Chemico-Biological Interactions 178 (2009) 215–220

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Cis- and *trans*-regulatory elements of 3α-hydroxysteroid dehydrogenase/carbonyl reductase as biosensor system for steroid determination in the environment

Guangming Xiong, Yijing Luo, Saihong Jin, Edmund Maser*

Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunswikerstr. 10, D-24105 Kiel, Germany

ARTICLE INFO

Article history:
Available online 19 October 2008

Keywords:
Comamonas testosteroni
3α-Hydroxysteroid
dehydrogenase/carbonyl reductase
Biosensor
Steroid determination

ABSTRACT

3α-Hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni* is a key enzyme in the degradation of steroids in the environment. The encoding gene, *hsdA*, is expressed only at very low levels in the absence of steroids, but undergoes a several fold induction in the presence of steroid substrates. In previous investigations, we have elucidated the mechanism of *hsdA* regulation that involves several activators and repressors. In the present study, the *hsdA* gene was replaced by the green fluorescent protein (GFP) gene which was inserted downstream from the *hsdA* regulatory region. By homologous integration into the chromosomal DNA, the *C. testosteroni* mutant strain CT-GFP5-1 was generated and used as fluorescence based biosensor system for steroid determination. With this cell-based system we could determine testosterone in a range between 57 and 450 ng/ml, estradiol between 1.6 and 12.8 ng/ml, and cholesterol between 19.3 and 154.4 ng/nl. Interestingly, the sensitivity of this bioassay could be further increased by using only the cytosol of the mutant. With the resulting cell-free system we could determine testosterone in a range between 28 and 219 pg/ml, estradiol between 0.029 and 0.430 fg/ml, and cholesterol between 9.7 and 77.2 fg/ml. The recovery ratio of the extraction was around 95% and the maximum fluorescence signals were obtained as early as after 30 min. Limitations of the established steroid biosensor system were quenching at higher steroid concentrations and the relatively high background of fluorescence, which are currently being improved in our lab. Combined, by exploiting the regulatory region of the gene *hsdA* that codes for the enzyme 3α-hydroxysteroid dehydrogenase/carbonyl reductase we have constructed a mutant *C. testosteroni* strain that can be used as a sensitive biosensor system for steroid determination in the environment.

1. Introduction

Natural (e.g., estradiol, testosterone) and synthetic (e.g., ethinylestradiol) 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]. Estrogens and other steroids were detected in seawater, lakes and rivers in different areas [2–4]. For example, steroidal estrogens have especially been shown to be the main contributors to the estrogenic activity observed in aquatic systems contaminated with sewage treatment work effluents. Moreover, estrogens and testosterone were observed in drinking water [3].

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 is a Gram-negative bacterium found in soil and water [5], but has also been found in humans [6]. 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 [7]. We have shown in earlier investigations that 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) is a key enzyme in the degradation of these complex ring structures by *C. testosteroni* [8–10]. The enzyme catalyzes the interconversion of hydroxy- and oxo-groups at position 3 of the steroid ring structure. Interestingly, 3α-HSD/CR is expressed only at very low and basal levels in the absence of steroids, but can undergo a several fold induced expression in the presence of steroids like testosterone [8,10,11].

Previously, we elucidated the complex regulation of the 3α-HSD/CR gene (*hsdA*) from *C. testosteroni* and identified a variety of *cis*- and *trans*-regulating elements for *hsdA* expression [12–14].

* Corresponding author. Tel.: +49 431 597 3540; fax: +49 431 597 3558.
E-mail address: maser@toxi.uni-kiel.de (E. Maser).

0009-2797/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2008.10.012

In the present work, we replaced *hsdA* within the *C. testosteroni* chromosomal DNA by the gene coding for the green fluorescent protein (GFP). In the resulting mutant, GFP was under transcriptional control by the *hsdA* upstream regulatory region, including the *cis*-acting promoter and operator sequences, and the corresponding *trans*-acting factors. Both the mutant cells as well as the cytosol thereof were used as new and sensitive fluorescence based biosensor systems for the determination and quantification of steroids like testosterone, estradiol and cholesterol.

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).

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 [15].

2.4. Generation of the steroid reporter plasmid

The reporter plasmid for steroid detection was prepared by two step PCR (Fig. 1) and subcloning the resulting fragment into plasmid pCR2.1-Topo (Fig. 2). In brief, a 5.257 kb *Eco*RI fragment of *C. testosteroni* chromosomal DNA was cloned into pUC18 to yield p6 (not shown) [14]. Plasmid p6 contained the entire region for 3α-HSD/CR expression, i.e. *hsdA* itself, the regulatory genes *repA* and *repB*, as well as the promoter and two operator sequences. Plasmid p6 was used as a template for the two step PCR mutagenesis. In the first PCR (Fig. 1), primers pPromL: GCAGCACCTGCATGAGGA and pPromR: *TCCTTTGCTAGCCAT*GTCTTGTCTCCTTT were used with template p6 to generate a 483 bp DNA fragment that contained the *hsdA* promoter and operator sequences (468 bp), as well as the 3′end sequence *TCCTTTGCTAGCCAT* that complemented to the 5′end sequence of the GFP gene. The resulting 483 bp fragment from this first PCR was isolated on an agarose gel and used as the 5′end primer in the second PCR together with the 3′end primer pGFPR: TTATTTGTAGAGCTCATC and the GFP gene (720 bp) in template plasmid pcDNA3.1/CT-GFP-TOPO (Invitrogen) (Fig. 1). The final PCR fragment (1188 bp) was cloned into plasmid pCR2.1-TOPO (Invitrogen) to yield plasmid pTOPO-3αGFP5, which can replicate in *E. coli* but not in *C. testosteroni*, for the biosensor studies (Fig. 2).

2.5. 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

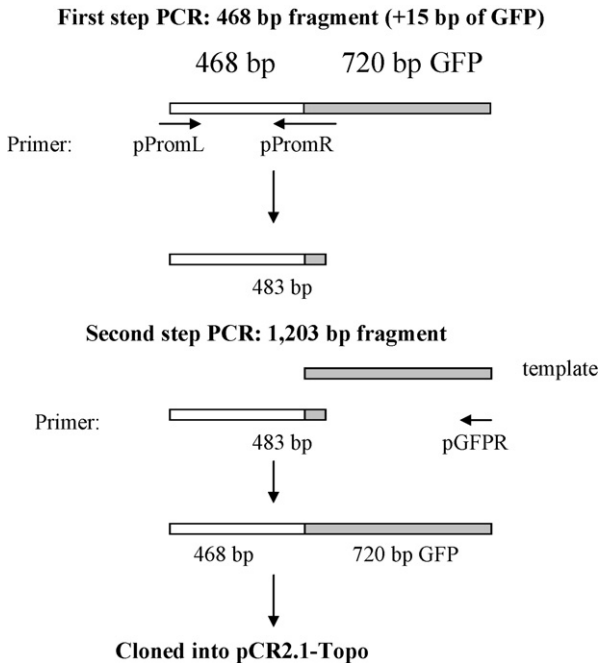

Fig. 1. Two step PCR to generate the steroid reporter construct. A 468 bp DNA region containing the upstream regulatory region of *hsdA* was fused to the GFP gene (720 bp). The resulting construct was cloned into vector pCR2.1-Topo to yield plasmid pTOPO-3αGFP5 (cf. Fig. 2).

C. testosteroni cells by electroporation (1.8 kv). *C. testosteroni* cells transformed with plasmid pTOPO-3αGFP5 represented the CT-GFP5-1 mutant strain and were used as the cell-based biosensor system. The cytoplasm prepared from these cells was used as the cell-free biosensor system.

2.6. Protein extraction

The probes for 3α-HSD/CR 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 lysis, 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.

2.7. Protein determination

Protein concentration was measured by the method of Bradford [16].

2.8. Elisa of 3α-HSD/CR

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

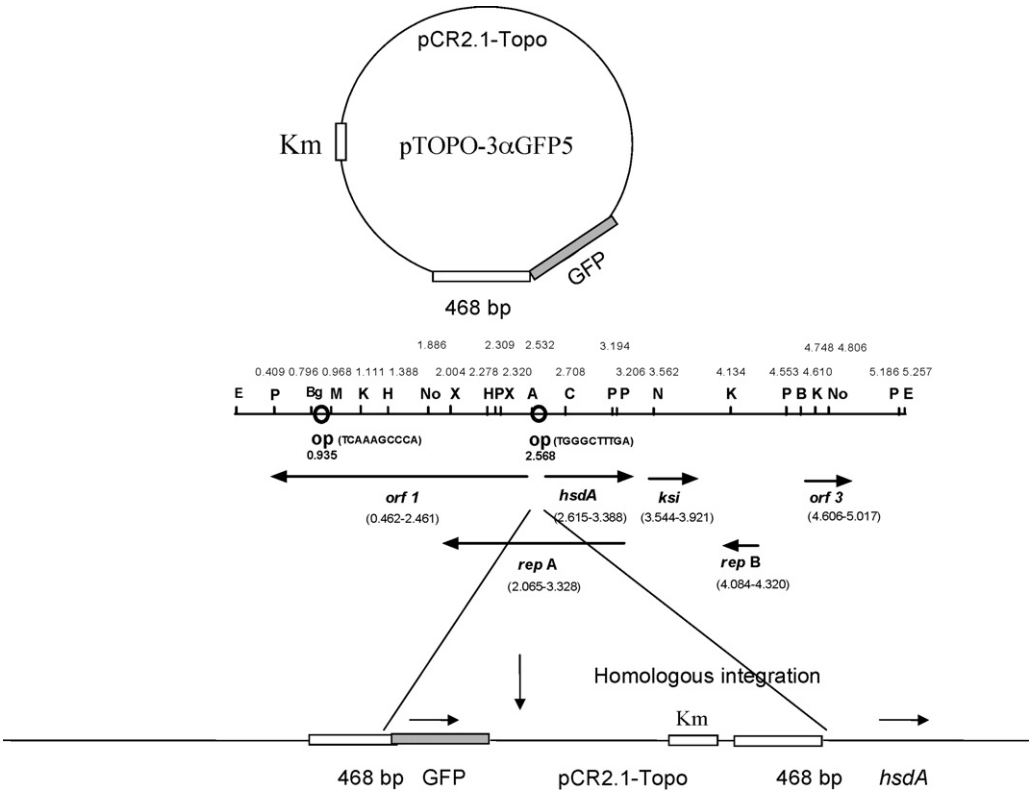

Fig. 2. The GFP gene was introduced into the chromosomal DNA of *C. testosteroni* by homologous integration. The vector was plasmid pTOPO-3αGFP5. The resulting mutant strain, CT-GFP5-1, was isolated due to its kanamycin resistance and used for further studies.

2.9. Fluorescence microplate assay

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. To 96-well black plates (100 μl wells) was added 50 μl $OD_{595\,nm}$ = 0.1 fresh cultured CT-GFP5-1 mutant cells or 50 μl 0.1 mg/ml cytoplasm of CT-GFP5-1 mutant cells, and 2 μl sample (in ethanol and SIN medium). Incubation was at room temperature for the time intervals indicated. 50 μl ELISA coating buffer (2×: 70 mM $NaHCO_3$, 30 mM Na_2CO_3, pH 9.6), was added into each well. The supernatant was discarded and the plate dried on tissue paper; then 50 μl of water was added. The results are given as RFU (relative fluorescence units) = sample with steroid–sample without steroid.

3. Results

3.1. Plasmid pTOPO-3αGFP5 preparation

As shown in Fig. 1, an 1188 bp DNA fragment was prepared by two step PCR. The fragment consisted of a 468 bp upstream region of *hsdA* containing the *hsdA* operator and promoter region. The downstream part represented the gene coding for GFP. The fragment was cloned into vector pCR2.1-TOPO to generate plasmid pTOPO-3αGFP5 which could be used as a steroid biosensor system (Fig. 2). Importantly, the resulting plasmid contained the kanamycin resistance gene and could replicate in *E. coli* but not in *C. testosteroni*.

3.2. Generation of a C. testosteroni based steroid biosensor system

C. testosteroni wild type cells were transformed with 20 μg of vector pTOPO-3αGFP5 by electroporation. Since pTOPO-3αGFP5 could not replicate in *C. testosteroni* cells, only those bacteria could grow in the kanamycin containing medium which harboured plasmid pTOPO-3αGFP5 integrated into their chromosomal DNA. Fig. 2 shows that the linearized pTOPO-3αGFP5 vector was integrated into the chromosomal DNA to yield *C. testosteroni* CT-GFP5-1 mutant cells. The CT-GFP5-1 mutants were cultured in SIN medium with 0.5 mM testosterone in 27 °C, 180 rpm overnight. Preliminary ELISA experiments showed that 3α-HSD/CR expression was increased (not shown). By use of a fluorescence microscope the green coloured bacteria could well be observed after testosterone induction (Fig. 3). To further check the system, mutant CT-GFP5-1 and wild type *C. testosteroni* cells were diluted to $OD_{595\,nm}$ = 1.0, 0.5, 0.25, 0.125, 0.063, 0.031. Microplate based fluorescence detection revealed that the fluorescence signals (given as relative fluores-

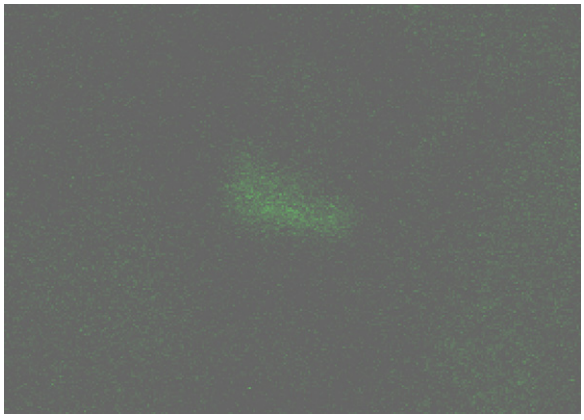

Fig. 3. Fluorescence signalling of *C. testosteroni* mutant strain CT-GFP5-1. The cells were photographed upon fluorescence microscopy after testosterone induction.

Table 1
Fluorescence detection of *C. testosteroni* CT-GFP-1 cells after testosterone induction.

$OD_{595\,nm}$	GFP5-1	*C. testosteroni*
1.0	47,674	29,605
0.5	29,274	22,442
0.25	21,176	18,124
0.125	16,761	15,827
0.063	13,687	13,724
0.031	12,676	12,512

Table 2
Time dependence of fluorescence detection with *C. testosteroni* CT-GFP-1 cells.

Estradiol (ng/ml)	5 min	10 min	30 min	60 min
13.3	41,802	41,257	47,674	46,402
6.65	40,751	40,516	47,434	46,236
3.33	40,130	39,926	46,739	45,703
1.67	39,578	39,211	45,818	44,858

cence units, RFU) indeed were higher in the CT-GFP5-1 mutants than in wild type cells (Table 1).

3.3. Steroid determination with a cell-based bioassay

The mutant CT-GFP5-1 cells were first cultured in SIN medium without steroid induction at 27 °C, 180 rpm, overnight and then adjusted to $OD_{595\,nm}$ = 0.1 with SIN medium. Testosterone, estradiol and cholesterol were diluted in SIN medium to final concentrations given below. Bacterial cells (50 μl) and the steroid containing solution (2 μl) were added into 96-well black plates (100 μl wells) and incubated at room temperature. Table 2 shows the time dependency of the incubation with estradiol as an example. Up to an incubation time of 30 min the fluorescence increased in this cell-based bioassay, and decreased again while approaching 60 min incubation. The results of testosterone, estradiol and cholesterol determination in this bioassay with different concentrations of the respective steroid are shown in Figs. 4–6. The determination range obtained was 57–450 ng/ml (0.195–1.56 μM) for testosterone (Fig. 4), 1.6–12.8 ng/ml (0.006–0.048 μM) for estradiol (Fig. 5), and 19.3–154.4 ng/nl (0.050–0.390 μM) for cholesterol (Fig. 6). Compared to testosterone, the sensitivities for estradiol and cholesterol were much higher, which is probably due to testosterone binding (scavenging effect) to RepB [13].

3.4. Steroid determination with a cell-free bioassay

A cell-free bioassay for steroid determination was established, since these are more stable than cell-based systems and do not require culturing of fresh cells before each detection [17]. Cytoplasm of mutant *C. testosteroni* CT-GFP5-1 cells was isolated and adjusted to 0.1 mg/ml protein with SIN medium. Plasmid pTOPO-3αGFP5 was added at 1 ng/ml concentrations into the cytoplasm. Cytoplasm (50 μl) and steroid (2 μl) were added into 96-well black plates (100 μl wells) and incubated at room temperature. After incubation, 50 μl ELISA coating buffer (2×) was mixed with cytoplasm. The supernatant was discarded and the plate dried on tissue paper; then 50 μl of water was added (Fig. 7). The results of testosterone, estradiol and cholesterol determination in this cell-free bioassay with different concentrations of the respective steroid are shown in Figs. 8–10. Importantly, the sensitivity of this cell-free assay was 2–3 orders of magnitude higher than that of the cell-based bioassay. The determination

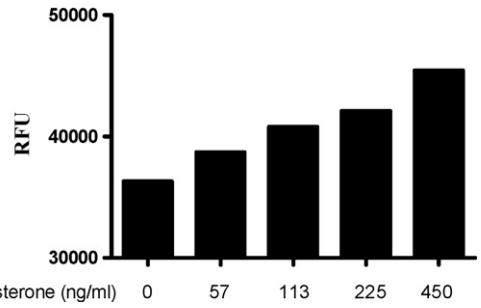

Fig. 4. Determination of testosterone with CT-GFP5-1 cells. RFU, relative fluorescence units.

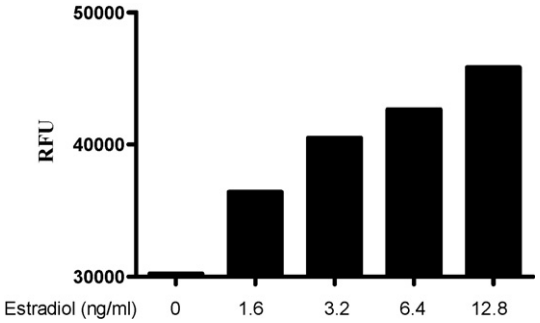

Fig. 5. Determination of estradiol with CT-GFP5-1 cells. RFU, relative fluorescence units.

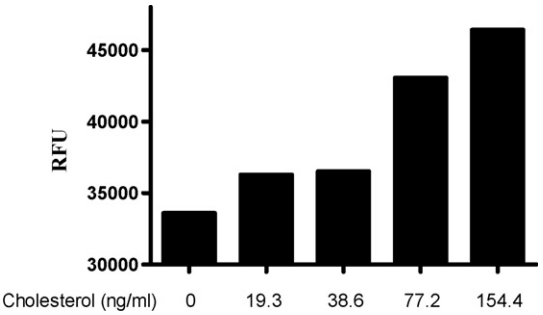

Fig. 6. Determination of cholesterol with CT-GFP5-1 cells. RFU, relative fluorescence units.

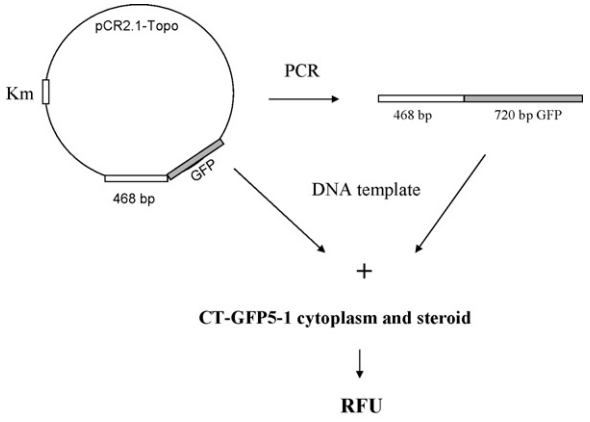

Fig. 7. Cell-free steroid determination system. The cytoplasm of mutant CT-GFP5-1 cells was isolated and incubated with the steroid samples. RFU, relative fluorescence units.

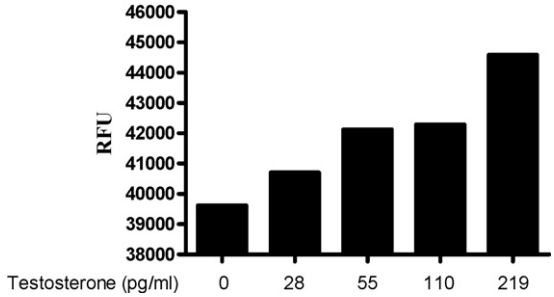

Fig. 8. Determination of testosterone with the cell-free detection system. The cytoplasm of mutant CT-GFP5-1 cells was incubated with the indicated concentrations of testosterone. RFU, relative fluorescence units.

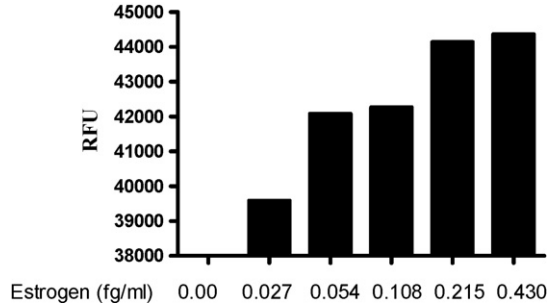

Fig. 9. Determination of estradiol with the cell-free detection system. The cytoplasm of mutant CT-GFP5-1 cells was incubated with the indicated concentrations of estradiol. RFU, relative fluorescence units.

range obtained was 28–219 pg/ml (0.1 –0.78 nM) for testosterone (Fig. 8), 0.029–0.430 fg/ml (0.1–1.53 fM) for estradiol (Fig. 9), and 9.7–77.2 fg/ml (24.5–195 fM) for cholesterol (Fig. 10). As observed for the cell-based assay, the sensitivities of estradiol and cholesterol were much higher compared to testosterone. The cytoplasm could be stored at −20 °C at least for 1 month.

3.5. Estradiol recovery after chloroform extraction

To check the system for accuracy and to simulate steroid extraction from natural sources, a definite amount of estradiol was extracted with chloroform and measured. An amount of 26.7 ng (2 μl 0.049 mM) estradiol was added into 1.0 ml SIN medium. 100 μl chloroform was mixed with the sample for 3 min. After 13,000 rpm centrifugation for 30 s and separation of the lower phase into a new eppendorf tube, the probe was vacuum centrifugated for 10 min to evaporate chloroform. The sample was solved in 10 μl ethanol, from which 2 μl was mixed with 50 μl SIN medium. After adding of 50 μl mutant *C. testosteroni* CT-GFP5-1

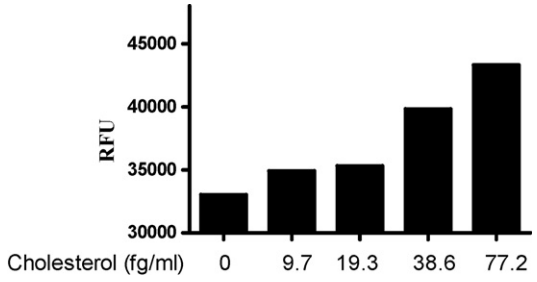

Fig. 10. Determination of cholesterol with the cell-free detection system. The cytoplasm of mutant CT-GFP5-1 cells was incubated with the indicated concentrations of cholesterol. RFU, relative fluorescence units.

Table 3
Estradiol recovery after chloroform extraction.

Estradiol (ng)	SIN + estradiol	SIN + estradiol extracted
26.7	43,315	42,992
13.3	40,138	40,083
6.7	39,158	39,363

cells ($OD_{595\,nm}$ = 0.1), RFU detection was performed. The protocol of extraction and detection revealed that estradiol recovery after chloroform extraction is more than 95% as shown in Table 3. Therefore, steroid extraction from environmental probes with chloroform should work in the above system, as should work steroid enrichment in samples where the concentration is lower than the assay sensitivity.

4. Discussion

There are several methods such as HPLC, GC, ELISA, and mass spectrometry to detect and quantify steroids like testosterone, estradiol and cholesterol in biological samples. Because of the relatively low sensitivity of these detection systems, the natural samples, especially water samples, have sometimes to be concentrated with different methods. This is why the steroid determinations are both time consuming and expensive.

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. In the course of our work, to solve the mystery of 3α-HSD/CR induction [8–10,12,18], we have reported on the identification of RepA and RepB that are involved in *hsdA* regulation (Fig. 2) [13,14]. RepA acts as a transcriptional repressor by binding to two operator sequences upstream of *hsdA* and preventing transcription of *hsdA* [14]. The gene coding for repressor RepB locates 932 nt downstream of *hsdA*. RepB also acts as a negative regulator of *hsdA* expression, but on the translation level [13]. We found that RepB binds to a 16 nt sequence downstream from AUG at the 5′ end of the 3α-HSD/CR mRNA, thereby preventing *hsdA* translation.

As shown in Figs. 1 and 2, the 720 bp GFP gene was inserted downstream from the *hsdA* regulatory region into the chromosomal DNA of *C. testosteroni* to yield the mutant CT-GFP5-1. Accordingly, the GFP gene is under the transcriptional control of RepA, but not of RepB, since the latter binds to the 3α-HSD/CR mRNA.

Recently, we reported the ability of several steroids to induce 3α-HSD/CR expression in *C. testosteroni* wild type cells: 17α-Hydroxyprogesterone, 11α-hydroxyprogesterone, 21α-hydroxyprogesterone, pregnenolone, androstandione, 1-dehydrotestosterone, 5-androsten-3β-17β-diol, deoxycorticosterone and testosterone [12]. Estradiol and cholesterol did not induce 3α-HSD/CR expression. However, in a sister paper we report on the ability of *C. testosteroni* to degrade estradiol and cholesterol when added to the culture. This means, that 3α-HSD/CR is obviously not involved in estradiol and cholesterol degradation and that regulation of the steroid degrading pathway might be different to that of testosterone.

For the present study this means that testosterone probably binds to both RepA and RepB, but estradiol and cholesterol bind only to RepA. As described above, RepB is an important repressor for 3α-HSD/CR wild type cells but it cannot regulate GFP gene induction in the CT-GFP5-1 mutant cells of the present study. Therefore, estradiol and cholesterol induced GFP expression but not 3α-HSD/CR expression (not shown). Furthermore, since testosterone binds to both RepA and RepB, which are both present in CT-GFP5-1 cells and CT-GFP5-1 cytosol, they might act as a kind of scavenging proteins for testosterone. On the other hand, estradiol and cholesterol bind

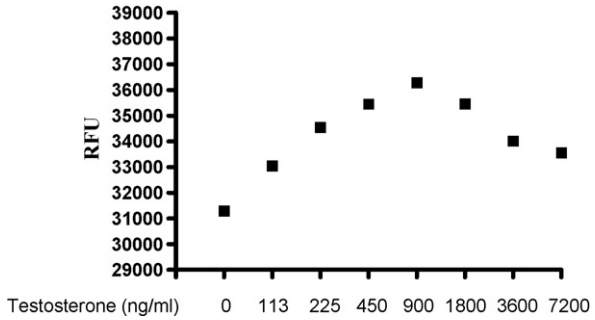

Fig. 11. Testosterone overload led to a decrease of fluorescence.

only to RepA, thus resulting in higher concentrations of free (i.e. unbound) steroid. This might be the reason for the lower amounts needed of estradiol and cholesterol for GFP gene expression compared to testosterone.

Interestingly, 2 μl of a 0.1 fM estradiol solution could be detected in the cell-free system which corresponds to only 600 molecules in the 50 μl reaction volume. This underlines the high sensitivity of the system, where steroid binding releases RepA from the operator such that transcription of the GFP gene may proceed. GFP gene transcription in turn functions as a magnifying mechanism for steroid determination and quantification. Another advantage of the cell-free system, in addition to its sensitivity, is its stability without the need for cell culturing [17]. The cell-free system can be stored at −20 °C for several months.

On the other hand, it seems that the concentration range of the steroid determination system is limited. As shown in Fig. 11, RFU increased dependent on the testosterone concentration between 0 and 900 ng/ml (1.56 mM). However, above 900 ng/ml of testosterone RFU decreased. The same effect was observed with estradiol and cholesterol (not shown) and might possibly be due to a quenching effect of the fluorescence signal. It should be noted that the current system does not allow distinguishing between different steroids. Another disadvantage is the high background of fluorescence observed in both the cell-based and cell-free systems. We are currently working to improve the assay conditions to get a larger determination range and to decrease the high background fluorescence.

In summary, we have fused the upstream regulatory region of the gene (*hsdA*) coding for the enzyme 3α-hydroxysteroid dehydrogenase/carbonyl reductase in *C. testosteroni* with that coding for GFP. The resulting *C. testosteroni* mutant cells and even more the cell-free cytosol of the mutants can be used as a sensitive biosensor system for steroid determination in the environment.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (MA 1704/4-1; MA 1704/4-2 and the Excellence Cluster “The Future Ocean” (to E. M.).

References

[1] T. Colborn, F.S. vom Saal, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environ. Health Perspect. 101 (1993) 378–384.
[2] K. Barel-Cohen, L.S. Shore, M. Shemesh, A. Wenzel, J. Mueller, N. Kronfeld-Schor, Monitoring of natural and synthetic hormones in a polluted river, J. Environ. Manage. 78 (2006) 16–23.
[3] L.S. Shore, M. Gurevitz, M. Shemesh, Estrogen as an environmental pollutant, Bull. Environ. Contam. Toxicol. 51 (1993) 361–366.
[4] A.M. Soto, J.M. Calabro, N.V. Prechtl, A.Y. Yau, E.F. Orlando, A. Daxenberger, A.S. Kolok, L.J. Guillette Jr., B.B. le, I.G. Lange, C. Sonnenschein, Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in Eastern Nebraska, USA, Environ. Health Perspect. 112 (2004) 346–352.
[5] E. Garcia Valdes, E. Cozar, R. Rotger, J. Lalucat, J. Ursing, New naphthalene-degrading marine Pseudomonas strains, Appl. Environ. Microbiol. 54 (1988) 2478–2485.
[6] G.R. Cooper, E.D. Staples, K.A. Iczkowski, C.J. Clancy, Comamonas (Pseudomonas) testosteroni endocarditis, Cardiovasc. Pathol. 14 (2005) 145–149.
[7] A.W. Coulter, P. Talalay, Studies on the microbiological degradation of Ring A, J. Biol. Chem. 243 (1968) 3238–3247.
[8] E. Möbus, E. Maser, Molecular cloning, overexpression, and characterization of steroid-inducible 3α-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*, J. Biol. Chem. 273 (1998) 30888–30896.
[9] 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.
[10] 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.
[11] C.T. Oppermann, K.J. Netter, E. Maser, Carbonyl reduction by 3 alpha-HSD from *Comamonas testosteroni*— new properties and its relationship to the SCAD family, Adv. Exp. Med. Biol. 328 (1993) 379–390.
[12] A. Göhler, 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.
[13] G. Xiong, H.J. Martin, E. Maser, Identification and characterization of a novel translational repressor of the steroid-inducible 3 alpha-hydroxysteroid dehydrogenase/carbonyl reductase gene in *Comamonas testosteroni*, J. Biol. Chem. 278 (2003) 47400–47407.
[14] G. Xiong, E. Maser, Regulation of the steroid-inducible 3alpha-hydroxysteroid dehydrogenase/carbonyl reductase gene in *Comamonas testosteroni*, J. Biol. Chem. 276 (2001) 9961–9970.
[15] J. Sambrook, D.W. Russel, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 2001.
[16] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of proteins using the principle of dye-binding, Anal. Biochem. 72 (1976) 248–254.
[17] T. Lamla, S. Hoerer, M.M. Bauer, Screening for soluble expression constructs using cell-free protein synthesis, Int. J. Biol. Macromol. 39 (2006) 111–121.
[18] C. Grimm, E. Maser, E. Mobus, G. Klebe, K. Reuter, R. Ficner, The crystal structure of 3alpha -hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni* shows a novel oligomerization pattern within the short chain dehydrogenase/reductase family, J. Biol. Chem. 275 (2000) 41333–41339.