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Steroid-inducible transcription of the 3β/17β-hydroxysteroid dehydrogenase gene (*3β/17β-hsd*) in *Comamonas testosteroni*

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Abstract

The expression of the *Comamonas testosteroni* gene, encoding 3β/17β-hydroxysteroid dehydrogenase enzyme (3β/17β-HSD), was analyzed at the transcriptional level. Northern blot analysis detected a 1 kb transcript in bacterial cells grown in minimum media supplemented with Casamino acids and testosterone. Also this transcript was observed when cells were grown in presence of 1-dehydrotestosterone, androstenedione and 1,4-androstadien-3,17dione, but not in presence of acetate, citrate, cholic acid, cholesterol, and cortisol. In addition, this effect was dependent on the presence of another carbon source in the growth medium used, revealing catabolite repression.

1. Introduction

Comamonas testosteroni can grow on a variety of steroid compounds as the sole carbon and energy source [1]. This Gram-negative bacteria can effect the complete oxidative degradation of the steroid skeleton by a series of enzymes, induced in the presence of these compounds in the culture medium [2]. Several of these enzymes have been purified and characterized biochemically [3–6] and the structural genes encoding Δ^5-3-ketosteroid isomerase [7,8], 3-oxosteroid Δ^1-dehydrogenase [9], 3-oxosteroid-Δ^4 (5α)-dehydrogenase [10], and 3α-hydroxysteroid dehydrogenase [11,12] have been cloned. In addition, we have isolated a 1,121 pb *Pst*I–*Hin*dIII DNA fragment, encoding 3β/7β-HSD enzyme, which catalyzes the conversion of testosterone into androstenedione [13]. Also, its nucleotide sequence has been determined (GeneBank accession number L08971) [14]. Although some of these steroid degradative enzymes have been well characterized, the molecular basis of their regulation is unknown. Several data emphasize the importance of clarifying the mechanisms of such a process. First, steroids play a particularly important role in certain prokaryotes, as they may simultaneously serve both as signal molecules and carbon source [12]. Second, testosterone also induces in *C. testosteroni* the synthesis of aromatic hydrocarbon-catabolizing enzymes [15]. This fact suggests that common regulation pathways may operate in the expression of steroid- and hydrocarbon-degrading enzymes. Third, we have recently described a novel transcriptional steroid-inducible gene (*stdC*) [16] which bears high homology with *Paracoccus denitrificans phaPRd* gene encoding a protein that is putatively involved in the metabolism of polyhydroxyalkanoic acids [17]. Interestingly, StdC protein was hypothesized as a possible transcriptional factor involved in the steroid degradation. All together these observations open a biotechnological interest in the study of the molecular mechanisms, controlling the expression of induced proteins by steroid compounds.

In the present report, we demonstrate that *3β/17β-hsd* gene is tightly controlled at the transcriptional

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level by several steroids. In addition, experimental evidence indicate that this gene is under catabolite repression.

2. Materials and methods

2.1. Chemicals and reagents

Restriction endonucleases were obtained from U.S. Biochemicals, Cleveland, OH. All other chemicals were of the highest purity available and were purchased from Sigma. [α-^{32}P] dATP was purchased from New England Nuclear, Boston, MA.

2.2. Bacterial strains and culture conditions

C. testosteroni cultures were grown at 30°C in either Luria Bertani broth or M9 minimal medium, supplemented with appropriate carbon sources. To test the effect of different carbon sources on the *3β/17β-hsd* gene expression, 0.25 μg/ml of either testosterone, cholic acid, cholesterol, 1-dehydrotestosterone, androstenedione or 4-pregnene-11β-17α-21-triol-20-one (cortisol) and 0.5% w/v of either acetate or citrate were added.

2.3. Northern blot analysis

C. testosteroni was grown in a culture medium as specified in each case and total RNA was extracted as described previously [16]. RNA samples (20 μg/lane) were electrophoresed on a 0.8% (w/v) agarose gel containing 1.2% formaldehyde and transferred to a nitrocellulose membrane [16]. Equal loading and transferring were assessed by methylene blue staining of the membrane. The membrane was prehybridized overnight at 37°C with 5 × NaCl/Cit (1 × NaCl/Cit contains 0.15 M NaCl and 0.15 M sodium citrate, pH 7), 50% formamide, 1% SDS, and 5 × Denhardt's solution and hybridized 6 h at 37°C in the same buffer with the ^{32}P-labeled 1,121 bp *Pst*I–*Hin*dIII restriction fragment of *p*SLGP1 [13]. The membrane was washed at 42°C with 0.1 × NaCl/Cit, 0.1% SDS three times for 30 min each and exposed to an X-ray film. The DNA fragments were labeled with [α-^{32}P]dATP (3000 Ci/mmol) by the random priming method [18].

2.4. Transcriptional induction kinetic

A 100 ml fresh overnight culture of *C. testosteroni* cells grown in LB medium was centrifuged, washed, resuspended in an identical volume of M9 minimal medium, and separated in four identical aliquots. Testosterone, androstenedione, 1-dehydrotestosterone or 1,4-androstadiene-3,17-dione were added to each aliquot to a final concentration of 0.25 mg/ml and RNA extraction was performed at different times: 0 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 10 h, and 24 h. Simultaneously, an aliquot of 1 ml was taken off and centrifuged at 12,000 *g* to identify the steroid recovered from the *C. testosteroni* cell cultures at each time. The cells were washed repeatedly with an equal volume of phosphate buffer saline (PBS), resuspended in PBS and sonicated with two pulses of 100 W for 30 s. The culture supernatant and sonicated cells were extracted three times with 3 volumes of ethyl-ether and evaporated under nitrogen stream. The residue was dissolved in 50 μl chloroform–methanol (1:1 v/v), submitted to thin layer chromatography (TLC) on silica gel GF 254, and developed using benzene–ethanol (95:5 v/v) as the solvent system. The plates were visualized using the UV 254 light.

2.5. Carbon catabolite repression analysis

A 75 ml fresh overnight culture of *C. testosteroni* cells grown in LB medium at 30°C was centrifuged,

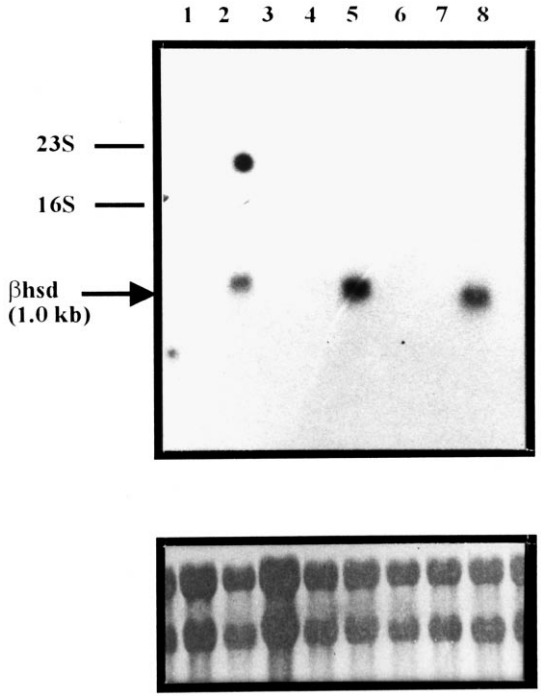

Fig. 1. Northern blot analysis. Total RNAs from *C. testosteroni* cells grown during 6 h in M9 minimal medium plus 0.1% casamino acids and: acetate (lane 1), testosterone (lane 2), cholic acid (lane 3), cholesterol (lane 4), 1-dehydrotestosterone (lane 5), citrate (lane 6), cortisol (lane 7) and androstenedione (lane 8) as carbon source were analyzed by electrophoresis on formaldehyde–agarose gels, transferred to a nylon membrane and stained with methylene blue (bottom panel). This membrane was hybridized with a probe spanning the *3β/17β-hsd* gene to yield the top panel. Marks on the left indicate the position of the 16 and 23 S rRNA bands, and the arrow indicates the position of the *3β/17β-hsd* transcript.

washed, resuspended in an identical volume of M9 minimal medium, and separated in three identical aliquots. Testosterone, testosterone plus acetate and testosterone plus citrate were added to each aliquot up to a final concentration of 0.25 mg/ml for testosterone and 0.5% for acetate or citrate. RNA extraction was performed at several times: 0, 15, 30, 45, 60, and 120 min.

3. Results

3.1. The 3β/17β-hsd gene is regulated at the transcriptional level

The expression of the *3β/17β-hsd* gene was examined at the transcriptional level. Northern blot analysis of total RNA, using the probe as 1,121 bp *Pst*I–*Hind*III fragment carrying the complete 3β/17β-HSD coding sequence as the probe, showed the presence of a single RNA transcript of approximately 1 kb. This transcript was detected in RNA samples obtained from cells grown in the presence of testosterone, 1-dehydrotestosterone or androstenedione (Fig. 1, lanes 2, 5 and 8, respectively). Conversely, no signal was observed in the RNA samples isolated from *C. testosteroni* cells grown in presence of acetate, cholic acid, cholesterol, citrate, and cortisol (lanes 1, 3, 4, 6 and 7, respectively).

3.2. Transcriptional induction kinetic of the 3β/17β-hsd gene

The levels of the *3β/17β-hsd* transcript were measured at several times after the addition of testosterone, androstenedione, 1-dehydrotestosterone or 1,4-androstadiene-3,17-dione. The results indicated that the four steroids tested are inducers of the *3β/17β-hsd* gene (Fig. 2). The densitometric data showed detectable *3β/17β-hsd* transcript in the range of 15–30 min (lane 2–3), after the addition of the steroids. Also, it was observed that *3β/17β-hsd* mRNA level in *C. testosteroni* cells grown in the presence of testosterone (Fig. 2(A)), reached a maximum after 2 h of culture and then decreased to undetectable levels. Similar kinetic *3β/17β-hsd* transcript patterns were obtained in cells grown in the presence of 1-dehydrotestosterone or 1,4-androstadiene-3,17-dione (Fig. 2(C) and (D)). In contrast, a high *3β/17β-hsd* mRNA level was obtained from cells grown in the presence of androstenedione even after 24 h of culture (Fig. 2(B)). In order to investigate, if a different metabolic kinetic pattern occurs in *C. testosteroni*, the steroids recovered from the cells as well as from the culture medium were analyzed by TLC. Fig. 3 shows the steroid patterns obtained from the cells indicating that the four steroids assayed were completely degraded in 10 h of culture. Similar results were found for the steroids recovered from the culture medium (data not shown).

3.3. Half life of 3β/17β-hsd mRNA

The stability of the *3β/17β-hsd* transcript was studied by inhibiting the transcription in cells cultivated in the presence of testosterone. *C. testosteroni* cells were induced for 2 h in M9 minimal medium containing testosterone as the sole carbon source; then rifampicin (100 μg/ml) was added to the culture. Northern blot experiments were performed with the total RNA

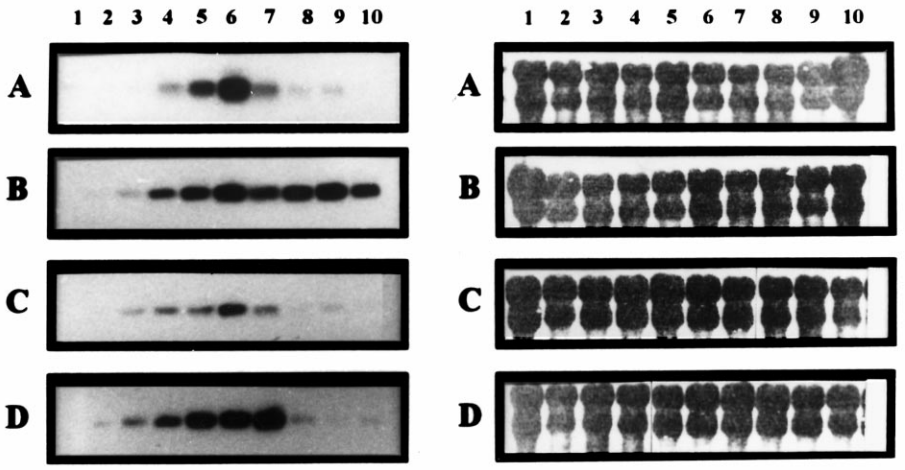

Fig. 2. Transcriptional induction kinetic of the *3β/17β-hsd* gene. An overnight culture of *C. testosteroni* cells grown in LB medium was centrifuged, suspended in M9 minimal medium and separated into four aliquots. Testosterone (A), androstenedione (B), 1-dehydrotestosterone (C), and 1,4-androstadien-3,17-dione (D) were added to each aliquot and RNA extractions were performed at different times: 0 (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 2 h (lane 6), 4 h (lane 7), 6 h (lane 8), 10 h (lane 9), and 24 h (lane 10). The RNA samples were analyzed on formaldehyde–agarose gels, transferred to a nylon membrane and stained with methylene blue (left panel).The membrane was hybridized with a probe spanning the *3β/17β-hsd* gene to yield the right panel. Data shown are representative of three independent experiments.

isolated from cells at several times after rifampicin addition (Fig. 4(A) and (B)). The *3β/17β-hsd* transcript levels were measured by conducting densitometry (Fig. 4(C)), indicating that its half life was 2 min (data in agreement with those reported for a typical prokaryotic mRNA) [19]. Similar results were obtained with the other steroids (data not shown).

3.4. 3β/17β-hsd Gene carbon catabolite repression control

In order to determine if another extra carbon source can affect the transcriptional induction mediated by testosterone, we performed Northern blot experiments with RNA obtained from *C. testosteroni* cells grown in the presence of testosterone plus acetate or citrate. Fig. 5 (left panel) shows that the simultaneous presence of an extra carbon source like acetate (Fig. 5(B)) or citrate (Fig. 5(C)) in addition to testosterone, inhibited the synthesis of *3β/17β-hsd* transcript indicating that this gene is under catabolite repression control.

4. Discussion

Inducible steroid degrading enzymes from *C. testosteroni* have been intensively studied during the last 40 years, but no information is available about the molecular mechanisms responsible of these induction phenomena. In the present work, we have shown that the *3β/17β-hsd* gene is tightly controlled at the transcriptional level as demonstrated by Northern blot analysis. The results indicate that *3β/17β-hsd* gene is expressed as a monocistronic 1 kb transcript, which is

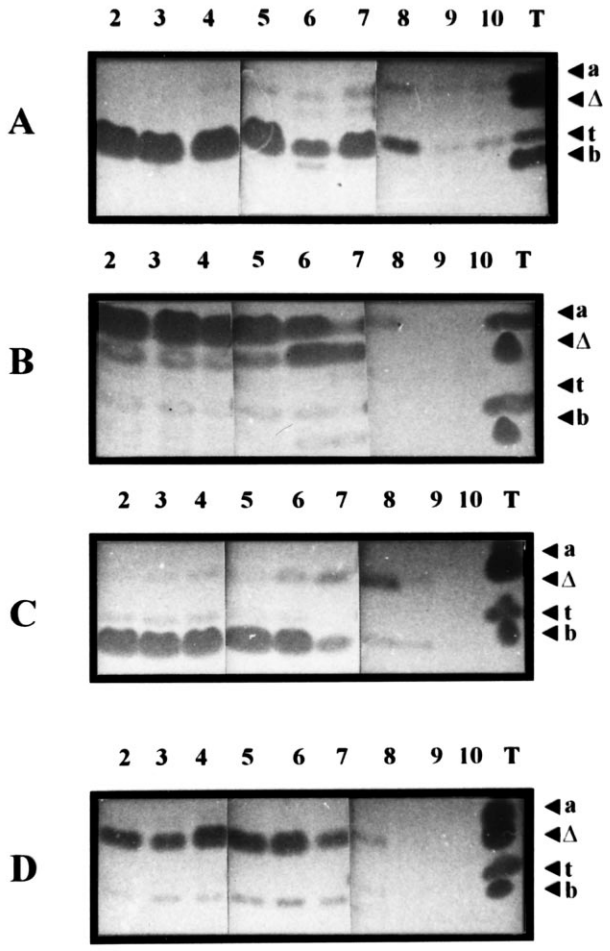

Fig. 3. TLC analysis of steroids recovered from *C. testosteroni* cell cultures. An overnight culture of *C. testosteroni* cells grown in LB medium was centrifuged, suspended in M9 minimal medium and separated into four aliquots. Testosterone (A), androstenedione (B), 1-dehydrotestosterone (C), and 1,4-androstadien-3,17-dione (D) were added to each aliquot and the steroids were extracted and analyzed as described in Material and Methods at different times: 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 2h (lane 6), 4 h (lane 7), 6 h (lane 8), 10 h (lane 9), and 24 h (lane 10) and T (control steroids). Marks on the right side indicate the position of the different control steroids: androstenedione (a), 1,4-androstadien-3,17-dione (Δ), testosterone (t), and 1-dehydrotestosterone (b). The experiment was repeated three times with similar results. Data shown are each from a representative experiment.

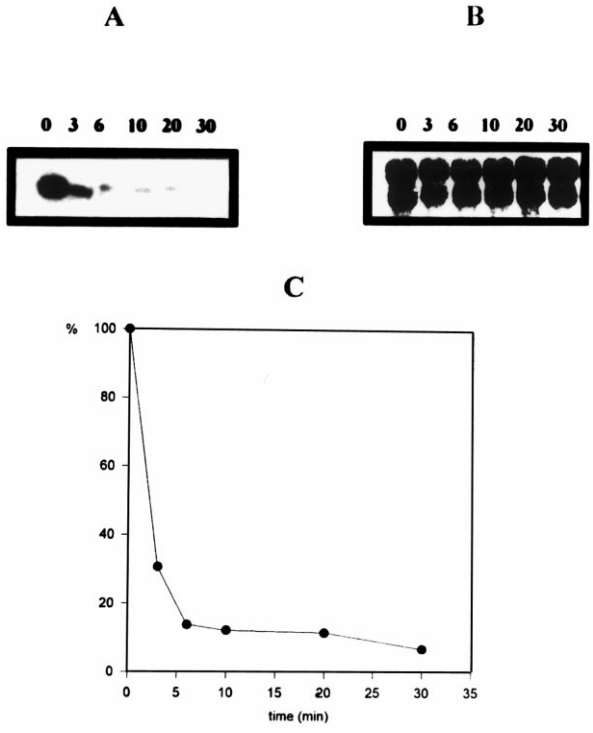

Fig. 4. *3β/17β-hsd* mRNA decay kinetic. *C. testosteroni* cells were induced after 2 h in M9 minimal medium containing testosterone as the sole carbon source. After that, rifampicin (100 μg/ml) was added and the RNA was isolated from the cells at the indicated times. The samples were analyzed on formaldehyde–agarose gels, transferred to a nylon membrane, and stained with methylene blue (B). The membrane was hybridized with a probe spanning the *3β/17β-hsd* gene to yield (A). The levels of *3β/17β-hsd* transcript were measured by densitometry (C). Each point represents the mean of two independent experiments.

abundant in cells grown in the presence of testosterone, 1-dehydrotestosterone or androstenedione but not in presence of cholic acid, cholesterol, and cortisol. These data and our previous report [16] in which it was demonstrated that *stdC* mRNA is induced by cortisol, suggest that more than one regulatory factors are involved in steroid-mediated transcription.

The *3β/17β-hsd* expression pattern observed in M9 minimal medium, 15–30 min after testosterone, 1-dehydrotestosterone or 1,4-androstadien-3,17-dione induction, reached a maximum level after 2 h and then decreased to undetectable values. This observation is not in agreement with a previously reported 3β/17β-HSD activity, in which, the enzyme activity appears after 6 h of *C. testosteroni* culture [5]. It is interesting to note that these experiments were performed with cells grown in the presence of testosterone plus yeast extract. As is demonstrated in the present report, the *3β/17β-hsd* gene is under catabolite repression control. So, it is reasonable to think that the delay in the synthesis of the 3β/17β-HSD enzyme was due to the negative effect of this extra carbon source present in the culture medium. Conversely, high *3β/17β-hsd* transcript level was observed in cells incubated in presence of androstenedione, even when all the steroids were completely degraded. Two alternative explanations are possible for understanding the sustained *3β/17β-hsd* expression. One is that androstenedione may be catabolized in a degradation pathway different from that of the other steroids assessed, generating compounds that are not detectable by TLC analysis and which could be transcriptional inducers of the *3β/17β-hsd* gene.

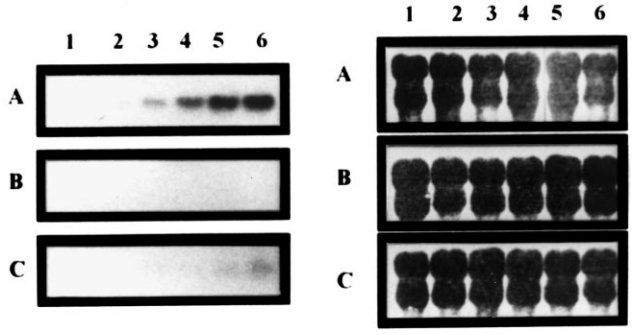

Fig. 5. Effect of additional carbon sources on the testosterone-mediated transcriptional induction of the *3β/17β-hsd* gene. An overnight culture of *C. testosteroni* cells grown in LB medium was centrifuged, suspended in M9 minimal medium and separated into three identical aliquots. Testosterone (A), testosterone plus acetate (B), and testosterone plus citrate (C) were added to each aliquot and RNA extraction was performed at different times: 0 (lane 1), 15 (lane 2), 30 (lane 3), 45 (lane 4), 60 (lane 5), and 120 min (lane 6). The RNA samples were analyzed on formaldehyde–agarose gels, transferred to a nylon membrane and stained with methylene blue (rigth panel). The membrane was hybridized with a probe spanning the *3β/17β-hsd* gene to yield the left panel. Data shown are representative of two independent experiments.

Another possibility is that the protein system responsible for the transcriptional activation of the *3β/17β-hsd* gene may have a higher affinity for androstenedione than for the other steroids, activating transcription even in the presence of undetectable androstenedione levels. The short half-life determined for *3β/17β-hsd* mRNA in *C. tesotseroni* cells, grown in presence of steroids, indicate, that the sustained level of the transcript was due to de novo synthesis and not because of transcript accumulation. Moreover, the low stability of the *3β/17β-hsd* transcript also suggests that post-transcriptional regulation could play an unimportant role in the *3β/17β-hsd* gene expression.

In conclusion, the present results demonstrate that the regulation of inducible *3β/17β-hsd* gene from *Comamonas testosteroni* is tightly controlled at the transcriptional level and is dependent on the carbon status of the cell. Molecular cloning of the regulatory proteins, responsible for the transcriptional induction of this gene, a requirement to elucidate the molecular basis of the data reported in this paper, is currently underway.

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