupted mutant did degrade testosterone, which has a 17β-hydroxyl group and 3-ketone group, though the growth was noticeably slower than that of the strain TA441 control [accepted, Horinouchi 2010]. These results suggested that the main role of the 3β,17β-hsd in TA441 cells is 3β-dehydrogenation and that there is at least one more dehydrogenase which acts on the 17β-hydroxyl group. Dehydrogenation/hydrogenation at the C-17 position is thought to be a reversible reaction because the major intermediate compounds isolated so far from TA441 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* may have more than one enzyme with 17β-dehydrogenating/hydrogenating activity to deal with intermediate compounds having considerable structural differences.

2-2. 3α-Dehydrogenation and negative regulation of 3α-dehydrogenation

Cholic acid and the derivatives, the main integrants of bile acid, and steroidal antibiotic fusidic acid are known for their anti-bacterial effect, but some steroid degrading bacteria including *C. testosteroni* are resistant with the ability of utilizing these compounds [38]. In contrast to testosterone, they have a α-oriented-hydroxyl

group at C-3 position. In steroid degradation by *C. testosteroni,* α-oriente-hydroxyl group at C-3 position also has to be converted to a ketone group before introduction of double bonds in the A-ring (Figure 4.).

3α-Dehydrogenase (3α-hsd) was purified in 1965 [39]. Then cloning of 3α-hsd gene using an antibody for 3α-hsd was reported in 1995 [40], but the deduced amino acid sequence at the N-terminal end showed c.a. 35% identity to ribosomal protein L10/P0 and the deduced amino acid sequence of the rest of the putative gene showed c.a. 50% identity to ribosomal protein L7/L12.

Another gene encoding 158 aa-putative 3α-hsd was cloned from strain ATCC11996 in 1998 [23, 41]. The deduced amino acid sequence of this 3α-hsd showed considerably high homology to many short-chain dehydrogenase/reductase (SDR) and the motives of 3α-hsd are conserved. The sequence of N-terminal of this 3α-hsd exactly matches to that of the enzyme purified directly from the *C. testosteroni* cells and 3-ketosteroid-Δ4(5)-isomerase (ksi) gene was found in the just downstream DNA region, both being included in steroid degradation hot spot of *C. testosteroni.*

3α-Hsd is induced by LuxR family positive regulator of *C. testosteroni* while genes encoding repressor of 3α-hsd, named RepA and RepB in ATCC11996, are found on the complementary strand of 3α-hsd and ksi (Figure 5.) [42]. In the absence of inducing steroids, RepA blocks transcription of 3α-hsd (named *hsdA* in ATCC11996) by preventing RNA polymerase to bind the promoter region of 3α-hsd gene. RepB blocks translation of the 3α-hsd/CR mRNA. In the presence of appropriate steroid compounds, the compounds both bind to RepA and RepB and release these proteins from the RNA polymelase and of the 3α-hsd/CR mRNA, respectively [43]. RepB of ATCC11996 is

reported as a protein consists from GNAT family acetyltransferase. Homology search

using deduced amino acid sequence of the putative RepB of strain TA441 and CNB-2

indicated that they are fusion proteins consisting both with GNAT family

acetyltransferase and an elongation factor, but whether fusion protein or not will not

affect much to the function.

2-3. Δ5,3-Ketosteroid isomeration

In mammals, transfer of a double bond at Δ5 to Δ4 is catalyzed by

3β-hydroxy-Δ5-steroid dehydrogenase at the same time as the dehydroxylation of

3β-hydroxyl group to ketone group, while in *C. testosteroni*, Δ5,3-ketosteroid isomerase

(ksi) just transfers a double bond at Δ5 of 3-ketosteroid to Δ4 (Figure 6). Ksi of *C.*

testosteroni has been under intensive studies since 1950s because of its interesting

mechanism and extraordinarily high catalytic efficiency [44]. Partial crystallization of *C.*

testosteroni ksi was performed in 1955 [19] and then complete crystallization and

analysis was reported in 1960s [45-47]. The amino acid sequence was determined in

1971 and the gene encoding ksi was cloned in 1987 [24, 25]. Complete crystal structure

was revealed in 1999 [48], and recent study indicated Tyr14, Asp38 and Asp99 or the

regions around them are key active site residues in ksi and Tyr14 is also significant as

the backbone of ksi [49].

A Δ5,3-ketosteroid isomerase-disrupted mutant of strain TA441 can grow on

dehydroepiandrosterone, which has a double bond at Δ5, but cannot grow on

epiandrosterone, which lacks a double bond at Δ5, indicating that *C. testosteroni* ksi is

responsible for transfer of the double bond from Δ5 to Δ4 and transfer of the double

bond by hydrogenation at Δ5 and following dehydrogenation at Δ4 is not possible [37].

2-4. Degradation of the side chain at C-17 position of cholic acid

C. testosteroni is unable to degrade majority of the steroid compounds with a large

side chain at C-17 position such as cholesterols and plant sterols [37], but effectively

degrades cholic acid and the derivatives. Cholic acid and the derivatives are not only

known for physiological activities on animals and anti-bacterial activity as the main

integrants of bile acid [50-59] but also other various functions that are expected to be

applied to medicals and their materials. Furthermore, they are reported to induce

defensive effects in plants [60, 61].

Analysis of degradation pathway of the side chain at C-17 position of cholid acid in *C.

teststeroni* is still in progress, but intermediate compounds identified so far from *C.

teststeroni* incubated with cholic acid suggested that the degradation pathway is similar

to those of *Pseutomonas* sp. NCIB 10590 and ATCC 31752 [62, 63] (manuscript on *C.

teststeroni* is in preparation). Degradation of the side chain at C-17 position starts with

removal of carbons at C-24 and C-23 position by β-oxidation followed by oxidative

removal of three carbons at C20, C21, and C22 position as a 2-oxo-propionic acid or the

derivative, resulting in production of a ketone group at C-17 position (Figure 7.)

Although degradation genes for the side chain at C-17 position of cholic acid in *C.

testosteroni* are yet to be isolated, a putative gene whose deduced amino acid sequence

shows around 70% identity to acyl-CoA-dehydrogenase CaiA of *Pseudomonas* sp. strain

Chol1 [64] was found in the hot spot of steroid degradation genes (indicated with bold

letters in Table 1). CaiA is an acyl CoA-dehydrogenase for 7α,12α

-dihydroxy-3-oxo-pregna-1,4-diene-20-carboxylate CoA-ester, a CoA-ester intermediate compound in colic acid degradation with two carbons at C-23 and C-24 position eliminated from cholic acid probably by β-oxidation. Moreover, a number of putative β-oxidation genes were found in the neighboring DNA region of the putative CaiA gene, suggesting that genes involved in degradation of the side chain at C-17 position locate in surrounding DNA region.

2-5. Inversion of an α-oriente-hydroxyl group to a β-oriente-hydroxyl group at C-12 position of cholic acid

Cholic acid has α-oriented-hydroxyl groups at C-7 position and C-12 position. After the degradation of the side chain at C-17 position to a ketone group, inversion of the α-oriented-hydroxyl group at C-12 position to the β-oriented-hydroxyl group is indispensable for aromatization of A-ring [28, 65]. The detailed mechanism is not clear yet, but C-9α position may be sterically-hindered from binding of the 9α-hydroxylase by α-oriented-hydroxyl group at C-12 position. The side chain at C-17 position of cholic acid is degraded to a ketone group to produce an intermediate compound 7α,12α-dihydroxyandrosta-1,4-diene-3,17-dione, which is converted to 7α-hydroxyandrosta-1,4-diene-3,12,17-trione by a dehydrogenase SteA, followed by converted to 7α,12β-dihydroxyandrosta-1,4-diene-3,17-dione by a hydrogenase SteB (Figure 8.). SteA is able to convert 7α-hydroxyandrosta-1,4-diene-3,12,17-trione to 7α,12β-dihydroxyandrosta-1,4-diene-3,17-dione a little, but the transformation of 7α,12α -dihydroxyandrosta-1,4-diene-3,17-dione to 7α,12β-dihydroxyandrosta-1,4-diene-3,17-dione is carried out far more effectively when

both SteA and SteB are involved together.

SteA and SteB are encoded in the DNA region upstream of a *meta*-cleavage enzyme gene *tesB* for cleavage of the aromatized A-ring (Figure 9.). These genes form a steroid degradation cluster, *steA,B,* ORF6,7, *tesB*, and the following ORFs, most of which are involved in B,C,D-ring degradation. Function of ORF6 and 7 is not clear, but they are though to be involved in steroid degradation.

Most of the α-oriented-hydroxyl group at C-7 still remains after the hydrolysis [28].

2-6. Aromatization of the A-ring accompanied by the cleavage of the B-ring, and the following degradation of the aromatized A-ring (steroid degradation gene cluster I)

In bacterial steroid degradation via aromatization of the A-ring, a ketone group at C-3 position and double bonds at Δ1 and Δ4 are indispensable for the aromatization of the A-ring (Figure 10). Δ1-Dehydrogenase (Δ1-DH) gene was cloned from *C. testosteroni* strain ATCC17410 in 1991 [26], and Δ4-dehydrogenase (Δ4-DH) gene was cloned from the same strain in 1996 [27]. Similar Δ1-DH (TesH) and Δ4-DH (TesI) genes were identified in strain TA441 with genes involved in A-ring degradation to reveal that Δ1-DH and Δ4-DH are adjacent each other and are contained in the same steroid degradation gene cluster, ORF18, ORF17, and *tesIHA2A1DEFG*. Most of the genes in this cluster are involved in aromatization and following degradation of the A-ring in steroid degradation [14] (Figure 11).

Testosterone degradation is initiated by dehydrogenation of the 17β-hydroxyl group to ketone group to produce 4-androstene-3, 17-dione (4-AD), which then undergoes

Δ1-dehydrogenation to 1,4-androstadiene-3, 17-dione (ADD). Following

9α-hydroxylation results in automatic cleavage of the B-ring and aromatization of the

A-ring to produce 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA).

ORF17 (Figure 11), which locates just downstream DNA region, is considered to encode

the reductase component of 9α-hydroxylase, because an ORF17-disrupted mutant

accumulates ADD when incubated with testosterone and deduced amino acid sequence

of OR17 shows more than 90% identity to flavodoxin reductase family 1 proteins though

the gene encoding oxygenase component was not found in this cluster.

   C-4 of 3-HSA is hydroxylated by TesA1A2 to yield

3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-HSA), which is

followed by cleavage between C-4 and C-5 by *meta*-cleavage enzyme (TesB in strain

TA441). Only the *meta*-cleavage gene is in another main steroid degradation gene cluster

(section 2-7). TesA1 shows the maximum over 90% amino acid sequence identity to

hydroxylases and TesA2 shows significant amino acid sequence identity to many

acyl-CoA dehydrogenase type 2-like proteins. Considered from the homology search

results, hydroxylation at C-4 position could be possible only with TesA1, but

gene-disruption experiments showed that both TesA1 and TesA2 are responsible for

hydroxylation at C-4 position [16].

   3,4-HSA is converted to

4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandorosta-1(10),2-dien-4-oic acid (4,9-DSHA)

by the *meta*-cleavage enzyme, followed by hydrolysis to produce

2-hydroxyhexa-2,4-dienoic acid (A-ring) and

9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (B,C,D-ring) by the hydrolase

TesD. 2-Hydroxyhexa-2,4-dienoic acid is degraded by TesEGF with a process similar to bacterial degradation of aromatic compounds such as biphenyl (Figure. 10). TesB,D,EFG, involved in the aromatized A-ring degradation, also shows 40-80% amino acid identity to corresponding enzymes in bacterial aromatic compound degradation, but the induction experiments showed that these enzymes are exclusively for steroid degradation.

Another product of hydrolysis of 4,9-DSHA, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (B,C,D-ring), is accumulated by ORF18-disrupted mutant incubated with testosterone and the homology search indicated ORF18-encoded enzyme to be CoA-transferase, suggesting that the degradation of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid is initiated by addition of CoA by ORF18-encoded CoA-transferase [28].

ORF18,17,*tesIHA2A1DEFG* and the downstream DNA region of *tesG* are in more than 100kb steroid degradation hot spot (table 1), while putative genes in the downstream DNA region of ORF18 are not considered to be involved in steroid degradation because of the homology and little influence of the gene-disruption on steroid degradation [28].

2-7. β-oxidation of B,C,D-rings and positive regulator of steroid degradation genes (steroid degradation gene cluster II)

Degradation of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid starts with addition of CoA by ORF18-encoded enzyme. The detail of further degradation is not revealed yet, but the analysis of intermediate compounds accumulated by gene-disrupted

mutants of strain TA441 suggested it to be mainly β-oxidation involving ORF1-33 on another main steroid degradation gene cluster *steA to tesR* (Figure 9 and table 1; *steA,B*,ORF7,6, *tesB,* ORF1-5, 21-23, 27, 28, 30-33, and *tesR*; *tesR* in strain TA441 and *teiR* in strain ATCC11996) (manuscript in preparation). Ongoing studies imply that degradation of C,D-rings of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid in *C. testosteroni* has significant difference to the proposed degradation pathway in *N. restrictus* [66], while degradation of testosterone to 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid are almost identical both in *C. testosteroni* and *N. restrictus.* Most of the ORFs1 to 33 are considered to be involved in 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid degradation because the gene-disrupted mutant accumulate putative intermediate compounds. ORF25 and ORF26 are not considered to be involved in the degradation due to the high homology of the deduced amino acid sequences to 6-aminohexanoate-cyclic-dimer hydrolases and the negligible influence of the gene-disruption on steroid degradation.

The gene which locates at the very end of this steroid degradation gene cluster, *tesR* in strain TA441 and *teiR* in strain ATCC11996, encodes transcriptional regulator indispensable for induction of most of the identified steroid degradation genes in *C. testosteroni* [17, 67-69]. This transcriptional regulator is a LuxR family protein and possesses helix-turn-helix motif at the C-terminal to bind DNA. It is an activator which induces 3β,17β-hsd, 3α-hsd, ksi, TesA2 to ORF18-encoded enzyme, TesA1 to TesG, SteA to ORF6-encoded enzyme, the *meta*-cleavage enzyme and the ORFs in the downstream DNA region [17, 67] when *C. testosteroni* strains are incubated with degradable steroids. Detail of induction mechanism and the induction substrate is not

clear, but at least steroidal four rings are suggested to be indispensable for induction of these steroid degradation genes because steroid degradation genes are not induced with 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, an intermediate compound with cleaved B-ring (unpublished data).

Homology search for putative steroid degradation genes in the genomic sequence data on the web using deduced amino acid sequences of *tesB* to ORF33 revealed the presence of putative steroid degradation gene clusters similar to *tesB* to ORF33 in bacteria of different genera. Among a number of candidate bacteria, ten strains of seven genera were selected for comparison (*C. testosteroni* CNB-1 [29], *C. testosteroni* KF1 (draft sequence, accession: NZ_AAUJ02000001) [70, 71], *Cupriavidu necator* (formally *R. eutropha*) JMP134 (accession: NC_007347) [72, 73], *Ralstonia eutropha* H16 (accession: NC_008314) [74], *Burkholderia cenocepacia* J2315 (accession: NC_011001) [75], *Burkholderia* sp. 383 (accession: NC_007511) [76], *Cu. taiwanensis* LMG 19424 (accession: NC_010530) [77], *Shewanella pealeana* ATCC700345 (accession: NC_009901), *S. halifaxensis* HAW-EB4 (accession: NC_010334) [78, 79].), and *Pseudoalteromonas haloplanktis* TAC125 (accession: NC_007481) [80, 81]), whose deduced amino acid sequence of putative steroid degradation genes correspond to *tesB* to ORF33 are showing higher homology than others (Figure 12.). All the selected strains are Gram-negative bacteria. Putative *meta*-cleavage enzyme gene and putative ORFs correspond to ORF1 to ORF33 except for ORF25 and ORF26 of strain TA441 are found in all the candidate steroid-degradation gene clusters of these bacteria, while genes not necessary for degradation of basic steroidal four rings, putative 6-aminohexanoate-cyclic-dimer hydrolase gene (ORF25 and ORF26), the regulator gene

(*tesR*/*teiR*), and the genes correspond to *steA*-ORF6, are found only in *C. testosteroni* strains but are often missing in bacteria of other genera. In seven strains out of ten, the gene order of genes correspond to *tesB* to ORF33 are quite similar to those of TA441, while those in *S. pealeana* ATCC700345, *S. halifaxensis* HAW-EB4, and *P. haloplanktis* TAC125 are very different, implying the considerable variety of bacterial steroid degradation genes even among Gram-negative bacteria. Between steroid-degrading Gram-positive bacteria and Gram-negative bacteria, corresponding steroid degradation enzymes share homology on some level, but gene localization is completely different [82-86].

Steroid degradation pathway and the genes in *C. testosteroni* are thought to be a common type of steroid degradation in Gram-negative bacteria. Further studies are expected for more information on bacterial steroid degradation.

3. References

[1]   R.M. Dodson, R.D. Muir, Microbiological transformations. II. Microbiological aromatization of steroids, J. Am. Chem. Soc. 80 (1958) 5004-5005.

[2]   R.M. Dodson, R.D. Muir, Microbiological transformations. III. The hydroxylation of steroids at C-9, J. Am. Chem. Soc. 80 (1958) 6148.

[3]   D.A. Shaw, L.F. Borkenhagen, P. Talalay, Enzymatic oxidation of steroids by cell-free extracts of *Pseudomonas testosteroni*: isolation of cleavage products of ring A, Proc. Natl. Acad. Sci. U.S.A. 54 (1965) 837-844.

[4]   R.M. Dodson, R.D. Muir, Microbiological transformations. IV. The microbiological aromatization of steroids., J. Am. Chem. Soc. 83 (1961) 4627-4631.

[5]   C.J. Sih, Mechanisms of steroid oxidation by microorganisms, Biochim. Biophys. Acta. 62 (1962) 541-547.

[6]   C.J. Sih, K.C. Wang, Mechanisms of steroid oxidation by microorganisms. II. Isolation and characterization of 3aα-*H*-4α-[3'-propionic acid]-7aβ-methylhezahydro-1,5-indanedione, J. Am. Chem. Soc. 85 (1963) 2135-2137.

[7]   K.C. Wang, C.J. Sih, Mechanisms of Steroid Oxidation by Microorganisms. Iv. Seco Intermediates, Biochemistry 2 (1963) 1238-1243.

[8]   C.J. Sih, S.S. Lee, Y.K. Tsong, K.C. Wang, 3,4-Diydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione. An intermediate in the microbiological degradation of ring A of androst-4-ene-3,17-dione, J. Am. Chem. Soc. 87 (1965) 1385-1386.

[9]   C.J. Sih, S.S. Lee, Y.K. Tsong, K.C. Wang, Mechanisms of steroid oxidation by microorganisms. VIII. 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, an intermediate in the microbiological degradation of ring A of androst-4-ene-3,17-dione, J. Biol. Chem. 241 (1966) 540-550.

[10]   A.W. Coulter, P. Talalay, Studies on the microbial degradation of steroid ring A, J. Biol. Chem. 243 (1968) 3238-3247.

[11]   S. Hayakawa, S. Hashimoto, (+)-(5R)-Methyl-4-oxo-octane-1,8-dioic Acid, microbiological degradation product from rings C and D of cholic acid, Biochem. J. 112 (1969) 127-128.

[12]   R.A. Leppik, R.J. Park, M.G. Smith, Aerobic catabolism of bile acids, Appl. Environ. Microbiol. 44 (1982) 771-776.

[13]   M. Horinouchi, T. Yamamoto, K. Taguchi, H. Arai, T. Kudo, *Meta*-cleavage enzyme gene *tesB* is necessary for testosterone degradation in *Comamonas testosteroni* TA441, Microbiology 147 (2001) 3367-3375.

[14]   M. Horinouchi, T. Hayashi, T. Yamamoto, T. Kudo, A new bacterial steroid degradation gene cluster which consists of aromatic compound degradation genes for seco-steroids and 3-ketosteroid dehydrogenase genes in *Comamonas testosteroni* TA441, Appl. Environ. Microbiol. 69 (2003) 4421-4430.

[15]   M. Horinouchi, T. Hayashi, H. Koshino, T. Yamamoto, T. Kudo, Gene encoding the hydrolase for the product of the *meta*-cleavage reaction in testosterone degradation by *Comamonas testosteroni*, Appl. Environ. Microbiol. 69 (2003) 2139-2152.

[16]   M. Horinouchi, T. Hayashi, T. Kudo, 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, J. Steroid Biochem. Mol. Biol. 92 (2004) 143-154.

[17]   M. Horinouchi, T. Kurita, T. Yamamoto, E. Hatori, T. Hayashi, T. Kudo, Steroid degradation gene cluster of *Comamonas testosteroni* consisting of 18 putative genes from *meta*-cleavage enzyme gene *tesB* to regulator gene *tesR*, Biochem. Biophys. Res. Commun. 324 (2004) 597-604.

[18]   M. Horinouchi, T. Hayashi, T. Kurita, T. Kudo, Identification of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid, 4-hydroxy-2-oxohexanoic acid, and 2-hydroxyhexa-2,4-dienoic acid and related enzymes involved in testosterone degradation in *Comamonas testosteroni* TA441, Appl. Environ. Microbiol. 71 (2005) 5275-5281.

[19]   P. Talalay, V.S. Wang, Enzymic isomerization of delta5-3-ketosteroids, Biochim Biophys Acta 18 (1955) 300-301.

[20]   P. Talalay, P.I. Marcus, Enzymatic formation of 3 alpha-hydroxysteroids, Nature 173 (1954) 1189-1190.

[21]   P. Talalay, M.M. Dobson, D.F. Tapley, Oxidative degradation of testosterone by adaptive enzymes, Nature 170 (1952) 620-621.

[22]   S. Genti-Raimondi, M.E. Tolmasky, L.C. Patrito, A. Flury, L.A. Actis, Molecular cloning and expression of the β-hydroxysteroid dehydrogenase gene from *Pseudomonas testosteroni*, Gene 105 (1991) 43-49.

[23]   E. Mobus, E. Maser, Molecular cloning, overexpression, and characterization of steroid-inducible 3α-hydroxysteroid dehydrogenase/carbonyl reductase from

Comamonas testosteroni. A novel member of the short-chain dehydrogenase/reductase superfamily, J. Biol. Chem. 273 (1998) 30888-30896.

[24]   A. Kuliopulos, D. Shortle, P. Talalay, Isolation and sequencing of the gene encoding Δ5-3-ketosteroid isomerase of *Pseudomonas testosteroni*: overexpression of the protein, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 8893-8897.

[25]   K.Y. Choi, W.F. Benisek, Cloning of the gene for Δ5-3-ketosteroid isomerase from *Pseudomonas testosteroni*, Gene 58 (1987) 257-264.

[26]   P. Plesiat, M. Grandguillot, S. Harayama, S. Vragar, Y. Michel-Briand, Cloning, sequencing, and expression of the *Pseudomonas testosteroni* gene encoding 3-oxosteroid Δ1-dehydrogenase, J. Bacteriol. 173 (1991) 7219-7227.

[27]   C. Florin, T. Kohler, M. Grandguillot, P. Plesiat, *Comamonas testosteroni* 3-ketosteroid-Δ4(5α)-dehydrogenase: gene and protein characterization, J. Bacteriol. 178 (1996), 3322-3330.

[28]   M. Horinouchi, T. Hayashi, H. Koshino, T. Kudo, ORF18-disrupted mutant of *Comamonas testosteroni* TA441 accumulates significant amounts of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid and its derivatives after incubation with steroids, J. Steroid Biochem. Mol. Biol. 101 (2006), 78-84.

[29]   Y.F. Ma, Y. Zhang, J.Y. Zhang, D.W. Chen, Y. Zhu, H. Zheng, S.Y. Wang, C.Y. Jiang, G.P. Zhao, S.J. Liu, The complete genome of Comamonas testosteroni reveals its genetic adaptations to changing environments, Appl. Environ. Microbiol. 75 (2009) 6812-6819.

[30]   R.M. Schultz, E.V. Groman, L.L. Engel, 3(17)β-Hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*. A convenient purification and demonstration of multiple molecular forms, J. Biol. Chem. 252 (1977) 3775-3783.

[31]   P. Minard, M.D. Legoy, D. Thomas, 3β, 17β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*. Kinetic evidence for the bifunctional activity at a common catalytic site, FEBS Lett. 188 (1985) 85-90..

[32]   S.J. Yin, N. Vagelopoulos, G. Lundquist, H. Joernvall, Pseudomonas 3β-hydroxysteroid dehydrogenase. Primary structure and relationships to other steroid dehydrogenases, Eur. J. Biochem. 197 (1991) 359-365.

[33]   J. Benach, C. Filling, U.C.T. Oppermann, P. Roversi, G. Bricogne, K.D. Berndt, H. Jornvall, R. Ladenstein, Structure of bacterial 3β/17β-hydroxysteroid dehydrogenase at 1.2 A resolution: a model for multiple steroid recognition, Biochemistry 41 (2002) 14659-14668.

[34]   J.E. Cabrera, G. Panzetta-Dutari, J.L. Pruneda, S. Genti-Raimondi, A New *Comamonas testosteroni* Steroid-inducible Gene: Cloning and Sequence Analysis, J. Steroid Bioche. Mol. Biol. 63 (1997) 91-98.

[35]   J.E. Cabrera, J.L. Pruneda Paz, S. Genti-Raimondi, Steroid-inducible transcription of the 3β/17β-hydroxysteroid dehydrogenase gene (3β/17β-hsd) in *Comamonas testosteroni*, J. Steroid Bioche. Mol. Biol. 73 (2000) 147-152.

[36]   J.L. Pruneda-Paz, M. Linares, J.E. Cabrera, S. Genti-Raimondi, Identification of a novel steroid inducible gene associated with the beta *hsd* locus of *Comamonas testosteroni*, J. Steroid Biochem. Mol. Biol. 88 (2004) 91-100.

[37]   M. Horinouchi, T. Kurita, T. Hayashia, T. Kudo, Steroid degradation genes in *Comamonas testosteroni* TA441: Isolation of genes encoding a Δ4(5)-isomerase and 3α- and 3β-dehydrogenases and evidence for a 100 kb steroid degradation gene hot spot, J. Steroid Biochem. Mol. Biol. 122 (2010) 253–263.

[38]   U.C.T. Oppermann, I. Belai, E. Maser, Antibiotic resistance and enhanced insecticide catabolism as consequences of steroid induction in the Gram-negative bacterium *Comamonas testosteroni*, J. Steroid Biochem. Mol. Biol. 58 (1996) 217-223.

[39]   P.I. Marcus, P. Talalay, Induction and purification of alpha- and beta-hydroxysteroid dehydrogenases, J Biol Chem 218 (1956) 661-674.

[40]   J.H. Abalain, S. Di Stefano, M.L. Abalain-Colloc, H.H. Floch, Cloning, sequencing and expression of *Pseudomonas testosteroni* gene encoding 3 alpha-hydroxysteroid dehydrogenase, J. Steroid Biochem. Mol. Biol. 55 (1995) 233-238.

[41]   U.C T. Oppermann, E. Maser, Characterization of a 3α-hydroxysteroid dehydrogenase/carbonyl reductase from the gram-negative bacterium *Comamonas testosteroni*, Eur. J. Biochem. 241 (1996), 744-749.

[42]   G. Xiong and E. Maser, Regulation of the steroid-inducible 3α-hydroxysteroid dehydrogenase/carbonyl reductase gene in *Comamonas testosteroni*, J. Biol. Chem. 276 (2001), 9961-9970.

[43]   G. Xiong, H.J. Martin, E. Maser, Identification and characterization of a novel translational repressor of the steroid-inducible 3α-hydroxysteroid dehydrogenase/carbonyl reductase gene in *Comamonas testosteroni*, J. Biol. Chem. 278 (2003), 47400-47407.

[44]   F.H. Batzold, A.M. Benson, D.F. Covey, C.H. Robinson, P. Talalay, The delta 5-3-ketosteroid isomerase reaction: catalytic mechanism, specificity and inhibition, Adv. Enzyme. Regul. 14 (1976) 243-267.

[45]   F.S. Kawahara, P. Talalay, Crystalline Delta 5-3-ketosteroid isomerase, J. Biol. Chem. 235 (1960) 1-2.

[46]   F.S. Kawahara, S.F. Wang, P. Talalay, The preparation and properties of crystalline delta5-3-ketosteroid isomerase, J. Biol. Chem. 237 (1962) 1500-1506.

[47]   P. Talalay, J. Boyer, Preparation of crystalline Δ-5-3-ketosteroid isomerase from *Pseudomonas testosteroni*, Biochim. Biophys. Acta. 105 (1965) 389-392.

[48]   H.S. Cho, N.C. Ha, G. Choi, H.J. Kim, D. Lee, K.S. Oh, K.S. Kim, W. Lee, K.Y. Choi, B.H. Oh, Crystal structure of Δ(5)-3-ketosteroid isomerase from *Pseudomonas testosteroni* in complex with equilenin settles the correct hydrogen bonding scheme for transition state stabilization, J. Biol. Chem. 274 (1999) 32863-32868.

[49]   H.J. Lee, Y.J. Yoon, S. Jang do, C. Kim, H.J. Cha, B.H. Hong, K.Y. Choi, H.C. Lee, 15N NMR relaxation studies of Y14F mutant of ketosteroid isomerase: the influence of mutation on backbone mobility, J. Biochem. 144 (2008) 159-166.

[50]   A.F. Hofmann, Bile acids, diarrhea, and antibiotics: data, speculation, and a unifying hypothesis, J. Infect. Dis. 135 Suppl. (1977) S126-132.

[51]   M. Podda, C. Ghezzi, P.M. Battezzati, E. Bertolini, A. Crosignani, M.L. Petroni, M. Zuin, Ursodeoxycholic acid for chronic liver diseases, J. Clin. Gastroenterol. 10 Suppl. 2 (1988) S25-31.

[52]   M. Podda, M. Zuin, P.M. Battezzati, C. Ghezzi, C. de Fazio, M.L. Dioguardi, Efficacy and safety of a combination of chenodeoxycholic acid and ursodeoxycholic acid for gallstone dissolution: a comparison with ursodeoxycholic acid alone, Gastroenterology 96 (1989) 222-229.

[53]   M. Podda, C. Ghezzi, P.M. Battezzati, A. Crosignani, M. Zuin, A. Roda, Effects of ursodeoxycholic acid and taurine on serum liver enzymes and bile acids in chronic hepatitis, Gastroenterology 98 (1990) 1044-1050.

[54]   G. Mazzella, P. Parini, F. Bazzoli, N. Villanova, D. Festi, R. Aldini, A. Roda, A. Cipolla, C. Polimeni, D. Tonelli, et al., Ursodeoxycholic acid administration on bile acid metabolism in patients with early stages of primary biliary cirrhosis, Dig. Dis. Sci. 38 (1993) 896-902.

[55]   S. Guldutuna, M. Leuschner, N. Wunderlich, A. Nickel, S. Bhatti, K. Hubner, U. Leuschner, Cholic acid and ursodeoxycholic acid therapy in primary biliary cirrhosis. Changes in bile acid patterns and their correlation with liver function, Eur. J. Clin. Pharmacol. 45 (1993), 221-225.

[56]   U. Leuschner, Ursodeoxycholic acid therapy in primary biliary cirrhosis, Scand. J. Gastroenterol. Suppl. 204 (1994) 40-46.

[57]   E. Roda, A. Cipolla, A. Salzetta, S. Marchetto, A. Pezzoli, E. Accogli, V. Novelli, C. Polimeni, C. Cerre, G. Mazzella, Influence of ursodeoxycholic acid on biliary lipids, Scand. J. Gastroenterol. Suppl. 204 (1994) 16-18.

[58]   F.M. Konikoff, T. Gilat, Effects of fatty acid bile acid conjugates (FABACs) on biliary lithogenesis: potential consequences for non-surgical treatment of gallstones, Curr. Drug Targets Immune. Endocr. Metabol. Disord. 5 (2005) 171-175.

[59]   M. Mikov, J.P. Fawcett, K. Kuhajda, S. Kevresan, Pharmacology of bile acids and their derivatives: absorption promoters and therapeutic agents, Eur. J. Drug Metab. Pharmacokinet. 31 (2006) 237-251.

[60]   J. Koga, H. Kubota, S. Gomi, K. Umemura, M. Ohnishi, T. Kono, Cholic acid, a bile acid elicitor of hypersensitive cell death, pathogenesis-related protein synthesis, and phytoalexin accumulation in rice, Plant Physiol. 140 (2006) 1475-1483.

[61]   T. Shimizu, Y. Jikumaru, A. Okada, K. Okada, J. Koga, K. Umemura, E. Minami, N. Shibuya, M. Hasegawa, O. Kodama, H. Nojiri, H. Yamane, Effects of a bile acid elicitor, cholic acid, on the biosynthesis of diterpenoid phytoalexins in suspension-cultured rice cells, Phytochemistry 69 (2008) 973-981.

[62]   M.E. Tenneson, J.D. Baty, R.F. Bilton, A.N. Mason, The degradation of cholic acid by *Pseudomonas* sp. N.C.I.B. 10590, Biochem. J. 184 (1979) 613-618.

[63]   R.J. Park, The major neutral products of the aerobic catabolism of cattle bile by *Pseudomonas* sp. ATCC 31752, Steroids 38 (1981) 383-395.

[64]   A. Birkenmaier, J. Holert, H. Erdbrink, H.M. Moeller, A. Friemel, R. Schoenenberger, M.J. Suter, J. Klebensberger, B. Philipp, Biochemical and genetic investigation of initial reactions in aerobic degradation of the bile acid cholate in *Pseudomonas* sp. strain Chol1, J. Bacteriol. 189 (2007) 7165-7173.

[65]   M. Horinouchi, T. Hayashi, H. Koshino, M. Malon, T. Yamamoto, T. Kudo, Identification of genes involved in inversion of stereochemistry of a C-12 hydroxyl group in the catabolism of cholic acid by *Comamonas testosteroni* TA441, J. Bacteriol. 190 (2008) 5545-5554.

[66]   S.S. Lee, C.J. Sih, Mechanisms of steroid oxidation by microorganisms. XII. Metabolism of hexahydroindanpropionic acid derivatives., Biochemistry 6 (1967) 1395-1403.

[67]   J.L. Pruneda-Paz, M. Linares, J.E. Cabrera, S. Genti-Raimondi, TeiR, a

LuxR-type transcription factor required for testosterone degradation in *Comamonas testosteroni*, J. Bacteriol. 186 (2004) 1430-1437.

[68]   A. Gohler, G. Xiong, S. Paulsen, G. Trentmann, E. Maser, Testosterone-inducible regulator is a kinase that drives steroid sensing and metabolism in *Comamonas testosteroni*, J. Biol. Chem. 283 (2008) 17380-17390.

[69]   M. Linares, J.L. Pruneda-Paz, L. Reyna, S. Genti-Raimondi, Regulation of testosterone degradation in *Comamonas testosteroni*, J. Steroid Biochem. Mol. Biol. 112 (2008) 145-150.

[70]   V. Rosch, K. Denger, D. Schleheck, T.H. Smits, A.M. Cook, Different bacterial strategies to degrade taurocholate, Arch. Microbiol. 190 (2008) 11-18.

[71]   D. Schleheck, T.P. Knepper, K. Fischer, A.M. Cook, Mineralization of individual congeners of linear alkylbenzenesulfonate by defined pairs of heterotrophic bacteria, Appl. Environ. Microbiol. 70 (2004) 4053-4063.

[72]   M.A. Sanchez, B. Gonzalez, Genetic characterization of 2,4,6-trichlorophenol degradation in *Cupriavidus necator* JMP134, Appl. Environ. Microbiol. 73 (2007) 2769-2776.

[73]   .H. Leveau, J.R. van der Meer, The *tfdR* gene product can successfully take over the role of the insertion element-inactivated TfdT protein as a transcriptional activator of the *tfdCDEF* gene cluster, which encodes chlorocatechol degradation in *Ralstonia eutropha* JMP134(pJP4), J. Bacteriol. 178 (1996) 6824-6832.

[74]   A. Pohlmann, W.F. Fricke, F. Reinecke, B. Kusian, H. Liesegang, R. Cramm, T. Eitinger, C. Ewering, M. Potter, E. Schwartz, A. Strittmatter, I. Voss, G. Gottschalk, A. Steinbuchel, B. Friedrich, B. Bowien, Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16, Nat. Biotechnol. 24 (2006) 1257-1262.

[75]   M.T. Holden, H.M. Seth-Smith, L.C. Crossman, M. Sebaihia, S.D. Bentley, A.M. Cerdeno-Tarraga, N.R. Thomson, N. Bason, M.A. Quail, S. Sharp, I. Cherevach, C. Churcher, I. Goodhead, H. Hauser, N. Holroyd, K. Mungall, P. Scott, D. Walker, B. White, H. Rose, P. Iversen, D. Mil-Homens, E.P. Rocha, A.M. Fialho, A. Baldwin, C. Dowson, B.G. Barrell, J.R. Govan, P. Vandamme, C.A. Hart, E. Mahenthiralingam, J. Parkhill, The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients, J. Bacteriol. 191 (2009) 261-277.

[76]   J.H. Leveau, S. Gerards, Discovery of a bacterial gene cluster for catabolism of the plant hormone indole 3-acetic acid, FEMS Microbiol. Ecol. 65 (2008) 238-250.

[77]   W.M. Chen, C.H. Wu, E.K. James, J.S. Chang, Metal biosorption capability of

Cupriavidus taiwanensis and its effects on heavy metal removal by nodulated *Mimosa pudica*, J. Hazard Mater. 151 (2008) 364-371.

[78]   J.S. Zhao, D. Manno, J. Hawari, Regulation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolism in *Shewanella halifaxensis* HAW-EB4 by terminal electron acceptor and involvement of c-type cytochrome, Microbiology 154 (2008) 1026-1037.

[79]   J.S. Zhao, D. Manno, C. Leggiadro, D. O'Neil, J. Hawari, *Shewanella halifaxensis* sp. nov., a novel obligately respiratory and denitrifying psychrophile, Int. J. Syst. Evol. Microbiol. 56 (2006) 205-212.

[80]   R. Papa, E. Parrilli, G. Sannia, Engineered marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125: a promising micro-organism for the bioremediation of aromatic compounds, J. Appl. Microbiol. 106 (2009) 49-56.

[81]   C. Medigue, E. Krin, G. Pascal, V. Barbe, A. Bernsel, P.N. Bertin, F. Cheung, S. Cruveiller, S. D'Amico, A. Duilio, G. Fang, G. Feller, C. Ho, S. Mangenot, G. Marino, J. Nilsson, E. Parrilli, E.P. Rocha, Z. Rouy, A. Sekowska, M.L. Tutino, D. Vallenet, G. von Heijne, A. Danchin, Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125, Genome Res. 15 (2005) 1325-13235.

[82]   R. van Der Geize, G.I. Hessels, R. van Gerwen, J.W. Vrijbloed, P. van Der Meijden, L. Dijkhuizen, Targeted disruption of the *kstD* gene encoding a 3-ketosteroid $\Delta(1)$-dehydrogenase isoenzyme of *Rhodococcus erythropolis* strain SQ1, Appl. Environ. Microbiol. 66 (2000) 2029-2036.

[83]   R. van der Geize, G.I. Hessels, R. van Gerwen, P. van der Meijden, L. Dijkhuizen, Unmarked gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid $\Delta1$-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker, FEMS Microbiol. Lett. 205 (2001) 197-202.

[84]   R. van der Geize, G.I. Hessels, R. van Gerwen, P. van der Meijden, L. Dijkhuizen, Molecular and functional characterization of *kshA* and *kshB*, encoding two components of 3-ketosteroid 9α-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* strain SQ1, Mol. Microbiol. 45 (2002) 1007-1018.

[85]   R. van der Geize, G.I. Hessels, L. Dijkhuizen, Molecular and functional characterization of the *kstD2* gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid $\Delta(1)$-dehydrogenase isoenzyme, Microbiology 148 (2002) 3285-3292.

[86]   R. van der Geize, G.I. Hessels, M. Nienhuis-Kuiper, L. Dijkhuizen,

Characterization of a second *Rhodococcus erythropolis* SQ1 3-ketosteroid 9α-hydroxylase activity comprising a terminal oxygenase homologue, KshA2, active with oxygenase-reductase component KshB, Appl. Environ. Microbiol. 74 (2008) 7197-7203.

[87]   D. Skowasch, E. Mobus, E. Maser, Identification of a novel *Comamonas testosteroni* gene encoding a steroid-inducible extradiol dioxygenase, Biochem. Biophys. Res. Commun. 294 (2002) 560-566.

4. Figure legend

Figure 1.   Proposed steroid degradation pathway of *Comamonas testosteroni* TA441.

Compound names are presented (R1=H, R2=H) unless others indicated:

3-oxo-5β-cholan-24-oic acid, (I); androsta-1,4-diene-3,17-dione or the derivative

(R1=α-OH or H, R2=α-OH or H), (II); androst-4-ene-3,12,17-trione, (III);

androsta-1,4-diene-3,17-dione or the derivative (R1=β-OH or H, R2=α-OH or H), (IV);

androsta-3,17-dione, (V); androst-5-ene-3,17-dione, (VI); androsta-1,4-diene-3,17-dione

or the derivative (R1=β-OH or H, R2=α-OH or H), (VII);

9-hydroxyandrosta-1,4-diene-3,17-dione, (VIII);

3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA), (IX);

3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, (X);

4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid, (XI);

2-hydroxyhexa-2,4-dienoic acid, (XII);

9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid, (XIII);

9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid CoA ester, (XIII). Enzyme names

are: 3β,17β-hydroxysteroid dehydrogenase; 3β,17β-hsd, 3α-hydroxysteroid

dehydrogenase; 3α-hsd, Δ5,3-ketosteroid isomerase; Δ5-ksi, 12α-hydroxysteroid

dehydrogenase;12α-hsd, 12β, hydrogenase;12β-ksh, Δ1-dehydrogenase; Δ1-DH,

Δ4-dehydrogenase; Δ4-DH. Bold numbers indicate the section numbers in the text where
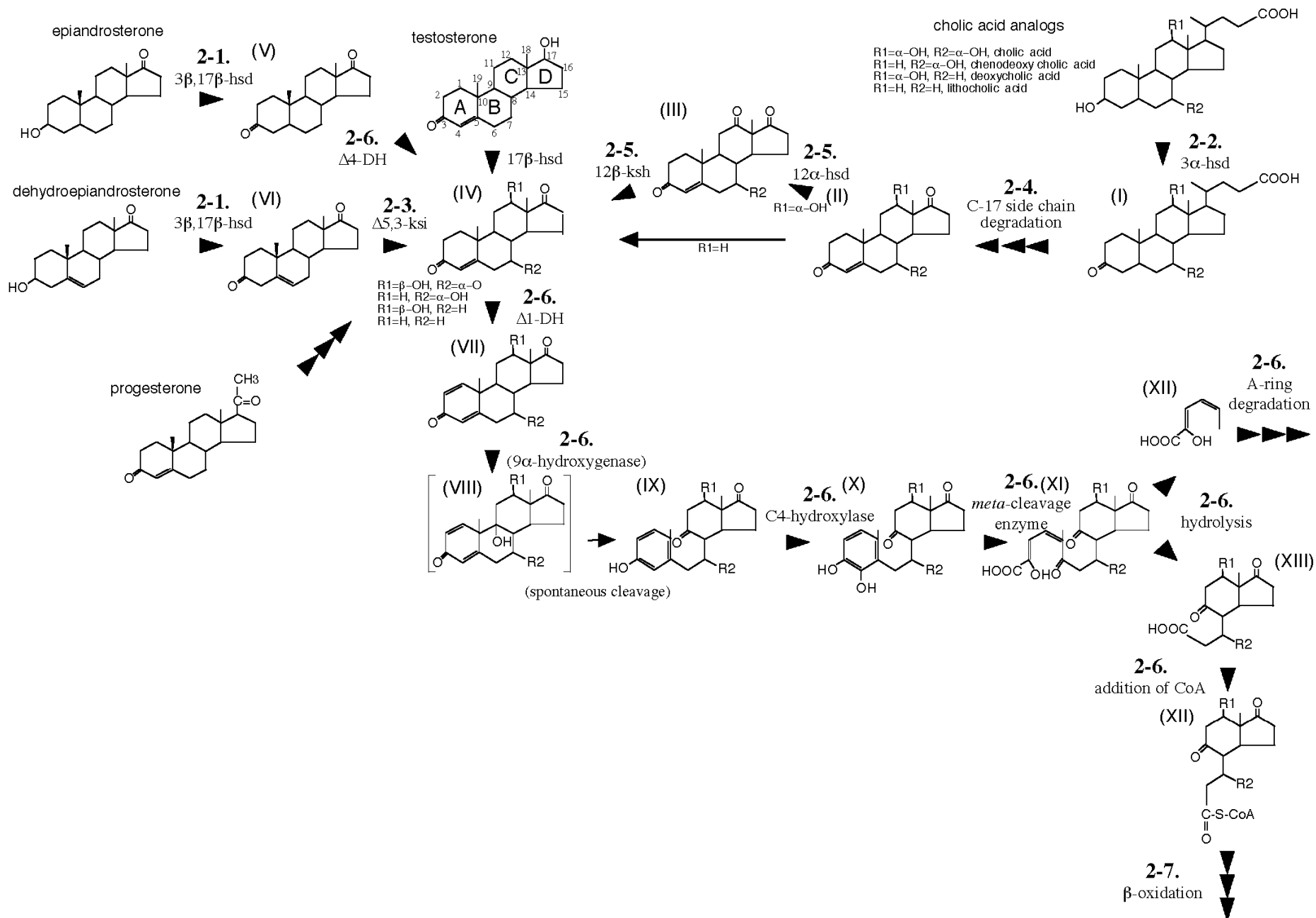
the detail is provided.

Figure 1.

epiandrosterone

2-1. (V)
3β,17β-hsd

testosterone

cholic acid analogs

R1=α–OH, R2=α–OH, cholic acid
R1=H, R2=α–OH, chenodeoxy cholic acid
R1=α–OH, R2=H, deoxycholic acid
R1=H, R2=H, lithocholic acid

2-6.
Δ4-DH

(III)

2-5.
12β-ksh

2-5.
12α-hsd
R1=α–OH (II)

2-4.
C-17 side chain
degradation

(I)

2-2.
3α-hsd

dehydroepiandrosterone

2-1. (VI)
3β,17β-hsd

2-3.
Δ5,3-ksi

(IV)

R1=β–OH, R2=α–O
R1=H, R2=α–OH
R1=β–OH, R2=H
R1=H, R2=H

2-6.
Δ1-DH

R1=H

progesterone

CH3
C=O

(VII)

2-6.
(9α-hydroxygenase)

(VIII)

(spontaneous cleavage)

(IX)

2-6.
C4-hydroxylase

(X)

2-6.(XI)
meta-cleavage
enzyme

2-6.
A-ring
degradation

(XII)

HOOC C OH

2-6.
hydrolysis

(XIII)

HOOC

2-6.
addition of CoA

(XII)

C-S-CoA
O

2-7.
β-oxidation

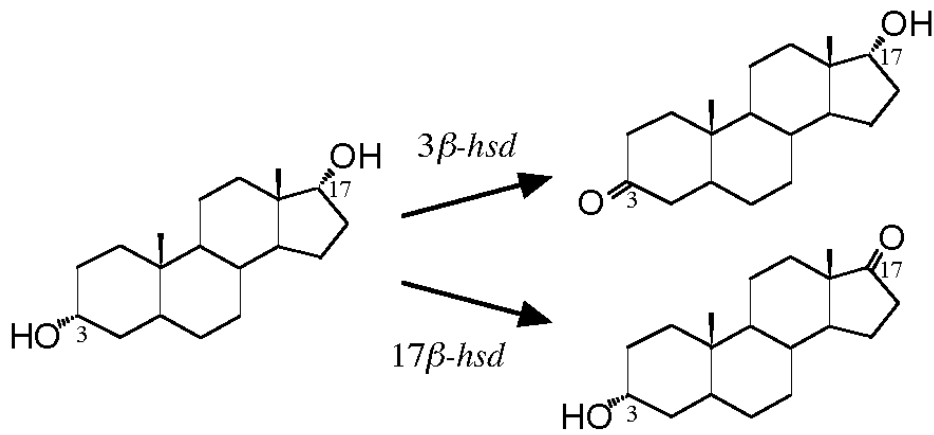**Figure 2.** Reaction of 3β,17β-hydroxysteroid-dehydrogenase (3β,17β-hsd)

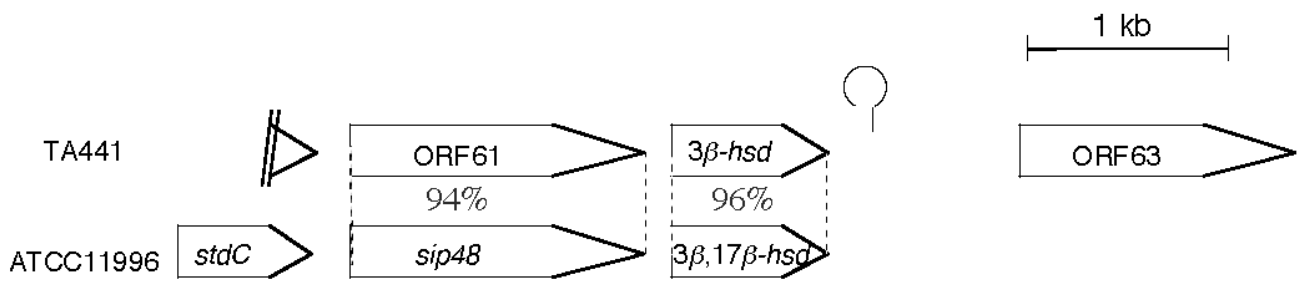**Figure 3.** 3β,17β-hydroxysteroid dehydrogenase (3β,17β-hsd) and surrounding genes

Figure 4.   Reaction of 3α-hydroxysteroid-dehydrogenase (3α-hsd)
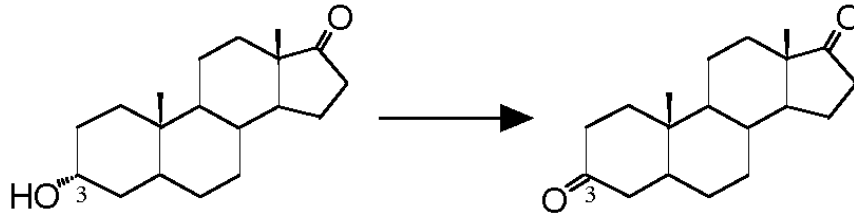

Figure 5.   3α-hydroxysteroid dehydrogenase gene (3α-hsd),

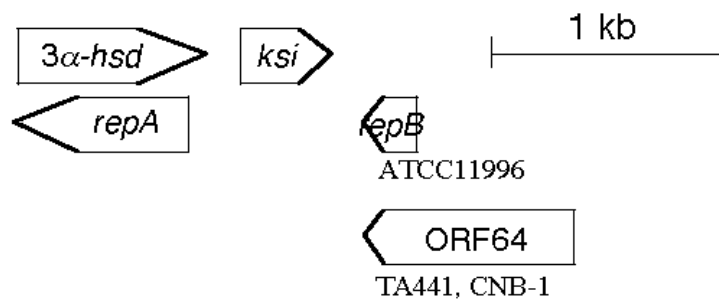Δ5,3-ketosteroid-isomerase gene (*ksi*), and repressor genes of 3α-hsd (*repA*, *repB*)

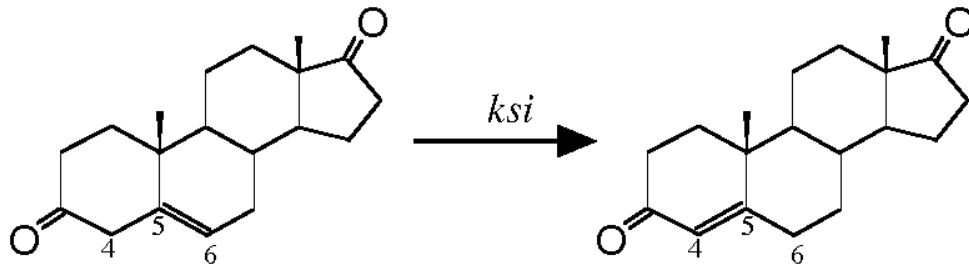**Figure 6.**   Reaction of Δ5,3-ketosteroid-isomerase (ksi)

Figure 7. Predicted degradation pathway of the side chain at C-17 position of cholic acid
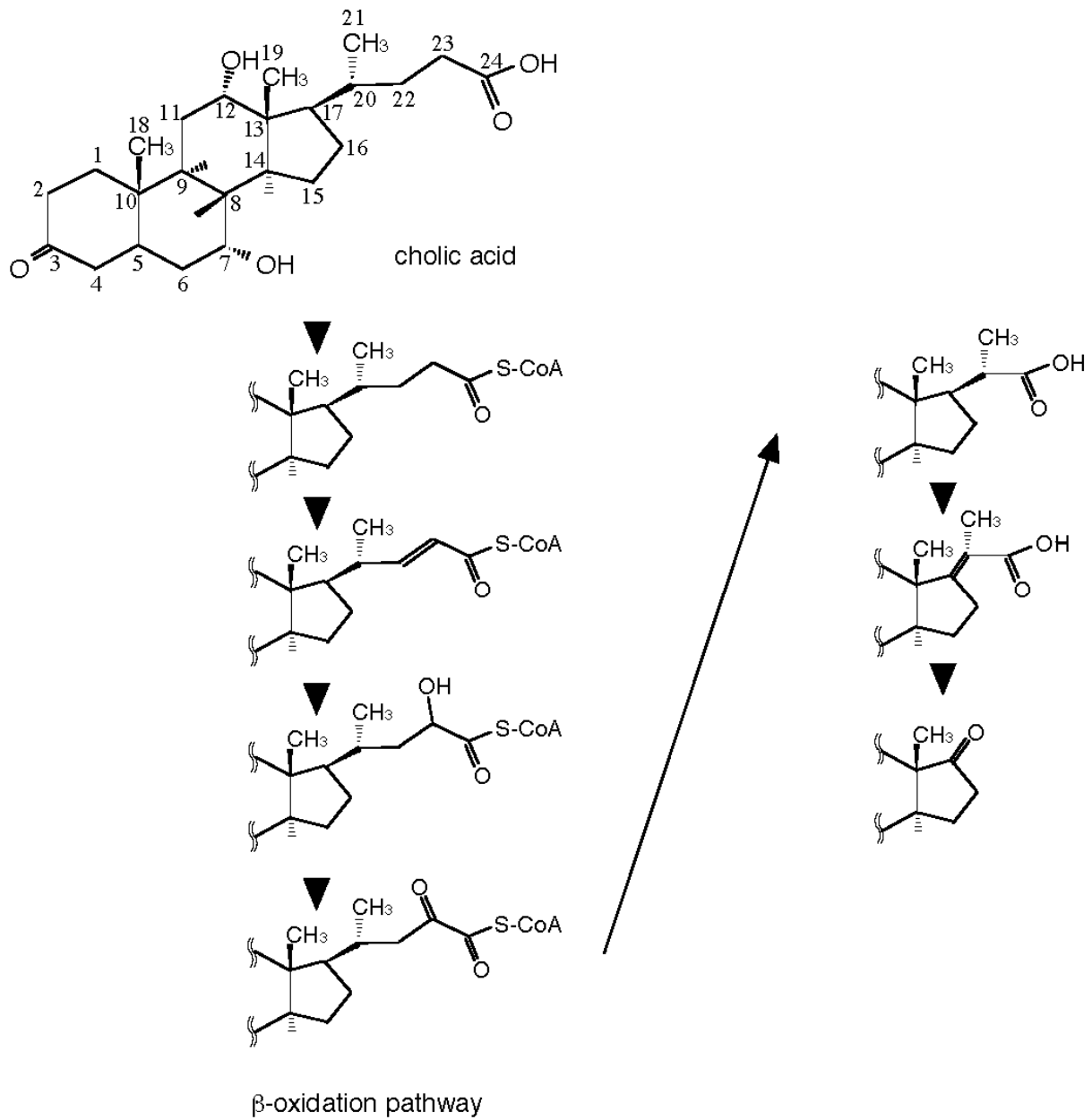

cholic acid

β-oxidation pathway

Figure 8. Chyral transformation of an *α*-oriented hydroxyl group to a *β*-oriented

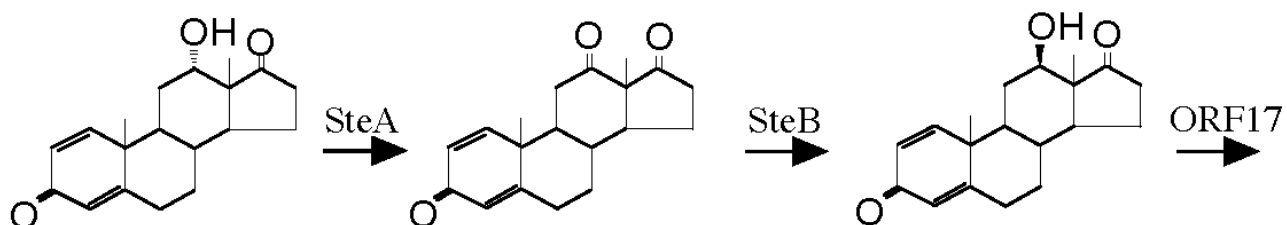hydroxyl group at C-12 position of cholic acid

Figure 9. Steroid degradation gene cluster consists of the genes mainly for aromatization, cleavage, and degradation of the A-ring

Figure 10. Aromatization, cleavage, and degradation of the A-ring in steroid
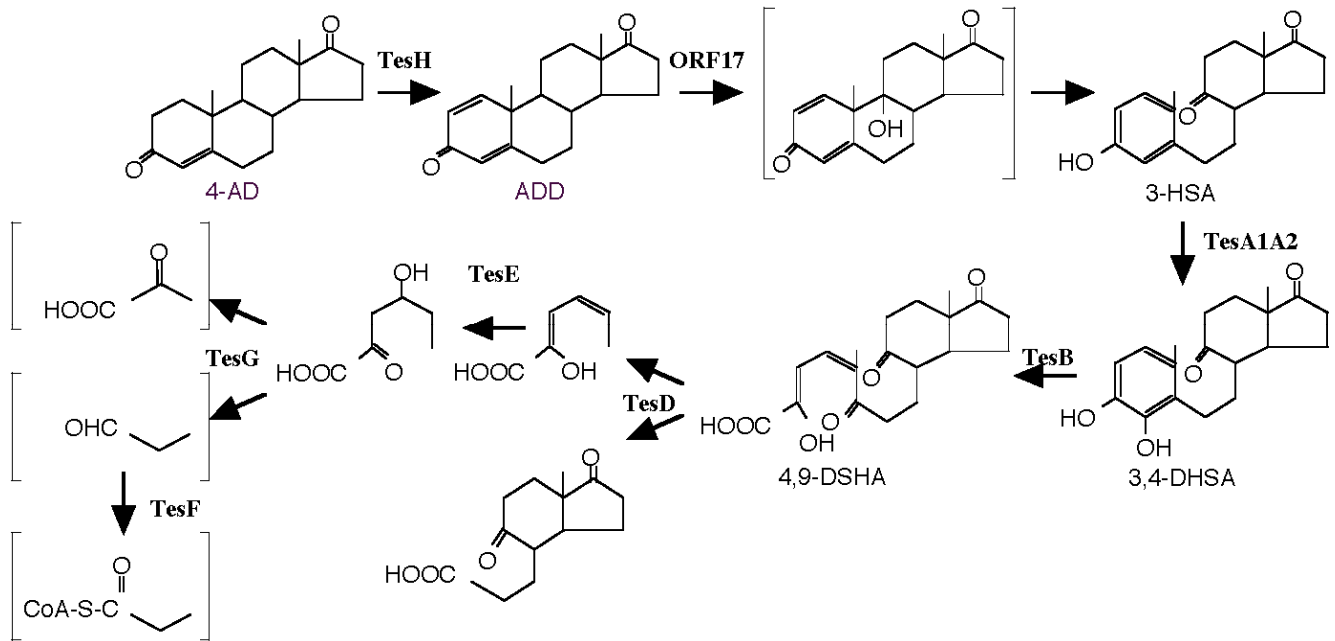
degradation pathway

Figure 11. Steroid degradation gene cluster consists of the genes mainly for
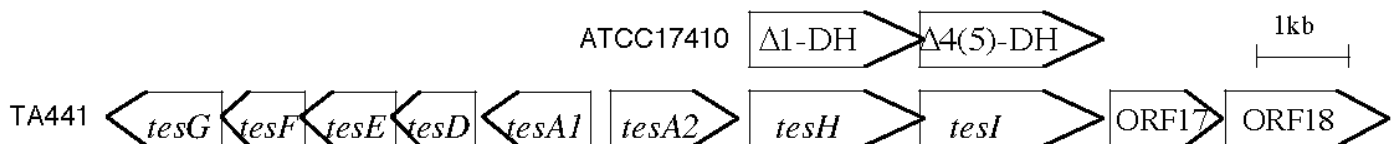
degradation of B,C,D-rings

Figure 12. Steroid degradation genes of *Comamonas testosteroni* TA441 (accession:

BAB15810) [17] with possible steroid degradation genes on the database which were

suggested by homology search using steroid degradation genes of TA441. The selected

bacteria are; *C. testosteroni* CNB-1 [29], *C. testosteroni* ATCC19966 (accession:

AAM77214, ACI39936, FJ215323, AAV40815) [67, 87], *C. testosteroni* KF1 (draft

sequence, accession: NZ_AAUJ02000001) [70, 71], *Cupriavidus necator* (formally

Ralstonia eutropha) JMP134 (accession: NC_007347) [72, 73], *R. eutropha* H16

(accession: NC_008314) [74], *Burkholderia cenocepacia* J2315 (accession:

NC_011001) [75], *Burkholderia* sp. 383 (accession: NC_007511) [76], *Cu. taiwanensis*

LMG 19424 (accession: NC_010530) [77], *Shewanella pealeana* ATCC700345

(accession: NC_009901), *S. halifaxensis* HAW-EB4 (accession: NC_010334) [78, 79],

and *Pseudoalteromonas haloplanktis* TAC125 (accession: NC_007481) [80, 81]. Arrows

indicate genes or ORFs, and striped arrows indicate that the genes or ORFs are probably

not involved in steroid degradation. Numbers (%) below the arrows indicate putative

amino acid sequences identity to the corresponding ORFs of TA441. Corresponding

genes and ORFs are connected with broken lines. Putative function is given below the

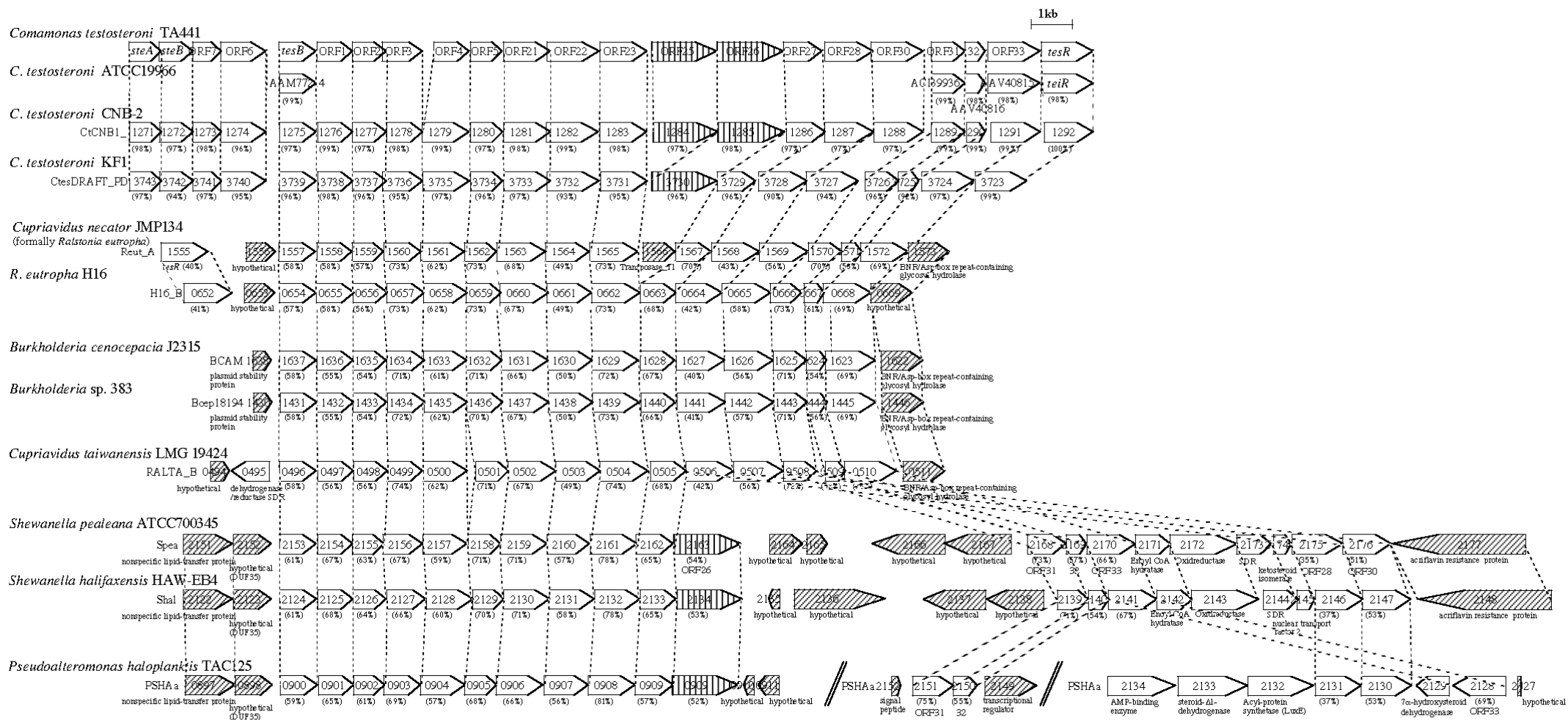ORFs when we thought it helpful.

Figure 12.

Table 1. steroid degradation genes in steroid degradation gene hot spot in *Comamomas testosteroni* strains

ATCC11996 Gene or accession No.	ATCC17410 Gene	TA441 Gene or ORF	Enzyme/ Putative product	CNB-2 Gene ID CtCNB1	DNA position (complementary)
-	-		transcriptional regulator, GntR family	1268	(1432546-1433037)
-	-		methylcitrate synthase/citrate synthase II	1269	(1433047-1434144)
-	-		transcriptional regulator, XRE family	1270	1434429-1435901
-	-	SteA	dehydrogenase for α-oriented hydroxyl group at C-12	1271	1436135-1436866
-	-	SteB	hydrogenase for ketone group at C-12 to β-oriented hydroxyl group	1272	1436879-1437646
-	-	ORF 7	hypothetical protein	1273	1437675-1438310
-	-	ORF 6	NADH:flavin oxidoreductase	1274	1438320-1439402
AAM77244 [83]	-	TesB	*meta*-cleavage enzyme for aromatized A-ring	1275	1439744-1440598
-	-	ORF1	CoA-transferase alpha subunit	1276	1440615-1441493
-	-	ORF2	CoA-transferase beta subunit	1277	1441506-1442276
-	-	ORF3	enoyl-CoA hydratase/isomerase family	1278	1442278-1443180
-	-	ORF4	enoyl-ACP reductase	1279	1443177-1444265
-	-	ORF5	enoyl-CoA hydratase/isomerase family	1280	1444288-1445046
-	-	ORF21	acyl-CoA dehydrogenase	1281	1445075-1446229
-	-	ORF22	acyl-CoA dehydrogenase	1282	1446247-1447386
-	-	ORF23	thiolase	1283	1447383-1448537
-	-	ORF25	6-aminohexanoate-cyclic-dimer hydrolase	1284	1448638-1450203
-	-	ORF26	6-aminohexanoate-cyclic-dimer hydrolase	1285	1450204-1451793
-	-	ORF27	short-chain dehydrogenase/reductase family	1286	1451853-1452728
-	-	ORF28	acyl-CoA dehydrogenase	1287	1452749-1453936
-	-	ORF30	acyl-CoA dehydrogenase	1288	1453933-1455111
ACI39936**	-	ORF31	short-chain dehydrogenase/reductase family	1289	1455327-1456112
AAV40816**	-	ORF32	MaoC domain protein	1290	1456115-1456600
AAV40815**	-	ORF33	acyl-CoA thiolase, acetyl-CoA acetyltransferase	1291	1456668-1457939
teiR [63]	-	TesR	LuxR-type transcription factor	1292	1457981-1459228
-	-		diguanylate cyclase	1293	1459749-1461293
-	-	-	carnitine dehydratase/bile acid-inducible protein	1294	(1461503-1462636)
-	-	-	(short-chain dehydrogenase/reductase SDR)	1295	1462821-1463564
-	-	-	(acetyl-CoA acetyltransferase)	1296	1463926-1465059
-	-	-	(3-hydroxyacyl-CoA dehydrogenase)	1297	1465077-1467236
-	-		(glucose-methanol-choline oxidoreductase)	1298	1467325-1469031
-	-		acyl-CoA dehydrogenase domain protein	1299	1469060-1471471
-	-		major facilitator superfamily MFS_1	1300	1471575-1472933
-	-		AMP dependent acyl-CoA synthetase and ligase	1301	1473197-1474822
-	-	(ND)	(hypothetical protein)	1302	(1474924-1475322)
-	-		amidohydrolase 3	1303	(1475610-1477325)
-	-		major facilitator superfamily MFS_1	1304	1477575-1478918
-	-		hypothetical protein	1305	1478915-1479643
-	-		rieske (2Fe-2S) domain	1306	(1479819-1480928)
-	-		short chain dehydrogenase/reductase SDR	1307	(1481087-1481923)
-	-		major facilitator superfamily MFS_1	1308	(1481945-1483279)
-	-		coniferyl aldehyde dehydrogenase	1309	1483404-1484828
-	-		short chain dehydrogenase/reductase SDR	1310	1485097-1485867
-	-		transcriptional regulator, LysR family	1311	(1486107-1487042)
-	-		DSBA oxidoreductase	1312	1487110-1487760
-	-	(ND)	(twin-arginine translocation pathway signal)	1313	1487811-1488710
-	-		ketosteroid isomerase like protein	1314	1488719-1489120
-	-		hypothetical protein	1315	1489139-1489399
-	-		streptomycin-3-kinase	1316	(1489452-1490153)
-	-		conserved hypothetical protein	1317	(1490567-1491058)
-	-		GCN5-related N-acetyltransferase	1318	(1491306-1491782)
-	-		protein of unknown function DUF925	1319	(1491906-1492490)
-	-		NADH:flavin oxidoreductase/NADH oxidase	1320	(1492713-1494839)
-	-		2,4-dienoyl-CoA reductase	1321	(1494856-1496871)
3α-*hsd* [38, 84] *hsdA* [85]	-	3α-*hsd*	3-hydroxysteroid 3α-dehydrogenase	1322	1497027-1497800
ksi [24, 25]		*ksi*	3-ketosteroid Δ4(5)-isomerase	1323	1497971-1498330
repA [86]		*repA*	repressor of 3-hydroxysteroid 3α-dehydrogenase		(1496985-1497803)
repB [86] -	-	ORF64	GreA/GreB family elongation factor	1324	(1498493-1499410)
-	-	(ND)	(GCN5-related N-acetyltransferase)	1325	(1499815-1500255)
-	-		transcriptional regulator, LysR family	1326	(1500300-1501211)

-	-		short-chain dehydrogenase/reductase SDR	1327	1501320-1502087
-	-		N-acyl-D-amino-acid deacylase		
-	-		glutathione-dependent formaldehyde-activating	1328	1502236-1502637
-	-		enoyl-CoA hydratase/isomerase	1329	(1502710-1503522)
-	-		short-chain dehydrogenase/reductase SDR	1330	1503622-1504422
-	-		protein of unknown function DUF925	1331	1504441-1505385
-	-		CaiA, acyl-CoA dehydrogenase-like protein	1332	1505401-1506504
-	-		MaoC-like dehydratase	1333	1506517-1506933
-	-		lipid-transfer protein	1334	1506957-1508138
-	-		**CaiA, acyl-CoA dehydrogenase-like protein**	**1335**	1508156-1509337
-	-		acyl-CoA synthetase	1336	1509380-1511191
-	-		beta-lactamase domain protein	1337	1511184-1512251
-	-		acyl-CoA dehydrogenase-like protein	1338	1512264-1513460
-	-		acyl-CoA dehydrogenase-like protein	1339	1513593-1514720
-	-		long-chain-fatty-acid-CoA ligase	1340	1514722-1516428
-	-		putative sigma E regulatory protein, MucB ,DUF1329	1341	1516711-1518090
-	-		protein of unknown function DUF1302	1342	1518126-1519637
-	-		dienelactone hydrolase	1343	1519751-1520812
-	-		RND efflux transporter	1344	(1520980-1521438)
-	-	(ND)		1345	(1521438-1523921)
-	-		BNR repeat containing glycosyl hydrolase	1346	(1523918-1524925)
-	-		nonspecific lipid-transfer protein	1347	1525136-1526350
-	-	-	acyl dehydrogenase protein of unknown function DUF35	1348	1526523-1527299
-	-		(ferric malleobactin transporter)	1349	1527577-1527855
-	-		TonB-dependent siderophore receptor, transporter	1350	1528207-1530306
-	-	TesG	4-hydroxy-2-oxo-hexanoate aldolase	1351	(1530465-1531505)
-	-	TesF	propionaldehyde dehydrogenase	1352	(1531520-1532443)
-	-	TesE	2-hydroxyhexa-2,4-dienoic acid hydratase	1353	(1532440-1533255)
-	-	TesD	hydratase for aromatized A-ring of steroids	1354	(1533260-1534099)
-	-	TesA1	hydroxylase at C-4 of aromatized A-ring	1355	1534179-1535156)
-	-	TesA2	hydroxylase at C-4 of aromatized A-ring	1356	1535436-1536632
-	Δ1-DH [26]	TesH	3-ketosteroid Δ1-dehydrogenase	1357	1536737-1538467
-	Δ4-DH [27]	TesI	3-ketosteroid Δ4-dehydrogenase	1358	1538483-1540219
-	-	ORF17	reductase component of C-9 hydroxylase	1359	1540303-1541370
-	-	ORF18	CoA-transferase at C-5 of cleaved B-ring	1360	1541374-1542996

(ND) :not detected in TA441
- :not sequenced or sequence incomplete