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Construction of a biosensor mutant of *Comamonas testosteroni* for testosterone determination by cloning the EGFP gene downstream to the regulatory region of the 3,17β-HSD gene

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ABSTRACT

Comamonas testosteroni (*C. testosteroni*) is able to catabolize a variety of steroids and polycyclic aromatic hydrocarbons. 3,17β-Hydroxysteroid dehydrogenase (3,17β-HSD) from *C. testosteroni* is a testosterone-inducible protein and a key enzyme in steroid degradation. 3,17β-HSD is a member of the short-chain dehydrogenase/reductase (SDR) superfamily. We found that a 2.4 kb regulatory DNA fragment upstream of the 3,17β-HSD gene (*βhsd*) responds to steroids and triggers *βhsd* gene induction. To exploit this *cis*-acting regulatory element for a steroid determination system, plasmids pK2.4-EGFP-4 and pBB2.4-EGFP-8 were constructed. Both plasmids contain the EGFP gene fused downstream to a 2.4 kb DNA fragment from *C. testosteroni*. However, whereas pK2.4-EGFP-4 could integrate into the chromosomal DNA of *C. testosteroni* and knock out the *βhsd* gene promoter, pBB2.4-EGFP-8 could replicate in *C. testosteroni* cells as a free plasmid DNA. After integration of pK2.4-EGFP-4 into the *βhsd* gene promoter 3,17β-HSD expression could not be induced such that EGFP expression in the mutant cells was at low levels. In contrast, in *C. testosteroni* cells transformed with pBB2.4-EGFP-8 the expression of EGFP was induced with testosterone. Our results showed that fluorescence counts (relative fluorescence units; RFU) increased in parallel with testosterone concentrations. Of note, estradiol and cholesterol could not induce the EGFP reporter gene. In summary, this new biosensor system might be used for the specific determination of testosterone.

1. Introduction

Natural and synthetic hormones are constantly excreted into the environment from human and animal sources. In the 1990s, some findings suggested that hormonally active agents may cause adverse health effects in humans and wild life [1]. Although the occurrence of steroid hormones in the environment has received a great deal of attention, there is still lack of exact data on their concentrations, because of the expensive and complex determination methods currently applied. Therefore, new methods for steroid detection and quantification are urgently needed that are cheap, easy to perform, and are sensitive enough to detect even lowest concentrations in the environment.

Comamonas testosteroni (*C. testosteroni*) is a Gram-negative bacterium found in soil and water [2], but has also been found in humans [3]. This bacterium is able to grow on steroids or aromatic hydrocarbons as sole carbon and energy source and may therefore play an important role in the biodegradation of these compounds in the environment [4]. In earlier investigations we found that 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) is a key enzyme in the degradation of these complex ring structures by *C. testosteroni* [5]. We have constructed a biosensor system to detect some steroid compounds with the 3α-HSD/CR gene promoter from *C. testosteroni* fused to the GFP gene in a reporter plasmid. However, this system is not suitable to detect a particular steroid specifically [6].

About 20 enzymes are involved in steroid degradation and most of their genes are inducible by steroids. To exploit the steroid inducible gene expression system from bacteria as a biosensor system for the specific detection of single steroid, we focused on the regulatory region of the gene coding for 3,17β-hydroxysteroid dehydrogenase (3,17β-HSD) from *C. testosteroni*. 3,17β-HSD is a member of the short-chain dehydrogenase/reductase (SDR) superfamily and is known as a testosterone inducible and key enzyme in steroid degradation [7,8].

Abbreviations: HSD, hydroxysteroid dehydrogenase; MCT, mutant *Comamonas testosteroni*; EGFP, enhanced green fluorescent protein.

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In this work, two plasmids pK2.4-EGFP-4 and pBB2.4-EGFP-8, both of which harbour the EGFP gene fused downstream to a 2.4 kb DNA regulatory fragment of the 3,17β-HSD gene (*βhsd*) from *C. testosteroni*, were prepared and transformed into *C. testosteroni* cells. Plasmid pK2.4-EGFP-4 integrated into the *C. testosteroni* chromosomal DNA, thereby leading to an interruption of the *βhsd* gene regulatory region. The resulting knock-out mutants (MCT = mutant *C. testosteroni* cells) could not respond to testosterone induction and served as negative control.

In contrast, plasmid pBB2.4-EGFP-8 could replicate in *C. testosteroni* as free plasmid and was shown to respond to testosterone induction via the *βhsd* promoter sequence. Since the cells with the replicating plasmid did only respond to testosterone, but not to estradiol nor to cholesterol, we conclude that they may serve as a cellular system for the specific detection and quantification of testosterone.

2. Materials and methods

2.1. Bacterial strains and plasmids

Host strains *Escherichia coli* (*E. coli*) HB101 (Promega) and *C. testosteroni* ATCC 11996 (Deutsche Sammlung für Mikroorganismen) were used for cloning and gene expression. Cloning of PCR fragments was carried out in pCR2.1-TOPO (Invitrogen). The target genes were subcloned into plasmid pUC19 (Invitrogen). Plasmid pK18 containing the kanamycin resistance gene was a gift from Ciba Pharmaceuticals Inc., Department of Biotechnology (Basel, Switzerland) [9]. Plasmid pBB1MCS-2 was a gift from Peterson and co-workers [10].

2.2. Growth media and growth conditions

Bacterial cells were grown in a shaker (180 rpm) in Standard I Nutrient broth medium (Merck, Darmstadt) or LB medium at 37 °C (*E. coli*) or 27 °C (*C. testosteroni*). Growth media contained 60 μg/ml ampicillin and 30 μg/ml kanamycin.

2.3. Restriction enzymes and other reagents

Restriction enzymes were obtained from Boehringer Mannheim, Biolabs, MBI and Amersham, and used according to the manufacturers' instructions. Ampicillin and kanamycin were from AGS, Heidelberg. Recombinant DNA work was carried out following standard techniques according to Sambrook and Russel [11].

2.4. Isolation of chromosomal DNA of C. testosteroni

Chromosomal DNA of *C. testosteroni* was isolated by phenol/chloroform extraction. One ml of overnight culture cells was harvested by centrifugation at 13,000 rpm for 20 s and then resuspended in 1 ml distilled water containing 1 μg lysozyme. Cells were lysed by freezing (−20 °C, 30 min) and thawing (at room temperature, 30 min) 3 times. DNA was then recovered from the lysate by phenol/chloroform extraction, followed by ethanol precipitation. The DNA was suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) and stored at 4 °C. The purified chromosomal DNA was used for target gene PCR.

2.5. PCR and plasmid construction

C. testosteroni chromosomal DNA was used as a template for PCR. PCR was performed according to the following conditions: 94 °C for 30 s, 68 °C for 180 s, 45 °C for 30 s; 40 cycles. Primers used for PCR are shown in Table 1. Resulting PCR fragments were cloned into plasmid pCR2.1-TOPO and subcloned into pUC19, pK18 and pBB1MCS-2.

Table 1
Primers used to perform PCR.

Primer name	Sequence
P1	AAGCTTGTTTGTCAGGGTTAC
P2	AAGCTTTCATCTATAGCCC
P3	AAGAGGAGACAGAGGGTGAGCAAGGGCGA
P4	AAGCTTTCATCATAGCCCCA
P2.409	TCAAGAGGAGACAGAGG
PEGFPR	TTACTTGTACAGCTCGTC
PEGFP-L	GTGAGCAAGGGCGAGGA
PEco-R	AGCTCCACCGCGGTGGCGGCC

2.6. Transformation of bacteria

All constructs were verified by restriction enzyme analysis of the resulting band patterns and/or were sequenced by MWG, Ebersberg. Plasmids were purified with the Tip-100 kit from Qiagen (Qiagen, Hilden). Ligated constructs were transferred into competent *E. coli* cells by the calcium chloride method or into competent *C. testosteroni* cells by electroporation (1.8 kv). *C. testosteroni* cells transformed with plasmid pK2.4-EFGP-4 represented the MCT (mutant *C. testosteroni)* strain in which the *βhsd* gene regulatory region was disrupted by insertion of the EGFP reporter construct. This strain represented the negative control.

In contrast, *C. testosteroni* cells transformed with plasmid pBB2.4-EGFP-8, harbouring the functional *βhsd* gene regulatory region upstream of the EGFP gene, served as the cell-based biosensor system.

2.7. Protein extraction

The probes for 3,17β-HSD ELISA detection were prepared by lysozyme digestion: 3 ml of bacterial cultures were centrifuged at 13,000*g* for 10 s. The pellet was washed three times with 1 ml PBS and resuspended in 200 μl PBS with 100 μg/ml lysozyme. To complete cell lyses, the suspension was frozen three times at −20 °C. Finally, the samples were centrifuged again at 13,000*g* for 20 min. The supernatant was used for protein determination and ELISA.

2.8. Protein determination

Protein concentration was measured by the method of Bradford [12].

2.9. ELISA of 3,17β-HSD

To quantify 3,17β-HSD protein expression, an ELISA was established and respective antibodies were generated. Rabbit antibodies directed against 3,17β-HSD from *C. testosteroni* were prepared according to standard methods. ELISA plates were coated with protein extracts containing 3,17β-HSD in coating buffer. The protein concentration of the samples was adjusted to 1 mg/ml. After 1:100 dilution with wash buffer 200 μl was added to each well. After washing, antibodies against 3,17β-HSD were added in a 1:10,000 dilution. The further procedure corresponded to that of the CAT ELISA kit from Boehringer, Mannheim.

2.10. Fluorescence microplate assay

In a total volume of 500 μl SIN medium, 50 μl *C. testosteroni* cells and 2 μl steroid sample (in ethanol and SIN medium) were incubated at 27 °C for 16 h. The cells were then washed with water

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and centrifugation. To 96-well black plates (100 µl wells) were added 100 µl $OD_{595\ nm}$ = 2.0 fresh cultured *C. testosteroni* wild type or mutant cells, and the resulting fluorescence measured. The results are given as RFU (relative fluorescence units) = sample with steroid – sample without steroid. GENios Pro Fluorescence Microplate Reader from Tecan, Austria GmbH was used to measure steroid concentrations in the samples, at excitation: 485 nm and emission: 535 nm.

3. Results

3.1. The βhsd gene could be induced by testosterone in C. testosteroni cells

Wild type *C. testosteroni* cells were cultured in the presence of 0.25 mM testosterone, estradiol or cholesterol, respectively. As shown in Fig. 1, ELISA results reveal that only testosterone could induce 3,17β-HSD expression in *C. testosteroni* wild type cells. Obviously, the promoter domain of the *βhsd* gene can be regulated by testosterone but not by estradiol nor cholesterol. According to our previous findings [8], the promoter domain of the *βhsd* gene extends over 2.407 kb (Fig. 2).

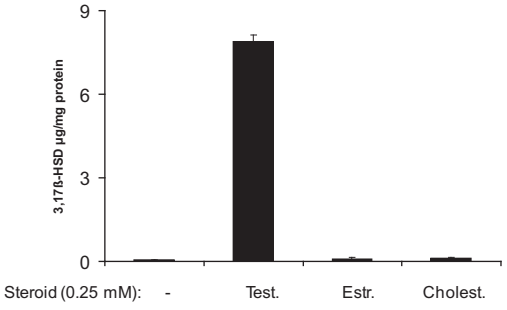

Fig. 1. Induction of the *βhsd* gene in *C. testosteroni* ATCC 11996 by testosterone. *C. testosteroni* ATCC 11996 cells were grown in the presence of 0.25 mM of different steroids. The results of ELISA show that only testosterone could induce 3,17β-HSD expression. The regulatory promoter region of the *βhsd* gene might therefore be used to generate a biosensor system for the specific determination of testosterone (Test = testosterone, Estr = estradiol, Cholest = cholesterol).

3.2. Isolation and cloning of a 3.184 kb DNA fragment containing the 3,17β-HSD gene together with its regulatory region

As shown in Fig. 2 (bottom), a 3.184 kb DNA fragment from *C. testosteroni* chromosomal DNA was prepared by PCR. Upstream of the entire 3,17β-HSD gene, this fragment contained the genes coding for RseB and PhaR [8], as well as two repeat sequences (RS1, RS2) and two promoters (promoter 1 and promoter 2) for 3,17β-HSD expressional regulation. Since the entire 3.184 kb DNA fragment could not be obtained by a single PCR with primers P1 and P4 from *C. testosteroni* cells nor *C. testosteroni* genomic DNA as template, the following PCR and cloning strategy was applied (cf. Fig 2; Table 1).

First, combining primer P1 with P3 and primer P2 with P4 yielded two DNA fragments (2.407 kb and 2.519 kb), respectively with *C. testosteroni* genomic DNA as template (Fig. 2). Second, with restriction enzyme *Nru*I the upstream part of the 2.407 kb (1.256 kb) and the downstream part of the 2.519 kb fragment (1928 bp, Fig 3) were fused and cloned into pUC19 to yield pUC-3.2-4, the latter containing the desired fragment of 3.184 kb (Fig. 3). Third, pUC-3.2-4 was finally used as a template, together with primers P1 and P4, to successfully amplify the 3.184 kb fragment by PCR (Fig. 2). The agarose gel (Fig. 2) reveals that the partial fragments (2407 bp and 2519 bp) could both be amplified with *C. testosteroni* cells or *C. testosteroni* genomic DNA as template, whereas the complete 3.184 kb fragment could only be obtained with plasmid pUC-3.2-4 as PCR template, although a faint 3.184 kb band was visible with *C. testosteroni* cells.

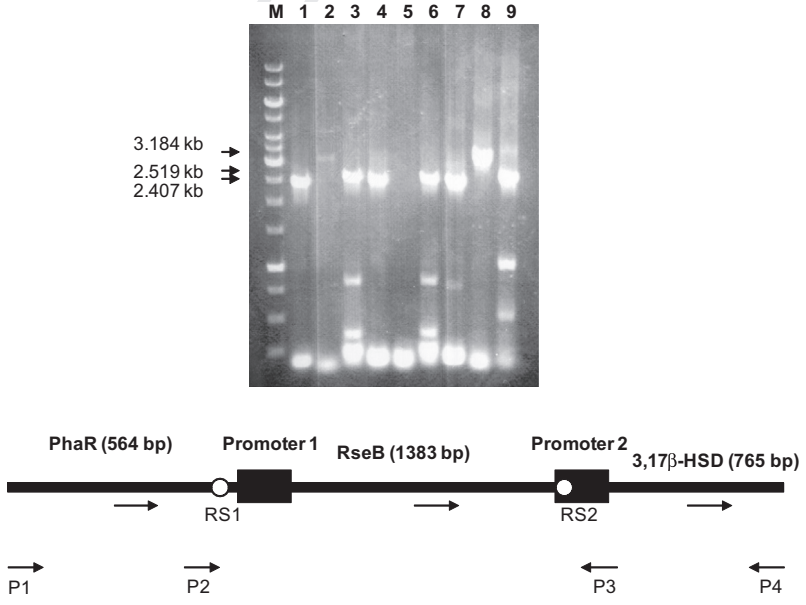

Fig. 2. PCR preparation of DNA fragments containing different parts of the *βhsd* gene regulatory promoter region. A variety of DNA fragments representing different parts of the *βhsd* gene regulatory promoter region was prepared by PCR (for primers used cf. Table 1). Lanes 1, 2, 3 = PCR fragments with *C. testosteroni* cells as template; lanes 4, 5, 6 = *C. testosteroni* chromosomal DNA as template; lanes 7, 8, 9 = plasmid pUC3.2-4 as template. Lanes 1, 4, 7 = PCR fragment with primers P1 and P3 (2.407 kb); lanes 2, 5, 8 = PCR fragment with primers P1 and P4 (3.184 kb); lanes 3, 6, 9 PCR fragment with primers P2 and P4 (2.521 kb). Both 2.407 kb and 2.519 kb DNA fragments were obtained by PCR with *C. testosteroni* cells, chromosomal DNA or plasmid pUC-3.2-4. However, the 3.184 kb fragment could only be multiplied with plasmid pUC-3.2-4 as template (lane 8), although a faint band at 3.184 kb appeared with *C. testosteroni* cells as PCR template (lane 2). The fragments were separated in a 0.8% agarose gel.

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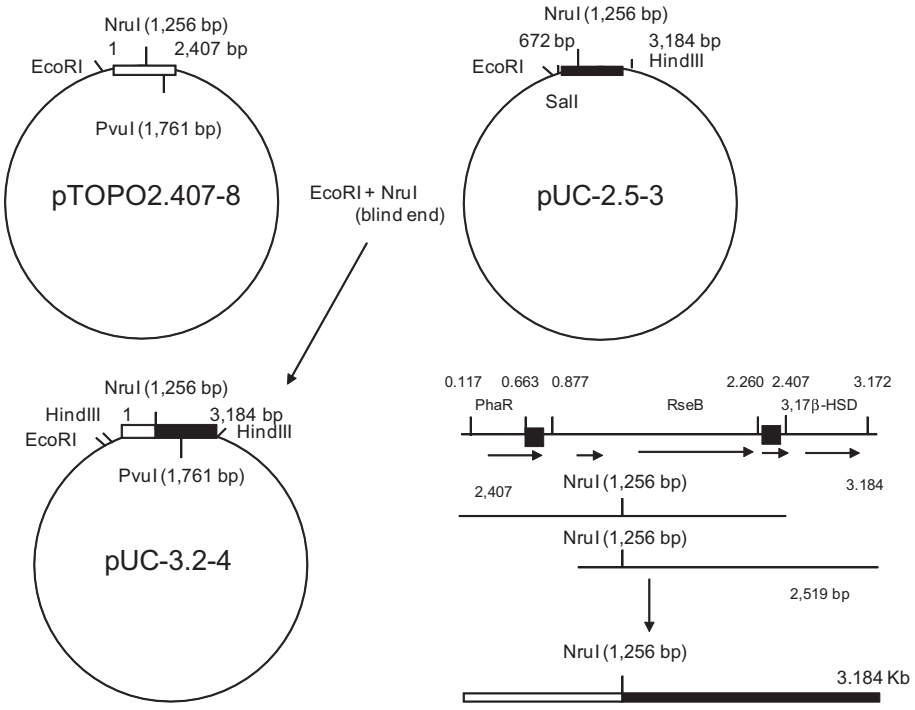

Fig. 3. Construction of a 3.184 kb fragment containing the entire regulating region of the *βhsd* gene. The 2.407 kb fragment and the 2.519 kb fragment (cf. Fig. 2) were cloned into plasmid pTOPO2.407-8 (upper left) and pUC-2.5-3 (upper right), respectively. Restriction enzyme *Nru*I was used to generate pUC-3.2-4 (lower left) which harbours the entire 3.184 kb regulatory region of the 3,17β-HSD gene (lower right).

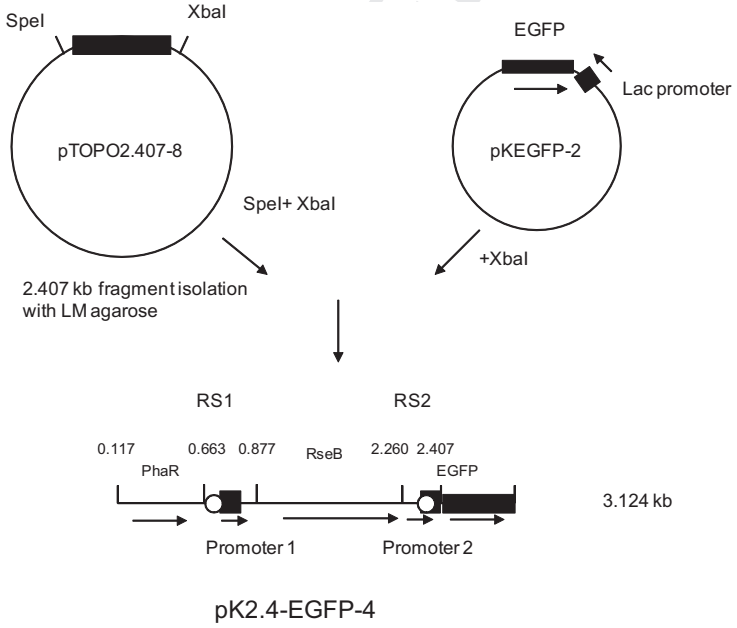

Fig. 4. Preparation of plasmid pK2.4-EGFP-4. A 2.407 kb fragment was digested from pTOPO2.407-8 with *Spe*I and *Xba*I. The fragment was cloned into pKEGFP-2 with *Xba*I digestion to yield plasmid pK2.4-EGFP-4. Plasmid pK2.4-EGFP-4 integrates into the chromosomal DNA of *C. testosteroni*, thereby leading to an interruption of the *βhsd* gene regulatory region. As a consequence, the resulting mutants (MCT = mutant *C. testosteroni*) could not respond to testosterone induction and served as negative control in the present study.

3.3. Subcloning and fusion of the 2.407 kb fragment with the EGFP gene into vectors pK18 and pBBR1MCS-2

Plasmid pK18 can replicate in *E. coli* but not in *C. testosteroni* cells. Plasmid pBBR1MCS-2, in turn, replicates in both *E. coli* and *C. testosteroni* cells. As shown in Fig. 4, the EGFP gene (717 bp) was cloned into pK18 to yield plasmid pKEGFP-2. The 2.407 kb fragment upstream of the 3,17β-HSD gene was then cloned into pKEGFP-2 and the new plasmid pK2.4-EGFP-4 was obtained which harbors the entire 3.124 kb reporter construct consisting of the βhsd gene regulatory region (2.407 kb) fused to the EGFP gene (717 bp). Plasmid pK2.4-EGFP-4 could integrate into the

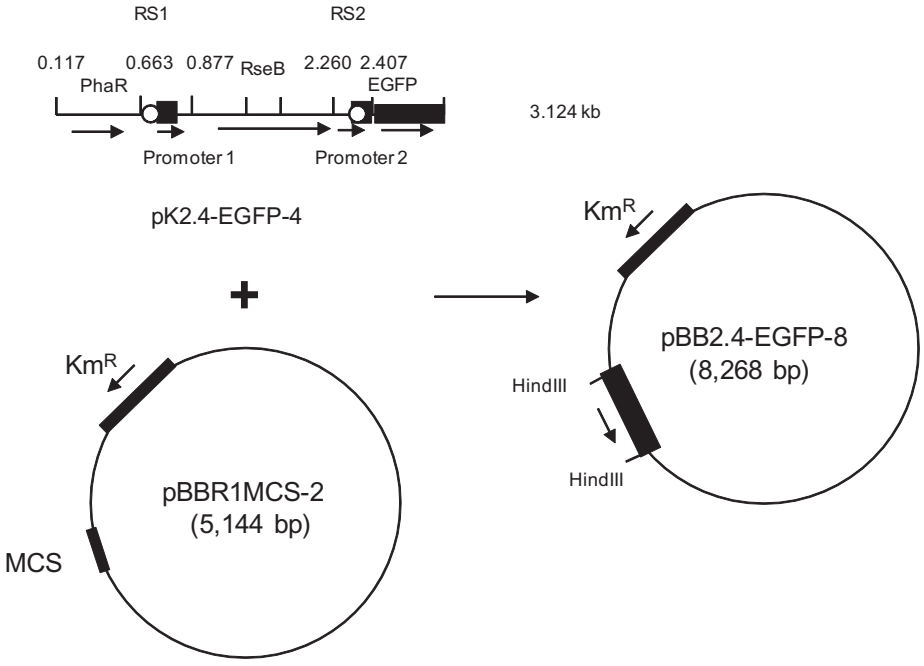

Fig. 5. Preparation of plasmid pBB2.4-EGFP-8. Plasmid pBBR1MCS-2 can replicate in both *E. coli* and *C. testosteroni*. The 2.407 kb and the EGFP gene fragment was digested with *Hind*III from pK2.4-EGFP-4 (cf. Fig. 4) and used to prepare the subclone plasmid pBB2.4-EGFP-8.

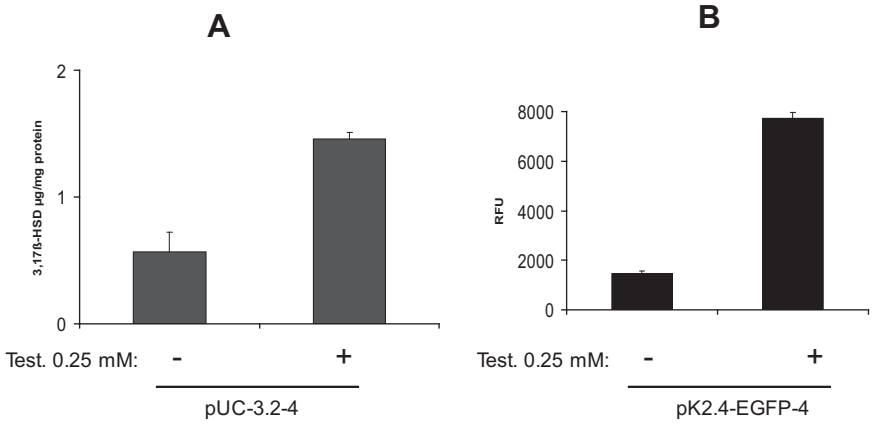

Fig. 6. *E. coli* HB101 cells transformed with pUC-3.2-4 or pK2.4-EGFP-4, both respond to induction by testosterone. *E. coli* HB101 cells transformed with either plasmid pUC-3.2-4 or plasmid pK2.4-EGFP-4 were grown in the presence of 0.25 mM testosterone. Fig. 6A shows the ELISA result of *βhsd* gene induction by testosterone in *E. coli* cells with pUC-3.2-4. Fig. 6B shows, likewise, the EGFP gene induction by testosterone in *E. coli* cells with pK2.4-EGFP-4.

chromosomal DNA of *C. testosteroni* by homologous cross linking and antibiotic pressure, because this plasmid is not able to replicate in *C. testosteroni* cells.

In a further approach, the entire insert (3.124 kb) of pK2.4-EGFP-4 was subcloned into pBBR1MCS-2 with restriction enzyme HindIII (Fig. 5) to yield plasmid pBB2.4-EGFP-8 (8.268 kb). This latter plasmid could replicate in *C. testosteroni* cells as a free plasmid and should confer antibiotic resistance to *C. testosteroni* cells as well as providing the ability to respond to testosterone.

3.4. Transformation of *E. coli* HB101 with plasmids pUC3.2-4 and pK2.4-EGFP-4 followed by testosterone induction

Plasmid pUC3.2-4 was transformed into *E. coli* HB101 cells. The *E. coli* culture was induced with 0.25 mM testosterone. ELISA was used to determine 3,17β-HSD expression in *E. coli* HB101 cells. As shown in Fig. 6A, the expression of the *βhsd* gene in pUC3.2-4 increased upon testosterone induction.

Plasmid pK2.4-EGFP was transformed into *E. coli* HB101 cells as well and the resulting fluorescence (RFU) was determined as a measure of EGFP gene induction via testosterone. Fig. 6B showed that the RFU likewise increased when 0.25 mM testosterone was added into the culture medium.

Other steroids such as estradiol and cholesterol were also tested in both systems. Interestingly, neither estradiol nor cholesterol addition to the culture medium lead to an induction of the genes coding for 3,17β-HSD (ELISA) or EGFP (RFU) (data not shown).

3.5. Transformation of *C. testosteroni* with plasmids pK2.4-EGFP-4 and pBB2.4-EGFP-8

C. testosteroni cells were transformed with plasmids pK2.4-EGFP-4 and pBB2.4-EGFP-8 by electroporation. Plasmid pK2.4-EGFP-4 could not replicate in *C. testosteroni* cells. As a consequence, only these transformed *C. testosteroni* cells could grow up in

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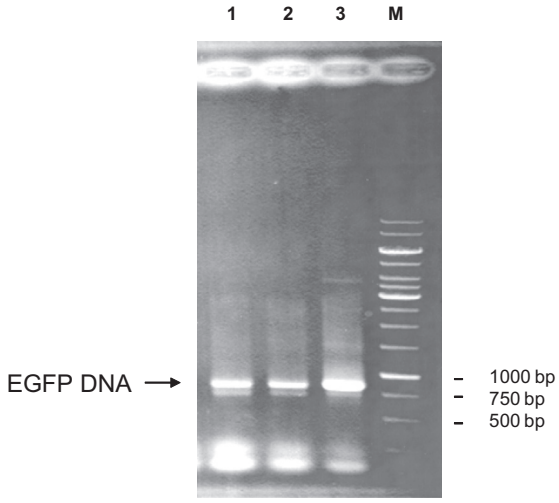

Fig. 7. PCR identification of transformed and integrated plasmids in *C. testosteroni*. To prove the successful plasmid transformation of *C. testosteroni*, primers of the EGFP gene were used for PCR, together with pK2.4-EGFP-4 mutated or pBB2.4-EGFP transformed *C. testosteroni* cells, respectively. Lane 1: the 760 bp fragment proved that plasmid pK2.4-EGFP-4 was integrated into *C. testosteroni* chromosomal DNA. Lane 2: the 770 bp fragment revealed that pBB2.4-EGFP-8 replicated in *C. testosteroni* cells. Lane 3: positive control PCR with plasmid pBB2.4-EGFP-8 as template. The fragments were run in a 0.8% agarose gel. The results reveal that the EGFP gene was present in all cells tested.

kanamycin and ampicillin medium if they contained plasmid pK2.4-EGFP-4 integrated in their chromosomal DNA. On the other hand, this plasmid integration lead to an interruption of the 3,17β-HSD gene regulatory region, such that *C. testosteroni* cells transformed with plasmid pK2.4-EGFP-4 could be considered as gene regulatory 3,17β-HSD knock-out cells which lost their ability to respond to testosterone induction.

PCR was used to prove plasmid pK2.4-EGFP-4 integration into *C. testosteroni* chromosomal DNA. As shown in Fig. 7 (lane 1), primers P2.409 and PEGFPR (Table 1) yielded the expected 760 bp fragment upon PCR, spanning the fusion of the *βhsd* gene regulatory region with the EGFP gene. This indicates that the EGFP gene had been integrated into the chromosomal DNA of mutant *C. testosteroni* (MCT) cells.

In contrast, plasmid pBB2.4-EGFP-8 could replicate in *C. testosteroni* cells. After transformation the free plasmid pBB2.4-EGFP-8 confers kanamycin and ampicillin resistance to *C. testosteroni* cells. Again, PCR was used to prove the presence of the replicating plasmid. By using primers PEGFP-L and PEco-R (Table 1), the resulting 770 bp fragment (Fig. 7, lane 2) indicates that plasmid pBB2.4-EGFP-8 replicates in the transformed *C. testosteroni* cells. As a control, plasmid pBB2.4-EGFP-8 itself was used as the template with the same pair of primers, finally yielding the corresponding 770 bp fragment (Fig. 7, lane 3).

3.6. Testosterone determination with the new biosensor system

Mutant *C. testosteroni* (MCT) cells as well as *C. testosteroni* cells transformed with pBB2.4-EGFP-8 were cultured in 0.5 ml SIN medium with kanamycin. The cells were induced with 0.25, 0.125, 0.063, 0.032, 0.016, 0.0078, 0.0039 mM ethanol diluted testosterone in 1.5 ml Eppendorf tubes. After 16 h of culture at 27 °C, cells were washed with water by centrifugation. Finally, the cells were resuspended in 220 µl water. The optical density of 100 µl bacterial suspension was adjusted to OD_{595} = 2.0 and used to determine the relative fluorescence units (RFU).

As shown in Fig. 8A, the RFU of *C. testosteroni* cells transformed with pBB2.4-EGFP-8 increased in parallel to the testosterone concentration applied. Whereas the negative control (only ethanol without testosterone) resulted in 17,237 RFU, these values increased to 42,029 when the testosterone concentration was 0.25 mM. Accordingly, *C. testosteroni* cells transformed with pBB2.4-EGFP-8 can be used as a biosensor system for testosterone determination.

In contrast, RFU values did not change with increasing testosterone concentrations in mutant *C. testosteroni* (MCT) cells (Fig. 8B). These mutants cannot be used to detect testosterone.

To test the specificity of the new biosensor system for testosterone, estradiol and cholesterol, in addition to testosterone, were added to *C. testosteroni* cultures transformed with pBB2.4-EGFP-8. Because estradiol and cholesterol did not lead to an induction of *βhsd* gene expression in *C. testosteroni* wild type cells (Fig. 1), we expected that, likewise, the RFU should not change with the EGFP reporter system. As to be seen in Fig. 9, indeed neither estradiol nor cholesterol caused any response, whereas testosterone again showed a strong increase in RFU. Thus, the results of the EGFP biosensor system with pBB2.4-EGFP-8 (Fig. 8) correspond to those obtained by ELISA with the 3,17β-HSD expression in wild type *C. testosteroni* cells (Fig. 1). Both results emphasize the specificity of the biosensor system for testosterone.

4. Discussion

There are several methods such as the classical analytical HPLC, GC, ELISA, and mass spectrometry or human nuclear steroid receptor systems to detect and quantify steroids like testosterone,

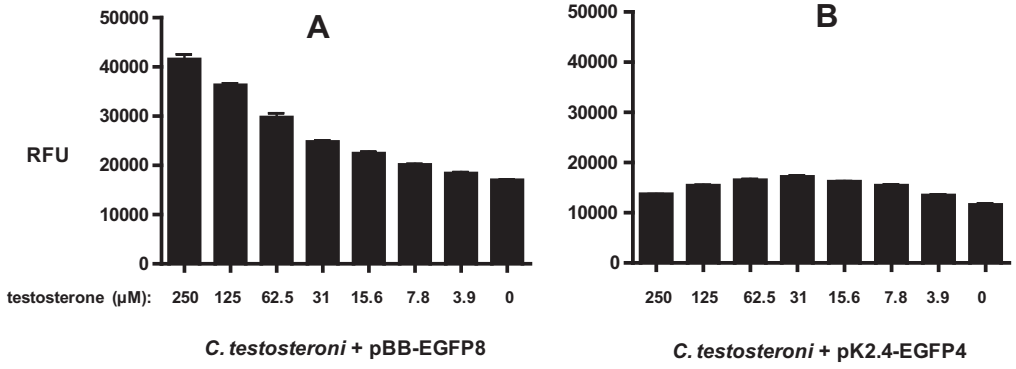

Fig. 8. Fluorescence determination upon induction with testosterone. *C. testosteroni* cells transformed with either pK2.4-EGFP-4 or pBB2.4-EGFP-8 were grown in the presence of different concentrations of testosterone and the resulting fluorescence measured (RFU = relative fluorescence units). RFU ($X \pm SD$, $n = 4$) was detected with excitation wavelength 485 nm and emission wavelength 535 nm. The cells with free plasmid pBB2.4-EGFP-8 showed a strong and concentration dependent increase in RFU upon testosterone induction. In contrast, after integration of pK2.4-EGFP-4 the cells responded only weakly to testosterone.

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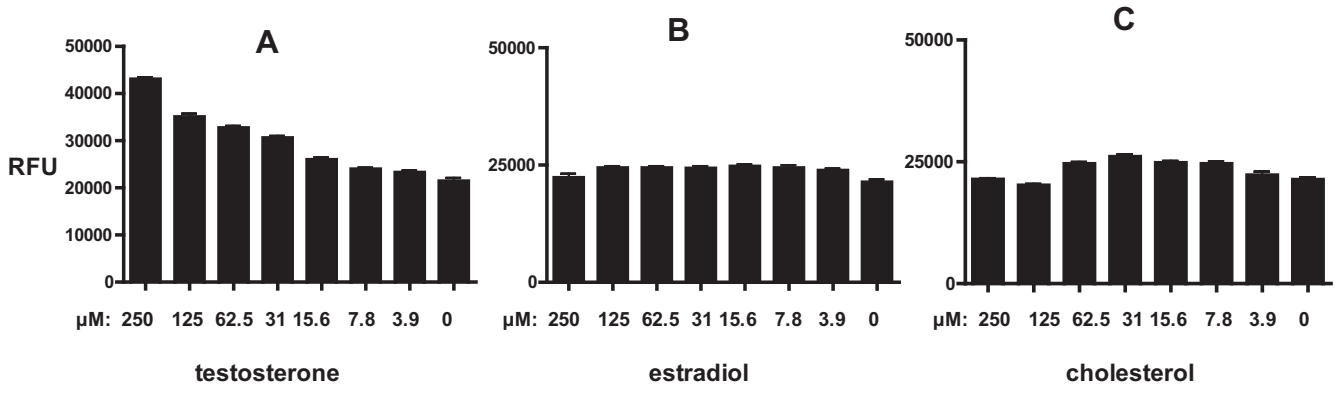

Fig. 9. Fluorescence determination upon induction with different steroids. *C. testosteroni* cells transformed with pBB2.4-EGFP-8 were grown in the presence of different concentrations of testosterone, estradiol or cholesterol and the resulting fluorescence measured (RFU = relative fluorescence units, $X \pm SD$, $n = 4$). Only testosterone leads to an induction of the EGFP gene via response to the *βhsd* gene regulatory region, whereas estradiol and cholesterol did not. Hence, the *βhsd* promoter/EGFP gene construct might be used for the specific determination of testosterone.

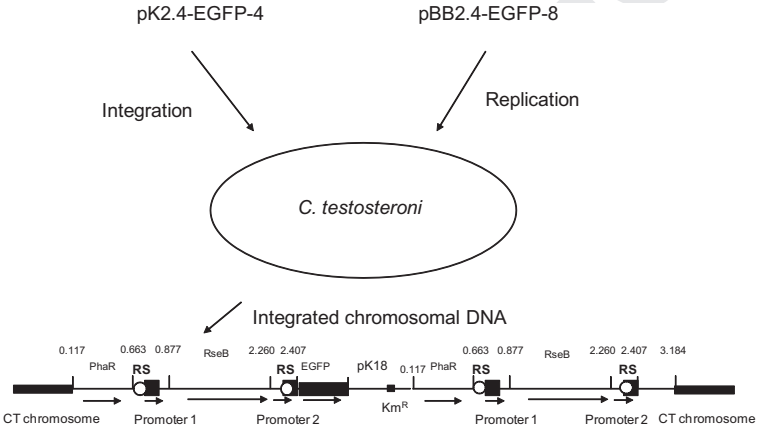

Fig. 10. Transformation and DNA plasmid integration of *C. testosteroni*. *C. testosteroni* cells were transformed with plasmid pK2.4-EGFP-4. This plasmid cannot replicate in *C. testosteroni* cells, but is able to integrate into its chromosomal DNA, thereby leading to an interruption of the 3,17β-HSD gene regulatory region. Upon kanamycin pressure only these *C. testosteroni* mutants (MCT) with the integrated plasmid can grow up and can be isolated. After transformation of *C. testosteroni* with pBB2.4-EGFP-8, this plasmid can replicate as free plasmid in *C. testosteroni* cells under kanamycin pressure.

estradiol and cholesterol in biological samples [13]. These methods are both time consuming and expensive.

We have previously reported the construction of a novel biosensor system with the bacterium *C. testosteroni* to detect steroid compounds [6]. This COSS (*C. testosteroni* steroid sensor) system was based on the upstream regulatory region of the 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) gene (*hsdA*). The expression of this gene is under multifactorial control of a variety of activators and repressors [5,14–16]. To construct the COSS system, we replaced the *hsdA* gene within the *C. testosteroni* chromosomal DNA by the gene coding for the green fluorescent protein (GFP) [6]. In the resulting reporter strain, GFP was under transcriptional control of the *hsdA* upstream regulatory region, including the *cis*-acting promoter and operator sequences together with the corresponding *trans*-acting factors [6]. The system could be used to detect testosterone, estradiol and cholesterol. However, while the system is easy to handle, cost efficient and enables the detection of several different steroids at the same time, it is not applicable to the determination and quantification of a specific steroid compound. For the latter approach, we seek more specific methods to detect a single steroid species such as testosterone, estradiol or cholesterol in natural samples.

The remarkable ability of *C. testosteroni* to use steroids both as carbon source and also as signal molecules for the induction of the appropriate steroid degrading enzymes has been matter of intense research. 3,17β-HSD in *C. testosteroni* is a testosterone inducible enzyme which is involved in steroid degradation [7,8]. In our work, we found that the encoding gene *βhsd* in *C. testosteroni* could only be induced by testosterone, but neither by estradiol nor cholesterol (Fig. 1). This is why we focussed on the *βhsd* gene regulatory region to construct a biosensor system specific for the determination of testosterone.

To generate appropriate reporter plasmids, we first aimed at preparing the complete upstream regulatory region of the *βhsd* gene by PCR. As shown in Fig. 2, this region contains, upstream of the *βhsd* gene, the genes coding for RseB and PhaR [8], as well as two repeat sequences (RS1, RS2) and promoter 1 and promoter 2. Because the entire 3.184 kb DNA fragment could not be obtained by a single PCR with respective primers from *C. testosteroni* cells nor *C. testosteroni* genomic DNA as template, we had to generate plasmid pUC-3.2-4. This plasmid contained the entire 3.184 kb region and was obtained by combining fragments from plasmid pTOPO2.407-8 und pUC-2.5-3 with restriction enzyme *Nru*I (Fig. 3). With plasmid pUC-3.2-4 as template, it was possible to

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generate the desired reporter plasmids pBB2.4-EGFP-8 (Fig. 5) and pK2.4-EGFP-4 (Fig. 6).

Although we performed several trials, to obtain the 3.184 kb fragment in a single PCR from *C. testosteroni* cells or from *C. testosteroni* genomic DNA, we failed. The reason for this failure is possibly the presence of the two repeat sequences RS1 and RS2 within the regulatory region. While the chromosomal DNA of *C. testosteroni* (in cells or purified) forms a loop structure via these two repeat sequences [8] and PCR is blocked, this loop structure is resolved when the DNA fragment becomes smaller, such as in plasmid in pUC-3.2-4. In other words, the loop structure within the 3.184 kb fragment is open such that PCR with template pUC-3.2-4 can proceed.

To enhance the sensitivity of the testosterone biosensor, we fused the *βhsd* gene regulatory region upstream to the EGFP (enhanced green fluorescence protein) gene to yield plasmids pK2.4-EGFP-4 (Fig. 4) and pBB2.4-EGFP-8 (Fig. 5). In a first set of experiments, the applicability of the resulting EGFP gene containing plasmids was tested in *E. coli* HB101 and compared to those baring the *βhsd* gene (Fig. 6). Indeed, testosterone leads to a higher extent of induction of the EGFP gene with plasmid pK2.4-EGFP-4 (RFU) as that of the *βhsd* gene obtained with plasmid pUC-2.54 (ELISA).

We then transformed *C. testosteroni* cells with plasmids pK2.4-EGFP-4 and pBB2.4-EGFP-8 and performed induction experiments with testosterone. While we were aware of the fact that plasmid pK2.4-EGFP-4 cannot replicate in *C. testosteroni* cells, it was a surprise to notice that pK2.4-EGFP-4 integrates into the chromosomal DNA of *C. testosteroni* (Fig. 7). Moreover, due the sequence identity of its *βhsd* gene promoter part, plasmid pK2.4-EGFP-4 integrated into the upstream regulatory region of the *βhsd* gene within the chromosomal DNA of *C. testosteroni* by homologous cross linking. The resulting mutant *C. testosteroni* (MCT) strain lost its ability to respond to testosterone induction (Table 1) and could be considered as regulatory 3,17β-HSD knock out. As shown in Fig. 2, *C. testosteroni* wild type cells contain two repeat sequences (RS1, RS2) upstream of the *βhsd* gene. The integration of plasmid pK2.4-EGFP-4 into this region leads to a doubling of these repeat sequences (Fig. 10) such that the DNA structure is altered and testosterone induction prevented. This MCT strain cannot be used as a biosensor system for testosterone detection (Fig. 8A).

In contrast, pBB2.4-EGFP-8 could replicate in *C. testosteroni* cells as free plasmid and was shown to provide *C. testosteroni* cells the ability to respond to testosterone induction in a concentration dependent manner (Fig. 8B). As the free plasmid pBB2.4-EGFP-8 contains the same *βhsd* gene promoter sequences as the chromosomal DNA of *C. testosteroni,* induction of the EGFP gene in plasmid pBB2.4-EGFP-8 resembles the induction of the *βhsd* gene in *C. testosteroni* and can be used as a biosensor system for testosterone detection.

Importantly, the new biosensor system seems to be specific for testosterone determination (Fig. 9A), because it did not respond to estradiol nor cholesterol in the same approach (Fig. 9B and C). The negative response towards estradiol and cholesterol with plasmid pBB2.4-EGFP-8 resembles our initial studies wild type *C. testosterone* cells, which did not show any enhanced expression of the *βhsd* gene in the presence of these two steroids (Fig. 1). In principle, this also means that the simultaneous presence of estradiol and cholesterol in a biological sample does not influence testosterone determination in this biosensor system.

In our study, we have established the detection system with testosterone concentrations in the low μM range. Relevant steroid concentrations occurring in the environment are probably lower, and the steroids may also be protein bound or particle adsorbed. This would limit the practicability of the assay in its current form, because the steroids need to be chloroform extracted and vacuum concentrated. However, we consider our system as a successful, first attempt to design and construct a biosensor for testosterone.

We have sequenced the genomic DNA of *C. testosteroni* ATCC 11996 [17]. From these and other data it is known that more than 20 enzymes are involved in the complete degradation of steroid compounds [5,18–21]. It can be speculated that the expression of some of the corresponding genes is also controlled by steroids, the regulatory region of which may therefore serve as future biosensor systems for the specific detection of steroids other than testosterone.

In summary, we have constructed a new bacterial cell based biosensor system for the specific detection and quantification of testosterone in biological samples. By fusing the regulatory region of the testosterone inducible 3,17β-HSD gene from *C. testosteroni* ATCC 11996 with the gene coding for EGFP, we successfully generated plasmid pBB2.4-EGFP-8 which is able to replicate in *C. testosteroni* and which provides the specificity of transformed *C. testosteroni* cells towards testosterone induction. Q6

Transparency Document

The Transparency document associated with this article can be found in the online version. Q7

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