Meta-cleavage enzyme gene *tesB* is necessary for testosterone degradation in *Comamonas testosteroni* TA441

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***Comamonas testosteroni* metabolizes testosterone as the sole carbon source via a meta-cleavage reaction. A meta-cleavage enzyme gene, *tesB*, was cloned from *C. testosteroni* TA441. The deduced N-terminal amino acid sequence of *tesB* matched that of the purified meta-cleavage enzyme which is induced in TA441 during growth on testosterone as the sole carbon source. The *tesB*-disrupted mutant did not show growth on testosterone, suggesting that *tesB* is necessary for TA441 to grow on testosterone. Downstream from *tesB*, three putative ORFs which encode products also necessary for growth of TA441 on testosterone were identified. The usual substrate of *TesB* is probably 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione. Although this compound was not identified in the gene disrupted mutants, accumulation of upstream metabolites of testosterone degradation, 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione, was shown by TLC analysis.**

Keywords: biodegradation, steroid hormone, seco-steroid

INTRODUCTION

Steroids are widespread in the environment, found as cholesterol, cholic acid, the sex and adrenal cortical hormones of mammals, and phytosterols in plants. In the environment, micro-organisms are considered to play a principal role in steroid degradation. Degradation of steroids such as testosterone by micro-organisms has attracted interest as a means to produce steroid hormone derivatives for use as hormonal drugs. In steroid-degrading micro-organisms, the catabolic enzymes for steroid degradation are usually not constitutively expressed but rather are induced by their respective substrates. *Comamonas testosteroni* is a Gram-negative bacterium which is able to grow using certain C₁₉ and C₂₁ steroids as well as many other aromatic compounds as the sole carbon and energy source. *C. testosteroni* metabolizes certain steroids through a complex metabolic pathway involving many enzymic steps and the synthesis of these enzymes is induced by certain steroids (Florin *et al.*, 1996; Möbus *et al.*, 1997; Möbus &

Maser, 1998). Degradation of testosterone in *C. testosteroni* is considered to be initiated by dehydrogenation of the 17 β -hydroxyl group to 4-androstene-3,17-dione (reaction 1 in Fig. 1) (Abalain *et al.*, 1993; Genti-Raimondi *et al.*, 1991), followed by desaturation of the A ring (Fig. 1). A pathway for degradation of 4-androstene-3,17-dione in *C. testosteroni* was proposed by Coulter & Talalay (1968), based on analysis of the compounds produced in culture of testosterone-grown *C. testosteroni*. In the degradation pathway, 4-androstene-3,17-dione undergoes Δ^1 -dehydrogenation (reaction 2 in Fig. 1) and 9 α -hydroxylation to produce 9 α -hydroxy-1,4-androstadiene-3,17-dione (compound II and reaction 3 in Fig. 1), then it is converted to 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (Coulter & Talalay, 1968). This compound is hydroxylated at the C-4 position to yield 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (Fig. 1), which is cleaved between C-4 and C-5 through a meta-cleavage reaction (Sih *et al.*, 1966). A number of the genes encoding enzymes involved in the degradation pathway of *C. testosteroni* have been identified in studies performed in the 1990s. The gene for the 17 β -dehydrogenase, which catalyses the initial dehydrogenation reaction (reaction 1 in Fig. 1), has been cloned and characterized (Abalain *et al.*, 1993; Genti-Raimondi *et*

Abbreviation: Km, kanamycin.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB040808.

Fig. 1. Proposed testosterone degradation pathway in *C. testosteroni* (Coulter & Talalay, 1968). Compound I, 9 α -hydroxy-4-androstene-3,17-dione; II, 9 α -hydroxy-1,4-androstadiene-3,17-dione; III, 2-oxo-*cis*-4-hexenoic acid; IV, 3 α ,4 β ,5,6,7,7a-hexahydro-7a β -methyl-1,5-dioxo-4-indanpropionic acid. The enzyme for reaction 1 is 17 β -dehydrogenase; 2, Δ^1 -dehydrogenase.

al., 1991). Δ^1 -Dehydrogenase and $\Delta^4(5\alpha)$ -dehydrogenase, which belong to the same transcription unit in *C. testosteroni* ATCC 17410, introduce a double bond into the A ring of 4-androstene-3,17-dione and 1-androstene-3,17-dione, respectively, to produce 1,4-androstadiene-3,17-dione (reaction 2 in Fig. 1; 1-androstene-3,17-dione is not indicated in Fig. 1) (Florin *et al.*, 1996; Plesiat *et al.*, 1991). In addition to these dehydrogenases, the genes for 3 α -hydroxysteroid hydrogenase and Δ^{5-3} ketosteroid isomerase from *C. testosteroni* have also been cloned and characterized (Abalain *et al.*, 1995; Möbus & Maser, 1998; Kuliopulos *et al.*, 1987; Choi & Benisek, 1988). Recently, the N-terminal sequences of about ten testosterone-inducible proteins have been determined (Möbus *et al.*, 1997). Some of them show high homology with catabolic enzymes involved in degradation of aromatic compounds, such as BphC, a *meta*-cleavage enzyme involved in biphenyl degradation (for example see Furukawa *et al.*, 1987; Kimbara *et al.*, 1989; Hofer *et al.*, 1993).

C. testosteroni TA441 was isolated from a termite, an organism which is well known as an effective wood degrader. TA441 has the ability to grow on aromatic compounds such as phenols, 3-(3-hydroxyphenyl)-propionic acid and their derivatives. TA441 can also utilize testosterone and some other steroids as the sole carbon and energy source. The *aph* genes and *mhp* genes, which

code for enzymes involved in the degradation of phenols and 3-(3-hydroxyphenyl)-propionic acid in TA441, have previously been identified and characterized (Arai *et al.*, 1998, 1999), whereas the genes encoding the enzymes involved in testosterone degradation have not yet been characterized. Cultures of TA441 show a yellow colour during growth on testosterone. This yellow colour suggests that testosterone degradation proceeds via a *meta*-cleavage reaction. TA441 has at least two *meta*-cleavage enzymes, *aphB* and *mhpB*. As an *aphB*⁻ *mhpB*⁻ double mutant of strain TA441 has been found to retain the ability to grow on testosterone and showed *meta*-cleavage activity (unpublished data), another *meta*-cleavage enzyme for testosterone degradation is therefore expected to exist.

In this paper, we report the cloning of the gene for the *meta*-cleavage enzyme which is produced during growth on testosterone and is necessary for testosterone degradation in TA441. The cloning of genes encoding three other proteins necessary for testosterone degradation, located downstream from the gene for the *meta*-cleavage enzyme, is also described.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used as the host strain for DNA

Table 1. Bacterial strains and plasmids used in this study

Ap; ampicillin, Km; kanamycin, Tc; tetracycline.

Strain or plasmid	Characteristics	Source or reference
Strains		
<i>C. testosteronei</i> TA441	Wild-type, Tes	Arai <i>et al.</i> (1998)
TesB ⁻	<i>tesB</i> ::Km ^r mutant of TA441	This work
ORF1 ⁻	ORF1::Km ^r mutant of TA441	This work
ORF2 ⁻	ORF2::Km ^r mutant of TA441	This work
ORF3 ⁻	ORF3::Km ^r mutant of TA441	This work
Plasmids		
pUC19	Ap ^r , <i>lacZ</i>	Vieira & Messing (1987)
pCPY	Ap ^r , pUC19 with 4.3 kb <i>Pst</i> I insert of <i>C. testosteronei</i> TA441	This work
pCP31	Ap ^r , pUC19 with <i>Pst</i> I- <i>Kpn</i> I insert from pCPY	This work
pCP311	Ap ^r , pUC19 with <i>Pst</i> I- <i>Apa</i> I insert from pCPY	This work
pTesB-Km ^r	Ap ^r , pCP311 derivative with Km-resistance gene in <i>Eco</i> RV site of <i>tesB</i>	This work
pCP312	Ap ^r , pUC19 with <i>Apa</i> I- <i>Eco</i> RI insert from pCPY	This work
pORF1-Km ^r	Ap ^r , pCP31 derivative with Km-resistance gene in <i>Eco</i> RV site of ORF1	This work
pORF2-Km ^r	Ap ^r , pCP31 derivative with Km-resistance gene in <i>Sac</i> II site of ORF2	This work
pORF3-Km ^r	Ap ^r , pCP312 derivative with Km-resistance gene in <i>Bam</i> HI site of ORF3	This work
pRWTesB	Tc ^r , pRW2 derivative carrying the same insert as pCP311	This work
pRWTesBp	Tc ^r , pRW2 derivative with 640 bp <i>Pst</i> I insert of pCP31	This work

manipulations. *E. coli* transformants were grown on LB medium at 37 °C. For plate cultures, 1.6% (w/v) agar was added to the media. Ampicillin (100 mg l⁻¹) was added for growth of transformants harbouring pUC19 derivative plasmids, tetracycline (5 mg l⁻¹) was added for growth of transformants harbouring pRW2 derivative plasmids, and kanamycin (Km) (30 mg l⁻¹) was added for growth of transformants harbouring plasmids containing the Km-resistance gene originating from pSUP5011. IPTG (100 mM) and X-Gal (20 mg l⁻¹) were added for colour selection. *Comamonas testosteronei* strains were grown at 30 °C in LB medium or C medium (Arai *et al.*, 1998) with suitable carbon sources. Carbon sources (testosterone, 1,4-androstadiene-3,17-dione or *p*-hydroxybenzoate) were added as a filtered DMSO solution at a final concentration of 0.1% (w/v). Growth of TA441 was monitored by counting colonies that appeared on LB plates, on which appropriately diluted cultures had been spread, after incubation at 30 °C. Km was added to media at 400 mg l⁻¹ to select recombinant strains of TA441, and a combination of 800 mg Km l⁻¹ and 300 µg carbenicillin l⁻¹ was added for further selection.

Partial purification and determination of the N-terminal amino acid sequence of TesB. *C. testosteronei* TA441 cells grown in testosterone-containing medium were centrifuged, resuspended in 10 mM potassium phosphate buffer (pH 7.5) and disrupted by sonication (Branson, TW3). The cell suspension was then centrifuged and the supernatant was applied to a DEAE ion-exchange column equilibrated with phosphate buffer. A protein fraction showing a yellow colour, corresponding to the *meta*-cleavage product of 2,3-dihydroxybiphenyl, was eluted with phosphate buffer containing 150 mM NaCl. 2,3-Dihydroxybiphenyl was used instead of the proposed substrate [3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione in Fig. 1], which was not commercially available. The enzyme in the fraction showing a yellow colour was partially purified by means of a Superose-12 gel filtration column and a Mono-Q HR 5/5

column using the Pharmacia LKB Biotechnology fast protein liquid chromatography system. The partially purified enzyme was run on a nondenaturing 12% acrylamide gel. The gel was stained with 2,3-dihydroxybiphenyl and a band showing a yellow colour corresponding to the *meta*-cleavage product was transferred to a PVDF membrane. The amino terminal sequence was determined by automated Edman degradation using the Applied Biosystems model 492 protein sequencing system.

DNA manipulation. Plasmid DNA was prepared from the *E. coli* host strain by the alkaline lysis method (Birnboim & Doly, 1979). Restriction endonucleases, the DNA ligation kit version 2 (Takara Shuzo) and the DNA blunting kit (Takara Shuzo) were used according to the manufacturer's instructions. DNA fragments were extracted by the glass powder method (GeneClean II kit, Bio101) as instructed by the manufacturer. Other DNA manipulations were performed according to standard methods (Sambrook *et al.*, 1989).

Cloning of genes encoding *meta*-cleavage enzymes. Total DNA of strain TA441 was digested with *Pst*I and ligated to pUC19 vector which had been digested with *Pst*I and treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals). *E. coli* JM109 was transformed with the resultant plasmids according to the method of Hanahan (1983). Ampicillin-resistant transformants were selected on LB agar plates. Transformants with *meta*-cleavage activity were selected on the basis of yellow pigmentation after spraying them with 2,3-dihydroxybiphenyl (50 mM in acetone).

Nucleotide sequence determinations. Deletion libraries of the reconstructed plasmids were generated using a DNA deletion kit (Takara Shuzo). DNA sequences were determined using an ABI model 373A automated DNA sequencer and the dye terminator sequencing protocols (Perkin-Elmer). The templates for dideoxy chain-termination reactions were prepared using the Wizard Plus Minipreps DNA Purification System (Promega). Both strands of the DNA were sequenced and the

nucleotide sequences of linking junctions of the fragments were determined using custom-designed oligonucleotides as primers. Alignment of the *meta*-cleavage enzyme and related proteins was performed according to the method of Higgins & Sharp (1989) with the PAM matrix of Dayhoff *et al.* (1978).

Construction of plasmids and mutant strains. Plasmid pSKKm^r was constructed by ligating a *Hind*III–*Sal*I fragment of pSUP5011, containing a Km-resistance gene, with *Hind*III- and *Sal*I-treated pBluescript KS–. The *tesB* gene in pCP311 was disrupted by insertion of a *Sma*I fragment from pSKKm^r, containing the Km-resistance gene, into the *Eco*RV site in *tesB*. The resultant plasmid, pTesB-Km^r, which encodes the Km-resistance gene in the same transcriptional direction as *tesB*, was used for inactivation of the *tesB* gene in TA441 by homologous recombination according to the method described previously (Arai *et al.*, 1998). A Km-resistant and carbenicillin-sensitive TA441 mutant was selected and designated strain TesB[–]. Insertion of the Km-resistance gene into the chromosome of TesB[–] was confirmed by hybridization using the Km-resistance gene and *tesB* as probes. ORFs 1, 2 and 3 in TA441 were individually disrupted in the same way. The Km-resistance gene was inserted into the *Eco*RV site in ORF1, the *Sac*II site in ORF2 and the *Bam*HI site in ORF3 (see Fig. 3).

Growth of TA441 and mutant strains on testosterone. TA441 and mutant strains were grown at 30 °C in 5 ml LB medium for about 15 h. The cells were centrifuged and the pellet was resuspended in C medium at an OD₆₀₀ of 10. A 50 µl portion of the resultant cell suspension was transferred as inoculum into 10 ml C medium supplemented with 0.1% (w/v) testosterone or *p*-hydroxybenzoate (positive control) as the sole carbon source and incubated at 30 °C for 24 h. The extent of growth was determined every 3 h; colonies that appeared on LB plates on which appropriately dilutions of the culture had been spread were counted after incubation at 30 °C for about 15 h. When several kinds of mutants were used (see Fig. 6 and Table 2), a 100 µl portion was used as inoculum.

Promoter activity. TA441 was transformed with pRWtesBp carrying the transcriptional fusion construct *tesB*:*lacZ*. The plasmid pRWtesBp is a broad-host-range plasmid pRW2 derivative encoding 640 bp *Pst*I fragment of TA441 total DNA (see Fig. 3). TA441(pRWtesBp) cells grown overnight in LB medium with 5 mg tetracycline l^{–1} were washed twice and resuspended in C medium at an OD₆₀₀ of 10. A 200 µl portion of the resultant cell suspension was transferred as inoculum into 100 ml C medium containing 0.1% testosterone, 1,4-androstene-3,17-dione or *p*-hydroxybenzoate (negative control) with 5 mg tetracycline l^{–1} and incubated at 30 °C. Changes in the activity of β-galactosidase, the *lacZ* gene product, were monitored every 2 h. The activity of β-galactosidase was measured by the protocol described by Miller (1992). Before A₄₂₀ and A₅₀₀ were measured, centrifugation was needed to remove testosterone or 1,4-androstene-3,17-dione. To measure OD₆₀₀, these compounds were removed from the culture by ethyl acetate extraction. The cells were collected by centrifugation and resuspended in the same volume of water.

TLC. Cultures grown for 24 h in the experiment shown in Fig. 5 were used for TLC analysis. The culture was extracted with a half volume of ethyl acetate, subjected to TLC (kieselgel 60 F254 plate, Merck KgaA) using benzene/methanol (19:1, v/v) as the solvent system and detected by UV absorption at 254 nm. Testosterone, 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione were used as standards.

RESULTS AND DISCUSSION

N-terminal sequence of the testosterone-inducible *meta*-cleavage enzyme

TA441 cultures became yellow in colour when the organism was grown with testosterone as the sole carbon source. This yellow colour suggests that TA441 degrades testosterone via a *meta*-cleavage reaction. Our preliminary experiments showed that TA441 had a *meta*-cleavage enzyme which is produced when TA441 is grown on testosterone, in addition to the *meta*-cleavage enzymes already characterized in this strain (*aphB* and *mhpB*) (Arai *et al.*, 1998, 1999), and that the *aphB*[–] *mhpB*[–] double knock-out mutant of TA441 still retained the ability to utilize testosterone as the sole carbon source. From these results, we considered that a *meta*-cleavage enzyme different from AphB and MhpB was involved in testosterone degradation in TA441, and we proceeded to partially purify the protein and determine the N-terminal sequence of the enzyme, as described in Methods. The sequence (MMEIRGLAYVVAESSDLD-RWVSYARDVLG) was not identical to the N-terminal sequences of AphB or MhpB; it was identical to the N-terminal sequence of the testosterone-inducible protein TIP1 of *C. testosteroni* ATCC 11996 (Möbus *et al.*, 1997). Cell-free extracts of TA441 cells grown with testosterone or with other compounds were analysed by PAGE and stained with 2,3-dihydroxybiphenyl (Fig. 2). The band corresponding to this *meta*-cleavage enzyme was not observed when TA441 cells were grown in the absence of testosterone.

Cloning of the *meta*-cleavage enzyme gene

E. coli JM109 was transformed with recombinant plasmids comprising a genomic library of strain TA441 and ampicillin-resistant transformants which showed

Fig. 2. PAGE of the cell-free extract of TA441 cells grown with testosterone, *p*-hydroxybenzoate or succinate. The gel was stained with 2,3-dihydroxybiphenyl and the *meta*-cleavage enzyme is presented with a yellow colour in the original gel.

Fig. 3. Partial restriction map of a 4.5 kb *Pst*I fragment, the insert in pCPY, encoding the testosterone-degrading enzyme genes of *C. testosteroni* TA441. The *meta*-cleavage enzyme gene *tesB* and putative ORFs are indicated by large white arrows. The black arrowhead indicates the promoter sequence. Deletion plasmids are indicated below the restriction map; gene segments still present are indicated as lines. Plasmids ptesB-Km^r to pORF3-Km^r were constructed to make gene-disrupted mutants. Restriction sites where the Km resistance gene was inserted are indicated by small white arrowheads. The small black arrowheads indicate the direction of transcription regulated by the *lac* promoter of the pUC19 vector. Abbreviations are: Ap, *Apal*; Bm, *Bam*HI; Bgl, *Bgl*II; ERI, *Eco*RI; EV, *Eco*RV; ET, *Eco*T22I; Kp, *Kpn*I; Nr, *Nru*I; Ps, *Pst*I; Scl, *Sac*I; SclI, *Sac*II; Xb, *Xba*I. The accession number of this sequence is AB040808.

yellow pigmentation attributable to *meta*-cleavage of 2,3-dihydroxybiphenyl were selected. 2,3-Dihydroxybiphenyl was used instead of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (Fig. 1), which was not commercially available. *Meta*-cleavage enzymes usually have broad substrate specificity, and in the case of strain TA441, the *meta*-cleavage activity of 2,3-dihydroxybiphenyl was observed when the cells were grown in the presence of testosterone, but not succinate (data not shown). Among the selected transformants, one transformant was found by hybridization to harbour a plasmid which showed homology with an oligonucleotide based on the N-terminal sequence of the testosterone-inducible *meta*-cleavage enzyme (ATG-ATGGAGATCCGCGGCCTGGCCTACGTGGTGG-CCGA) and the restriction map of the DNA insert differed from that of *aphB* or *mhpB*. These results suggested that the cloned *meta*-cleavage enzyme gene was the expected one. This plasmid was designated pCPY. Other transformants showing the yellow colour were found to carry plasmids encoding *aphB* or *mhpB*. Fig. 3 shows the restriction map of the 4.5 kb pCPY insert; the region constituting the structural gene for the *meta*-cleavage enzyme was identified by examination of the activity displayed by each of a series of deletion mutants. Among them, a transformant carrying pCP311, which contained a 1.3 kb *Pst*I–*Apal* fragment, showed *meta*-cleavage activity (Fig. 3).

Nucleotide sequence of the *meta*-cleavage enzyme gene

We determined the nucleotide sequence of the 1.3 kb *Pst*I–*Apal* insert in pCP311 and identified an ORF by computer analysis. The deduced N-terminal amino acid sequence of this ORF matched that of the purified testosterone-inducible *meta*-cleavage enzyme exactly.

Fig. 4. Phylogenetic tree for TesB and *meta*-cleavage enzymes for 2,3-dihydroxybiphenyl. The tree was constructed using CLUSTAL W with the PAM matrix. AphB, a *meta*-cleavage enzyme for catechol in *C. testosteroni* TA441, is used as an outgroup. Abbreviations are: CarB2, CarB2 of *Pseudomonas* sp. strain CA10 (Sato *et al.*, 1997) (accession no. D89065); EdoB, EdoB of *Rhodococcus rhodochrous* NCIMB 13064 (Kulakov *et al.*, 1998) (AJ003244); BphC1 (TA421), BphC1 of *Rhodococcus* sp. TA421 (Kosono *et al.*, 1997) (D88013); BphC7, BphC7 of *Rhodococcus* sp. TA421 (Kosono *et al.*, 1997) (D88019); BphC1 (P6), BphC1 of *R. globerulus* P6 (Asturias *et al.*, 1994) (P47231); BphC (KKS102), BphC of *Pseudomonas* sp. strain KKS102 (Kimbara *et al.*, 1989) (M26433); BphC (KF707), BphC of *P. pseudoalcaligenes* KF707 (Furukawa *et al.*, 1987) (P08695); BphC (LB400), BphC of *Burkholderia cepacia* LB400 (Hofer *et al.*, 1993) (P47228). Identities (%) between TesB and the *meta*-cleavage enzymes are: CarB2, 55; EdoB, 46; BphC1 (TA421), 42; BphC7, 42; BphC1 (P6), 42; BphC (KKS102), 39; BphC (KF707), 41; BphC (LB400), 42.

As this result and the results of experiments involving gene disruption, described below, strongly indicated that the cloned *meta*-cleavage enzyme was involved in testosterone degradation in TA441, we named this gene

Fig. 5. Growth of TA441 and TesB⁻ on testosterone as the sole carbon source. The strains were grown in 10 ml C medium supplemented with 0.1% (w/v) testosterone (TA441, Δ ; TesB⁻, $\blacktriangle$), *p*-hydroxybenzoate (TA441, $\square$; TesB⁻, $\blacksquare$) (positive control) or without any carbon source (TA441, $\circ$; TesB⁻, $\bullet$) (negative control).

tesB. The deduced amino acid sequence of *tesB* showed the most similarity (55% identity) to CarB2, a *meta*-cleavage enzyme from *Pseudomonas* sp. CA10 (Sato *et al.*, 1997). TesB also showed 46% identity with EdoB from *Rhodococcus rhodochrous* NCIMB 13064 (Kulakov *et al.*, 1998) and 39–42% identity with BphCs from *Rhodococcus* and *Pseudomonas* sp. strains. A phylogenetic tree for TesB and these *meta*-cleavage enzymes is shown in Fig. 4. BphC1 from *Rhodococcus globerulus* P6 (Asturias *et al.*, 1994), *Pseudomonas* sp. strain KKS102 (Kimbara *et al.*, 1989), *Pseudomonas pseudoalcaligenes* KF707 (Furukawa *et al.*, 1987) and *Burkholderia cepacia* LB400 (Hofer *et al.*, 1993) are *meta*-cleavage enzymes in the biphenyl degradation pathway which utilize 2,3-dihydroxybiphenyl as a sub-

strate, whereas the substrates of CarB2, EdoB, BphC1 and BphC7 from *Rhodococcus* sp. TA421 (Kosono *et al.*, 1997) have not yet been identified. The phylogenetic tree for TesB and the *meta*-cleavage enzymes implies that these enzymes are divided into two groups: BphCs and the other *meta*-cleavage enzymes whose substrates are unknown. Conserved motifs in BphC, three amino acid residues functioning as metal-binding ligands (H₁₄₆, H₂₁₃ and E₂₆₄) and three involved in active sites (H₁₉₈, H₂₄₄ and Y₂₅₄) (Han *et al.*, 1995), are also conserved in TesB.

Disruption of *tesB* in TA441 and growth of the mutant on testosterone

To confirm that TesB is necessary for testosterone metabolism in TA441, *tesB* in TA441 was disrupted with a Km-resistance gene by homologous recombination. Insertion of the Km-resistance gene into *tesB* in the mutant strain was confirmed by hybridization (data not shown), and the resultant TesB mutant strain was designated TesB⁻. The results obtained by comparing the growth of TesB⁻ and TA441 on testosterone as the sole carbon source are shown in Fig. 5. With *p*-hydroxybenzoate, which is metabolized by Mhp proteins, as the sole carbon source, TesB⁻ grew as well as TA441, whereas with testosterone as the sole carbon source, the growth of the mutant was negligible, the same as the growth observed without any carbon source. The absence of significant growth of TesB⁻ on testosterone indicates that TesB is necessary for growth of TA441 on testosterone. The ability to grow on testosterone was partially recovered in the TesB⁻ mutant harbouring pRWtesB, a derivative of a broad-host-range plasmid pRW2 encoding TesB (Table 2). From this result, it is confirmed that the reduction of the growth of TesB⁻ mutant on testosterone was not caused by a polar effect.

Sequence analysis of genes downstream from *tesB*

As *tesB* is necessary for testosterone degradation in TA441, gene segments downstream from *tesB* were characterized. The DNA sequence of the insert in pCPY (Fig. 3) was found to contain three putative ORFs just

Table 2. Growth of mutant strains on testosterone

The extent of growth was determined at the beginning of the cultivation period and after about 24, 48 and 72 h. The data are the mean of more than three independent experiments.

Strain	Plasmid	c.f.u. (SE) (cells ml ⁻¹)			
		0 h	24 h	48 h	72 h
TA441	—	2.7 × 10 ⁷ (9.4 × 10 ⁶)	2.6 × 10 ⁸ (2.8 × 10 ⁸)	—	—
TesB ⁻	pRWtesB	9.8 × 10 ⁶ (2.1 × 10 ⁶)	7.7 × 10 ⁷ (1.1 × 10 ⁷)	2.6 × 10 ⁸ (2.6 × 10 ⁷)	—
TesB ⁻	—	4.3 × 10 ⁷ (1.6 × 10 ⁷)	7.7 × 10 ⁷ (1.7 × 10 ⁷)	—	7.3 × 10 ⁷ (3.4 × 10 ⁷)
ORF1 ⁻	—	2.7 × 10 ⁷ (6.4 × 10 ⁵)	1.4 × 10 ⁸ (3.1 × 10 ⁷)	—	1.9 × 10 ⁸ (4.0 × 10 ⁷)

Fig. 6. Growth of TA441 (▲) and mutant strains ORF1⁻ (□), ORF2⁻ (◇), ORF3⁻ (○) and TesB⁻ (△) on testosterone as the sole carbon source. ORF1⁻ to TesB⁻ are gene disrupted mutants of ORF1 to *tesB*, respectively. The strains were grown in 10 ml C medium supplemented with 0.1% (w/v) testosterone.

downstream from *tesB*. These ORFs were designated ORF1 to 3. The deduced N-terminal amino acid sequence of the ORF2 product (SEIALVDRVICAARA-WENDGEVLATGIGL) was almost identical to that of TIP6 (SEIALVDRVIEAARA-WENDGEVLATGIGL; 29 of 30 amino acid residues were the same), one of the testosterone-inducible proteins in *C. testosteronei* ATCC 11996 (Möbus *et al.*, 1997). The deduced amino acid sequences of ORF1 and ORF2 showed 26.9% identity with subunit A of glutamate CoA-transferase, which is involved in glutamate metabolism in *Acidaminococcus fermentans*, and 22.4% identity with subunit B, respectively (Mack *et al.*, 1994; Jacob *et al.*, 1997). Glutamate CoA-transferase produces acetate and glutaconyl-CoA from acetyl-CoA and (E)-glutaconate. The deduced amino acid sequence of ORF3 showed about 30% identity with glutaconyl-CoA hydratase from *E. coli* (Eichler *et al.*, 1994) and other enoyl-CoA hydratases. Although the homologies are low, all the ORFs have some homology with enzymes for CoA compounds, implying the concern of these ORFs to CoA compounds. The distance between them is very short (16 bp between *tesB* and ORF1, 12 bp between ORF1 and ORF2, and 1 bp between ORF2 and ORF3), suggesting that *tesB* and these ORFs may be co-transcribed.

ORF1 to 3 in TA441 were individually disrupted by insertion of a Km-resistance gene, yielding the mutant strains ORF1⁻ to ORF3⁻, respectively. Fig. 6 shows the growth of TA441 and the mutant strains on testosterone as the sole carbon source. In this experiment, twice the number of the cells (compared to the experiment shown in Fig. 5) were added as the initial inoculum, which

Fig. 7. TLC of cultures of the mutant strains: TesB⁻ (lane 1), ORF1⁻ (lane 2), ORF2⁻ (lane 3), ORF3⁻ (lane 4) and TA441 (lane 5). Cultures grown for 24 h in the experiment shown in Fig. 6 were extracted with a half volume of ethyl acetate, subjected to TLC (kieselgel 60 F254 plate, Merck KgaA) using benzene/methanol (19:1, v/v) as the solvent system and detected by UV absorption at 254 nm. Testosterone (Ts), 4-androstene-3,17-dione (4-A) and 1,4-androstadiene-3,17-dione (1,4-A) were used as the standards.

probably caused the increase of cell numbers in the initial 6 h. After 6 h, the mutant strains showed little growth on testosterone, indicating that ORF1 to 3 are involved in testosterone metabolism in TA441.

Cultures of the ORF1-, 2- or 3-disrupted mutants grown on testosterone did not show the accumulation of the characteristic yellow colour of *meta*-cleavage products (data not shown). These results probably indicate that the enzymes encoded by ORF1 to 3 do not act on the *meta*-cleavage compound produced by TesB. The ORF1⁻ mutant showed slight growth on testosterone when it was cultured for several days (Table 2). This suggests that ORF1 is probably involved in the degradation pathway at a step after the cleavage of testosterone into compounds III and IV.

TLC analysis of the culture of the mutants

TesB is presumed to catalyse the *meta*-cleavage of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, because the TesB⁻ mutant showed no significant growth on testosterone, suggesting that TesB catalyses a reaction at an early step in the testosterone degradation pathway in TA441. The mutant cultures grown for 24 h in the experiment shown in Fig. 6 were acidified with HCl, extracted with ethyl acetate, and the ethyl acetate fraction was analysed by TLC (Fig. 7). Accumulation of 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione and another unknown compound (indicated by an arrow in Fig. 7) was observed in the culture media of all the mutants. 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione was not identified. It may be the unknown compound, or may remain in the water fraction, not extracted into the ethyl acetate fraction under the present extraction conditions. All the mutants

Fig. 8. Transcriptional activity of the *tesB* promoter in *C. testosteroni* TA441 cultured with testosterone (○), 1,4-androstadiene-3,17-dione (□) or *p*-hydroxybenzoate (△, negative control). TA441 was transformed with pRWtesBp carrying the transcriptional fusion construct *tesB::lacZ* (see Fig. 3). Changes in the activity of β -galactosidase, the *lacZ* gene product, were monitored. The data are means of at least three representative results of independent experiments. ●, Growth of TA441(pRWtesBp) on testosterone; ■, 1, 4-androstadiene-3,17-dione; ▲, *p*-hydroxybenzoate. The error bars of growth data are not presented.

accumulated 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione, which are upstream metabolites of testosterone degradation, indicating that ORF1 to 3 are also involved in testosterone degradation. One possibility for why these compounds were particularly accumulated in all the mutant cultures is that only these compounds are detectable under present analytical conditions. But it seems that these compounds tend to be accumulated in testosterone degradation, as we detected these compounds in the culture of TA441 at an early growth phase (around 8 h) and also in the cultures of other newly isolated testosterone-utilizing bacteria grown with testosterone (data not shown).

Induction of TesB

A putative promoter sequence was found upstream from *tesB* (133–182 in the sequence under accession number AB040808, indicated by the black arrowhead in Fig. 3). The transcriptional activity of this putative promoter was assessed by monitoring the expression of β -galactosidase from the *lacZ* fusion plasmid pRWtesBp (Fig. 3). The result is shown in Fig. 8. Significant induction was observed in TA441 cells grown in the presence of testosterone or its degradation intermediate 1,4-androstadiene-3,17-dione. This result implies that ex-

pression of *tesB* is induced by an intermediate compound formed in the course of testosterone degradation.

A putative terminal signal was not found in the sequenced region. Instead, there is another putative ORF just downstream from ORF3, whose stop codon was not found in the sequenced region, indicating that this probable gene cluster continues downstream. Isolation and characterization of genes upstream and downstream from the gene segment *tesB* to ORF3 are under way.

In this study, we have isolated four genes for testosterone degradation from *C. testosteroni* and showed that they are induced by a testosterone metabolite and are necessary for testosterone degradation. Our results will effectively facilitate investigation of the entire testosterone degradation pathway of *C. testosteroni*. Further studies are required to confirm the nature of the substrates and products of the enzymes derived from these cloned genes. Characterization of other genes involved in testosterone degradation will also be important to clarify the features of the testosterone degradation pathway in *C. testosteroni*.

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