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**ISOLATION AND IDENTIFICATION OF TWO
REPRESSORS, TetR AND LuxR, FOR $3,17\beta$ -*hsd* AND 3α -*hsd/cr*
GENE REGULATION IN *Comamonas testosteroni***

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Summary

In the 1950s, the importance of microbial degradation was acknowledged with the emergence of bioinformatics approaches. Numerous reports have investigated the elimination of steroid hormones from contaminated ecosystems by microbial degradation. Due to its capability of degrading multiple toxic aromatic contaminants, *Comamonas testosteroni* (*C. testosteroni*) is widely recognised as a significant environmental bacterium for metabolising various toxic aromatic pollutants, and it has been proven to be a prospective biocatalyst for environmental pollution. Enzymatic biodegradation and biosynthesis are important intracellular processes. However, the expression of some enzymes could be inhibited directly by many environmental pollutants, thus interfered by endogenous active steroid hormone levels. The hydroxysteroid dehydrogenases (HSDs) are a set of steroidogenic enzymes with a major role in steroid biosynthesis and metabolism [1]. Thus, research on the regulation of HSDs has been a recent trend. 3,17 β -Hydroxysteroid dehydrogenase (3,17 β -HSD) from *C. testosteroni* is a pivotal HSD enzyme in steroid degradation. To elucidate the complete molecular regulation of *C. testosteroni*, improving the understanding of the 3,17 β -*hsd* gene induction mechanism is essential. Sequencing of the *C. testosteroni* ATCC11996 genome showed that there are two repeat sequences (RS; 16 bp), RS1 and RS2, located upstream of 3,17 β -*hsd*. Between RS1 and RS2, there are 1,661 bp. In this work, a *tetR* repressor (522 bp) downstream of 3,17 β -*hsd* was identified. A bioinformatics analysis showed that the TetR family of proteins plays a critical role in transcriptional repressors, which control the regulation by environmental signals *in vivo*. Results also shown that *C. testosteroni* can metabolise steroid compounds by a group of enzymes involved in environmental responses. Here it is proposed that TetR might be a repression factor for inhibiting the expression of 3,17 β -*hsd* in *C. testosteroni*. The overarching goal of this dissertation was to identify the *tetR* regulation mechanism and its pathway for 3,17 β -*hsd*, so *tetR* was cloned into various plasmids with different fragments of the 3,17 β -*hsd* gene, with and without the *enhanced green fluorescent protein* (*egfp*) as a reporter gene, to infer the functional mechanism and/or overexpression. The results demonstrated that TetR plays a repressor role in 3,17 β -*hsd* gene expression. In addition, various steroids can induce 3,17 β -*hsd* expression, especially testosterone. In order to further confirm that TetR acts as a repressor to inhibit the 3,17 β -*hsd* expression, a *tetR* knockout mutant of wild type *C. testosteroni* was

synthesised. The expression of *3,17 β -hsd* in the *tetR* knockout mutant was low, as in wild-type cells. Interestingly, testosterone induced a strong increase in the expression of *3,17 β -hsd*, especially in *tetR* knockout mutants. In principle, the results of *tetR* knockout mutants support the hypothesis that TetR is a repressor for the *3,17 β -hsd* expression, but the exact role of testosterone in this research remains unclear. Finally, *3 α -hsd/cr* is another gene in *C. testosteroni* that has been detected previously. Therefore, the relationship between *tetR* and *3 α -hsd/cr* was studied here in a similar way. The results indicated that *tetR* does also mediate the regulation of *3 α -hsd/cr*.

However, besides *tetR*, some other factors seem to regulate *3,17 β -hsd* expression. The *luxR* gene, located 1,125 bp upstream of *3,17 β -hsd* was identified by a bioinformatics analysis to be also involved in the regulation of *3,17 β -hsd*. It is also a potent enzyme in steroid degradation and sensitive to environmental signals. Moreover, the LuxR protein plays a vital role in quorum sensing (QS), which coordinates various genes' expression for cell densities, including genes encoding bioluminescence, virulence factors, sporulation, antibiotic biosynthesis, nitrogen fixation, biofilm formation and so on [2]. The characteristics and roles of the LuxR family members, especially in *C. testosteroni*, which located 57,915 bp upstream of *3,17 β -hsd* was detected. To investigate *luxR* regulation, *luxR* was cloned into plasmids for functional characterisation. The data indicated that LuxR indeed acts as a repressor for *3,17 β -hsd* expression. Testosterone, in turn, which is known to induce *3,17 β -hsd* expression, could not resolve *luxR* repression, except with promoter 2.

In summary, this dissertation adds important information to the understanding of regulation of steroid hormone degradation in bacteria.

Zusammenfassung

Seit 1950 mit dem Aufkommen der Biologischen Informationstheorie die Bedeutung des mikrobiellen Abbaus erkannt wurde, haben zahlreiche Berichte sich mit der Eliminierung von Steroidhormonen aus kontaminierten Ökosystemen durch mikrobiellen Abbau auseinandergesetzt. Aufgrund der Fähigkeit zum Abbau zahlreicher toxischer aromatischer Kontaminanten wird *Comamonas testosteroni* (*C. testosteroni*) weitgehend als bedeutendes Umweltbakterium für die Metabolisierung verschiedener toxischer aromatischer Schadstoffe angesehen und hat sich als prospektiver Biokatalysator für Umweltverschmutzungen erwiesen. Unter ihnen sind der enzymatische biologische Abbau und die Biosynthese wichtige intrazelluläre Prozesse, jedoch könnte die Expression einiger Enzyme direkt durch einige Umweltschadstoffe inhibiert werden, wodurch die endogenen aktiven Steroidhormonspiegel gestört werden. Die Hydroxysteroid-Dehydrogenasen (HSDs) sind eine Reihe von steroidogenen Enzymen, die eine wichtige Rolle bei der Steroidbiosynthese und dessen Metabolismus spielen [1]. Daher beschäftigt sich die Forschung mit der Regulierung von HSDs. 3,17 β -Hydroxysteroid-Dehydrogenase (3,17 β -HSD) aus *C. testosteroni* ist ein entscheidendes Enzym beim Steroidabbau. Um die vollständige molekulare Regulation von *C. testosteroni* aufzuklären, ist die Verbesserung des Verständnisses des 3,17 β -*hsd*-Induktionsmechanismus eine wesentliche Voraussetzung. Die Sequenzierung des *C. testosteroni* ATCC11996-Genoms zeigt, dass zwei repetitive Sequenzen (RS; 13 bp), lokalisiert upstream von 3,17 β -*hsd* zwischen der Wiederholungssequenz 1 (RS1) und der Wiederholungssequenz 2 (RS2) mit 1.661 Basenpaaren vorhanden sind. Darüber hinaus identifizieren wir ein *tetR* (522 bp) downstream von 3,17 β -*hsd*. Eine bioinformatische Analyse zeigt, dass Proteine der TetR-Familie eine entscheidende Rolle als transkriptionelle Repressoren spielen und Regulierungen von Umweltsignalen in vivo kontrollieren.

Außerdem kann *C. testosteroni* Steroidverbindungen durch eine Gruppe von Enzymen metabolisieren, die von Umwelthormonen beeinflusst werden. Es wird vorgeschlagen, dass TetR daher ein Repressionsfaktor zur Hemmung der Expression von 3,17 β -*hsd* in *C. testosteroni* sein könnte. Das übergeordnete Ziel dieser Dissertation ist es, die *tetR*-Genregulation und ihre Rolle für 3,17 β -*hsd* zu charakterisieren, dafür wurde das *tetR*-Gen in verschiedene Plasmide mit verschiedenen Fragmenten von 3,17 β -*hsd* kloniert, sowohl mit *egfp*-Reportergen zur funktionellen Charakterisierung als auch durch Überexpression. Die Ergebnisse zeigten, dass TetR eine Rolle als Repressor in der 3,17 β -*hsd* Expression

spielt. Zusätzlich zu der Tatsache, dass verschiedene Steroide die *3,17 β -hsd* Expression induzieren können, insbesondere die des Testosterons, konnten wir indirekt durch Zugabe von Testosteron zur *tetR*-Expression zeigen, dass TetR ein Repressor für die *3,17 β -hsd* ist. Um nachweisen zu können, dass TetR als ein Repressor wirkt, der die Expression von *3,17 β -hsd* inhibiert, wurde eine *tetR*-Gen-Knockout-Mutante von normalem *C. testosteroni* synthetisiert. Die Expression von *3,17 β -hsd* in der *tetR*-Gen-Knockout-Mutante war geringer als in Wildtyp-Zellen. Interessanterweise induzierte Testosteron einen starken Anstieg der Expression von *3,17 β -hsd*, insbesondere in *tetR*-Gen-Knockout-Mutanten. Grundsätzlich stützen die Ergebnisse der Versuche mit *tetR*-Gen-Knockout-Mutanten unsere Hypothese, dass TetR ein Repressor für die Expression von *3,17 β -hsd* ist, aber die genaue Rolle von Testosteron bleibt unklar. Das *3 α -hsd/cr* -Gen ein weiteres Gen in *C. testosteroni*, das zuvor bereits identifiziert wurde. Daher untersuchten wir die Beziehung zwischen dem *tetR*-Gen und dem *3 α -hsd/cr*-Gen in ähnlicher Weise. Das Ergebnis zeigte, dass das *tetR*-Gen vermutlich auch an der Regulation des *3 α -hsd/cr* -Gens beteiligt ist. Neben tetR regulieren einige andere Faktoren die *3,17 β -hsd* Expression.

Das *luxR* (1,125 bp), das sich upstream von *3,17 β -hsd* befindetet, ist ein weiteres neues Gen, das durch bioinformatische Analyse als Transkriptionsregulator identifiziert wurde, es ist ein potentes Enzym im Steroidabbau und empfindlich gegenüber Umweltsignalen, weiterhin spielt es eine wichtige Rolle beim Quorum Sensing (QS), das die Expression verschiedener Gene für Zelldichten koordiniert, darunter auch Gene, die für Biolumineszenz, Virulenzfaktor, Sporulation, Antibiotikabiosynthese, Stickstofffixierung, Biofilmbildung usw. kodieren [2]. Um die Eigenschaften und Rollen dieser Familie, insbesondere in *C. testosteroni*, in dem es 57.915 bp upstream von *3,17 β -hsd* identifiziert wurde und um die *luxR*-Regulation zu erforschen, wurde das *luxR* in Plasmide zur funktionellen Charakterisierung kloniert. Die Daten zeigen, dass LuxR tatsächlich als ein Repressor für die *3,17 β -hsd* Expression wirkt. Testosteron wiederum, von dem bekannt ist, dass es eine *3,17 β -hsd* Expression induziert, konnte die LuxR-Repression nicht überwinden, außer mit Promotor 2.

Diese Dissertation liefert wichtige Informationen, um unsere Forschung zu fördern und unser Verständnis der Umweltregulierung des Steroidhormon-Abbaus weiter zu vertiefen, um die besten Abbaewege zu erkennen und den Abbau von Umweltverschmutzung durch biologische Methoden zu entwickeln.

Abbreviations

ATCC	American Tissue Culture Collection
APS	Ammonium persulphate
AFP	α-Fetoprotein
bp	Base pair(s)
BSA	Bovine serum albumin
d	Day
DHEA	Dehydroepiandrosterone
ELISA	Enzyme-linked immunosorbent assay
EGFP	Enhanced green fluorescent protein
EDCs	Effects of endocrine-disrupting compounds
E1	Oestrone
E2	17β-Oestradiol
E3	Oestriol
EE2	17α-Ethinylestradiol
GC/MS	Gas chromatography coupled with mass spectrometry
HPLC	High-pressure liquid chromatography
HTH	Helix-turn-helix
h/hrs	Hour/hours
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilo base pair
LB	Luria broth
MS	Mass spectrometry
mM	Millimolar
min	Minute
nm	Nanometre

Abbreviations

Ni-NTA	Nickel-nitrilotriacetic acid
ori	Origin of DNA replication
OP.	Operon
OD	Optical density
PBS	Phosphate, NaCl-buffer (phosphate-buffered saline)
PCR	Polymerase chain reaction
Pr.	Protein
PCOS	Polycystic ovary syndrome
QS	Quorum sensing
rpm	Revolutions per minute
RT	Room Temperature
RFU	Relative fluorescence units
SAP	Shrimp alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIN	Standard I Nutrient broth medium
SLS	Sodium lauroyl sarcosinate
STP	Standard temperature and pressure
TAE	Tris-acetate-EDTA
TBE	Tris-boric acid-EDTA
TEMED	Tetramethylethylenediamine
Tet.	Testosterone
Tris	(Hydroxymethyl)aminomethane
TM	<i>tetR</i> knock-out mutant
v/v	Volume per Volume
w/v	Weight per Volume
µl	Microlitre

1. Introduction

1.1 *Comamonas testosteroni*

In 1987, Tamaoka et al. [3,4] reclassified *Pseudomonas testosteroni* (the original name) into *C. testosteroni* based on a phylogenetic analysis. *C. testosteroni* (Fig. 1) is a bacterial species assigned to the β -*Proteobacteria*. In particular, the bacterium *C. testosteroni* ATCC 11996 acts as a the representative strain [5]. The *C. testosteroni* is of particular research interest because they have been revealed to have their significance in the biochemical sewage treatment process [6,7]. Therefore, they are thought as the wastewater primary surface colonisers [8,9]. *C. testosteroni* are Gram-negative, aerobic, non-pigmented and rod-shaped bacteria. Commonly found as environmental microorganisms in contaminated environmental samples, including sediment, soil and water [10,11]. They often utilise steroids and aromatic compounds without sugars [12] and manifest their resistance to multiple heavy metals and drugs [13]. Furthermore, *C. testosteroni* have been reported to be promising organisms because they are capable of mineralising complex xenobiotic compounds [14]. To study the pathway of their metabolic capabilities, a novel insight has been provided into their metabolic abilities, the genome sequences of several *Comamonas* strains. In previous studies, even though *C. testosteroni* strains ATCC 11996, S44 and CNB-2 were shown to lack the key genes needed to assimilate carbohydrates, the chromosomes of these strains were shown to encode catabolic enzymes for the biodegradation of aromatic compounds [12,15]. In addition, Xiong's study [13] showed that 22% of all genes are transporter genes, while 6% are involved in signalling regulation. The proportions of genes are high for these functions found in the complete genomes of *C. testosteroni*, ensuring their adaptation to changing surroundings. *C. testosteroni* strains are also interrelated with heavy metal resistance, as several operons have been detected to code superfluous metal transporters [15,16].

C. testosteroni strains utilised testosterone, acetate and lactate as the sole carbon sources [17], whereas glucose and the majority of the carbohydrates are rarely used [12,18]. Since *C. testosteroni* strains are capable of degrading contamination and resist multiple metals, researchers have given attention on them in order to analyse the regulation of these mechanisms of metabolism. Being as common bacteria in the environment, most strains of *C. testosteroni* have been isolated from sludge [10,12,19] and heavy metal-polluted mining soil [13]. So far, four *C. testosteroni* strains' genomic sequences have been published. Gong

et al. [12] published the total genome sequence of *C. testosteroni* ATCC 11996 and analysed its general genome characteristics which express many functions (e.g. metabolising aromatic compounds, regulating elements that induce or repress the expression of catabolic enzymes and resisting heavy metals). In regard to the use of carbohydrates, studies of the genome of the *C. testosteroni* ATCC 11996 strain indicate that the genes encoding hexokinase and glucokinase are missing [10,12]. However, a comprehensive genomic analysis of the *C. testosteroni* strains is still absent yet to be made [13].

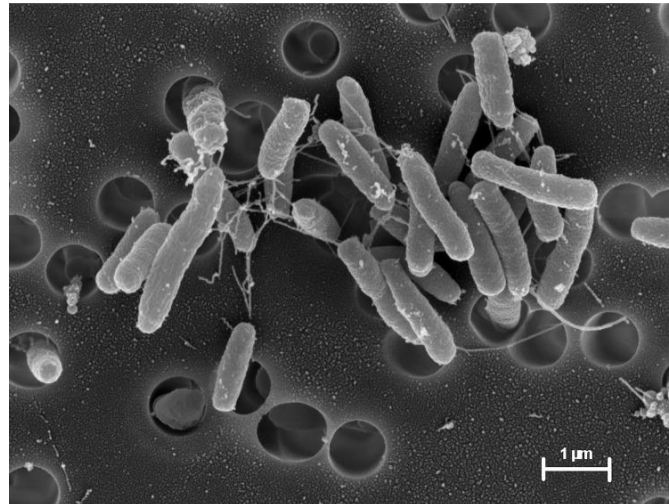


Fig. 1 Scanning electron micrograph image of *C. testosteroni* [20].

1.2 Steroid hormones in environment - environmental and public health concerns for humans and livestock

Steroid hormones are widely distributed in the diverse environmental models, such as soil, sediment, groundwater and surface water. They are used on humans and livestock for various pharmaceutical and medical purposes, such as effective contraception, cancer treatment and endocrine-disrupting disease therapy [21,22]. In spite of that, excess hormones discharged into the environment or human bodies can result in metabolic imbalances, give rise to an accumulation of abnormalities in the initial stage of growth. From recent studies, many influential retrospective observations related to the adverse effects of endocrine-disrupting compounds (EDCs) [21,23]. A brief pollution route is shown in Fig. 2, with the main effective objects being humans and wildlife.

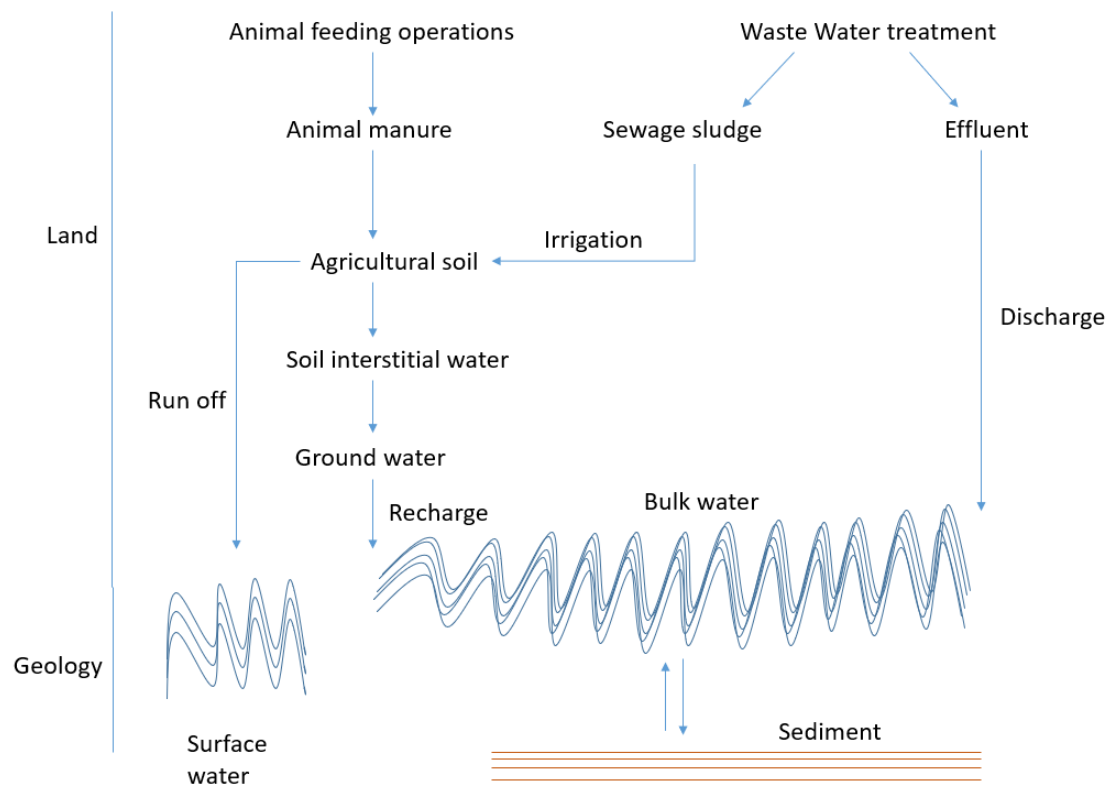


Fig. 2 Schematic presentation of main source of contamination.

For human beings the inimical effects (e.g. increasing occurrence rate of prostate and testicular cancer in males, mammary cancer and polycystic ovary syndrome in females, physical and mental growth changes in children of superfluous steroid hormones can be attributed to endocrine disruption) [24,25]. Adverse effects have also been found in a large number of wildlife species, including female masculinity, male feminisation, changes in sex ratio and intersexuality in fishes [26,27]. Moreover, steroid hormones, and even their intermediate products, are highly harmful to human health. Concerned with their high threats on human health, the European Union (EU) has imposed a prohibition on the administration of all natural and synthetic steroids in edible animals [28,29]. Meanwhile, the development of new rapid detection methods is promptly encouraged [30].

Apart from humans' environmental pollution continues to be a worldwide threat affecting the health of wildlife populations. Hazardous levels of various environmental contaminants are closely associated with high mortality rate, reproductive disorders, developmental abnormalities and a rising rate of disease outbreaks in wild animals [31,32]. It is worth noting that a low concentration (several ng/L) of oestrogens, such as oestrone (E1), 17β -oestradiol (E2) and 17α -ethinylestradiol (EE2), causes the feminisation of male fish [33-35]. Androgens, for example androstenedione, can also be associated with abnormalities of

reproduction in fishes and frogs under the same concentration [36-40]. Sorensen et al. [41] reported that androstenedione, a male pheromone of goldfish, may play a physiological role in maintaining the milt of male goldfish. For example, the amphoteric white sucker (*Catostomus commersonii*) accounts for 18% to 22% of the population at wastewater discharge sites [27]. More notably, excess oestrogen exposure in white sucker affects its gonadal differentiation during the early stage of growth. In further detail, extra oestrogen lead to gender bias [42], the occurrence of hermaphrodite [43] and a declined functional reproductive capacity in later life [10,19]. Another example is EE2, the exposure to EE2 with a concentration ranging from 1–25 ng/L in a zebrafish's adolescent days may cause an increase in its dose-dependent vitellogenin concentration [10].

Environment related hormone concentrations showed that the reproductive success of long term breeding zebrafish and other species with very low concentrations of EE2 are susceptible to serious harmful effects [13,42,43]. Kidd et al. [42] showed that low concentrations of EE2 (i.e. 5 to 6 ng/L) led to the feminisation of male zebrafish and oogenesis (i.e. transgender process). These observations indicate that the concentration of oestrogens and their analogues in fresh water can affect the sustainability of the wild fish population. In a recent study, EE2 instantaneous exposure in the zebrafish gonadal differentiation period (from 20–60 days after fertilisation) affects subsequent reproductive behaviour in male and female zebrafish [44]. Moreover, through exposure to EE2 with a concentration of 2.76 ng/L, the probability of a spawned fish being male is increased in competitive situations. In contrast, for female zebrafish exposed to EE2 with a concentration of 9.86 ng/L in their early lives, the rates of subsequent reproductive success is reduced in competitive spawning.

Guillette et al. [45,46] found that the reproductive capacity of male zebrafish spermatozoa was decreased after 8 weeks of β E2 induction. They also reported one of the most widely studied and documented cases of endocrine disorders, and examined a number of cases on the steroidogenic activity of runoff obtained from contaminated or control lakes in central Florida with juvenile alligators. The relative testosterone level of β E2 in male alligators was very low, while that of female alligators was high. Conjunction. The testosterone levels of alligators living in polluted lakes were significantly higher than those of alligators living in control lakes, which was due to the increase in β E2 synthesis. A comparison of the ovaries of alligators hatched in polluted lakes with those of alligators hatched in control lakes revealed that there was a significant difference in the synthesis of

β E2 in vitro between the two.

Based on this evidence, it is the changes in the reproductive and endocrine systems may be partly due to the changes in gonadal steroidogenic activity and the synthesis of plasma, which contains containing sex hormone-binding proteins. A strong relationship between hormone exposure and reproductive dysfunction was observed in other studies [47-50].

1.3 General structure and significance of steroid hormones

In general, steroids are characterised by four fused rings of the carbon skeleton (a cyclopentanoperhydrophenanthrene ring) (Fig. 3).

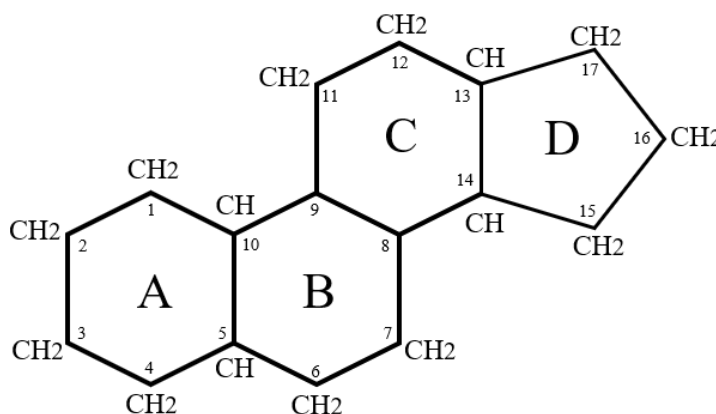


Fig. 3 Basic structure of steroid hormones. The steroid skeleton consists of four fusion rings labelled from A to D. Each carbon atom is bonded to 29 hydrogen atoms by 1 to 17 bonds.

The steroids are composed of the functional groups on the four core rings and the oxidation states of these rings. Hundreds of steroids were found in plants, animals and fungi. All steroids are produced by cells containing sterol, lanosterol (animals and fungi) or cycloartenol (plants) (Table 1). Table 1 Most common steroid nomenclature by fundamental structure

1. Introduction

	Carbon atoms present (Fig. 3)	Naturally occurring general classes	Examples shown in text
Gonane	1–17	None	Gonane*
Estrane	1–18	Estrogens	Estradiol
Androstane	1–19	Androgens	Androstane* ; Testosterone Androstenedione
Pregnane	1–21	Gestogens and adrenal steroids	Progesterone ; Cortisol; Aldosterone ; Batrachotoxin
Cholane	1–24	Bile acids	Cholic acid ; Sodium glycocholate
Cholestane	1–27	Sterols	Choleste; Scymnol
Ergostane	1–28	Sterols	Ergosterol; Cyasterone
Stigmastane	1–29	Sterols	Stigmasterol; Antheridiol
Lanostane	1–27; 30–32	Trimethyl sterols	Lanosterol; 22,25-Oxidoholothurinogenin
Cardanolide	1–23	Cardiac glycosides	Digitoxigenin
Bufanolide	1–24	Toad poisons	Bufotoxin
Spirostan	1–27	Sapogenins	Dioscin

*Gonane and androstane themselves do not occur in nature.

Most of the protein-bound steroid hormones are in water only soluble in water in small percentage, moderately hydrophobic and show low volatility in weak acids [51,52]. There are three categories of steroids: oestrogens, androgens and progestogens. Oestrogens (e.g. E1) are hormones occurring in female vertebrates predominantly, which affect femininity significantly, ovulation, mating, reproduction and somatic cell function [36]. Androgens (e.g. testosterone, androstenedione) play an important role in masculine characteristics, mating, reproductive behaviour and muscle growth [53]. Progestogens (e.g. progesterone) are deemed hormonal balancers and help preserve pregnancy [54]. The amounts of steroids secreted by humans and animals have many different effects, such as gender, physiology and development, as well as the impact of animal species [55,56]. Human females secrete a large amount of oestrogen and acid water-soluble glucuronide binding 95% a day mainly through urine oestrogen secretion (Table 2). During pregnancy, oestrogen excretion is detected to be at the highest level, but the amount and concentration depend on the stage of

pregnancy. Human males excrete 2.1–3.1 mg/d of androgens (mainly testosterone and androstenedione). Lange et al. [55] steroid excretion secretion from farm animals, calculating the excretions per animal based on millions of animals using the existing data in the United States (US) and the EU statistics for the year 2000 (Table 3).

Table 2 Human production and excretion of hormone [57]

Steroid hormone	Amount excreted in urine ($\mu\text{g}/\text{d}$)	Amount produced ($\mu\text{g}/\text{d}$)	Gender (reproductive status)
Estrone	0.3–	82-95	Female (cycling)
Estrone	13	13	Female (prepubertal)
Estrone	1.5	48	Male
Estrone	6.5	6.5	Male (prepubertal)
17 β-estradiol	3-5	3-5	Female (pregnant)
17 β-estradiol	0-2	97-110	Female (cycling)
17 β-estradiol	41	41	Female (prepubertal)
17 β-estradiol	3	88	Male
Estriol	35	35	Male (prepubertal)
Androgens	2100-3100	6500 (testosterone)	Male
Androgens	500-800	240 (testosterone)	Female

Table 3 Estimated annual secretions of steroid hormones by farm animals in EU and US - Year 2000 [55]

Million head	Estrogens (tons)	Androgens (tons)	Gestagens (tons)	Million head	Estrogens (tons)	Androgens (tons)	Gestagens (tons)
82	26	4.5	185	98	45	1.9	253
122	3.0	1.0	79	59	0.83	0.35	22
112	1.3	—	58	7.7	0.092	—	3.9
1002	2.8	1.6	—	1816	2.7	2.1	—
1318	33	7.1	322	1981	49	4.4	279

— The data was not found.

Steroids are hormones that promote the growth of livestock by exceeding recommended use. They play a main role in biology, medicine and chemistry. Steroids include all sex hormones, adrenocortical hormones, bile acids, invertebrate sterols, insect ecdysone and many other plant and animal physiologically active substances. Steroid residues are mainly derived from the extensive use of steroidal anti-inflammatory drugs, growth-stimulating compounds and oral contraceptives for treatment [56].

In all vertebrates and many invertebrates, steroids, including cholesterol, are likely synthesised through the same procedure. Cholesterol is a central steroid hormone precursor. The biosynthesis of cholesterol in the liver of vertebrates is especially potent, but it also occurs in the gonads, intestines, skin and immature brain. Cholesterol is almost undetectable in the adult brain. Insects, such as some annelids, molluscs and protozoans do not synthesise cholesterol but must acquire cholesterol or related sterols from their diets.

Progesterone is secreted by the corpus luteum of the ovary, but it is further metabolised to corticosteroids, such as cortisol and aldosterone, in the adrenal cortex. In the testes, progesterone and its derivative testosterone are further transformed into androstenedione. In the ovaries, androstenedione is changed to oestradiol (Fig. 4). In general, each organ secretes its own characteristic hormones, but under certain diseased states conditions/illness,

such as genetic defects and endocrine gland tumours, these forms can be severely distorted [56].

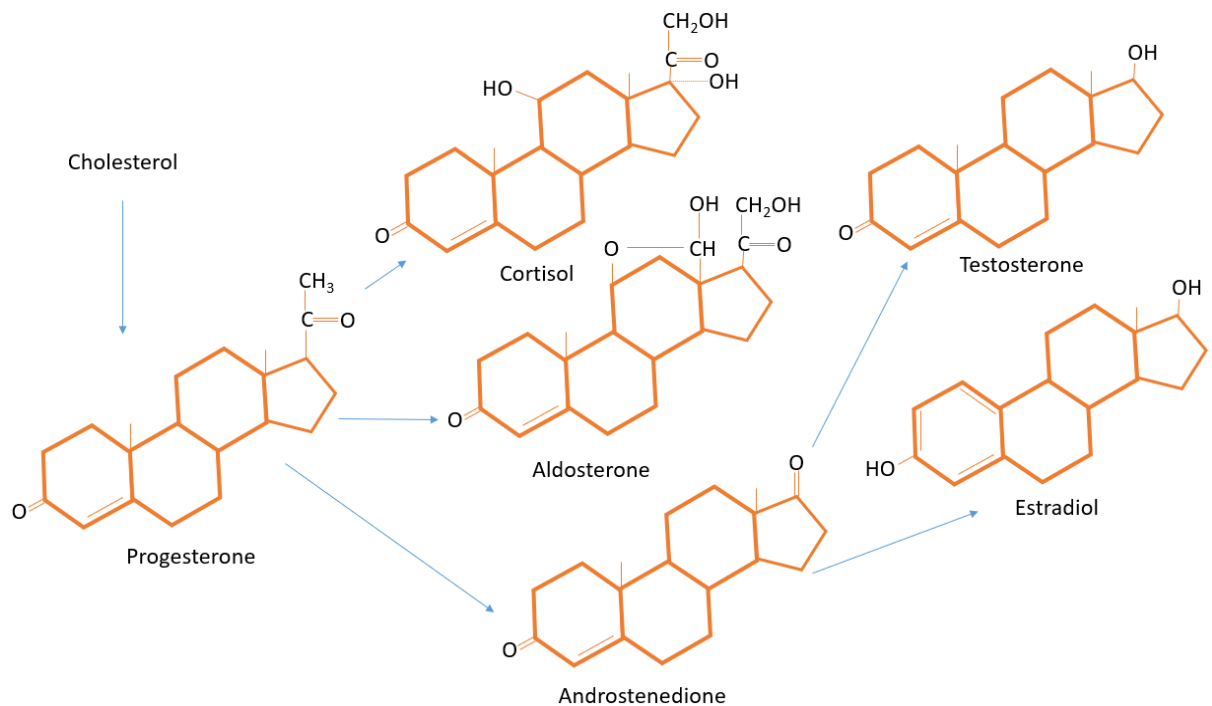


Fig. 4 Biogenetic relationships of principal vertebrate steroid hormones. Steroids containing phenolic rings (i.e. aromatic rings accompanied by hydroxyl groups) are intrinsic products of vertebrate ovaries.

These are oestrogens, in which oestradiol is the most effective. They are mainly used to maintain the female reproductive tissue in a fully functional state, promote or prepare for the state of oestrus or mating and stimulate the development of the mammary glands and other feminine features. Oestrogenic steroids have been isolated from the placenta, adrenal tissue and urine of pregnant female mammals of many species. Unexpectedly, oestrogenic steroids can also be isolated from stallion testes and urine [56].

In addition to steroids' central physiological effects, all steroids have potent influences on the entire physical metabolic system. These are regarded as strong pharmacological side effects when bodies are exposed to excessive natural steroid hormones during either hormone therapy or endocrine abnormality. In some synthetic analogues of the natural hormones, their activity is considerable and prominent, and the others are minimised. Moreover, different biological activity of natural hormones (androgens, oestrogens, glucocorticoids, and mineralocorticoids) can cause antagonism-to occur regularly.. Many

steroid analogues also include some of the natural hormone inhibitors, especially, testosterone [58,59].

1.3.1 Steroid hormones in female vertebrates - progesterone

The corpus luteum is a temporary endocrine structure during ovulation in vertebrates (release of a mature egg cell from the ovary), which produces progesterone and its derivatives. Progesterone is also secreted by the adrenal glands and the placenta. It can combine with oestrogen, thereby regulating the metabolism in the mammal uterus to allow the completion of implantation and subsequent growth activity of the fertilised ovum. Oestrogens and progesterone can stimulate the development of the fallopian tubes and the secretion of albumin in birds. Oestrogens and progesterone suppress ovulation; this is actually what forms the basis of steroid anti-fertility drugs. Oestrogens and progesterone may also act in invertebrates, but their functions in these animals are vague [56].

In many tissues, mainly in the liver, steroid hormones are decomposed to inactive physiological products and finally discharged through urine, although some of them are excreted via the bile and eventually excreted via faeces. In pregnancy, the main metabolites of progesterone can be detected in the urine, mainly through measuring the concentration of placental progesterone output. The detection of progesterone can help spot abortion early, therefore progesterone monitoring is essential.

1.3.2 Steroid hormones in male vertebrates - androgens

Androgens increase male sexual behaviour and aggression, promote muscle development and facial and physical hair growth and deepen the voice. Testosterone and androstenedione are the main androgens in which testosterone is the more effective one. In the sexual organs, testosterone is converted to 5 α -dihydrotestosterone, which is a more potent androgen [56].

Urinary steroids analysis assist the diagnosis of endocrine abnormalities. Urinary 17-ketosteroid (androstane derivatives of a C=O function at C17) is mainly increased in the liver by the oxidation of adrenal steroid hormones. Thus, adrenal gland secretions are used for measurement rather than a measurement of testicular secretion.

1.3.3 Metabolism of steroid hormones in plants

The early steps of steroid biosynthesis are the same in plants and animals. However, in plants, lanosterol is replaced by cycloartenol-related compound, which contains a ternary ring (C9, C10 and C19), replacing the nuclear double bonds of lanosterol. The side chains

of phytosterols, such as lent sterol, ergosterol from yeast and other fungi contain extra carbon atoms that bind to S-adenosine methionine, thus contribute to methyl formation in many biological processes. Although most plant tissues contain only small amounts of cholesterol, this sterol is an important biological precursor of Phyto steroids, such as sapogenins, glycosides and alkaloids. Since pregnane derivatives are intermediates in these transformations, plants and animals appear to have important characteristics of steroid metabolism in general [56].

1.4 Pathways of steroid hormone degradation

1.4.1 Abiotic degradation of steroid hormones

In natural waters, photodegradation provides an alternative for abiotic degradation [60]. The photodegradation of oestrogens (E1, β E2, E3 and EE2) in pure water [61] and river water [62] has been analysed. The half-life of oestrogen is 2 to 3 hrs in rivers, while in air-saturated purified water, the half-life is 5 to 42 hrs. That is, the photolysis is sped up by the photosensitisation of dissolved organic matter (DOM) in river water [62]. The TiO₂-assisted photodegradation efficiency of E1 and β E2 can be affected by the pH values of humic acid [63]. In recent years, the degradation of testosterone under UV irradiation has been studied frequently in phosphate buffers with different excitation wavelengths and in natural water, and the transformation products have been identified [64]. Under laboratory conditions, testosterone can be transformed very quickly, and the rate of phototransformation is not influenced by the water's pH value. What is more, many researchers use manganese oxides, which contain much redox potential in soil and sediment, to oxidise or catalyse organic and inorganic compounds [65,66]. Manganese oxide (MnO₂) can be used as an oxidative removal substrate. Rudder et al. [65] analysed this aspect and concluded that the MnO₂ reactors can remove 81.7% of oestrogenic activity at a concentration of about 15 μ g/L of EE2. Many researchers have studied the oxidative transformation and the reaction pathway of β E2 in aqueous solution by MnO₂ [67]. Under different conditions, some researchers analysed the reaction kinetics and oestrogenic activity removal of β E2 by MnO₂ [66]. Li et al. [68] found that the oxidation transformation of β E2 by MnO₂ could not exhibit pseudo-first-order kinetics over 30 min. Moreover, they showed that metal ions and carboxylic acids inhibited the initial reaction rate and reduced the active surface sites with the co-solutes.

1.4.2 Steroid hormone microbial deconjugation and degradation

Humans and livestock secrete natural and synthetic steroids, which are non-functional glucuronic acid or sulphonide conjugates that can be decomposed and split in standard temperature and pressure (STP) in manure (storage and treatment) [69]. Because high-density microorganisms exist in STP, one can assume that common faecal microorganisms (e.g. *E. coli*) can hydrolyse conjugates into unconjugated forms by sulphatase and glucuronidase enzymes [70,71]. Probably because faecal bacteria (*E. coli*) produce many β -glucuronidase enzymes, glucuronate oestrogens can be readily deconjugated in wastewater, which has been verified in a laboratory biodegradation test [72]. E2 can be deconjugated more rapidly at higher temperatures because of the increased hydrolysis, which is the result of a positive effect on microbes producing aryl-sulphates and glucuronidases [73]. These findings reveal the importance of considering conjugated oestrogens in animal wastes, since the oestrogens can deconjugate conjugated oestrogens in animal wastes into active free oestrogens via microorganisms.

Through microbial degradation, it is expected that the steroid hormones can be removed from the environment. Many biodegradation researchers have paid much attention to steroid hormone degradation. However, little is known about which factors influence the degradation rates and pathways. Many conditions influence steroid hormone (i.e. β E2 and testosterone) degradation, such as dissolved oxygen, temperature, pH and the existence of specific degrading bacteria. Generally, it has been shown that hormones can be degraded more readily under aerobic conditions [74] and at higher (up to 30°C) temperatures [75,76], correlated to non-pure water content [77]. β E2 was biodegraded at temperatures of 10°C, 20°C and 30°C, and the first-order reaction kinetics of β E2 were 2.63, 3.49 and 3.98/h, respectively by *Pseudomonas aeruginosa* (*P. aeruginosa*) TJI isolated from aerobic activated sludge in the presence and absence of glucose. Li et al. [78] conducted experiments on semi-continuous batch degradation and found that the easily biodegradable substrate glucose may inhibit the degradation of β E2 and E1.

The biodegradation of steroid hormones, especially oestrogens, has been assessed by many researchers in agricultural soil [79] and soil modified with manure or biosolids/sewage sludge [78,80], river water and sediment [16,81] and pure culture medium [82]. Matsuoka et al. [83] observed the fast biodegradation of natural oestrogen in Japanese river water. Bacteria degraded β E2 in river water within five days in the summer, while it completely

degraded E1 within seven days in the winter. In contrast, it was observed that the synthetic oestrogen EE2 is less degradable (i.e. it needs a fortnight or longer) than natural oestrogens, and no complete degradation was observed. Jürgens et al. [81] observed similar results in UK rivers.

In recent years, in aerobic studies, such as on river sediment, it was found that β E2, E1 and testosterone were mineralised and the biodegradation rate of oestrogens was lower than that of testosterone. In this research, it is important to consider microbial processes in sediment, as researchers attempt to divide the transformation of relatively hydrophobic compounds into sediment phases in rivers [16].

A field-scale study was conducted and indicated that some bacteria, such as Gram-positive bacteria (*Bacilli*, *Nocardia*, *Erythrococca*, *Mycobacteria*) and Gram-negative bacteria (*Comamonas* and *Pseudomonas*), degrade androgens, oestrogens and progesterone [82,84,85]. In connection with this article, it is worth mentioning that Roh et al. [86] reported that the strain *SphingomonasKc8* using β E2 has the ability to degrade testosterone and to use testosterone as a further growth substrate. In most studies, hormone conversion has been shown to occur under aerobic conditions [87].

Research on steroid hormones in the natural environment metabolised through biodegradation has been reported by many species of bacteria that can convert cholesterol and phytosterols found at high concentrations into other steroid hormones. For example, phytosterols can be transformed into progesterone and androstenedione [88].

Therefore, by considering sources, adsorption mechanisms and biodegradation pathways, one can predict the fate of many steroid hormones in the environment.

1.5 Advantages of steroid biodegradation

Considering the potential negative ecological effects of raised steroid hormones from human and animal waste, some researchers greatly raised their interest in the mobility and persistence of environmental steroid hormones.

Adsorption and biodegradation need to act together to achieve the expected result of removing steroid hormones from water, sediment and soil. Along with studying the absorption of oestradiol in river sediment, Jürgens et al. [81] showed that less than 1% of the existing steroids are expected to be wiped off from the aqueous phase by suspended sediment. Andersen et al. [89] reported that the absorption of oestradiol and oestrogen in

active sludge and anaerobic sludge occurs in a narrow range and the absorptive steroid hormone reduces slowly along the treatment.

Steroids with similar chemical properties (Table 4) display low water solubility characteristics and relatively high melting points [69,90,91].

Table 4 Selected physicochemical properties of steroidal hormones

Steroid hormone	Molecular weight [g/mol]	Water solubility [mg/l]	Log Kow	Melting point [°C]
17 β -Estradiol	272.4	3.9-13.3	3.1-4.0	171
Estrone	270.4	0.8-12.4	3.1-3.4	259
17 α -Ethinyl estradiol	296.4	32.0	3.6-4.1	183
Testosterone	288.4	18.0-25.0	3.2	155
4-Androstene-3,17-dione	286.4	37.0-41.0	-	173
Cholesterol	386.6	2.0	-	149

Steroid degradation products are widely used in the synthesis of sex hormones and other organic compounds with many pharmacological applications. In recent years, it has been recognised that biosensor technology represents a variety of methods to analyse and solve food and agricultural safety problems, including environmental safety, pharmacology and medical problems.

Recently, Varriale et al. [30] developed a fluorescence polarisation method for the direct detection of penicillin G in milk and patulin in juice. In this work, the synthesised 17 β -oestradiol hemisuccinate-glutamine-binding protein (GLNBP) was labelled with fluorescent probe (Biotium CF®647) and the polyclonal monoclonal antibody of 17 β -oestradiol jointly. On this basis, 17 β -oestradiol in a non-pre-treatment milk sample was determined by competitive immunoassay. Bacteria can take advantage of the metabolic activity of sex steroid hormones through some unique enzymes, which adjust the balance between active and inactive steroid hydroxysteroid dehydrogenases (HSDs). A bioinformatics analysis already identified the genomes that encode HSDs in distinct bacteria that constitute the normal gastrointestinal microbiota [92-94].

Although the natural steroid hormones can be degraded by abiotic methods, such as photolysis, the biodegradation process by biota is mainly responsible for the elimination of E2 and testosterone, which comprises their metabolites in the soil as well as in the receiving water. Studies of Culucci et al. [77,95] showed that the natural steroid hormone compounds are rapidly degraded under adequate moisture and suitable temperature.

1.6 The introduction of hydroxysteroid dehydrogenases (HSDs)

Hydroxysteroid dehydrogenases (HSDs) belong to the short-chain dehydrogenases/reductases super family (SDR). This is a large super family of proteins that share about 250 to 300 amino acid residues, and which comprise NADPH/NAD⁺-dependent oxidoreductases.

HSDs are widely expressed as diverse isoforms with different positions and functions in all steroidogenic organs. They are key enzymes involved in reproduction and growth that regulate the strong steroid concentration of hormone-dependent diseases as suitable targets.

As their names indicate, they are active at different positions of the steroidal skeleton (3 α -, 3 β -, 11 β -, 17 β -, 20 α - and 20 β -position). HSDs are a major component of the systemic (endocrine) and local (endocrine) mechanisms. They convert inactive steroids into the corresponding active forms in target tissues, and vice versa, thereby regulating the activation of steroid hormone receptors or other non-genomic signal transduction pathways. Thus, HSDs act as a molecular switch that allows the pre-regulation of steroid hormone receptors [96].

It is clearly recognised that humans and some other primate species are unique. They have adrenal glands to secrete many inactive steroid precursors, including dehydroepiandrosterone (DHEA). These inactive steroids do not bind to their cognate receptor unless they are converted into active oestrogens and/or androgens in target organs [97]. Gender steroid hormones cause imbalance; for example, androgens and oestrogens can lead to diverse severe diseases. Hormone-dependent cancer can occur in both women and men. These diseases are usually fatal, and according to reports from Western countries, breast cancer is the most common cancer in females, while prostate cancer is the most prevalent cancer in males. In addition, there are various other prevalent hormone-dependent diseases, such as endometriosis and polycystic ovary syndrome (PCOS). Nevertheless, people still poorly understand the causes of these diseases, and there is a lack of effective pharmacological treatment. Using pharmaceutical compounds to regulate local sex steroid hormone production is a new therapy option for hormonal diseases with tremendous therapeutic value [98].

In addition to non-HSD enzyme inhibition (aromatase and 5 α - reductase) [99], recent attempts have promoted the development of HSD inhibitors as a therapeutic strategy. However, several HSD enzymes are also considered promising upon drug targeting

therapies [100], such as 17β -HSD enzyme isoforms [101]. Therefore, researchers are focus on the localisation/tissue distribution of the main HSDs (3β -HSD, 11β -HSD, 17β -HSD) and the regulation of their isoforms. The role of HSDs in normal and pathological processes can also be investigated by experimental models.

1.7 Bacterial hydroxysteroid dehydrogenases (HSDs)

HSDs are widely found in a variety of microorganisms, including bacteria and archaea where they play a role as a signal molecule to modulate gene expression. From the interaction between host and microorganisms, HSDs play a key role in regulating steroid signalling by binding to specific intracellular receptors that are ligand-dependent transcription factors or membrane receptors that stimulate multiple signal transduction pathways. In addition, HSDs can also affect the metabolism, pathogeny or growth of bacteria.

To date, the genetics and biochemistry of microbial steroid degradation have mainly been studied in a few model bacteria, and the diversity of this metabolism remains largely unexplored.

3,17\beta-*hsd* is a key enzyme in the Gram-negative bacterium *C. testosteroni*, but the enzyme expression and regulation remain unknown. So far, researchers examined *3,17\beta*-*hsd* regulation. In Pruneda-Paz's survey [102], *3,17\beta*-*hsd* can be induced by a variety of steroid substrates, such as testosterone. The report also concluded that, two promoters (promoter 1 and promoter 2) involved in *3,17\beta*-*hsd* transcription. They are particular DNA sequences located directly nearby the transcription gene, and play a role in the initiation of gene transcription. Moreover, the *rseB* gene (1383 bp) locates upstream of *3,17\beta*-*hsd* could be induced and expressed together with *3,17\beta*-*hsd*. Li's research [59] proved that *phaR* is a repressor for *3,17\beta*-*hsd* expression and located on upstream of *3,17\beta*-*hsd*. In addition, two repeat (16 bp) sequences were also found on upstream of *3,17\beta*-*hsd*, which possibly form a ring structure (i.e. loop structure) to regulate *3,17\beta*-*hsd* expression (Fig. 5).

1. Introduction

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AAGCTTGTGTGTCAGGGTTACTGCTTCCGATGAGGGTGTGTCAGCTGCTAAAAATCTGTGTCAGTCAATGCTCAATTGACGACGGGGCCGATCCCCAGAACATA
-35 -10
AAGGACTTGGC (117 bp)
SD
----Repressor phaR (546 bp)
ATGCAAGAAACAAAGAGGGCAAGAGGGTGTATGTCAGGTGGGGAAATCAGCGAATCATCAAGAAGTACCCCAATCGTCGCCTCTACGACACCAATACCTCTTCATACATCACGCTGGCCGAGGTCAAGCAACT
GGTCAATGGATAGCGGAGCCGGTCTCATCAGGGATGCCAAGACCAATGAAGACCTGACCGCTGCCATCTCTGTCAGATCATTCTGGAGGAAGAAGCGGGCGGGCCCCATGTTCTCCGAGGCCATGCTGGCCAA
CATCATCCCTTCTATGGTCAATGCCATGCAAGGGCTTCATGGGGAGCTACATGAAAGAAAGTATGTCAGATGTTCCAGAGCTTCCAGAGCAAGCTGGCGGGCCAGCCAGGGAGTGAACCCGAGGCCCTGGAAGCA
GTTCAATGAGCATGCACCTCAAGCCATGCAAGGGCTTGATGGCAAGCTATATGGAGCAAGCTCCAGAACATGTTCCAGCCAGATGCAGGAGCAGATGCAAAAGCAGACAGAGCAGATGCTGGGTGTGTTGGCATCA
GCGCTGA

ACGAAATCTGCTTTCCGAGCGGGCCAAATGCCCCCTTTTGTGCTGTGCTGGCTAGTCTAATTGACGATGCTTGGCTTCCCAACGGT
RS (16 bp)
CGAACAAATGATTTCTGCTTCGCAATTGCGAGTCGGCAAGGAAGAAGAACGATAAGAGAAGTTCAATGACAGGTAGATGCATGGCAGATGCATCCAAAGGAGACTAGGC (214 bp)
-10 SD

----rseB (1383 bp, zip48)
ATGAAGTTAGGAGTGGGGCCCCCGTTCCGCTGGTGTGCTGGTGCAGGTGTATTGTTGGCGGGGAGGTTGGGCCAAGGCGACGCCCGATGAACCTGGCCAGGCTGGGCAAGTGCCTAACCTGCACGGGGGGAGAA
AAAGCGGGCTCAACCAGTGGTGTGCCGAGTTACAGGGAAATGGCTGGGTACACCACCGGCAATCAATACAACCCGATGCTGGCCAGCACCCCGTAGACCCCTATGCCCGTGAAAGCCGTTGCTCACCATC
ACGGCTGAGAATATGGCGGAGTATGCCGAGCGCTGAGCGAGGGCCAGAAGGCCATGTTGGCAAAATACCCAGGACCTATCGCATCCGGTATCAGGGGCATCCGCACTTCGCTTTTCGGATGCGCTGTGCG
CGCGGGCCGAAAGAACCGCCAGGATGCGGTGATGAACCGAGACGGTCAAGGGCAACAAGGGTGTGGTCAAAAGGAGCGCTGCCCTTCCCGTTCCCCAGAAACGGCACTTGAGCTGGCTTCAACAATCTTTCGCTT
CGCTGCTTTCACCGAAACACAGCTGCGTGAACAACGCCAAATGTGCTGGCCGATGGCAGCATCGTCTGGGGTCCGCGCAATCGCGCTTCAAGTCAAGTCAACGCCCGCCAAACGCAAGTCAACCCCTGAGCA
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TGCGCCAGATTCGCGAATACGGTTTCGATCAGCCCTATCGGGCACAGCGGCAAGCTGACGATTGACTCCGATCGCCGTGTTCAACGGCTCGCCGAGCGTACAACTGGAAGTCCGCTGGGCAAGAGGAAAGTCT
ATGTCGGGCCAATGCTACAAAGATTCACGGCAGCAACGTTGAAGTATGCCGATGCTGCTAAACCCCGTCAATGAAAACCCGACTACATGCGCTACGAGCTGGCCGTGTCTGGGTGCTGGAGGCTTCGCTCAAG
ATGGCTATCGCCACATGTTGGCAAGCGCTGCTGTTCTCGATGAAGATACCGGTGAGCGCTGATGAGCGATTACTACGATGCCCGTGGCCAGTTGGCTGCGAGCGGTGGTCAATCAATTAAGCTTACGCTTCGA
CGCCCGATCTGGCATGGCGGCAACAGCTTATCAGACCTGAACTCCGGCGGTATGTGGCTACAACCTGTTCCAGGAAACGCCCCAGGGGGCCGCTTTGAACAAGGGCAATATGACTGGCCCATGTTTACCG
CGAAGCGGGCGGTAACCGCAGCAACTAA

AAAAATGAATCGGGCAACACGCTGTGGCTACTTGTGTGGCTGTGGGCTTTTGCATCAAGCAAGGAGCGCTTCCGCTCCTTTTATGTTGGCCGTAGCAAT
-35 RS (16 bp) -10
GCCCTGTTACAGGGTGTGACAAAATCAAGAGGAGCAGAGG (147 bp)
SD

----3,17β-hsd (765 bp)
ATGACAAATCGTTGACAGGTAAGGTGGCCGTGTCAGTGGTGTGCCAGCGGTGTGGGCTTGAAGTGGTGAAGTGCCTTCTGGCGAAGGCGCCAAAGTCCGATTACAGCATATCAATGAAGCGGGGGGAG
CAACTGGCGGCTGAACCTGGGGCAACGCTCCATGTTTCCGCCATGACGTGAGCAGCGAGGCGGACTGGACGCTTGTGATGGCGCGGTGCAGCGGCGTCTGGGACGCTCAATGTGCTGGTCAACAATGCGCGC
ATCCTGCTGCTGGTGAATGGAACAAGGGCGTCTGGAGGATTTACGCCGCTGCTCAAGATCAACACCGAGTCAAGTCTTCAATGGTTGCCAGCAGGGCATTGCGCGATGAAGGAGACAGGAGGCTCCATCATC
AATATGGCTCGGTATCAGAGTGGCTGCCATAGAGCAATACGCGGCTATAGCCGCAAGGCGCGGTGTGGCACTGACCCGCGCGCTCAGCTGAGCTGTGAAAGCAAGGCTATGCGATTCGCGTCAAT
TCCATTATCCCGATGGCATCTATACGCCATGATGCAAGGCTTCGCTGCAAAAGGGCGTGAAGCAAGGAAATGGTCTTGCATGACCCCAAGCTCAACCGTGCAGGCGCGGCTATATGCCCCGAGGCTATCGCGCAGC
TGGTGTGTTCTGGCCAGCGACGATCCAGTGTGATGAGCGGTGAGCGAGTGCATGCAGATAACTCGATTCTGGGCATGGGGCTATAG
ATGAAAGCTTA
```

Fig. 5 The sequences of *3,17β-hsd* and the two repressor genes, *PhaR* and *rseB*, upstream of *3,17β-hsd* [59]. *PhaR* and *rseB* are two genes located upstream of *3,17β-hsd*, two promoters (p1 and p2) locate nearby to the repeat sequences (RS1 and RS2).

1.8 TetR-type transcriptional regulator family

Tetracycline antibiotics are a group of broad-spectrum antibiotics that can be used to treat the infection of Gram-negative bacteria, Gram-positive bacteria, spirochetes, chlamydia, rickettsia, mycoplasma, actinomycetes and amoeba. These compounds usually bind to bacterial ribosomes and terminate their protein synthesis to kill bacteria. The Tet Repressor Protein (TetR) is a group of proteins that can provide resistance towards tetracycline antibiotics. Tetracycline repressor proteins (TET regulators) are extensively used in molecular genetics to regulate gene expression.

Under environmental conditions, the proteins of the TetR family are involved in the adaptation to complex changes in the environment. This correlates with the fact that many members of the TetR family are found among microbes with abundant extra cytoplasmic functional sigma factors to recent structural insights into promoter recognition and regulation [82,103]. They are also commonly used as control elements to regulate gene expression in higher eukaryotes [104]. In addition, they are common single-component prokaryotic signal transduction systems [105]. TetR family members exhibit high sequence similarity in the DNA-binding domain [106]. This regulator contains a conserved helix turn helix (HTH) motif at the N-terminal DNA binding end of the protein and a ligand binding

pocket at the C-terminal end [105]. In the absence of ligands, they often bind to DNA and inhibit gene expression, in order to prevent transcription. The effects on the C-terminal pocket or molecular binding can lead to structural changes in proteins, resulting in the release of regulatory factors from the DNA [105].

In this study, a new transcriptional factor, *tetR*, was identified in *C. testosteroni*. *tetR* is located 3.319 kb downstream of the *3,17β-hsd* gene on the *C. testosteroni* ATCC 11996 chromosome [12] with an orientation opposite to *3,17β-hsd* (Fig. 6). The open reading frame of *tetR* consists of 522 bp and translates into a protein of 174 amino acids, which is a member of the TetR family as revealed by sequence analyses. The location of *tetR* is on contig 58, 79551-80072 (Bioinformatics analysis) of the *C. testosteroni* ATCC 11996 chromosome.

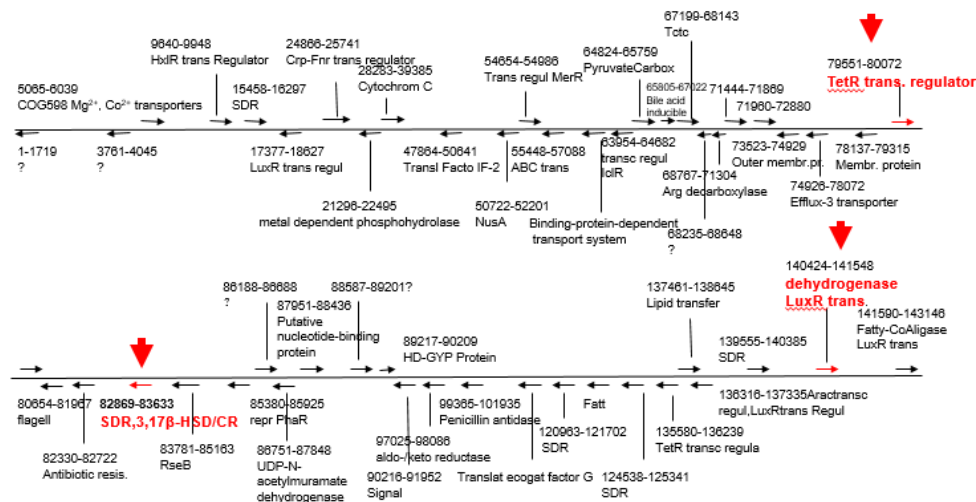


Fig. 6 Genetic structure and regulatory elements of *3,17β-hsd*. A novel transcriptional regulator gene, *tetR*, was identified 3.319 kb downstream of *3,17β-hsd*, while *luxR* was located 56,792 kb upstream of *3,17β-hsd*.

1.9 LuxR-type transcriptional regulator family

The LuxR protein is closely linked with quorum sensing (QS) phenomena. QS is regarded as a bacterial language with which bacteria can communicate with each other or themselves through individual cells that respond to specific signal metabolites. In QS, different autoinducer synthases generate these particular signals, and cells respond to them by mediating the receptor/activator proteins. These constitute the evolutionarily conserved families of regulatory proteins known as the LuxI and LuxR families, respectively [107].

The LuxR-type protein contains 252 amino acids, and this family of DNA-binding proteins is characterised by the presence of a specific regulatory helix-turn-helix (HTH) domain. The HTH domain is a structural motif of protein which occurs in many proteins that regulate gene expression. LuxR has two domains: an N-terminal domain that binds pheromone AHL and occupies two-thirds of the entire protein and a C-terminal domain that contains a conserved HTH structure and binds to DNA [108,109]. Choi et al. [108] indicate that the C-terminal domain contains all the sites essential for LuxR-DNA and LuxR-RNA polymerase interactions.

This family of regulators is involved in many bacterial physiological processes, such as bioluminescence, plasmid transformation, biofilm formation and the production of extracellular enzymes, virulence factors and secondary metabolites. Therefore, research of LuxR proteins has broad application prospects in environmental monitoring, agricultural pest control, medicine and microbial fermentation and so on. For example, in the fields of environmental monitoring and food detection, environmental microbiological detection can be used by transferring the luminous bacterial QS system into an appropriate phage and detecting the number of active host bacteria instructed by the phage [110]. This method can also be used to detect antibiotics or other substances harmful to cell metabolism in milk [111]. In medicine, it can interfere with bacterial QS signals by enzymes, antibodies or structural analogues to inhibit the LuxR-mediated QS, thereby facilitating the elimination of pathogenic bacteria from the infected host [109,112]. It can also inhibit the function of the LuxR protein family to achieve the purpose of reducing bacterial pathogenicity [113]. Moreover, the overexpression of *luxR* can be used for the large-scale production of antibiotics.

The LuxR family comprises a variety of bacteria and functionally diverse transcription factors that could act as both activators and repressors. Case et al. [114] analysed 265 species of *Proteobacteria*. Their results showed that at least 113 kinds of protea contained LuxR protein analogues. LuxR regulatory pathways usually involve fine-grained control. In some cases, the bacteria achieve this fine control through different regulatory proteins [115,116]. In other cases, this fine control is achieved by other regulatory mechanisms [117].

In many cases, a bacterium can have more than one LuxR protein required to react diversely to various signalling molecules. This is a complex process to coordinate and balance between these different reactions. The signal exchange between the bacterial cells is also a

1. Introduction

very complicated process. Many signalling pathways regulated by LuxR family proteins produce not only mutual synergistic effects but also antagonistic effects.

In this study, a novel transcription factor, *luxR*, is found in *C. testosteroni* which is located 56,792 kb upstream of the *3,17 β -hsd* gene on the chromosome of *C. testosteroni* ATCC 11996. It has an opposite direction to that of *3,17 β -hsd* (Fig. 6). The open reading frame of *luxR* consists of 1125 bp, and it is transformed into a 367-amino-acid protein belonging to the LuxR family by sequence analysis. Moreover, *luxR* is located on contig 58, 140424-141548 (Bioinformatics analysis) of the *C. testosteroni* ATCC 11996 chromosome.

2. Aim of the present dissertation

This dissertation aims to bring forward the understanding of gene regulation in *C. testosteroni* by steroid hormones. Up to now, little is known on how *C. testosteroni* respond to testosterone mineralisation and the degrading mechanisms, which are caused by regulation of gene expression. The purposes of this dissertation are given below in detail: Therefore, my dissertation is dedicated for the following purposes::

- (I) To elucidate the gene regulation by *tetR* of two important HSD enzymes, *3,17 β -hsd* and *3 α -hsd/cr*.
- (II) To analyse the *tetR* regulatory mechanism on *3,17 β -hsd*.
- (III) To investigate the LuxR function on *3,17 β -hsd*.
- (IV) To locate and describe the LuxR acting site for *3,17 β -hsd* regulation.
- (V) To analyse the influence of temperature on *3,17 β -hsd* expression.
- (VI) To investigate the relationship between testosterone and *tetR* expression.
- (VII) To investigate the relationship between testosterone and *luxR* expression.

These studies should help not only to understand the mechanism of *3,17 β -hsd* regulation, but also to offer an idea in identifying new environmental biomarkers with regard to *3,17 β -hsd* expression, as well as to identify new disease biomarkers in body fluids, cells and tissues.

3. Materials and methods

3.1 Bacterial strains, media and growth conditions

For molecular cloning and gene expression, host strains *E. coli* HB101, *E. coli* BL21 (DE3)pLysS and *C. testosteroni* ATCC 11996 were used. Detailed information about these strains and related mutant strains is shown in Table 5.

Table 5 Bacterial strains used in this study

Strain	Relevant genotype	Origin/Reference
<i>E. coli</i> HB101	F ⁻ mcrB mrr hsdS20(r _B ⁻ m _B ⁻)recA13 LeuB6ara-14 proA2 lacY1 galK2 xyl5 mtl-1 rpsL20(Sm ^R) glnV44 λ ⁻	Promega (Mannheim, Deutschland)
<i>E. coli</i> BL21(DE3)pLysS	F ⁻ ompT gal dcm lon hsdS _B (r _B ⁻ m _B ⁻)λ(DE3)pLysS(cm ^R)	Promega (Mannheim, Deutschland)
<i>C. testosteroni</i> ATCC 11996	Strain ATCC11996	ATCC/DSMZ*

*ATCC: American Type Culture Collection; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

Bacterial cells were grown in Standard I Nutrient (SIN), which is a broth medium obtained from Merck (25 g/l, containing 15.0 g of peptones, 3.0 g of yeast extract, 6.0 g of sodium chloride and 1.0 g of D (+) glucose). When necessary, bacterial cells were incubated at 37°C (*E. coli*) or 27°C (*C. testosteroni*) in a shaker at 180 rpm. Growth media contained 100 µg/ml ampicillin and/or 30 µg/ml kanamycin.

3.2 Primers

Primers were synthesized by “Eurofins”

Table 6 Primers used in this study

Primer	Sequence (5'→3')	gene	modification
pHsdR-F	<u>CATA</u> TGGATTTCAATGCGC	<i>hsdR</i>	<i>NdeI</i>
pHsdR-R	GGAT <u>CC</u> AAGAGCGGTCATGC	<i>hsdR</i>	<i>BamHI</i>
pGFP-L	ATGGCTAGCAAAGGAG	<i>gfp</i>	
pGFP-R	TTATTTGTAGAGCTC	<i>gfp</i>	
<i>ptetR</i> -EL	CGCC <u>CATA</u> TGGCCCTGGCCCTGGTC	<i>tetR</i>	<i>NdeI</i>
<i>ptetR</i> -ER	CGCG <u>GATC</u> CTCAGGCTGTCGCCGTTGC	<i>tetR</i>	<i>BamHI</i>
<i>luxR</i> -EL	CGCC <u>CATATG</u> ATGGATAGCGACATA	<i>luxR</i>	<i>NdeI</i>
<i>luxR</i> -ER	CGCG <u>GATCC</u> CTTTTCGCCCAATAG	<i>luxR</i>	<i>BamHI</i>

As shown in Table 6, primers *pHsdR*-F and *pHsdR*-R were used to prepare the *3,17β-hsd* gene. Primers *pGFP*-L and *pGFP*-R were used to prepare *gfp*, primers *ptetR*-EL and *ptetR*-ER were used to prepare *tetR*. Primers *luxR*-EL and *luxR*-ER were used to prepare *luxR*. *NdeI* and *BamHI* acted as the restriction enzymes used in this study.

3.3 Plasmids

The plasmids used are as below:

Table 7 Plasmids used in this study

Plasmid	Phenotype	Reference
pCR2.1-TOPO	TA cloning vector. Ap ^r Km ^r	Invitrogen
pUC19	multicopy-cloning vector. Ap ^r	Invitrogen
pK18	multicopy-cloning vector. Km ^r	Invitrogen
pET15b	expression vector for purification of proteins by nickel affinity chromatography. Ap ^r	Novagen
p6	5.257-kb <i>EcoRI</i> fragment containing <i>hsdA</i> and its regulatory elements cloned into pUC18. Ap ^r	Xiong and Maser, 2001
pUC3.2-4	whole sequence with two promoters and <i>β</i> <i>hsd</i> expressed in pUC19. Ap ^r	Mingtang Li, Xiong and Maser, 2013
pUC1126-5	promoters together and <i>β</i> <i>hsd</i> expressed in pUC19. Ap ^r	Mingtang Li, Xiong and Maser, 2013
pUC912-10	promoter 2 and <i>β</i> <i>hsd</i> expressed in pUC19. Ap ^r	Mingtang Li, Xiong and Maser, 2013
pET-T3	<i>tetR</i> expressed in pET15b. Ap ^r Km ^r	this study
pK-LuxR	<i>luxR</i> expressed in pK18. Km ^r	this study
pK2.4-EGFP4	<i>egfp</i> expressed in pK18. Km ^r	Mingtang Li, Xiong and Maser, 2013
pTOPO-TetR3	<i>tetR</i> expressed in pCR2.1-TOPO. Ap ^r Km ^r	this study
pKT3	<i>tetR</i> expressed in pK18. Km ^r	this study
pTopo-TM2	<i>tetR</i> mutant expressed in pCR2.1-TOPO. Ap ^r Km ^r	this study

As shown in Table 7, pCR2.1-TOPO was used to clone *3,17β-hsd*, *tetR*, and *luxR* from *C. testosteroni* ATCC 11996. Plasmids pUC19 and pK18 were the control vectors. Plasmid pET15b was the vector to prepared for target gene expression, protein purifications and *egfp* determination. p6 was used to clone *3α-hsd/cr* and its regulatory elements. To research the *3,17β-hsd* regulation, some plasmids were also made. pUC3.2-4 contains *3,17β-hsd* and the fragment before it, while pUC1126-5 includes the same gene sequence with pUC3.2-4, except the fragment between two promoters. pUC912-10 contains *3,17β-hsd* and only promoter 2 and repeat sequence 2. pK2.4-EGFP-4 comprises *egfp* and the upstream gene sequence before *3,17β-hsd*. *egfp* is used to replace *3,17β-hsd*. Simultaneously, a number of plasmids which contains *tetR* were also constructed (pTOPO-TetR3, pKT3, pET-T3, pTOPO-TM2). pTOPO-TetR3 was prepared for PCR reactions, while pTOPO-TM2 was cloned for the *tetR* mutant preparation. pK-LuxR was the vector containing *luxR*. pK2.4-EGFP4 was the vector containing the DNA sequence on upstrem of *3,17β-hsd* (without *3,17β-hsd*) and *egfp* gene. pTOPO-TetR3 was the plasmid containing *tetR* for PCR reactions. pKT3 contains the *tetR* gene. pTOPO-TM2 was cloned for the *tetR* mutant preparation.

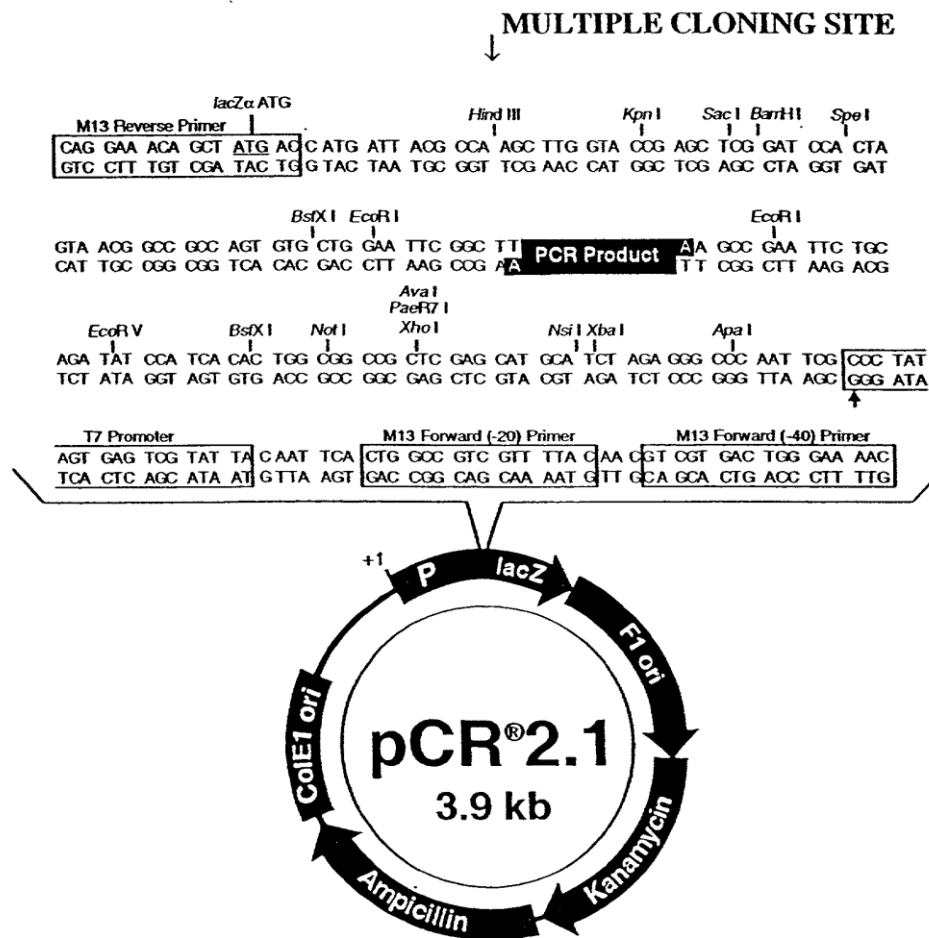


Fig. 7 Plasmid map of of pCR 2.1-TOPO (Invitrogen). Plasmid pCR2.1-TOPO was used to clone a PCR fragments with overhangs A (prepared by Taq DNA polymerase). As shown in the Fig 7, pCR 2.1-TOPO contains a 115 bp polylinker with cloning sites for 16 restriction enzymes. In addition, pCR 2.1-TOPO contains the *LacZ* promoter, as well as kanamycin resistance and ampicillin resistance genes.

3. Materials and methods

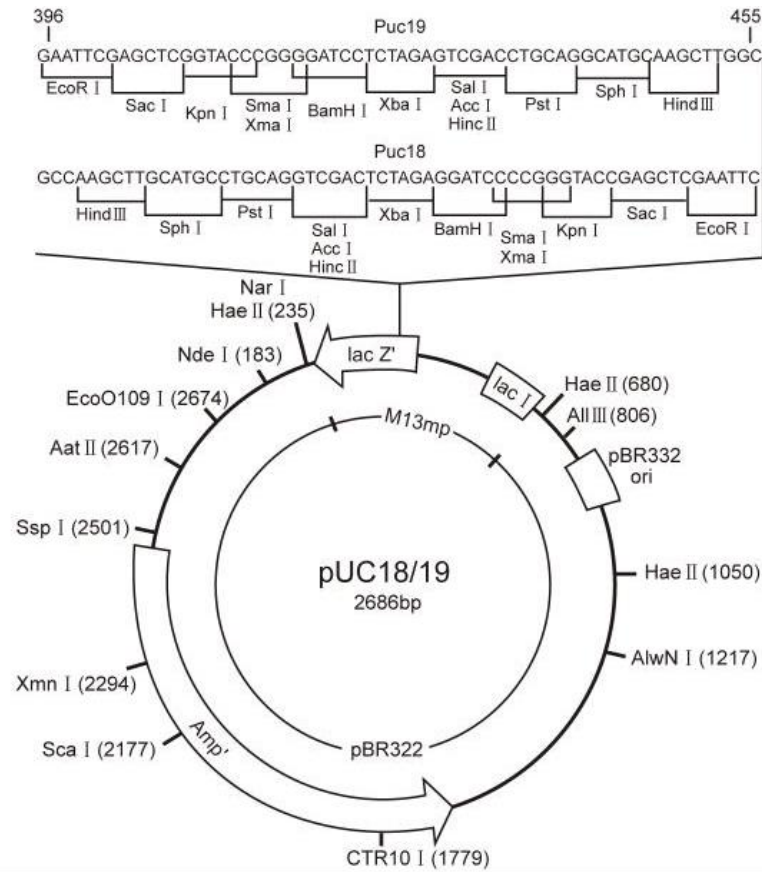


Fig. 8 Plasmid map of pUC19 (Solarbio). As shown in Fig. 8, pUC19 is 2686 bp and contains a 54 bp polylinker sequence (with 13 restriction enzyme sites). Ampicillin resistance gene could be used to select the clones. The plasmid could be replicated in *E. coli* but not in *C. testosteroni*.

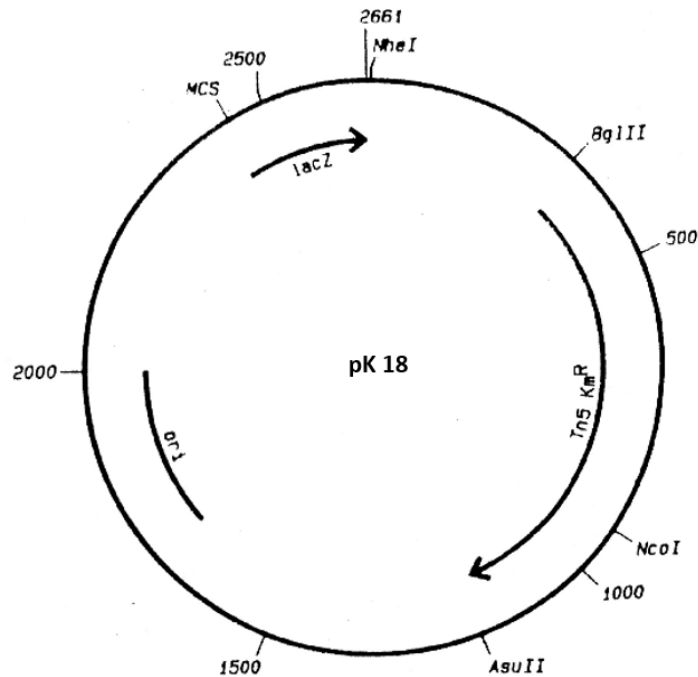
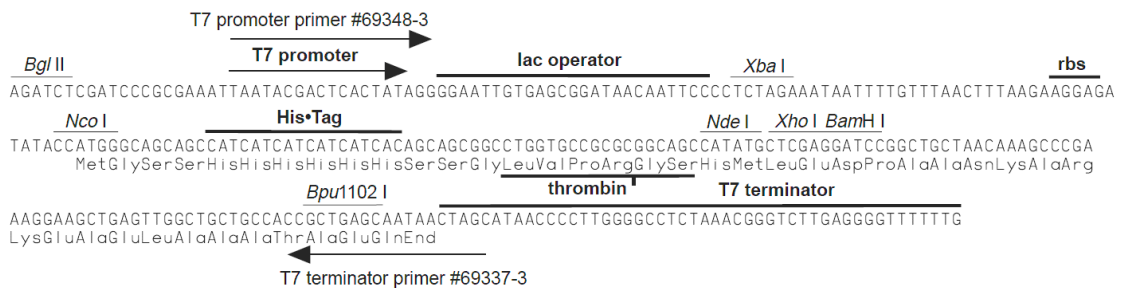
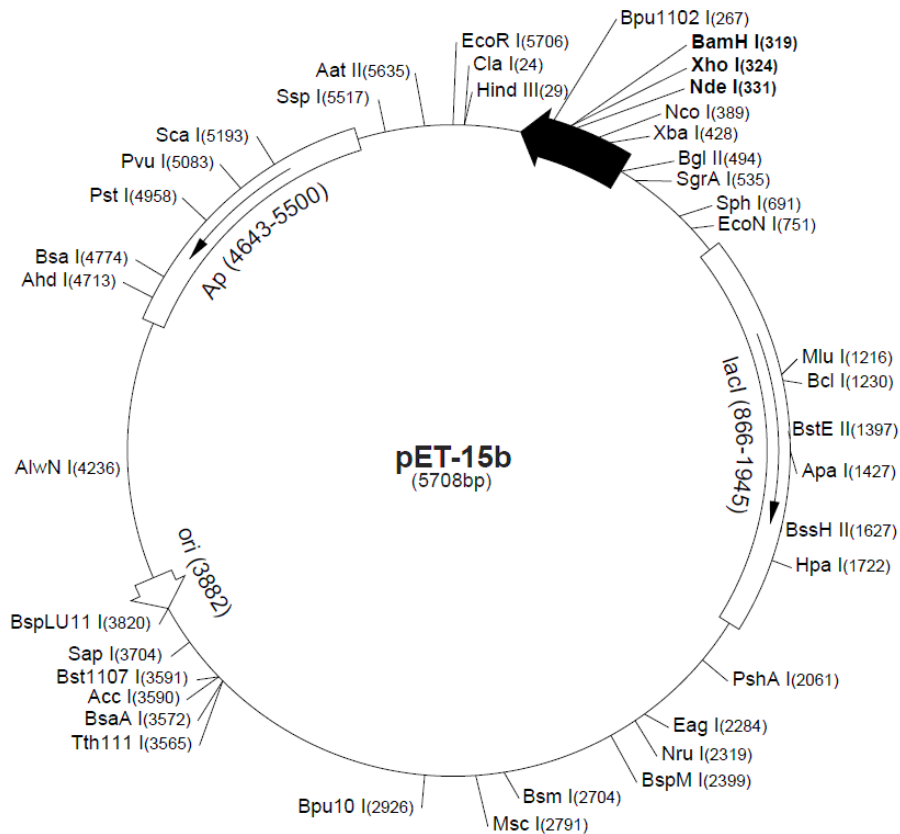


Fig. 9 Plasmid map of pK 18 (Invitrogen). The pK 18 plasmid contains a kanamycin resistant gene and is a high-copy-plasmid. The plasmid could be replicated in *E. coli* while not in *C. testosteroni*, and also used to prepare gene knock out mutant of *C. testosteroni*.



pET-15b cloning/expression region

Fig. 10 Plasmid map of pET-15b(Novagen). As shown in Fig. 10, pET-15b contains an N-terminal His.Tag sequence followed by a thrombin site and three cloning sites. The Lac operator and the T7 promoter are included in the cloning region. The plasmid is used to express and purify target protein.

3.4 Equipment

Assay Plate 96 well	CoStar, Germany
Centrifuge Biofuge	Schnakenberg GmbH, Germany
DNA electrophoresis	BIO-RAD; Germany
GENios Pro Fluorescence Microplate Reader	Austria GmbH, Tecan
iMARK Microplate Reader	BIO-RAD; Japan
ImageMaster	Pharmacia Biotech, Sweden
LabGard Nu-813	NuNRE, Germany
Microscope	Bresser; Rhede, Germany
PCR-Cycler-Mastercycler	Eppendorf, Germany
Protein electrophoresis	Major Science, Taiwan
24 Place rotor centrifuges	Heraeus Biofuge Pico, Germany
SDS-PAGE electrophoresis apparatus	Roche Diagnostics, Germany
Scanner PowerLook III	Umax Digital, Germany
Single Channel Pipette	Eppendorf, Germany
Thermal Imaging System FTI-500	Pharmacia Biotech, Germany
Thermo mixer	IKAMAG, Germany
Vortex GENE 2 Digital	Scientific Industries, America
Water Bath Circulator Heater	HAAKE N3; Germany

3.5 Chemicals

Agarose	Carl Roth GmbH, Germany
Acetic acid	Carl Roth GmbH, Germany
Ammonium persulfate (APS)	SERVA, Germany
Ampicillin	AppliChem GmbH, Germany
Bovine serum albumin (BSA)	Behringwerke AG; Germany
Bradford reagent	Sigma-Aldrich Chemie GmbH, Germany

3. Materials and methods

Chloroform	Carl Roth GmbH, Germany
dNTPs	Fermentas GmbH; Germany
1 kb DNA ladder	Fermentas GmbH; Germany
EDTA	Sigma-Aldrich Chemie GmbH, Germany
Estradiol	Sigma-Aldrich Chemie GmbH, Germany
Gel 40	Sigma-Aldrich Chemie GmbH, Germany
Glycerin	Biotium, Inc., Germany
Glycine	Merck KGaA, Germany
Hydrochloric acid (HCl)	Carl Roth GmbH, Germany
Isopropanol	Merck KGaA, Germany
Low melting point agarose	Invitrogen, Darmstadt, Germany
KH_2PO_4	Merck KGaA, Germany
Kanamycin	CALBIOCHEM, Germany
MgCl_2	Fermentas GmbH, Germany
Methanol	Merck KGaA, Germany
NaCl	Merck KGaA, Germany
NaOH	Merck KGaA, Germany
Na_2HPO_4	Merck KGaA, Germany
Phenol	Carl Roth GmbH, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH, Germany
Standard I Nutrient Broth medium (SIN)	Merck KGaA, Germany
Tween-20	Sigma-Aldrich Chemie GmbH, Germany
Testosterone	Sigma-Aldrich Chemie GmbH, Germany
TEMED	Sigma-Aldrich Chemie GmbH, Germany
Tris base	Merck KGaA, Germany

3.6 Restriction enzymes and kits

Restriction enzymes and Vent DNA polymerase were from New England Biolabs. Shrimp alkaline phosphatase was from Usb. T4 DNA ligase and Taq DNA polymerase were from Fermentas (Massachusetts, USA). RNase A was from Boehringer (Mannheim, D). TOPO TA cloning kit was from Invitrogen. Plasmid Midi Kit and nickel-nitrilotriacetic acid (Ni-NTA) Spin Kit were from QIAGEN (München, D). DIG DNA labelling, and Detection Kit was from CARL Roth (Karlsruhe, D).

3.7 PCR

The amplification of specific DNA fragments is facilitated by polymerase chain reaction (PCR). Primers that served as specific primers to amplify the DNA of interest are listed in Table 6. The reaction was done in a total volume of 20 µl containing 10 ng of template DNA, 1 µl of forward primer (Eurofin, 100 pM), 1 µl of reverse primer (100 pM), 1 µl of dNTPs (100 mM), 10 µl of MgCl₂ (25 mM), 5 µl of 10× PCR buffer and H₂O (adding to 50 µl). The PCR program was as follows: 2 min at 95°C for pre-denaturation; 25 thermal cycles involving denaturation at 95°C for 30 sec., annealing at 45°C for 30 sec., then extension 30 sec., finally at 72°C for 10 min and storage at 4°C. The PCR product was then analysed by agarose gel electrophoresis.

3.8 Agarose gel electrophoresis

Agarose gel electrophoresis is commonly used to separate DNA fragments of more than 50 bp in size based on the size of the fragments, that is, longer fragments move more slowly in the gel than smaller fragments. For proper separation of fragments with different lengths, 0.8% - 2% of agarose in 1× TAE buffer was used as running buffer. After running for 30 min, the gel was transferred into the staining solution containing 10 µg/ml ethidium bromide, which can incorporate into the DNA and enables detection of the loaded DNA. Following staining for 5 min, the gel was washed with water for another 5 min, subsequently the gel was documented with UV light in a UV-transilluminator and the picture was printed out.

3.9 Digestion of DNA with restriction endonucleases

Digestion of restriction endonuclease was performed with the enzymes and buffers according to the instruction given in the manuals. Reactions were generally performed in a

total volume of 20 μ l. Double digestions were done with suitable buffer which is compatible with the both enzymes. All digestions were performed at 37°C for 1 hr.

To prevent religation of the linearized vector in the subsequent ligation reactions, the 5' end of the vector DNA was dephosphorylated. The DNA to be dephosphorylated was subjected to shrimp alkaline phosphatase (SAP) treatment after restriction reaction, where one-unit SAP was used in a 60 μ l reaction volume containing 4 μ l of 10 \times SAP reaction buffer and incubated at 37°C for 15 min. Inactivation of the enzyme was performed through incubation at 65°C for 5 min.

3.10 Phenol/chloroform extraction of DNA

After digestion with restriction enzymes, phenol/chloroform extraction was used to eliminate proteins from DNA solutions. First, the DNA solution was filled up with H₂O to a final volume of 200 μ l and subsequently an equal volume of phenol was added then vigorously shaken and incubated at RT for 5 min. After centrifugation at 13,000 rpm for 1 min, the down phase was moved into a new EP tube, and 200 μ l of chloroform was added. Mixing and centrifugation were repeated, and the down phase was discarded. After a second chloroform-step, 1/20 volume of 5 M NaCl and 2 volumes of absolute ethanol were added, and centrifugated at 13,000 rpm for 10 min. The supernatant was discarded and the sedimented nucleic acids were washed with 1 ml of 70% ethanol. Finally, the pellet was dissolved in 10 μ l H₂O.

3.11 Ligation of DNA fragments

Ligases catalyse the formation of phosphodiester bonds between the directly adjacent 3'-hydroxyl and 5'-phosphoryl termini of nucleic acid molecules, therefore, ligation of DNA fragments requires the presence of compatible ends and DNA ligase. 50 ng of restriction enzyme-digested vector DNA was incubated with a three- to five-fold molar excess of insert depending on the insert size. One unit of T4 DNA ligase was added in a final reaction volume of 10 μ l. The ligation mixtures were incubated at 22°C for 3 hrs, and inactivation of T4 DNA ligase was performed at 65°C for 3 min and immediately placed on ice for 5 min.

3.12 Transformation

Before transforming plasmid or ligation mixtures into competent *E. coli*, chemical competent *E. coli* was prepared with CaCl₂. Two ml of SIN medium was inoculated with 20 µl of overnight *E. coli* culture. After it reached up to an OD₆₀₀ 0.4-0.6, one ml cells are harvested by centrifugation 13,000 rpm for 20 sec.. The precipitated cells were gently resuspended in 1 ml of 0.1 M CaCl₂ and incubated on ice for 30 min. After centrifugation 13,000 rpm for 20 sec., the precipitated bacterial cells were resuspended in 0.5 ml or 1 ml of CaCl₂ and incubated on ice for additional 30 min. For transformation, 1 µl plasmid DNA or ligation mixtures was added into 100 µl of chemical competent cells and incubated on ice for 30 min. During the incubation plasmid DNA attaches to the outer membrane of the cells. The cells were heatshocked in 42°C waterbath for 90 sec. and immediately placed on ice for 5 min. During heatshock the plasmid DNA was picked up into the bacteria. After addition of 400 µl of SIN medium, the competent cell mixtures were shaken at 110 rpm for 1 h at 37°C. The cell mixtures were plated on SIN agar plates (100 µl/ml ampicillin and/or 30 µg/ml kanamycin depending on the type of antibiotic resistance gene used). After 10 min, the plate was inverted and incubated at 37°C overnight.

3.13 Mini preparation of plasmid DNA from *E. coli*

Mini preparation of plasmid DNA was performed according to the alkaline lysis method. One ml of SIN medium containing respective antibiotics was inoculated with a single colony of bacteria and incubated at 37°C with continuous shaking for 5 hrs at 180 rpm. The cell culture was centrifuged at 13,000 rpm for 20 sec., and the supernatant was discarded. The pellet was resuspended by adding 100 µl of buffer P1 (50 mM Tris/HCl, pH8.0, 10 mM EDTA, and 100 µg/ml RNase A). After addition of 100 µl of buffer P2 (200mM NaOH and 1% SDS) and gently shaking for 1 min, the mixtures were incubated at room temperature (RT) for 5 min. In this step the cellular membranes of the bacteria were lysed. To neutralize the mixtures and to precipitate chromosomal DNA and proteins, 100 µl of buffer P3 (3 M potassium acetate, PH 5.5) was added and the mixtures were mixed gently. After centrifugation at 13,000 rpm for 1 min, the supernatant containing plasmid DNA was transferred into another tube. Two volumes of absolute ethanol were added into the supernatant, the mixtures were shaken and then centrifuged at 13,000 rpm for 7 min at RT. The pellet of plasmid DNA was washed with 70% ethanol to dissolve salts. Then after centrifuging at 13, 000 rpm for 5 min, the pellet was dissolved in 10 µl H₂O or TE buffer

and stored at 4°C.

3.14 Cloning of *tetR* from *C. testosteroni* and subcloning of *tetR* fragments

As shown in Table 8, the *tetR* was cloned from *C. testosteroni* ATCC 11996 chromosomal DNA by using forward primer pTetR-L containing a *NdeI* site and reverse primer pTetR-R containing a *BamHI* site (Table 8). The full length *tetR* gene was then cloned into PCR2.1-TOPO to yield plasmid pTOPOTetR, which after sequence confirmation (Eurofins) was used as template for further PCR reactions. To generate *tetR* controlled by the *lac* promoter, plasmid pTOPOTetR was digested with *EcoRI* and then the resulting fragment was ligated into either pK18 in the same direction with the *lac* promoter to yield pKT3 (which was used in the experiment) or in the reverse direction with the *lac* promoter to yield pKT2. To overexpress the recombinant TetR protein, the *BamHI-NdeI* fragment of pTOPOTetR was subcloned into pET15b downstream of the N-terminal His-Tag coding sequence to yield pET-T3, which was used for recombinant TetR protein preparation.

3.2-4-EL and 3.2-4-ER were used to prepare the DNA fragment containing the *3,17 β -hsd* gene and two operators in front of the *3,17 β -hsd* gene. Primers *tetR*-EL and *tetR*-ER were used to prepare the *tetR* fragment for PCR. Primers *tetR*-ML and *tetR*-MR were used to prepare the *tetR* mutant pTOPO-TM2. Primers *tetR*-IML and *tetR*-IMR were used to identify the pTOPO-TM2. Primers *luxR*-EL and *luxR*-ER were used to clone the *luxR* for PCR.

Table 8 Primers for DNA fragment preparations

Name	Sequence	Direction	Usage
3.2-4-EL	CGCCATATGCAAGAAAACAAA <i>NdeI</i>	Forward	pUC3.2-4
3.2-4-ER	CCCAAGCTTCTATAGCCCCATGCC <i>HindIII</i>	Reverse	
<i>tetR</i> -EL	CGCCATATGGCCCTGGCCCTGGTC <i>NdeI</i>	Forward	pCR2.1-TOPO cloning
<i>tetR</i> -ER	CGCGGATCCTCAGGCTGTCGCCGTTGC <i>BamHI</i>	Reverse	
<i>tetR</i> -ML	TAGGGCCCTGGCCCTGGTCA	Forward	Mutant construction pTOPO-TM2
<i>tetR</i> -MR	AGATCGCATTTTCTCCATG	Reverse	
<i>tetR</i> -IML	GACAACACAGCAATCCTC	Forward	Mutant identification
<i>tetR</i> -IMR	ATATCTGCAGAATTCGCC	Reverse	
<i>luxR</i> -EL	CGCCATATGATGGATAGCGACATA <i>NdeI</i>	Forward	pCR2.1-TOPO cloning
<i>luxR</i> -ER	CGCGGATCCCTTTTCGCCCAATAG <i>BamHI</i>	Reverse	

3.15 Construction of a *tetR* knock-out mutant strain *C. testosteroni*

A *tetR* disrupted mutant of *C. testosteroni* was prepared through homologous integration. A DNA fragment containing the full length *tetR* was generated by PCR using forward primer *tetR*-ML and reverse primer *tetR*-MR (Table 8) with plasmid pCR2.1-TOPO as template. The 300bp fragment was cloned into PCR2.1-TOPO containing the kanamycin resistance gene to yield pTOPO-TM2. Since the plasmid pTOPO-TM2 cannot replicate in *C. testosteroni* and the sensitivity of wild type *C. testosteroni* to kanamycin, only mutants of *C. testosteroni* with kanamycin resistance gene of plasmid pTOPO-TM2 can be recombined with the chromosomal DNA of *C. testosteroni*. The mutant could grow in medium containing kanamycin. According by 10 µg of pTOPO-TM2, which contained *tetR* sequences homologous to the chromosomal DNA of *C. testosteroni*, was transformed into *C. testosteroni* by electroporation (1.8 kV, 1-cm cuvette, BIO-RAD). The cells were spread on 30 µg/ml kanamycin SIN agar plates and cultured at 27°C overnight. The colonies were proved by PCR for homologous integration (Fig. 27).

3.16 Homologous recombination – cross linking integration

To prepare the knock-out mutant of *C. testosteroni*. 75 µl *C. testosteroni* cells in 2 ml SIN medium were incubated at 27°C for 6 hrs. After reaching to an OD600 of 0.4-0.6, 1 ml cells were harvested by centrifugation (13,000 rpm for 30 sec.). The pellet was resuspended by adding 1 ml 10% glycerol. The cells were incubated on ice for 5 min. After centrifugation (13,000 rpm for 30 sec.), the resulting pellet was resuspended in 500 µl 10% glycerol. 100 µl *C. testosterone* glycerol was mixed with 10 µl plasmid. The mixture was put into Bio-Rad GenePulser electroporators: 1.8 kV, 4.9 ms. 400 µl SIN medium was added into the mixture and cultured 4 hrs at 27°C. Then the mixture was spreader onto the Kanamycin-resistant petri dish and cultured at 27°C overnight. To the end, monoclonies were selected and cultured in liquid LB medium.

3.17 Growth of wild type *C. testosteroni* and a *tetR* or *luxR* knock-out mutant strain in the presence of steroids

To determine if the mutants without *tetR* or *luxR* affects the utilization of steroids as the carbon source of *C. testosteroni*, wild type *C. testosteroni* and *tetR* or *luxR* knock-out mutant strains were incubated with 0.5 mM testosterone. After incubation at 27°C overnight, growth was recorded through measurement of the optical density of the sample.

3.18 Overexpression and purification of proteins

Overexpression of TetR proteins was performed in *E. coli* strain BL21(DE3) pLysS with plasmid pET-T3, and recombinant protein was extracted under denaturing conditions and purified by the 6× His-tag sequence. In brief, cells transformed with recombinant pET-T3 plasmid were grown at 37°C in a shaker (180 rpm) in SIN medium supplemented with 100 µg/ml ampicillin. 100 µl of the overnight culture was used to inoculate 2 ml of fresh SIN medium. When the bacteria had grown to an optical density of 0.4-0.6 at 595 nm, target protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.25 mM. After induction for 4h at 37°C or overnight at RT, cells were harvested by centrifugation, the cell pellet was either stored at -80°C for further usage or directly suspended in 200 µl of lysis buffer (50 mM sodium dihydrogenphosphate, 300 mM sodium chloride, and 10 mM imidazole, pH 8.0) containing different concentrations of sodium lauroyl sarcosinate (SLS). Cells were lysed by freezing (-20°C, 30 min) and thawing (RT, 30min) 3 times, and the resulting mixtures were centrifuged by 13,000 rpm

for 20 min. The supernatant was applied to a mini nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography column. After washing 2 times with 600 μ l of washing buffer (8 M urea, pH 4.5), the TetR protein was eluted from the column by applying 100 μ l of elution buffer (8 M urea, pH 3.5) for 4 times.

3.19 Immunization and preparation of antisera against TetR

On the first day, rabbits were injected subcutaneously with an emulsion of 0.5 ml ddH₂O and 0.5 ml complete Freund's adjuvant. For immunization, one microgram of purified TetR protein was dissolved in an emulsion of 0.5 ml dd H₂O and 0.5 ml incomplete Freund's adjuvant, and rabbits were immunized on the following days: 1 d, 30 d, 60 d and 67 d. The antiserum was collected at day 74 and antibody titer determination in the rabbit serum was performed by ELISA.

3.20 Protein determination

Protein concentration was determined by the method of Bradford [118] with Roth-Quant solution using bovine serum albumin (BSA) as standard. The principle of this method is based on the observation that the maximum absorption for an acidic solution of Coomassie Brilliant Blue G - 250 shifts from 465 nm to 595 nm when binding to protein occurs. Each time the assay was performed, a standard curve was prepared using BSA as protein standard (0-1 mg/ml). Photometer with 595 nm was corrected for the blank. 20 μ l of appropriately diluted samples or BSA was mixed with 980 μ l 1:5 dilution, and the absorbance of the solution at 595 nm was measured at RT with a spectrophotometer after 10 min.

3.21 SDS-PAGE

In order to analyse the purity of recombinant proteins, polyacrylamide gels containing the strong anionic detergent SDS were used. The polypeptides bind SDS and become negatively charged, and the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide independent of its sequence [119]. Thus, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

In most cases, SDS-PAGE is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a pH and ionic strength different from that of the buffer used to cast the gel [119]. The sample and the stacking gel contain Tris-HCl (pH 6.8), the upper

and lower buffer reservoirs contain Tris-glycine (pH 8.3), and the resolving gel contains Tris-HCl (pH 8.8). All components of the system contain 0.1% SDS [119]. 15% resolving gel (10 ml) is composed of the following components: 3.75 ml of 40% acrylamide:bisacrylamide solution, 2.5 ml of 1.5 M Tris-HCl (pH 8.8), 100 μ l of 10% ammonium persulfate (APS), 100 μ l of 10% SDS, 4 μ l of TEMED and 3.55 ml of H₂O (Sambrook and Russel, 2001). The 5% stacking gel (5 ml) contained 0.625 ml of 40% acrylamide:bisacrylamide, 0.63 ml of 1 M Tris-HCl (pH 6.8), 50 μ l of 10% APS, 50 μ l of 10% SDS, 5 μ l of TEMED, and 3.73 ml of H₂O[120]. Electrophoresis was performed using the Roth electrophoresis apparatus Max Fill.

To load onto the polyacrylamide gel, samples were mixed with 5 \times loading buffer (250 mM Tris-HCl, pH 6.8, 500 mM dithiothreitol, 10% (w/v) SDS, 0.5% bromophenol blue and 50% (v/v) glycerol) and heated at 98°C for 2 - 3 min. Subsequently, the samples were loaded onto the gel. The electrophoresis chamber was completely filled with Tris/glycine-electrophoresis buffer (25 mM Tris base, 250 mM glycine, pH 8.3, and 0.1% SDS). The electrophoresis was run with a voltage of 100 V until the bromophenol blue reached the bottom of the resolving gel. Protein markers of known molecular weight were used upon SDS-PAGE. After electrophoresis, the proteins were visualized by staining the gel at RT for 30 min in staining solutions (dissolving 1.25 g of Coomassie Brilliant Blue R-250 in 1 L of methanol: acetic acid solution containing 500 ml of methanol, 400 ml of H₂O, and 100 ml of glacial acetic acid)[120]. Destaining was carried out at RT for 30 min with destaining buffer (250 ml of methanol, 75 ml of glacial acetic acid, and 675 ml of H₂O).

3.22 ELISA for 3,17 β -HSD

Protein for 3,17 β -HSD ELISA detection was prepared from 2 ml of bacterial cell culture and subsequent centrifugation at 13,000 rpm for 10 sec. The pellet was washed 3 times with 1 ml water and resuspended in 200 μ l of blocking buffer (PBS with 5% BSA, 0.1% Tween 20) with 100 μ g/ml of lysozyme. The suspension was frozen at -20°C and thawed at RT 30 min for 3 times. Finally, the samples were centrifuged again at 13,000 rpm for 20 min. The supernatant was diluted into 1mg/ml of total protein and used for 3,17 β -HSD detection.

To quantify 3,17 β -HSD protein expression, an ELISA was established, and respective antibodies against 3,17 β -HSD from *C. testosteroni* were prepared in rabbits. ELISA plates were coated with protein samples containing 3,17 β -HSD diluted in coating buffer (NaCO₃ 1.59 g, NaHCO₃ 2.93 g, and NaN₃ 0.2 g, pH 9.6 in 1 L). As a standard 200, 100, 50, 25,

12.5, 6.25 ng of purified 3,17 β -HSD was mixed in coating buffer into and applied the wells. After washing (washing buffer: NaCl 8 g, KH₂PO₄ 0.2 g, Na₂HPO₄ 1.5 g, KCl 0.2 g and Tween 20 0.5 ml, pH 7.4 in 1 L), antibodies against 3,17 β -HSD were added in a 1:1000 dilution. Following incubation for additional 30 min and washing 3 times, the secondary antibodies peroxidase-conjugated swine anti-rabbit immunoglobulin were used in a 1:1000 dilution. ABTS is used to be substrate solution to yield the results, and incubated the result with ABTS at 37°C for 1 hr. The further procedure corresponded to that of the chloramphenicol acetyl transferase ELISA kit from Roth.

3.23 Fluorescence microplate assay

In a total volume of 500 μ l SIN medium, 50 μ l *C. testosteroni* cells and 2 μ l of 0.1 mM steroid sample (in ethanol and SIN medium) were incubated at 27°C for 16 hrs. The cells were washed with water and centrifugation. To 96-well black plates (100 μ l wells) was added 100 μ l OD_{595 nm} = 2.0 fresh cultured *C. testosteroni* cells. GENios Pro Fluorescence Microplate Reader was used to measure EGFP expression in the samples, at excitation: 485 nm and emission: 535 nm. The results are given as relative fluorescence units (RFU).

3.24 Sequencing

Manufactured synthesis of primers and DNA sequencing were performed by MWG (Ebersberg, D). Before sub cloning, fragments prepared by PCR were cloned into pCR2.1-TOPO and then checked the sequence by “Eurofins”.

3.25 Statistics

The experiments were repeated at least four times (n=4). The data are expressed as mean \pm SD and are derived from the “Microsoft Excel” software.

4. Results

4.1 Isolation and cloning of the TetR protein of *C. testosteroni*

A new transcriptional factor, *tetR*, containing 522 bp was identified from the genome sequence of *C. testosteroni* ATCC11996 and located on contig 58 (Bioinformatics analysis). The polymerase chain reaction (PCR) was used to copy *tetR*. As shown in Table 8, primers *tetR-EL* and *tetR-ER* were used to generate and amplified the *tetR* 522 bp fragment. The result showed that a strong 522 bp band upon agarose gel electrophoresis (Fig. 11).

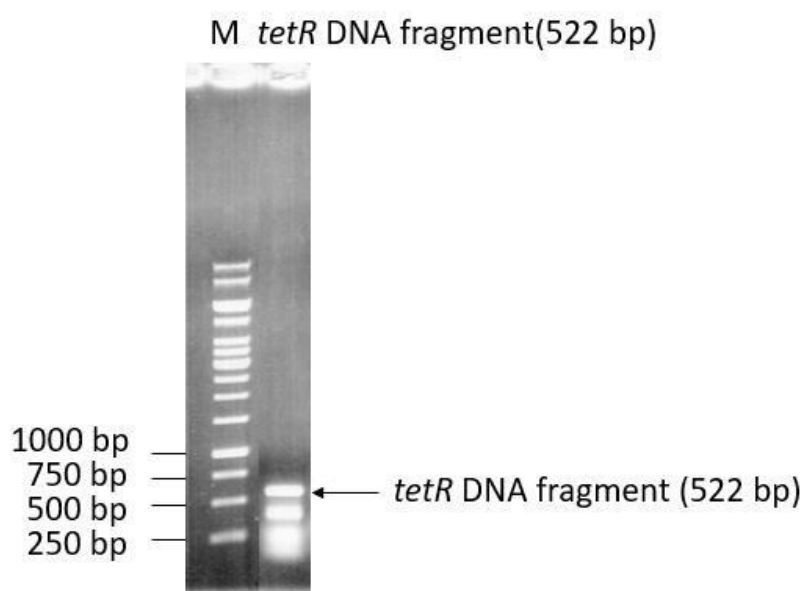


Fig. 11 Identification of *tetR* DNA fragment. For identification, the *tetR* DNA fragment was run on a 0.8% agarose gel and compared with standard marker. A *tetR* DNA fragment, comprising 522 bp was found on the gel.

The *tetR* cloning is based on TA cloning (Fig. 12). More notably, TA cloning is a well-known subcloning technique that avoids the use of restriction enzymes and is simpler and faster than traditional subcloning. Therefore, it is a beneficial and typical type of cloning. This technique relies on the ability of adenine (A) and thymine (T) (complementary base pairs) to hybridise on different DNA fragments and to ligate together in the presence of ligases. PCR products are often amplified by Taq DNA polymerase. The polymerase preferentially adds an adenine to the 3' end of the product. Thus, the PCR-amplified insert fragment was cloned into a linearised vector with

complementary 3' thymidine overhangs [121].

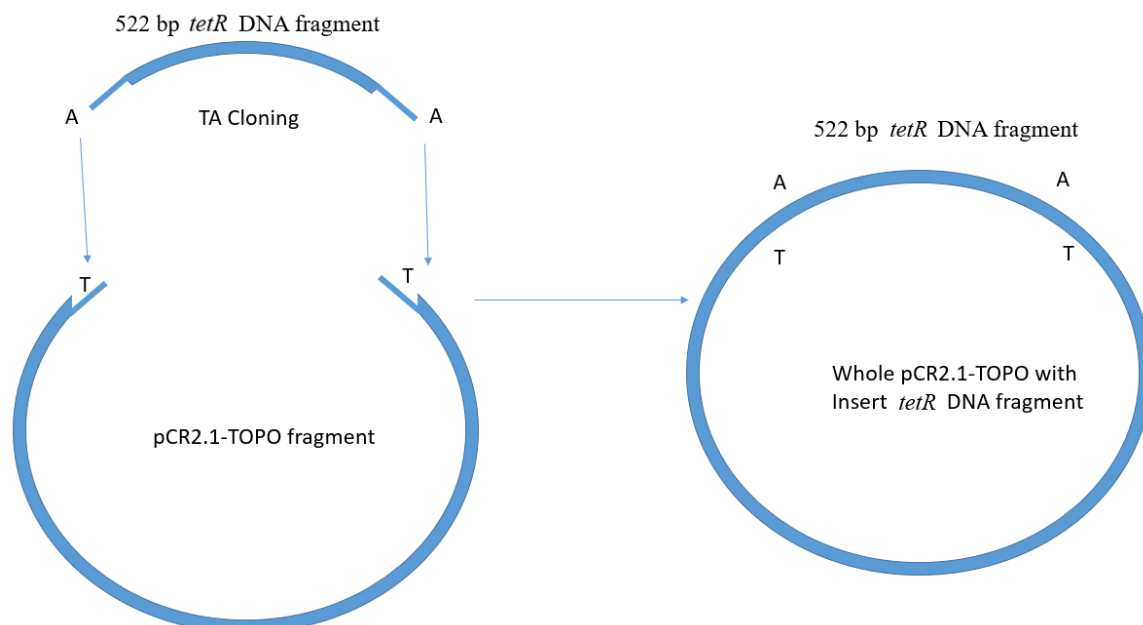


Fig. 12 Physical diagram of TA cloning. T vectors facilitate the cloning of DNA fragments that have been generated by PCR. Taq polymerase adds a single deoxyadenosine (A) nucleotide to the 3' ends of the PCR-*tetR* products. This activity is template independent. The T vector generated in this protocol contains a single deoxythymidine (T) residue at the 3' ends. This T hybridises to the single A overhang on the PCR-*tetR* product and increases the efficiency of ligation.

In the present study, *tetR* was identified from *C. testosteroni* by sequencing and amplified by PCR (primers are shown in Table 8). The results of agarose gel electrophoresis are shown in Fig. 11. After analysing the *tetR* sequence, the results showed that there was 48% similarity (81 of 167 amino acids) to the TetR family repressor from *P. aeruginosa* [122]. The gene fragment was then cloned into plasmid pCR2.1-TOPO to yield pTOPO-T3 (Fig. 13). The restriction enzymes *NdeI* and *BamHI* were used to cleave the 522 bp *tetR* DNA fragment from pTOPO-T3. The agarose gel electrophoresis showed that a strong 522 bp PCR fragment was obtained with the pTOPO-T3 as template (Fig. 14). Plasmid pTOPO-T3 could be replicated in both *E. coli* and *C. testosteroni*.

4. Results

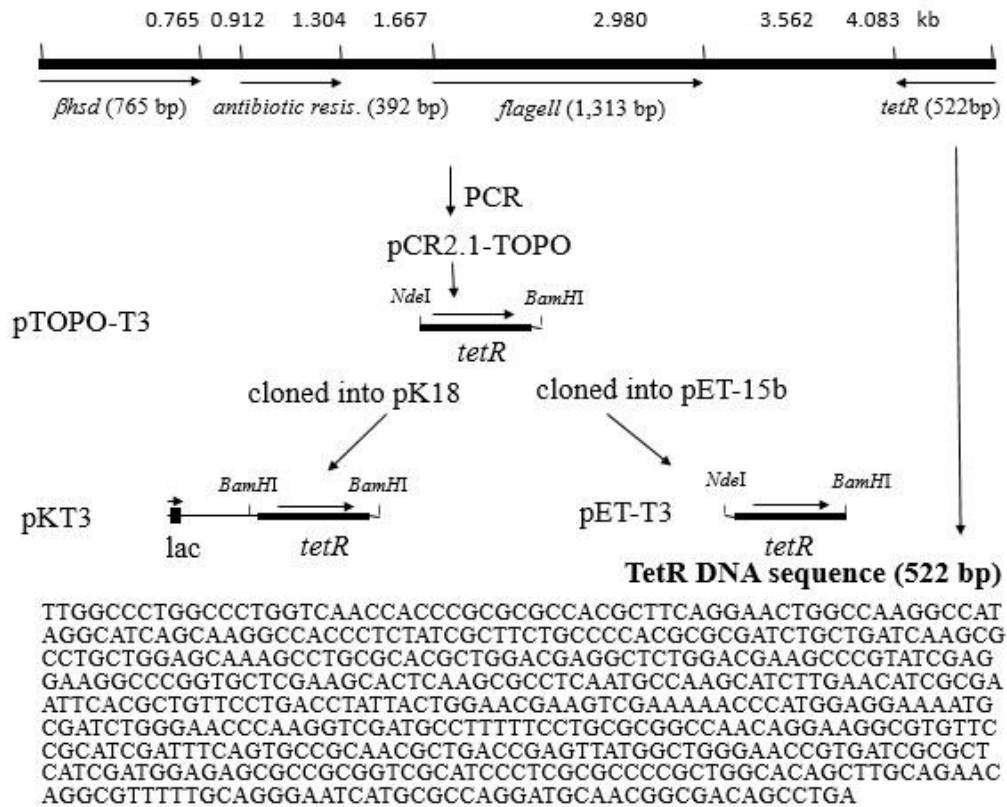


Fig. 13 *tetR* DNA fragment and PCR. The *tetR* DNA fragment (522 bp) was amplified by PCR and cloned into plasmid PCR2.1-TOPO to yield pTOPO-T3. Then, to ensure the plasmid coexistence in future experiments, *tetR* was cloned from pTOPO-T3 and subcloned to pK18 and pET15b separately to yield pKT3 and pETT3.

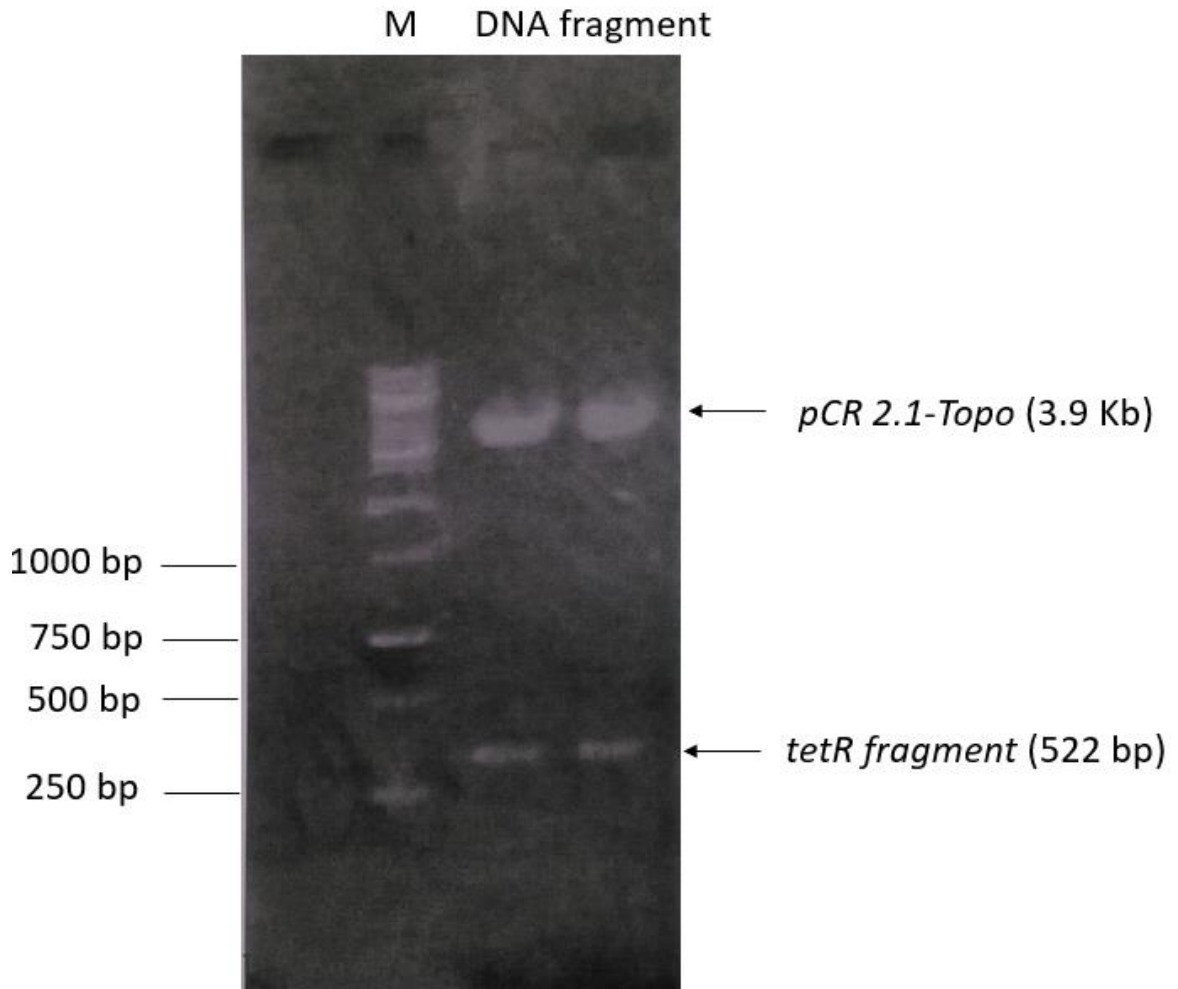


Fig. 14 Low-melting (LM) agarose gel electrophoresis to detect the *tetR* DNA fragment. The *tetR* fragment was cleaved by *Bam*HI and *Nde*I from pTOPO-T3.

Low melting agarose including the *tetR* DNA fragment was taken (Fig. 15), and phenol/chloroform extraction was used to obtain the DNA solution to prepare the subcloned plasmid pKT3.

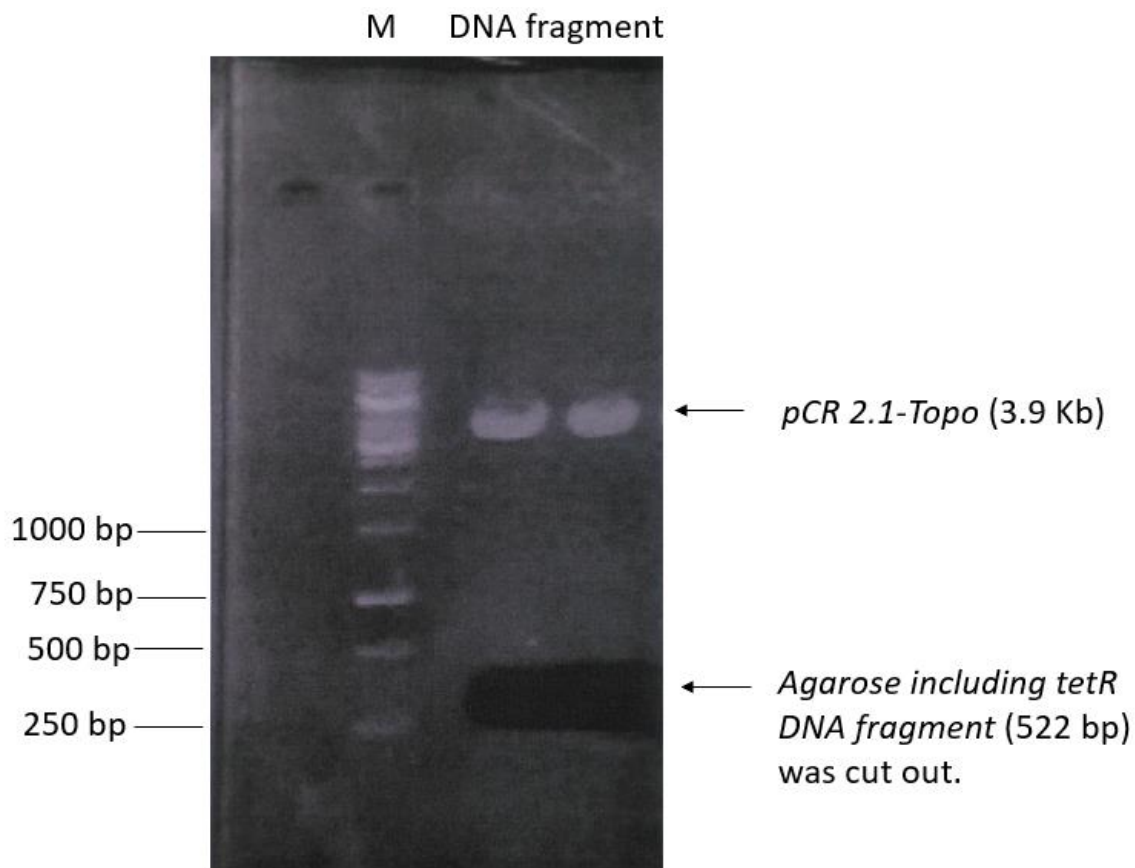


Fig. 15 Plasmid pKT3 preparation. LM agarose gel electrophoresis was used to purify the *tetR* DNA fragment. Upon comparing this photo with Fig. 14, it shows that the *tetR* DNA fragments had been cut out from the agarose gel in order to elute the fragments for further analysis.

The resulting 522 bp fragment, which was checked by DNA sequencing, was subcloned into plasmid pK18 to yield plasmid pKT3 (Fig. 16) and identified on a 1.5% agarose gel (Fig. 17).

4. Results

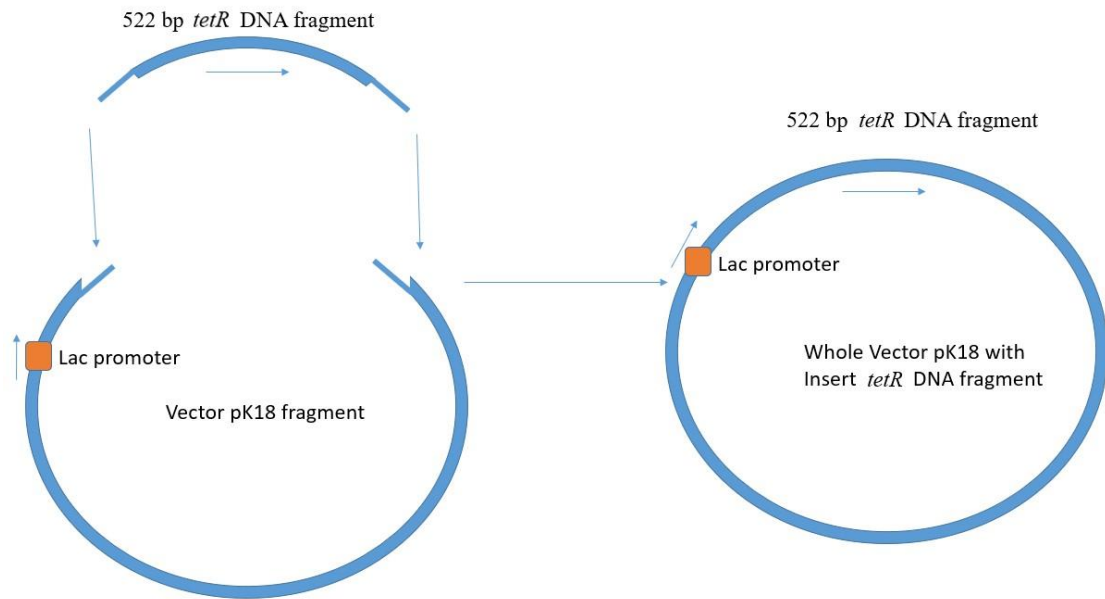


Fig. 16 Diagram of plasmid pKT3 (Right one). The *tetR* fragment was cloned to the pK18 plasmid. The lac promoter was situated upstream of the *tetR* fragment, and its orientation was the same as that of the *tetR* fragment.

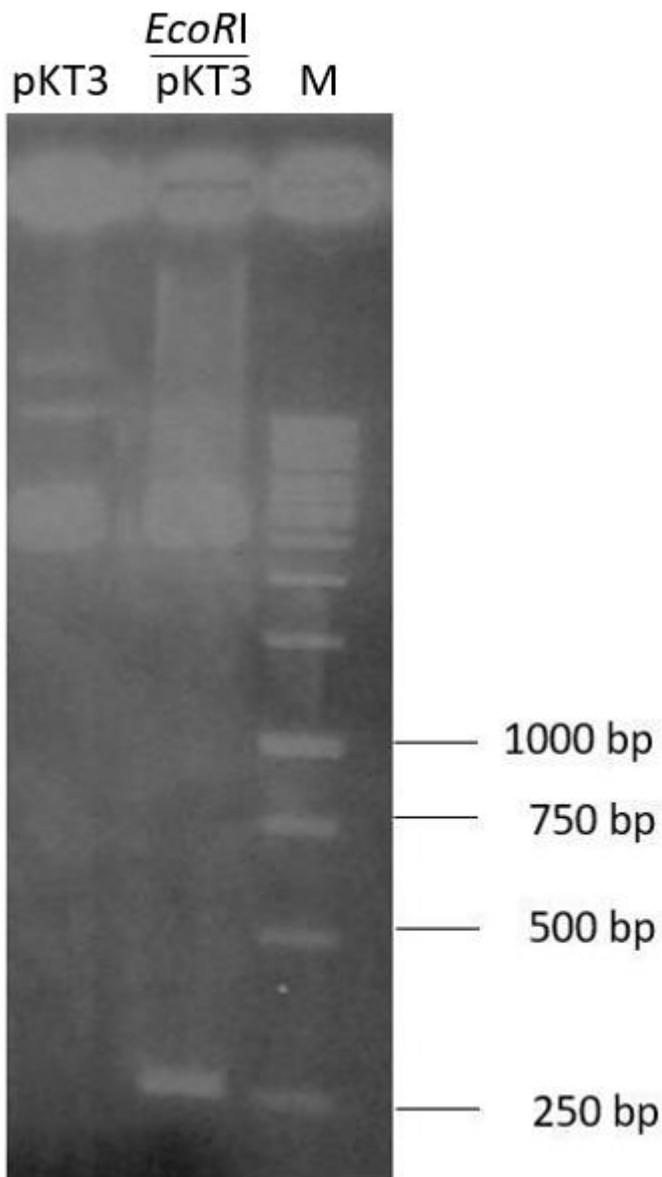


Fig. 17 Plasmid pKT3 identification. For identification, the plasmid pKT3 was run on a 1.5% agarose gel and compared with the marker, and the DNA fragment was cleaved with *EcoRI*.

In plasmid pKT3, the *lac* promoter of pK18 was located upstream of *tetR*, and the system served as a control for *tetR* expression and function. Simultaneously, *tetR* was subcloned into pET15b with *Bam*HI and *Nde*I digestion, resulting in plasmid pET-T3 (Fig. 18), which was used to express *tetR* in *E. coli* BL21 cells, and identified on a 1.5% agarose gel (Fig. 19).

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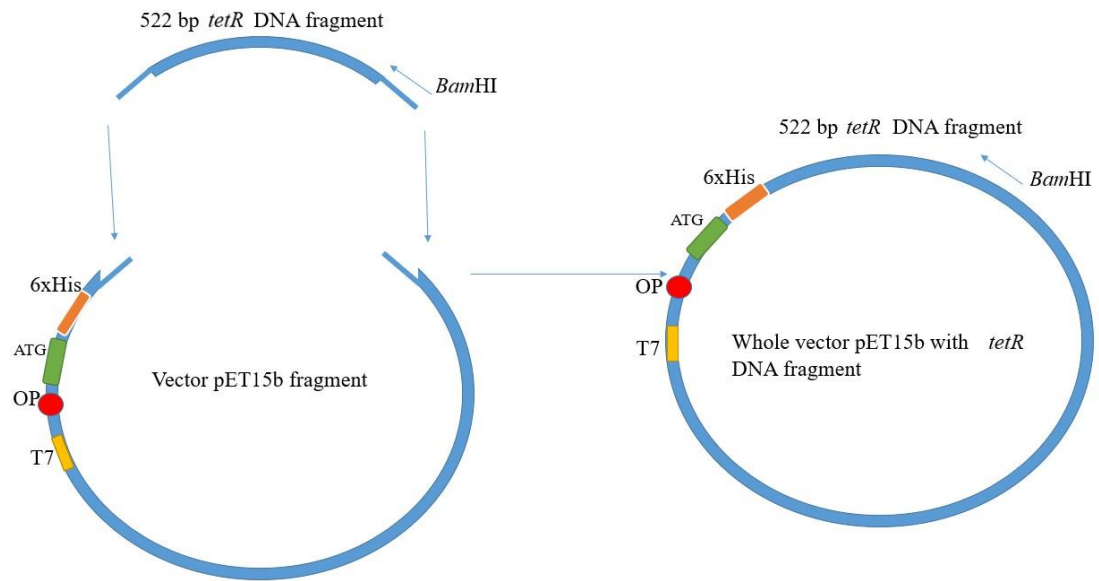


Fig. 18 Diagram of plasmid pET-T3. The *tetR* fragment was cloned to the pET15b plasmid. The T7 promoter and operon were upstream of the *tetR* fragment. pET-T3 was prepared for protein purification and fluorescence microplate assays.

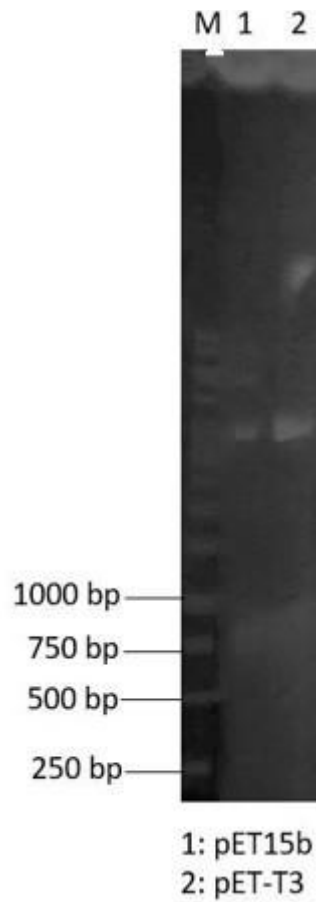


Fig. 19 Plasmid pET-T3 identification. For the identification of plasmid pET-T3, pET-T3 and pET15b (as control) were run on a 1.5% agarose gel.

4.2 Overexpression and purification of TetR

In the pET-T3 plasmid, *tetR* was cloned into vector pET15b, which has a N-terminal His-tag sequence downstream for the expression from *C. testosteroni* ATCC11996. The pET-T3 plasmid was transferred into BL21 cells. After induction with 0.25 mM IPTG at RT 180 rpm for 5 to 6 hrs, the overexpression of TetR was first dissolved in 200 μ l of 'B' buffer of the 'QIAGEN tip 20 (25)' kit. The recombinant protein (KDa) was then purified by nickel-chelate chromatography under denaturing conditions. The molecular mass was identified on an SDS-polyacrylamide gel to predict the amino acid sequence (Fig. 20). The molecular mass of the purified TetR protein plus the His-tag was about 21.9 kDa. The purified protein was used for the preparation of polyclonal antibodies.

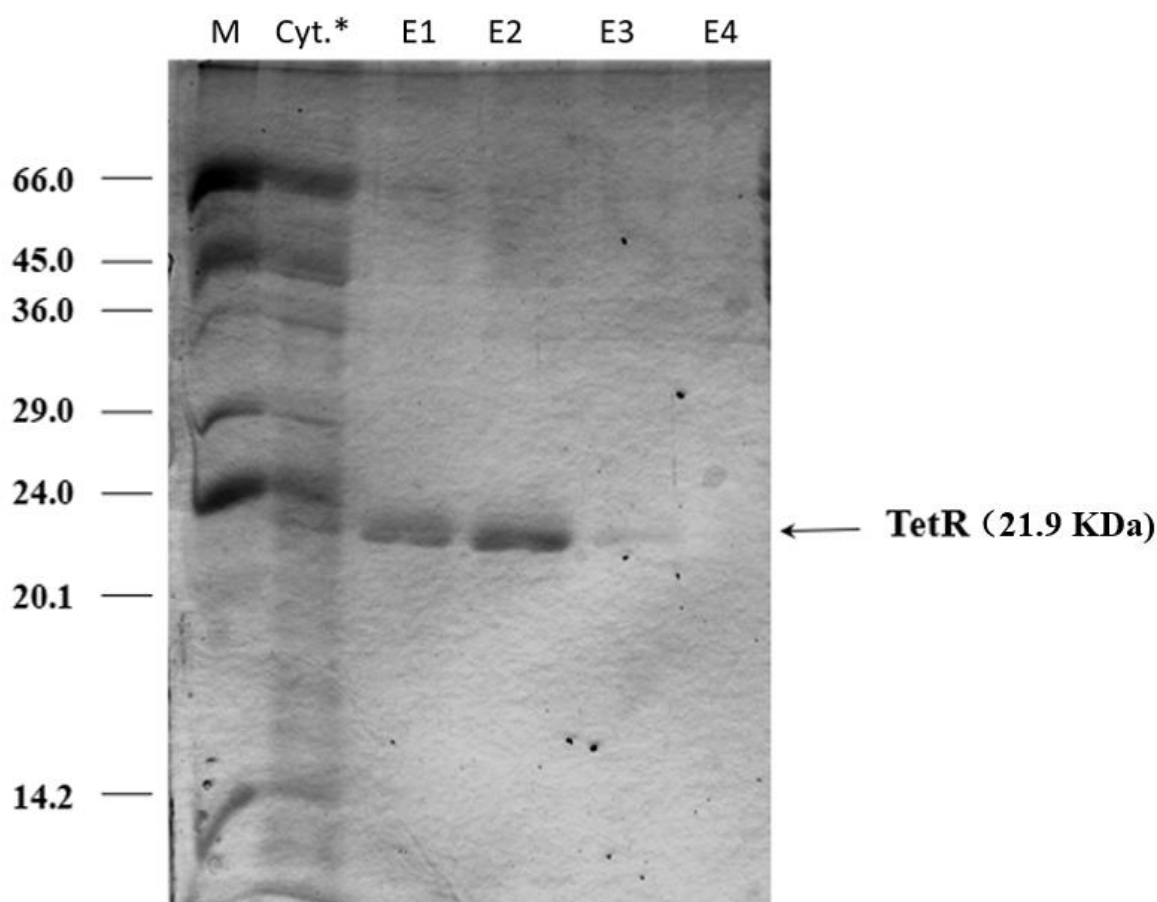


Fig. 20 SDS-PAGE (15%) of TetR protein. The protein was electrophoresed on a 15% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). M: protein marker; lane TetR Mix: cellular lysate; lane E1: elution 1; lane E2: elution 2; lane E3: elution 3; lane E4: elution 4. (*Cyt. = Cytoplasm after IPTG induction)

4.3 *3,17 β -hsd* expression regulated by TetR protein

To roughly characterise the TetR cis-acting domain in the regulatory region of *3,17 β -hsd*, three plasmids, pUC3.2-4 (containing *3,17 β -hsd*, promoters 1 and 2 and the sequence between them, RS1, RS2 [two RSs], the *PhaR* and the *RseB*), pUC1126-5 (containing *3,17 β -hsd* with promoters 1 and 2 together without the sequence between them, RS1, RS2, the *PhaR* and the *RseB*) and pUC912-10 (containing *3,17 β -hsd* with only promoter 2 and one RS [RS2], the *PhaR* and the *RseB*) were generated (Fig. 21) [59]. The *E. coli* strain was chosen, because this bacterium does not contain the steroid induction system of *C. testosteronei*. This strategy should help to focus on the TetR and the effect of testosterone in the *3,17 β -hsd* regulatory region. To avoid possible temperature effects, transformed *E. coli* HB101 cells were cultured both at 27°C (temperature optimum for growth of *C. testosteronei*) and at 37°C (temperature optimum for growth of *E. coli*). Expression of *3,17 β -hsd* was determined by ELISA. Moreover, for a further comprehensive determination, plasmid pK2.4-EGFP-4 was constructed to characterise the complete upstream regulatory region of *3,17 β -hsd* with a fluorescence reporter gene (*egfp*) instead of *3,17 β -hsd*. Considering the advantage of expression, plasmids were transformed into *E. coli* BL21 cells and used RFU detection.

Li et al. [59] found that testosterone clearly induced the expression of *3,17 β -hsd*. Thus, here, testosterone was used to induce *3,17 β -hsd* expression.

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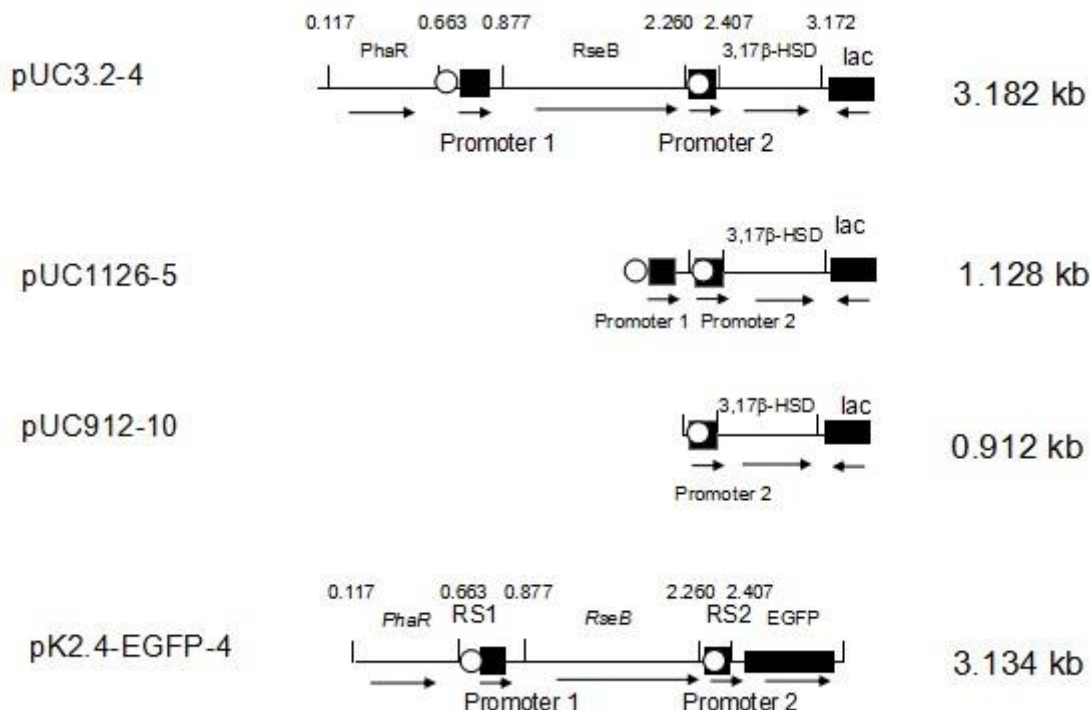


Fig. 21 Construction of series of plasmids for ELISA and RFU determination. Different fragments of *3,17β-hsd* were cloned into plasmid pUC19 and plasmid pKEGFP-2 separately, called pUC3.2-4 and pK2.4-EGFP-4. pUC3.2-4 included not only the whole *3,17β-hsd*, but also the promoters 1 and 2, the sequence between them, RS1, RS2 [two RSs] and the *PhaR* and *RseB*, which locate in front of *3,17β-hsd*. However, pUC1126-5 contained the same genes as pUC3.2-4, except the two RSs, RS1 and RS2, and the sequences between them. pUC912-10 contained the *3,17β-hsd* fragment with only promoter 2 and one RS (RS2).

As shown in Fig. 22, when plasmid pUC3.2-4 was cotransformed with either pK18 (control) or the *tetR*-containing plasmid pKT3 into HB101 cells cultured at 37°C, amounts of *3,17β-hsd* protein could be detected up to 2.136 μg/mg in cells with pK18. After cotransformation with pKT3, *3,17β-hsd* expression decreased about 25%. At 27°C, *3,17β-hsd* expression was also repressed by TetR. The result reveals that TetR is a repressor for *3,17β-hsd* expression, and at this temperature, testosterone could induce *3,17β-hsd* expression, especially in the presence of TetR.

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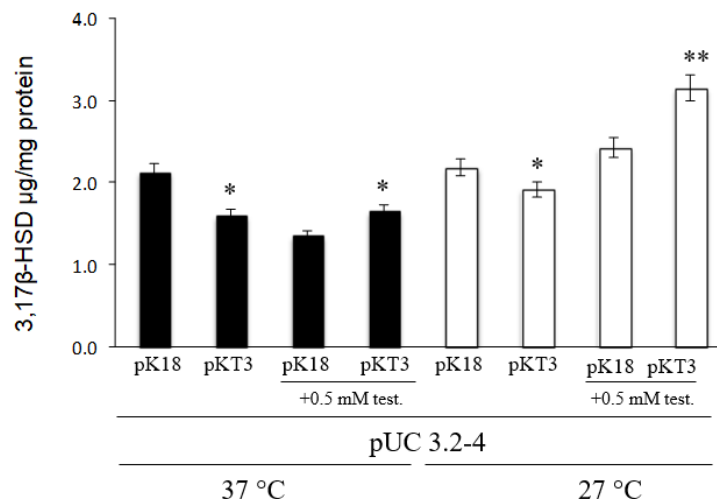


Fig. 22 TetR activity determination in *E. coli* HB101 with pUC3.2-4. Plasmid pUC3.2-4 was cotransformed into HB101 with either pKT3 (containing *tetR*) or pK18 (control) at 37°C or 27°C. Testosterone (0.5 mM) was used for *3,17β-hsd* induction. *3,17β-hsd* was determined by ELISA.

Using the same approach, pUC1126-5 was cotransformed into HB101 cells with either pK18 or pKT3. As shown in Fig. 23, levels of *3,17β-hsd* expression did not change in the absence or presence of TetR. However, testosterone addition led to a considerable decrease with pK18 or pKT3 at 37°C. Whereas no TetR and/or testosterone activity were seen at 27°C, *3,17β-hsd* expression at 27°C was generally lower compared to that at 37°C.

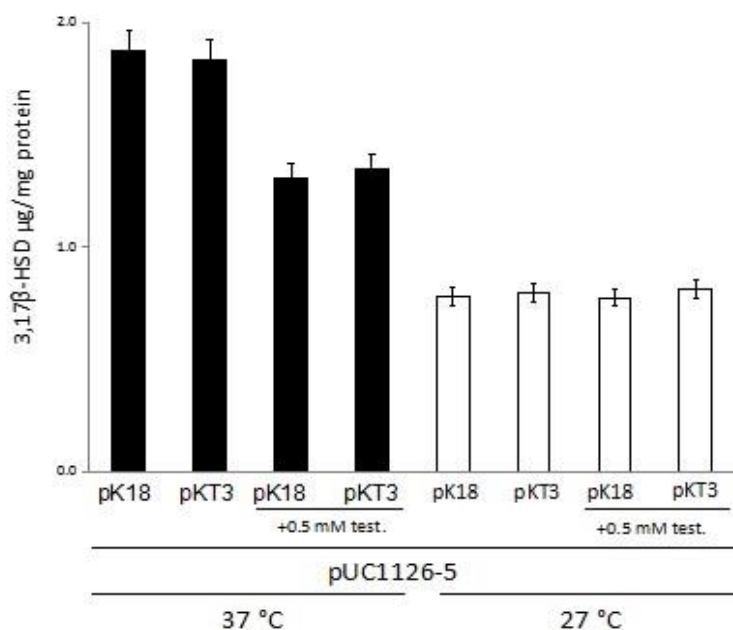


Fig. 23 TetR activity determination in *E. coli* HB101 with pUC1126-5. Plasmid

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pUC1126-5 was cotransformed into HB101 with either pKT3 (containing *tetR*) or pK18 (control) at 37°C or 27°C. Testosterone (0.5 mM) was used for *3,17β-hsd* induction. *3,17β-hsd* was determined by ELISA.

The result indicates that TetR is a repressor that could inhibit *3,17β-hsd* expression. After the induction of testosterone, no more TetR activity was found. Therefore, the inhibition of TetR was lost by adding testosterone to the SIN medium. Additionally, in plasmid pUC1126-5, a 1,165 bp fragment (Fig. 23) was deleted between promoter 1 and promoter 2. The result of ELISA showed that there was no TetR activity to *3,17β-hsd* expression when there were no RSs (RS1, RS2) or the sequence between them. The induction of testosterone was also lost at 37°C and 27°C. Therefore, the 1,165 bp fragment between RS1, RS2 and RS1, RS2 might be important for TetR function and induction.

Likewise, pUC912-10, containing only promoter 2 and one RS (RS2), was cotransformed with K18 or pKT3 into *E. coli* HB101 (Fig. 24). Whereas the same result was seen with TetR and/or testosterone as that with pUC1126-5 (Fig. 23) at 37°C, a marked temperature effect could be observed at 27°C. At 37°C, *3,17β-hsd* expression was lower than at 27°C, but also here, testosterone did not induce, but rather decreased, enzyme expression.

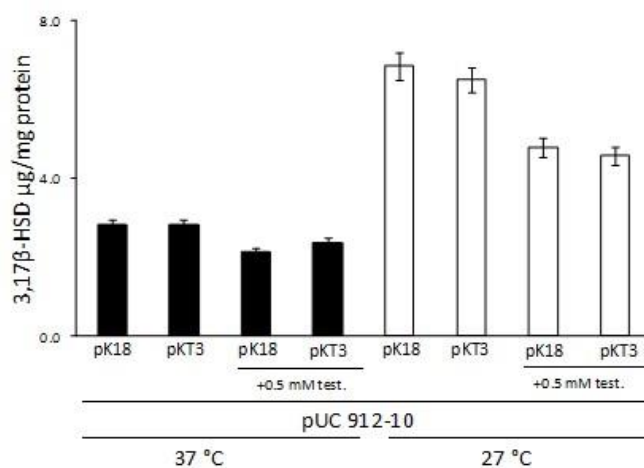


Fig. 24 TetR activity determination in *E. coli* HB101 with pUC912-10. Plasmid pUC912-10 was cotransformed into HB101 with either pKT3 (containing *tetR*) or pK18 (control) at 37°C or 27°C. Testosterone (0.5 mM) was used for *3,17β-hsd* induction. *3,17β-hsd* was detected by ELISA.

According to these results, there is a special structure involved in *3,17β-hsd* regulation by *tetR*. After analysing the sequence of *3,17β-hsd*, two RSs were found upstream of the two promoters. The first RS, RS1, was ‘GCCCGCTTTTTTGTGT’, and the other one,

RS2, was 'GCCTCCTTTTTTATGT'. There were 1,165 bp between them. In some cases, a DNA loop structure that occurs spontaneously in nucleosomes subjected to superhelical stress is involved in gene regulation. These loop structures are not stable, and they can be affected by temperature. At 27°C, the suitable temperature for *C. testosteronie*, TetR can bind on the loop structure to yield a complex that exhibits low specificity in DNA binding. However, at 37°C, this loop structure could be disrupted, thus the TetR repression function could be lower.

4.4 TetR negatively regulates expression of 3,17β-hsd with egfp in E. coli HB101

An *egfp* containing plasmid pK2.4-EGFP-4, which harbours the complete upstream regulatory region of 3,17β-*hsd* (promoters 1 and 2, two RSs [RS1, RS2] and the genes coding for *PhaR* and *RseB*) (Fig. 21), was cotransformed with pET15b (control) or pET-T3 (containing *tetR*) into the *E. coli* BL21 strain. pET15b could not express the *egfp* without IPTG induction in *E. coli* BL21 cells, even though it was cotransformed with plasmid pK2.4-EGFP-4. In this research, *E. coli* HB101 or *E. coli* BL21 was used as the host strain. Because *E. coli* BL21 contains T7 polymerase upon IPTG induction, they were used for plasmid transformation. The *egfp* was used as the reporter system instead of 3,17β-*hsd*. The cells were cultured at 37°C and induced by IPTG (0.25 mM), which led to the overexpression of TetR via pET-T3. The results (given as RFU) reveal that *tetR* expression without IPTG already led to a slight decrease in *egfp* expression, which was compared to that with empty vector pET15b (Fig. 25). With the IPTG induction of *tetR*, however, a strong decrease in RFU expression was observed. The results strongly indicate that TetR has repressor activity for 3,17β-*hsd* expression, especially when the entire upstream regulatory region of 3,17β-*hsd* is present.

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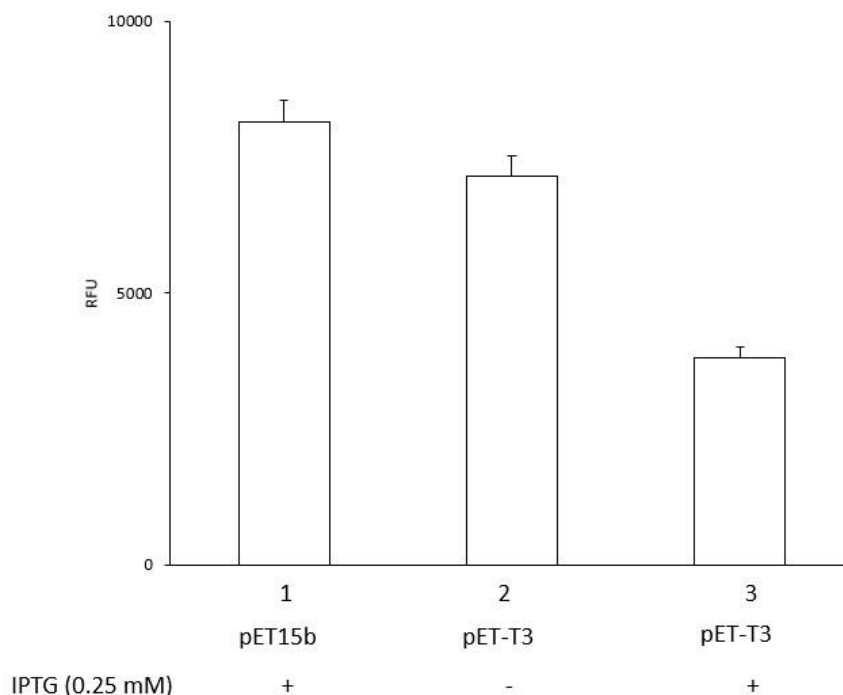


Fig. 25 TetR activity determination in *E. coli* BL21 with pK2.4-EGFP-4. pK2.4-EGFP-4 was cotransformed into *E. coli* BL21 with pET-T3 (containing *tetR*) or pET15b (control). *3,17 β -hsd* expression was quantified by RFU. IPTG was used to induce the expression of *tetR*.

In Fig. 25, the cells with pET15b do not contain *tetR* (column 1), and in column 2, pET-T3 contains *tetR*, but without IPTG induction, *tetR* in plasmid pETT3 was not expressed. In column 3, TetR is expressed at a high level. The RFU of column 3 was lower than that of column 1 and column 2. The result proved that TetR is a repressor of *3,17 β -hsd* expression.

According to an ELISA with TetR antibodies, *tetR* expression in *C. testosteroni* could not be induced by testosterone (data not shown).

Plasmid pK2.4-EGFP-4, which was constructed in previous experiments, was obtained by cloning the *egfp* into pK18 to yield plasmid pKEGFP-2 [123]. The 2,407 kb fragment upstream of *3,17 β -hsd* was then cloned into pKEGFP-2 to yield plasmid pK2.4-EGFP-4. The *egfp* was used here as a reporter gene instead of *3,17 β -hsd*. Plasmid pET15b and *E. coli* BL 21 cells are used to achieve stable and high levels of external *tetR* via T7 promoters. T7 promoters can produce high levels of transcription in *E. coli* when T7 RNAP is present, and they are not recognised by *E. coli*. RNAP makes T7 promoters capable of independent regulation from *E. coli* promoters. Plasmid pET15b is a T7 promoter vector. The T7 promoter must be recognised by the T7 phage RNA polymerase

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from the T7 phage and transcript. Therefore, when using the T7 promoter in the expression vector, the receptor bacteria that could produce the T7 phage RNA polymerase must be used as the host. *E. coli* BL21 has a gene encoding T7 RNA polymerase, so it can express T7 RNA polymerase, bind to the T7 promoter of the pET vector and start transcription (Fig. 26).

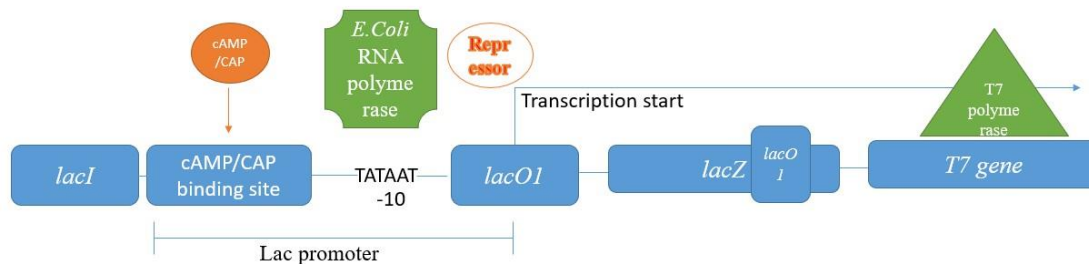


Fig. 26 Transcriptional control of T7 in BL21.

IPTG is a molecular biology reagent. This compound is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon, and it is therefore used to induce protein expression where the gene is under the control of the *lac* operon. Unlike allolactose, the sulphur atom creates a chemical bond that is non-hydrolysable by the cell, preventing the cell from metabolising or degrading the inducer. Moreover, IPTG uptake by *E. coli* can be independent of the action of lactose permease, since other transport pathways are also involved [124]. At low concentration, IPTG enters cells through lactose permease, but at high concentrations (typically used for protein induction), IPTG can enter the cells independently of lactose permease [125]. On the one hand, IPTG was able to induce *E. coli* BL21 to produce T7 RNA polymerase. On the other hand, the inhibition of the *lac* operon on the pET vector was inhibited by IPTG so that the *tetR* transcription could be smoothly controlled. The function of *tetR* could be detected via controlling the amount of *tetR* expression.

The transcription of T7 (encoding T7 RNA polymerase) in pET system expression hosts (BL21) is controlled by the *lac* promoter. T7 is transcribed as the second gene in bicistronic mRNA (the first gene contains an N-terminal fragment of *lacZ* that includes the α -peptide coding region). Positions of the three mutations of the wild-type *lac* promoter region are indicated by coloured circles. The lac repressor (*lacI* product) binds to *lacO1* and then interacts with pseudo-operons *lacO2* and *lacO3* to prevent transcription by *E. coli* RNA polymerase. The inducer IPTG binds to the repressor,

reducing its affinity for *lacOI* and thus enabling transcription to occur. When cAMP levels are sufficiently high (e.g. in the absence of glucose), the CAP/cAMP complex is formed and binds immediately upstream from the promoter to fully stimulate transcription. In the presence of glucose, CAP/cAMP is not formed and transcription is decreased.

4.5 Preparation of *tetR* knockout mutant

In order to understand the biological function of TetR, a *tetR* knockout mutant of *C. testosteroni* was prepared by the homologous integration method. Using chromosomal DNA of *C. testosteroni* as a template to prepare a reading frameshift mutation with primer *tetR-ML* by PCR, an additional ‘G’ was added after ‘TAG’ (Table 8). The 301 bp PCR fragment was cloned into plasmid pCR2.1-TOPO to yield pTOPOTM2. pTOPOTM2 was used to transform *C. testosteroni* cells. As shown in Fig. 27, after homologous integration, the *tetR* from the knockout mutant ‘TM’ was cut into two parts by pTOPOTM2. The resulting knockout mutant TM also contained the kanamycin resistance gene for further selection under antibiotic pressure. The successful knockout insertion of plasmid pTOPOTM2 was verified by 1.5% agarose gel (Fig. 28).

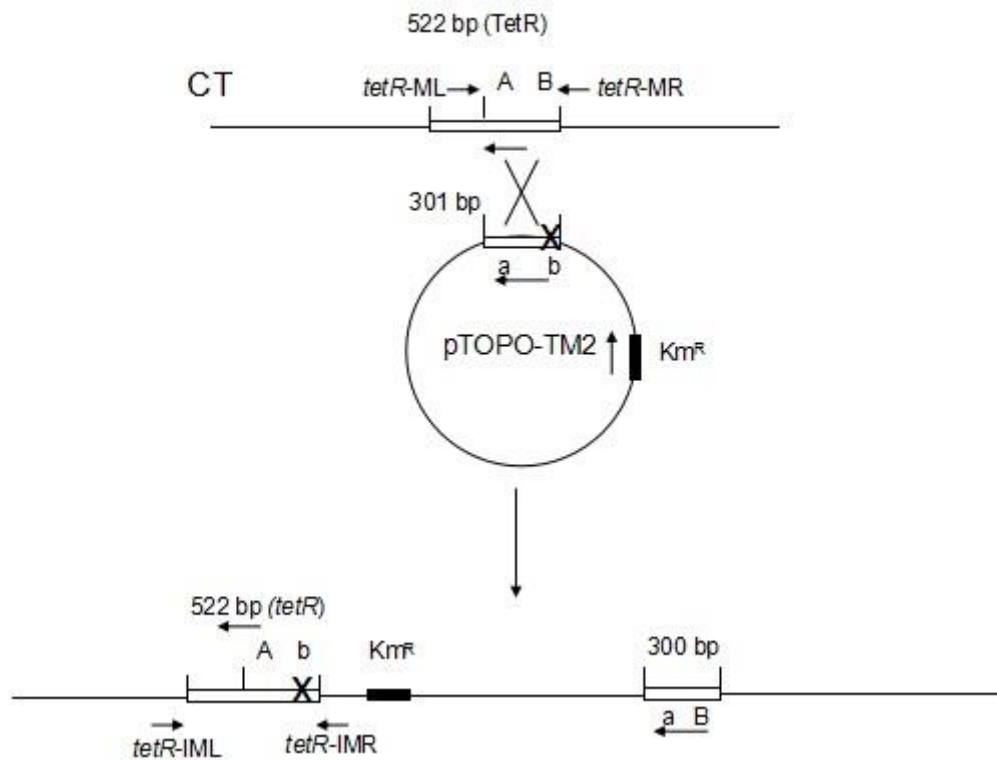


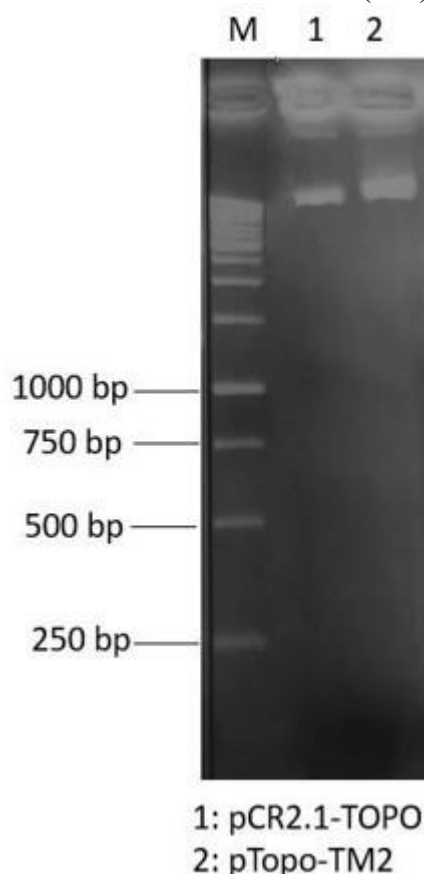
Fig. 27 Generation of a *tetR* knockout mutant (TM) of *C. testosteronei*.

Fig. 28 Plasmid pTOPOTM2 verification. For the verification of plasmid pTOPOTM2, pTOPOTM2 and pCR2.1-TOPO (as control) were run on the 1.5% agarose gel.

4.6 *3,17 β -hsd* and *3 α -hsd/cr* expression in *tetR* knockout mutants of *C. testosteronei*

According to the results obtained in this study with *E. coli* systems, TetR appears to be a repressor of *3,17 β -hsd* expression. With the *tetR* knockout system of *C. testosteronei* (Fig. 27), further evidence for this hypothesis should be provided. Since it was found in earlier studies [126] that the gene coding for *3 α -hsd/cr*, which is also a key enzyme involved in the degradation of steroids and xenobiotic carbonyl compounds, likewise undergoes complex regulation in *C. testosteronei*, it was suspected that TetR might regulate *3 α -hsd/cr* as well. Wild-type *C. testosteronei* and the knockout mutant (TM) were cultured in SIN medium with or without 0.5 mM testosterone. After overnight culture, *3,17 β -hsd* and *3 α -hsd/cr* expression was tested by ELISA (Fig. 29).

4. Results

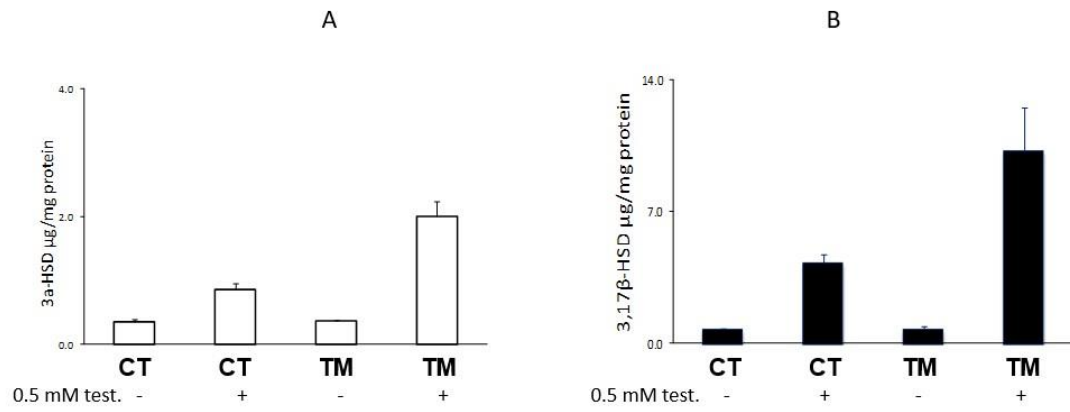


Fig. 29 Determination of TetR activity on expression of *3a-hsd/cr* and *3,17β-hsd*. Wild-type *C. testosteronei* and *tetR* knockout mutants (TM) were induced by testosterone expression. *3a-hsd/cr* (A) and *3,17β-hsd* (B) expression levels were detected by ELISA.

As shown in Fig. 29, both enzymes could be induced by testosterone in wild-type *C. testosteronei* as well as in the *tetR* knockout mutant TM. Interestingly, compared to wild-type *C. testosteronei*, the amounts of 3 α -HSD/CR(A) and 3,17 β -HSD (B) after testosterone induction were considerably higher in the *tetR* knockout mutant TM. The result supports the hypothesis that TetR is a repressor for both *3 α -hsd/cr* and *3,17 β -hsd* expression. Notably, without testosterone induction, *3,17 β -hsd* and *3 α -hsd/cr* were almost not expressed in both wild-type *C. testosteronei* and knockout mutant TM. This result reveals that TetR is a repressor, and its function can be resolved by testosterone. Moreover, it reveals that some other regulators are involved in the regulation of both *3,17 β -hsd* and *3 α -hsd/cr* expression in *C. testosteronei*.

4.7 Isolation and identification of LuxR positively regulates *3,17 β -hsd* expression

A new transcriptional factor, named LuxR for ‘luminescence regulation protein’, was identified in *C. testosteronei*, and supposed to be involved in *3,17 β -hsd* regulation. *luxR* is located 56.792 kb upstream of *3,17 β -hsd* with an orientation opposite to *3,17 β -hsd* on the *C. testosteronei* ATCC 11996 chromosome (Fig. 6). *luxR* was amplified from *C. testosteronei* chromosomal DNA by PCR (primers are shown in Table 8) and identified by sequencing (Fig. 30) and agarose gel electrophoresis (Data is not shown here). The open reading frame of *luxR* consists of 1,125 bp and is translated into a protein of 367 amino acids. It is a member of the LuxR family as revealed by sequence analyses. The location of *luxR* is contig 58, 140429-141548 (Bioinformatics analysis) of the *C. testosteronei* ATCC 11996 chromosome. The gene was then cloned into plasmid pCR2.1-TOPO to yield pTOPOluxR. The resulting 1,125 bp fragment was subcloned into plasmid pK18 to yield plasmid pKLuxR. In plasmid pKLuxR, the lac promoter of pK18 is located upstream of *luxR*, and the system served as a positive control for *luxR* expression and function (Fig. 30).

4. Results

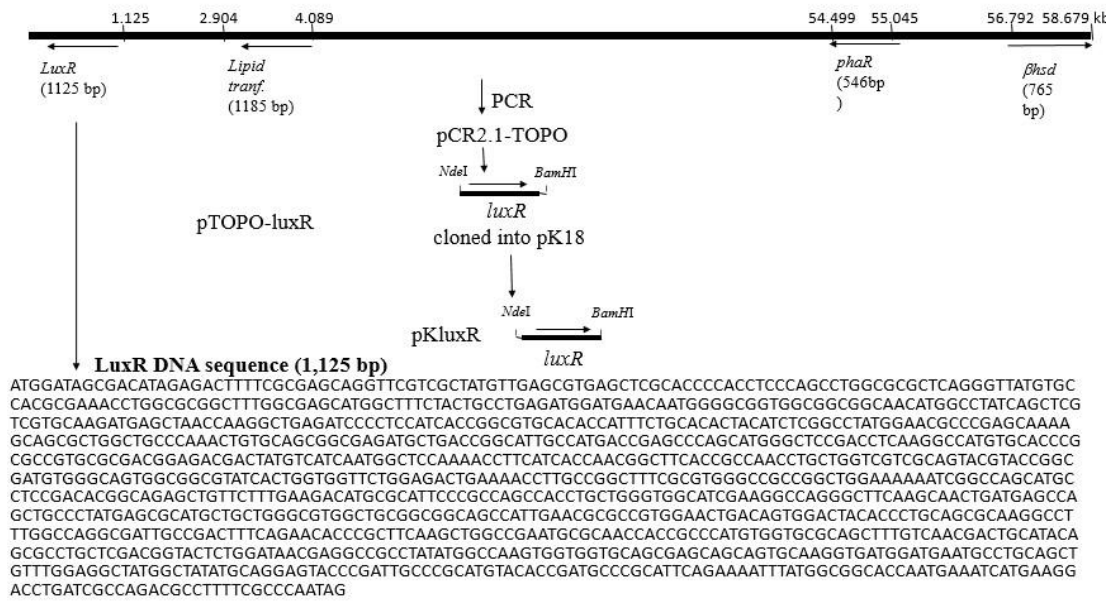


Fig. 30 The sequence of *luxR* (1,125bp). The *luxR* (1,125bp) was amplified by PCR and cloned into plasmid PCR2.1-TOPO to yield pTOPOluxR. The gene was further subcloned into pK18 to obtain pKLuxR.

4.8 Repression of *3,17β-hsd* expression by LuxR in *E. coli* system

To roughly characterise the LuxR cis-acting domain in the regulatory region of *3,17β-hsd*, three plasmids, pUC3.2-4, pUC1128-5 and pUC912-10, and the *E. coli* system were chosen. pKLuxR was cotransformed into *E. coli* strain HB101 with these three plasmids separately, and pK18 served as the control. The culture medium contained 100 µg/ml ampicillin and 30 µg/ml kanamycin if necessary. To avoid the effect of temperature, the cells were cultured at 27°C (Fig. 31) or 37°C (Fig. 32), respectively. Testosterone was also used to induce *3,17β-hsd* expression (Fig. 33).

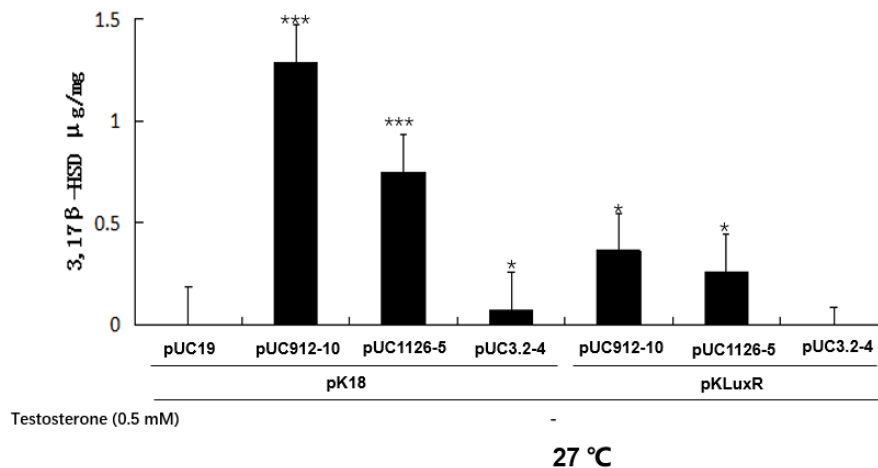


Fig. 31 LuxR activity determination in *E. coli* HB101 without testosterone at

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27°C. *3,17β-hsd* expression in *E. coli* HB101. Cells were cotransformed with plasmids pUC912-10, pUC1128-5 and pUC3.2-4 plus pK18 (control) or pKLuxR separately. The cells with pUC19 plus pK18 were also used as controls. ELISA results revealed that *luxR* is a repressor for *3,17β-hsd* expression in *C. testosteroni*.

At 27°C, without testosterone and pKLuxR, plasmid pUC912-10 (containing promoter 2), pUC1128-5 (containing promoter 1 and promoter 2 in close proximity) and pUC3.2-4 (containing promoter 1 and promoter 2, the whole sequence between them, *RS1*, *RS2* [two RSs] and the genes coding for *PhaR* and *RseB*) were cotransformed with pK18 (control) separately. The amount of *3,17β-hsd* protein was detected (Fig. 31). After combining pUC 912-10 and pK18, a large amount of *3,17β-hsd* fragments (up to 1.288 µg/mg protein) was expressed in the cells. On the other hand, the expression with pUC1128-5 and pK18 decreased by 42% in comparison with the expression with pUC 912-10, while that with pUC 3.2-4 decreased by nearly 95%, which suggested that promoter 1 had the main function of regulating the expression of *3,17β-hsd*. More notably, some structures between promoter 1 and promoter 2 were also needed.

Li [59] reported that a repressor, *PhaR*, is present in 3.182 kb fragments (Fig. 5). The *PhaR* activity needs *RS1* and *RS2*. pUC 912-10 contained one *RS2*, so *PhaR* could not bind to the DNA. In pUC 1128-5, a 1383 bp DNA fragment was deleted, and the DNA conformation was changed. Therefore, *3,17β-hsd* expression decreased (without *LuxR*).

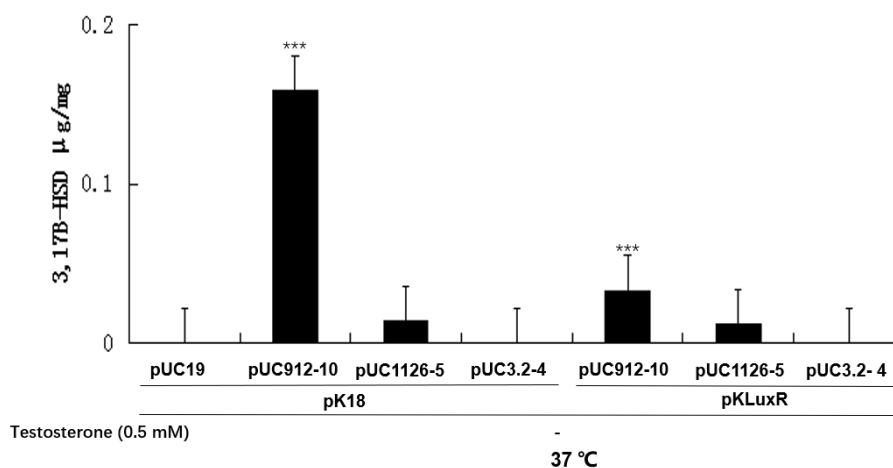


Fig. 32 *LuxR* activity determination without testosterone induction in *E. coli* HB101 at 37°C. Plasmids pUC912-10, pUC1128-5 and pUC3.2-4 were cotransformed into *E. coli* HB101 with either pKLuxR (containing *luxR*) or pK18 (control) at 37°C separately. *3,17β-hsd* was detected by ELISA.

As shown in Fig. 32, levels of *3,17β-hsd* expression at 37°C are much lower than those

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detected at 27°C, as Pruneda-Paz [102] reported previously. Comparing the *3,17β-hsd* expression without *luxR* (pK18 as control) in Fig. 31, it decreased from 0.159 μg/mg to 0.033 μg/mg in cells with the pUC 912-10 and pKLuxR combination. The expression was too low (lower than 0.015 μg/mg) to detect the change in the absence or presence of LuxR in the cells with pUC1128-5 or pUC3.2-4.

Thus, these results revealed that LuxR could inhibit *3,17β-hsd* expression in *E. coli* at 37°C, and promoter 2 may have the main function of regulating the expression of *3,17β-hsd*.

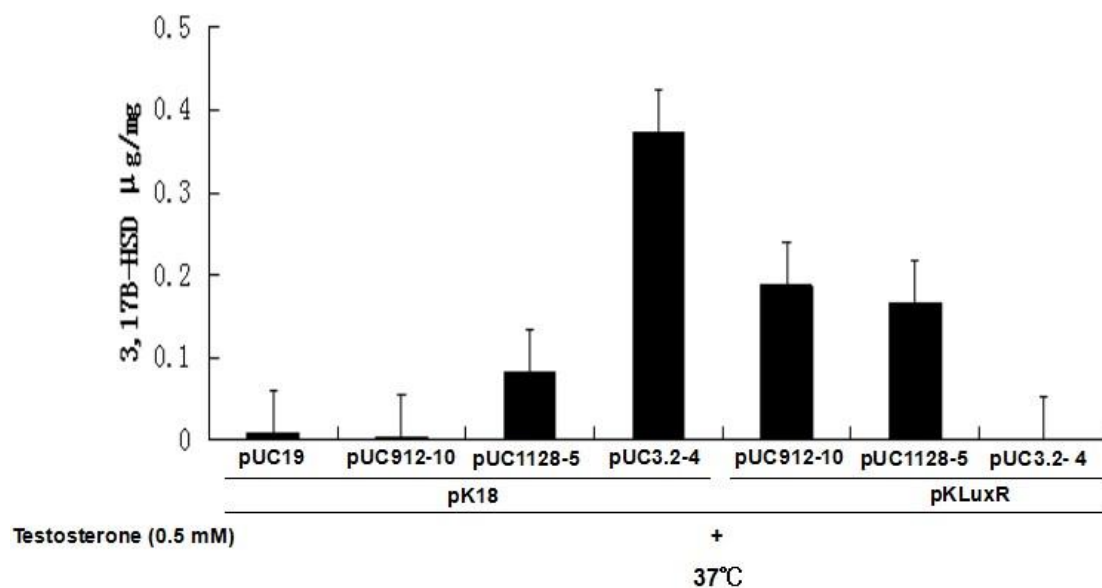


Fig. 33 LuxR activity determination with testosterone induction in *E. coli* HB101 at 37°C. Plasmids pUC912-10, pUC1128-5 and pUC3.2-4 were cotransformed into HB101 with either pKLuxR (containing the *luxR*) or pK18 (control) at 37°C separately.

For *3,17β-hsd* induction, 0.5 mM testosterone was used. *3,17β-hsd* was detected by ELISA. Interestingly, without *luxR*, *E. coli* cells expressed larger amounts of *3,17β-hsd* induction after testosterone. However, in the presence of LuxR, the *E. coli* cells containing pUC912-10 and pUC1128-5 expressed more *3,17β-hsd* than the cells without *luxR*. No induction could be found when the cells contained pKLuxR and pUC3.2-4 after induction. At 37°C, the LuxR protein might clearly bind to operator.

5. Discussion

Natural and synthetic steroid hormones are persistently released into the environment. They are regarded as contaminants that pollute diverse areas, such as soil and water, even at low concentrations. The continuous exposure of steroid hormones negatively influences the endocrine system of all kinds of animals [127]. In the 1990s, studies on detecting steroid contamination in different areas appeared [128]. The processing of hazardous environmental pollutants caused by steroid hormones has taken on great significance for researchers. Non-biological measuring methods of hormones in the environment include liquid chromatography coupled with fluorescence measurement or mass spectrometry (MS), high-pressure liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC/MS) [129,130]. However, as the sample preparation is time consuming, the concentrations are insufficient and expensive to detect hormones in the environment, these methods seem unsuitable [123].

Therefore, new methods for detecting and quantifying steroids that are cheap, easy to perform, and sufficiently sensitive in the environment. Offering a broader spectrum of different substances in one assay are urgently needed. Thus, exploring how to deal with these dangerous environmental pollutants in the field using biological methods is a new vision which is driving a great deal of research. The current research is focused on helping to understand microbial degradation by considering the biochemical, genetic and physiological aspects of these microorganisms [131,132]. The inducer of *3,17 β -hsd* might be used to prepare a new biosensor system in future.

Steroids can be degraded by certain bacterial species. However, only a few of them are isolated from seawater, cultured and investigated in terms of biochemical and molecular characterisation. *C. testosteroni* ATCC11996, which we used frequently in our previous studies [132-135], is a Gram-negative Proteobacteria found in soil and water. It is capable of using steroids or aromatic hydrocarbons as the sole carbon and energy source of growth, and it may be used for the bioremediation of contaminated areas.

5.1 Usage and advantage of TA cloning

TA cloning, performed using the Original TA Cloning Kit, is also known as rapid cloning. This method was used to clone *tetR*. Thus, the insert (*tetR* and primer *tetR*-EL, primer *tetR*-ER) is created by PCR using Taq DNA polymerase, and the Taq polymerase

functions simultaneously as a terminal ligase. As shown in Fig. 12, pCR2.1-TOPO was used as a T vector to perform the TOPO cloning, the rapid cloning of PCR products that combines topoisomerase with a T vector. It does not require the ligation of DNA ligase. TOPO TA cloning using topoisomerase's own properties can greatly increase the TA recombination rate by more than 95%. Thus, it could ensure that the PCR of the target gene, *tetR* fragment, was more accurate. This process is much simpler and faster than traditional subcloning, because restriction enzymes are not required except for linearised vectors. There is also no need to add restriction sites when designing primers, so shorter primers can be used. In addition, in the absence of a viable restriction site for traditional cloning, TA cloning is often used as an alternative.

5.2 3,17 β -*hsd* expression regulated by TetR

Tetracycline is an antibiotic used to treat a number of bacterial infections. Resistance to tetracycline in *E. coli* first emerged in the mid-1960s [136]. Protein factors that regulate and induce tetracycline resistance (e.g. TetR) were partially purified 10 years later [137]. *tetR* sequences and many molecular details about the regulation of tetracycline resistance were unveiled in the 1980s [138-142]. TetR family transcriptional regulators (TFTRs) are present in a large number of bacterial genomes with soil-dwelling bacteria encoding the highest numbers [106]. This family has been most completely characterised genetically and biochemically, while their specific gene regulatory function and the identity of their target genes are in some cases not completely understood [143,144]. More than 10,000 proteins are found in non-redundant protein databases, and most bacteria contain at least one TFR [145]. The regulation paradigm was first described in *E. coli*. TetR is the repressor of the tetracycline efflux pump encoded by *tetA* (Fig. 34). In the absence of tetracycline, a pair of TetR dimers binds to overlapping operator sequences in the intergenic region between the divergently transcribed *tetR* and *tetA*. When tetracycline is present, it binds directly to TetR, trapping it in a conformation that is incompatible with DNA binding [146]. This allows the transcription of both *tetR* and *tetA*.

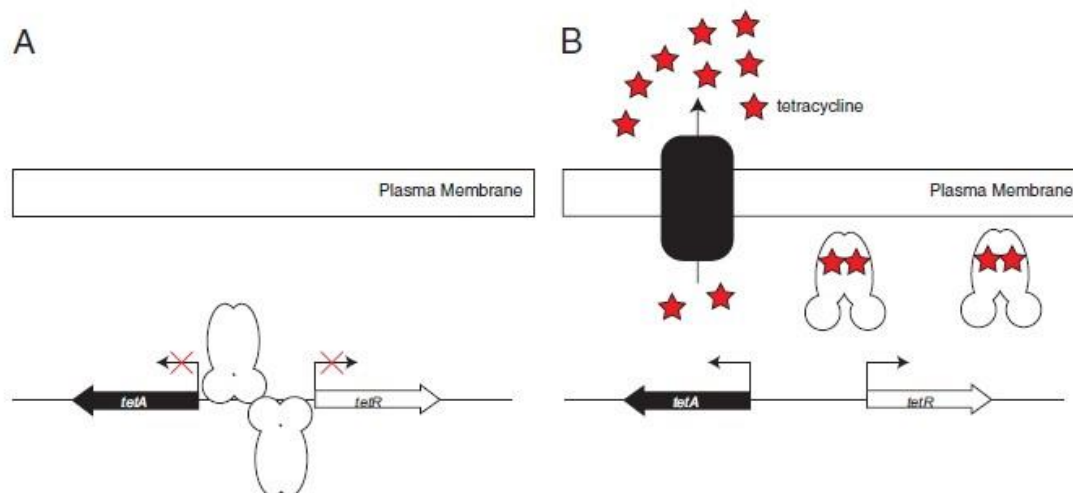


Fig. 34 TetR regulates expression of tetracycline resistance determinant encoded by *tetA* [147]. (A) In the absence of tetracycline, a pair of TetR dimers binds to repeated palindromic sequences in the intergenic region between the *tetR* gene and the *tetA* gene. (B) When present, tetracycline binds to TetR, causing a conformational change such that TetR can no longer bind DNA. This allows for the expression of the tetracycline efflux pump encoded by *tetA*.

The range of TFTR-controlled functions include carbon metabolism, nitrogen metabolism, co-factor metabolism, cell-to-cell signalling and cell division [147]. The genes coding for TFRs are often located near their target genes, and many family members are repressors that are de-repressed upon binding small-molecule ligands [44,122].

TFTRs that do not conform to the paradigm and act as activators [148,149], serve as global regulators [150], interact with peptide ligands [151] and even regulate enzyme activity post-translationally [152] are being described. TFRs regulate adaptive responses in most bacteria in that they permit the microorganisms to express transcripts of the genes (and proteins) only when they are required [152,153]. These observations clearly suggest that there is still much to be learned about this ubiquitous family.

The bacterium *C. testosteroni* can adapt to steroids as a sole source of carbon and energy which may be a causative factor for their wide distribution in nature, including soil, wastewater and even humans. Steroid hormones, which are excreted from animal and human sources and which are of environmental concern, may undergo bioremediation by *C. testosteroni*, thereby limiting their endocrine-disrupting potential [53]. Research on the steroid-degrading capability of *C. testosteroni* and their adaptation to steroid substrates on the molecular level is important and may lead to a targeted application of

these bacteria to clean steroid-contaminated soil or wastewater.

In the present dissertation, the focus was on the molecular regulation of *3,17 β -hsd* from *C. testosteroni*. *3,17 β -HSD* of *C. testosteroni* is an important enzyme in steroid decomposition [55-57,61,102,154]. More than five isoforms of *17 β -HSD* enzymes have been discovered, and they have been found in different hormone metabolism pathways. *3 β -HSD* is essential to androgen metabolism. *3,17 β -hsd* catalyses the oxidoreduction at carbon 3 and 17 of the steroid nucleus, which is the prerequisite for steroid breakdown. Importantly, *3,17 β -hsd* provides the adaptation of *C. testosteroni* to steroids as the sole carbon source, since it is only expressed in the presence of steroid substrates.

By a bioinformatics approach and using the genome sequence data from *C. testosteroni* ATCC11996 [12], a potential repressor gene, *tetR* was identified. It is located 3,319 bp downstream of *3,17 β -hsd* and its protein product, TetR, was hypothesized to be involved in *3,17 β -hsd* regulation. A phylogenetic analysis revealed close similarity to TetR proteins in other bacteria.

The *tetR* gene was amplified from *C. testosteroni* chromosomal DNA by PCR and sequencing. For characterisation of the TetR function, an *E. coli* system which did not contain the entire steroid induction system of *C. testosteroni* was used and the *tetR* was cloned into different plasmids, including an *egfp* reporter system. The TetR activity is dependent on the size and components of the upstream *3,17 β -hsd* regulatory region. In more detail, with the full upstream regulatory region of *3,17 β -hsd*, such as in plasmid pUC3.2-4, the *3,17 β -hsd*-repressing potential of TetR was indicated. Shorter promoter fragments, containing condensed spaces between both promoters and both RSs (pUC1126-5) or only promoter 2 and RS2 (pUC912-10), did not respond to TetR repression. With the *egfp* as the reporter system and the full upstream regulatory region of the *3,17 β -hsd* in plasmid pK2.4-EGFP-4, the same result was achieved. Moreover, when IPTG was added to the *E. coli* system to strongly induce TetR expression via the cotransformed plasmid pETT3, the clear repressor activity of TetR towards the entire *3,17 β -hsd* regulatory region was seen.

Previously, it was hypothesised that the two repeat sequences, RSs (RS1, RS2), upstream of promoter 1 and promoter 2, respectively, form a loop structure that is involved in *3,17 β -hsd* expression [59]. It is quite possible that TetR acts by binding to this region.

This is the supposition to explain that the effect of repressor can be found only in the presence of the entire *3,17 β -hsd* upstream regulatory region. The role of testosterone in this system remains obscure, perhaps because the *E. coli* strain does not contain other regulatory factors present in *C. testosteroni*.

According to some studies, temperature can also be an external environmental factor that determines which genes are turned on and off, thereby influencing the way an organism develops and functions [155-157]. This result was also found in *tetR* expression. Only at 27°C with pUC3.2-4, testosterone led to an induction of *3,17 β -hsd* in the presence of TetR. This is interesting, because 27°C is the suitable temperature for the growth of *C. testosteroni*, whereas *E. coli* grows well at 37°C. Some structures which are unstable with temperature maybe involved in the *3,17 β -hsd* regulation.

5.3 Expression in *tetR* knockout mutant

A gene knockout is a genetic technique in which one of an organism's genes is made inoperative ('knocked out' of the organism). With its sequence interrupted, the altered gene in most cases is translated into a non-functional protein, if it is translated at all. Therefore, a *tetR* knockout mutant of *C. testosteroni* was constructed to infer the TetR activity under more physiological conditions (*C. testosteroni*; 27°C). It turned out that the low basal levels of *3,17 β -hsd* expression did not change after the *tetR* had been knocked out. As expected, testosterone led to the induction of *3,17 β -hsd* in wild-type *C. testosteroni*. However, upon the addition of testosterone to *tetR* knockout mutants, *3,17 β -hsd* expression increased several-fold compared to the control and was even more than twofold higher than that in wild-type *C. testosteroni*. This clearly indicates that TetR acts as a negative regulator of *3,17 β -hsd* expression in *C. testosteroni*. Obviously, the repressor activity is regulated by testosterone. This is logical, because, as mentioned above, the TFRs provide bacteria the ability to adapt to changes in the environment (e.g. altered substrate sources).

5.4 Expression regulated by TetR

In previous studies, the complex molecular regulation of the *3a-hsd/cr*, which involves a variety of activator and repressor genes [126] had been elucidated. Interestingly, from the results, *3a-hsd/cr* expression can be induced by testosterone more than twofold, but in *tetR* knockout mutants, the expression can be induced more than fourfold. This result

proved that TetR can also regulate *3a-hsd/cr* expression, thereby controlling the metabolism of steroids with an A/B-ring structure or trans-ring fusion in *C. testosteronei* as well. It should be kept in mind that *3a-hsd/cr* expression is also regulated by, among others, a loop structure, where testosterone plays a key role [126].

In summary, the identification of TetR in the regulation of the steroid metabolism pathway in *C. testosteronei* reveals a new aspect of TFRs in the adaptive response to environmental challenges. Obviously, TetR regulates the expression of the key genes in steroid metabolism, *3,17 β -hsd* and *3a-hsd/cr*, via their upstream regulatory promoter regions and can therefore be considered an important factor in the steroid-dependent signalling network of *C. testosteronei*. The detailed connection between TetR and testosterone in the system of *3,17 β -hsd* regulation remains to be elucidated.

5.5 Expression regulated by LuxR

Several studies and the sequencing of many bacterial genomes have proved the presence of many *luxR*-type genes [76]. LuxR homologues are known to have a modular structure consisting of an *N*-terminal autoinducer-binding domain and a *C*-terminal HTH DNA-binding domain [77,158]. The roles of LuxR arise in bacteria, and some of them may be important players in-intra-species and inter-kingdom signalling. They could also be highly diverse in terms of the types of ligands they respond to as well as their mechanisms of target gene regulation [159]. By a bioinformatics approach and using the genome sequence data from *C. testosteronei* ATCC11996, *luxR* was suspected and found to be a potential regulator gene in *β -hsd* regulation, so it was amplified from *C. testosteronei* chromosomal DNA by PCR. As with TetR, LuxR activity is dependent on temperature and components of the upstream *3,17 β -hsd* regulatory region.

In more detail, *E. coli* containing pUC3.2-4 (containing two promoters and *3,17 β -hsd*, *PhaR*, *RseB*), pUC1128-5 (containing two promoters together and *3,17 β -hsd* DNA fragment) or pUC912-10 (containing promoter 2) was cultivated at 27°C separately, with pUC19 as the control. Without testosterone and pKLuxR, the *3,17 β -hsd* expression with pUC912-10 was the highest. In contrast with the value in *E. coli* with pUC1128-5 or pUC3.2-4, the expression showed a gradual declining trend. Interestingly, in both pUC1128-5 and pUC3.2-4, there were two promoters, while in pUC912-10, there was only one promoter. Therefore, there is perhaps some structure involved in *3,17 β -hsd*

regulation. As suspected, pUC3.2-4 may automatically form a loop structure between two promoters. There is a short sequence between pUC1128-5 and pUC3.2-4, so there may be an unstable loop structure between the two promoters. With pKLuxR, *3,17 β -hsd* expression with pUC912-10 decreased significantly, while the change in *3,17 β -hsd* expression with pUC1128-5 or pUC3.2-4 was too low to detect. Therefore, LuxR may bind to a sequence near promoter 2. Thus, through binding near promoter 2 and the loop structure, LuxR may be able to keep a stable DNA structure and regulate *3,17 β -hsd* expression.

Moreover, in the same way at 37°C, the expression was much lower than it was at 27°C. This phenomenon reported before is important, because the optimum temperature for *C. testosteroni* is 27°C, while that of *E. coli* is 37°C.

After obtaining the result without testosterone, the research proceeded further. *3,17 β -hsd* expression was investigated with 0.5 mM testosterone. *3,17 β -hsd* expression of pUC3.2-4 with pK18 could be induced by testosterone, because as previous report [59], testosterone could bind to the repressor located upstream of *3,17 β -hsd* to relieve its repressor function. Furthermore, the expression of pUC1128-5 or pUC912-10 with pKLuxR could be induced by testosterone. This result indicated indirectly that LuxR is a repressor for *3,17 β -hsd* and that promoter 2 sequence may be an important *3,17 β -hsd* regulatory domain.

5.6 Primer design to clone target gene

Primer design is a primary task for investigating genes and their regulation. A primer is a single strand of DNA (generally about 20 bases) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyse this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3' end of the primer and synthesis at the opposite strand.

For this dissertation, primers were designed, the primers were designed by using NCBI, Primer Premier 5.0. Manual monitoring showed the left primer for *tetR* as being 'CGCCATATGGCCCTGGCCCTGGTC' (24 bp) and the right primer as being 'CGCGGATCCTCAGGCTGTCGCCGTTGC' (27 bp). They were combined with the principles of primer design, such as keeping the primer 'GC' composition about 60% to

make the melting temperature close to 72°C. Suitable temperature and primer length can make sure that the TaqDNA polymerase could react. What is more, for improving the activity of enzyme restriction in future, before adding the restriction sites, an additional 'CGC' was added at the beginning of the primers [160].

5.7 Advantage of using low melting (LM) agarose

Low melting (LM) agarose is an agarose with a high resolution to DNA fragments, including PCR products. LM agarose can recover undamaged nucleic acid at denaturation temperature. The low gel temperature ensures the application of gel in remelting agarose and avoids the difficult extraction of DNA caused by the absence of *T4* DNA ligase inhibitor. LM agarose is an ideal method for agarose digestion. It is easy to recover DNA fragments suitable for cloning or enzymatic treatment, and even though it is more expensive and its visualisation in photos is worse than that of regular agarose, the target gene can be extracted from LM agarose but not from regular agarose.

Beyond that, 0.8% or 1.5% agarose gels were chosen as good separation or resolution of large 5–10 kb DNA fragments. *tetR* is included in this range and yielded clear results.

5.8 Application of plasmids and bacteria

In this study, to investigate the regulation of genes accurately, appropriate plasmids and bacteria was selected to transcribe and increase the target gene. A plasmid is a small-cycle DNA molecule that can autonomously replicate in the cell and is used for cloning, transferring and manipulating target genes. Often, the genes carried in plasmids provide bacteria with genetic advantages, such as antibiotic resistance. Considering plasmid compatibility and high copy numbers, pK18 and pUC 19 plasmids were used. The pK18 plasmid includes the kanamycin resistance gene, while the pUC 19 plasmid includes the ampicillin resistance gene. Since they use different replication systems, they are able to coexist in the same host. Thus, we used the pK18 plasmid to clone *tetR* and the pUC 19 plasmid to clone *3,17β-hsd* and then transferred them into the same *E. coli* HB101 cells.

In this research, *egfp* was cloned into the plasmid pKEGFP-2 instead of *3,17β-hsd* and named it pK2.4-EGFP-4. This plasmid was cotransformed with pET15b, one kind of popular bacterial expression system, into *E. coli* HB101 or BL21, two kinds of bacteria

commonly used in this type of study. It is worth noting that BL21 was used bacteria for pET systems. BL21 included the bacteriophage T7 promoter, which can control the transcription of the target gene by the lac operon contained in the pET system, while HB101 did not include the bacteriophage T7 promoter.

5.9 Global regulation of *3,17 β -hsd* expression

HSD is an essential enzyme in all mammalian steroid biosynthesis and forms an important prereceptor signalling pathway [161]. This constitutes the basis of a new concept for potential therapeutic value in the development of inhibitors in the treatment of diseases (e.g. obesity, diabetes and hormonal-dependent cancer) as well as in fertility control. Since most of these pharmacological targets are composed of SDR-type HSD, it is necessary to study the structure, gene-activity relationships and mechanisms of the SDR-type HSD family. *3,17 β -hsd* belongs to the group of HSD proteins, and investigating *3,17 β -hsd* regulation can help us to understand HSD protein actions. *3,17 β -HSD* is a redox enzyme that acts on the donor CH-OH group with NAD or NADP⁺ as the receptor. This enzyme catalyses the following enzymatic reactions:



3 or *17 β -HSD* can also act on the other *3 β* hydroxysteroids or *17 β* hydroxysteroids. *3,17 β -HSD* exhibits *3 β -* and *17 β -OH* dehydrogenase as well as keto reductase activities depending on the respective steroid. As Benach [162] showed, the probe-accessible surface of the *3,17 β -HSD* protein for the APO structure can reveal a deep cavity open to the bulk solvent and close to the catalytic triplet. The surface of the β -side is irregular, whereas two small subcavities occurring on the β -side of the steroid skeleton (at C10 and C13) accommodate the angular methyl group. These characteristics provide the structural basis for the enzyme to favour β -hydroxysteroids rather than α -hydroxysteroids. The specificity of *3 β -* and *17 β -hydroxysteroids* can be attributed to the overall elongated shape of the cavity. In addition, these clefts are suitable for steroids with ring A/B cis fusion or near planar trans-A/B ring structures (i.e. testosterone).

In addition, *3,17 β -hsd* mediated the activity of morphine dehydrogenase [163-165]. Previous research has shown that there are two operator sequences, promoter 1 and promoter 2, located upstream of *3,17 β -hsd*.

In this research, it is detected-that only whole *3,17 β -hsd* can be induced by testosterone, while the gene without promoter 1 or the sequence between the two promoters cannot. Normally, inducible genes contain the promoters for the binding of regulator proteins. To this operator domain(s), repressor(s) may bind and block target gene expression [166,167]. On the other hand, inducer molecules (e.g. testosterone) might form a complex with the repressor protein(s) to block repression and to promote target gene transcription. This is the classical mechanism of gene induction originally proposed by Monod in the operon model [123,168].

From the results, it concluded that there is some structure interfering with testosterone induction. There are two promoters in front of *3,17 β -hsd*. What is more, there are two RSs before *3,17 β -hsd*. Promoter 1 is between these two RSs. They are located at 1.383 kb and 2.409 kb upstream of *3,17 β -hsd*, respectively. While RS1 overlaps with RS2, a loop structure was suspected to be formed and involved in *3,17 β -hsd* expression.

RS1

ACGAAATCTGCTTCCCGAGCGGGCCAATG**GCCCGCTTTTTTGTGT**CTGTTGCT
GGCGTTTGCCTGCGCTAGTCCTAATTGACGATGCTTGCCTTGCCAACGGTTCGAACAATG
ATTCGTTGCTTCGCAATTGCGAGTCGGCAAGGAAGAAGAACGATAAGAGAAGTTCAAT
GACAGGTAGATGCATGGCAGATGCATCCAAGGAGACTAGGC (1383BP)

AAAATGAATGCGGCAAACAGCCTGTGGCGTACTTTGTGTGGCTGTGGGCTTTTGCCAT

RS2

TCAAGCAAGGAGGCGCTTGC**GCCTCCTTTTTTAAGT**GGCCGTAGCAATGCCCTG
TTCAGGGCTTGACAAAATCAAGAGGAGACAGAGG (2409 BP)

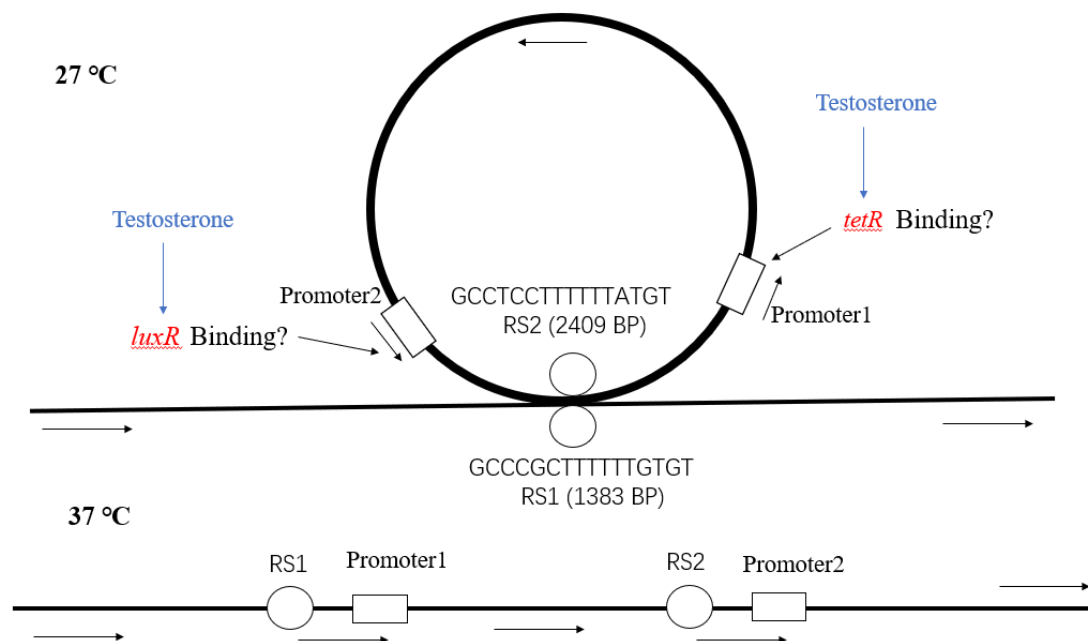


Fig. 35 Two RSs upstream of *3,17β-hsd* [59]. Two repeat sequences, RSs (13 bp; in bold letters in the upper panel; unfilled circles in the lower panel), were found upstream of *3,17β-hsd*. Between these RSs, there are 1661 bp, which may form a DNA loop structure (A) or represent two separated RSs (B) to which the repressor (e.g. *PhaR*) may bind.

5.10 Temperature effect

C. testosteroni ATCC11996 can survive at 27°C, while it cannot survive at 37°C. HB101, a chemically competent *E. coli* cell, is suitable for many molecular biology applications, including routine subcloning and cloning with limited amounts of DNA, and it can survive at both 27°C and 37°C. Therefore, *3,17β-hsd* can be expressed in HB101, but as the result, the whole expression quantity of *3,17β-hsd* fragments at 27°C was lower than that at 37°C. (Refer to Fig. 22 with plasmid pK18, in contrast to pUC3.2-4 [including whole *3,17β-hsd*] expression in HB101 at 37°C). The expression at 27°C increased weakly, while the expression of pUC912-10 (without the DNA sequence between the RSs and promoter 1) increased by almost 60%. This result concluded that there is some structure (i.e. loop structure) and/or-promoter 1 that is interfering with the ability of *C. testosteroni* to survive at 37°C. From the expression of plasmid pUC1126-5 (Fig. 23, only without the DNA sequence between the RSs), the situation is contrary to the expression of plasmid pUC3.2-4 and pUC912-10. That is, it declines by almost 60% at 27°C. Compared with the situation of pUC1126-5 and pUC3.2-4, the structure between the two RSs (i.e. loop structure) plays an important role in *3,17β-hsd*

expression at different temperatures. Comparing pUC1126-5 and pUC912-10, promoter 1 is more significant than promoter 2 in *3,17 β -hsd* expression for *tetR* regulation, while promoter 2 is more important than promoter 1 in *3,17 β -hsd* expression for *luxR* regulation. These suggestions are also in line with the results. Beyond these results, it is hypothesised that the loop structure, which plays an important role in the metabolic regulation of steroids in *C. testosteroni*, will be disturbed when the temperature is higher than that at 27°C.

5.11 Stability of testosterone concentration in SIN media

In this research, bacteria cells were cultured in SIN medium with or without 0.5 mM testosterone at 27°C or 37°C separately at 180 rpm overnight. To ensure the equilibrium property, 0.5 mM testosterone was added to the SIN medium before separating the medium with different cells. Moreover, it ensured that the solubility of testosterone in each SIN medium was the same by the low final concentration of testosterone. Xiong [169] proved that the determination of testosterone by a bioassay range obtained was 57–450 ng/ml (0.195–1.56 μ M). When testosterone was expended during the culturing or biological response, the surplus was diluted in the medium to balance the solution concentration.

5.12 Measurement and measurement units in the present research

In this work, the concentration of protein is given in [μ g/mg], which is often used in biological methods, as well as in many virus infections and endocrine diseases. For example, α -fetoprotein (AFP) determination is a common task to examine liver function. It is an auxiliary inspection tool used to assess liver cancer, maternal foetal health and so on. At present, an AFP value of more than 400 μ g/ml is one of the conditions for the diagnosis of liver cancer [170]. At present, the normal reference value of males is 15–200 μ g/L, and that of females is 12–150 μ g/L. Values outside this range may suggest iron deficiency anaemia, malnutrition, infection, cancer and other diseases [171].

Given the binding characteristics of proteins and the fact that protein concentrations are measured in micrograms, ELISA was chosen as the method to detect the target gene expression by the specific binding between antigens and antibodies. It has the advantages of high sensitivity and strong specificity.

The functional identifications of TetR and LuxR proteins in this thesis were published in *Chemico-Biological Interactions*. The article on TetR was named ‘Isolation and identification of a repressor TetR for *3,17β-hsd* expressional regulation in *C. testosteroni*’ and published in Jan. 2015 [172]. The article on LuxR was named ‘Functional analysis of a novel repressor LuxR in *C. testosteroni*’ and published in Oct. 2017 [173].

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Kiel, 2018

Tianyuan Pan

Erklärung

Erklärung

Eidesstattliche Erklärung:

Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation mit dem Titel **“Isolation and identification of two repressors, TetR and LuxR, for 3,17 β -*hsd* and 3 α -*hsd/cr* gene regulation in *Comamonas testosteroni*”** selbständig und ohne unerlaubte Hilfe angefertigt habe und dass ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt habe.

Kiel, 2018

Tianyuan Pan