

Aus dem Institut für Toxikologie und Pharmakologie für Naturwissenschaftler  
Universitätsklinikum Schleswig-Holstein Campus Kiel

**Identification of a New Marine Steroid-degrading  
Bacterium S19-1 and Isolation of Estradiol-inducible  
Genes and a Novel Promoter from this Bacterium**

Dissertation  
zur Erlangung des Doktorgrades  
der Agrar- und Ernährungswissenschaftlichen Fakultät  
der Christian-Albrechts-Universität zu Kiel

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Kiel, 2011

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Tag der mündlichen Prüfung: 09.02.2012

Gedruckt mit der Genehmigung der Agrar- und Ernährungswissenschaftlichen  
Fakultät der Christian-Albrechts-Universität zu Kiel

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## Summary

Environmental estrogens in water have been reported to be associated with abnormal sexual development and abnormal feminizing responses in some animals. Estrogen contamination of sea water is an ever growing problem and impacts population dynamics of all kinds of sea animals. Researches about elimination of estrogens from the contaminated environment have become a major issue in environmental research and policy. It has been demonstrated that biological processes play an important role in the removal of these stable compounds. Thus, 12 strains of steroid-degrading bacteria were isolated by our group from the Baltic Sea at Kiel, Germany in 2008.

In the present work, one of these steroid-degrading bacteria, strain S19-1, was identified. Strain S19-1 was characterized to be a Gram-negative bacterium. Phylogenetic analysis based on 16S rRNA sequence showed that strain S19-1 is closely related to the members of the genus *Buttiauxella* of the *Enterobacteriaceae* family. The optimal growth conditions of strain S19-1 require the presence of NaCl (2.1%) and a temperature of 20°C. High performance liquid chromatography (HPLC) results showed that 80% of estradiol could be degraded by S19-1 after 48 h incubation under these conditions. The improved growth of strain S19-1 was obtained by the presence of testosterone, estradiol or cholesterol in the minimal medium. Moreover, S19-1 was identified to be sensitive to antibiotics kanamycin, ampicillin, chloramphenicol, carbenicillin and streptomycin, but resistant against erythromycin. After transformation into strain S19-1, a series of plasmids including pK18 were found to be able to replicate in this bacterium.

Since S19-1 can degrade estradiol, there must be some genes involved in the degradation of estradiol in this bacterium. To analyze the estradiol degradation pathway in this marine isolate, fluorescence microplate assay (FMA) based on substrate-induced gene-expression was used to isolate genes involved in estradiol metabolism, in which chromosomal DNA of strain S19-1 was first digested with

restriction enzyme *SalI* and the resulting fragments were cloned into pKEGFP-2. *E. coli* cells harboring the recombinant plasmids were induced by 4 fmole estradiol and relative fluorescence units (RFU) of bacteria were measured. Compared to negative control pKEGFP-2, 37 plasmids out of meta-genomic library containing 323 plasmids could be induced by estradiol. Relative fluorescence units-increased amount percentage (RFU-IAP) of 6 plasmids among these 37 plasmids was about 6-7%, which is higher than the other 31 inducible plasmids (about 4-5%). So the insert fragments of these 6 plasmids were sequenced and then analyzed by BLAST search. Sequence analysis showed that the inserts of these plasmids comprise transcriptional regulators, substrate transporters, kinases and catabolic enzymes.

Not only catabolic enzymes and relevant regulatory elements, but inducible promoters could also be obtained by fluorescent microplate assay. Unexpectedly, bacteria containing one of those estradiol-inducible plasmids, plasmid p302, became green. This suggested that a strong promoter may exist in this fragment. Since a strong promoter from a new marine bacterium may be very useful in our future work, a series of deletion mutants of p302 were constructed to determine this new promoter. Finally, this promoter was identified to locate adjacent to the EGFP gene in the plasmid p302 and a 108-bp promoter sequence was obtained, the putative -10 region and -35 region of which were predicted.

## Zusammenfassung

Umweltöstrogene werden als Ursache einer abnormen Geschlechtsentwicklung im Sinne einer Verweiblichung gesehen, insbesondere bei aquatischen Lebewesen. Die Belastung des Meerwassers mit östrogen-wirksamen Verbindungen ist ein immer größer werdendes Problem, das die Populationsdynamik von Meerestieren aller Art beeinflusst. Die Erforschung von möglichen Eliminationswegen dieser Stoffe aus der Umwelt ist ein wesentlicher Gegenstand von Umweltforschung und politischem Handeln. Es wurde gezeigt, dass biologische Prozesse eine wichtige Rolle bei der Entfernung dieser stabilen Substanzen spielen. Daher wurden von unserem Arbeitskreis im Jahr 2008 Steroid-abbauende gram-negative Bakterienstämme aus der Ostsee bei Kiel (Deutschland) gesucht und 12 Stämme isoliert.

In dieser Arbeit wurde einer der Steroid-abbauenden Stämme, S19-1, bearbeitet. S19-1 wurde als gram-negatives Bakterium identifiziert. Eine phylogenetische Analyse der 16S-rRNA ergab, dass S19-1 in enger Verwandtschaft zur Gattung *Buttiauxella* aus der Familie der *Enterobakterien* steht. Optimale Wachstumsbedingungen erfordern eine Natriumchloridkonzentration von 2,1 % bei einer Temperatur von 20 °C. Mittels HPLC wurde ein 80%-iger Abbau von Estradiol durch S19-1 nach einer 48h dauernden Inkubation unter diesen Bedingungen detektiert. Verstärktes Wachstum wurde in Anwesenheit der Steroide Testosteron, Estradiol oder Cholesterol im Minimalmedium beobachtet. Weiterhin erwies sich S19-1 als sensitiv gegenüber den Antibiotika Kanamycin, Ampicillin, Chloramphenicol, Carbenicillin und Streptomycin, aber resistent gegen Erythromycin. Nach erfolgter Transformation zeigte sich, dass eine Reihe von Plasmiden, darunter pK18, in S19-1 repliziert werden.

Da S19-1 Estradiol abbauen kann, muss es die benötigten Proteine, bzw. die Gene, die diese kodieren, besitzen. Um den Abbauweg von Estradiol und die induzierten Gene

in diesem aus dem Meer isolierten Bakterium zu untersuchen, wurde ein auf Fluoreszenz durch substratinduzierte Genexpression basierender Mikroplatten-assay etabliert. Dazu wurde aus S19-1 chromosomale DNA mit dem Restriktionsenzym *SalI* verdaut und die resultierenden Fragmente in pKEGFP-2 kloniert. Mit den rekombinanten Plasmiden transformierte *E. coli* Zellen wurden mit 4 fM Estradiol inkubiert und die Fluoreszenz der Zellen gemessen (relative fluorescence units, RFU). Im Vergleich zur negativ-Kontrolle pKEGFP-2 zeigte sich bei 37 Plasmiden von insgesamt 323 eine Induktion durch Estradiol. Die Fluoreszenz-Zunahme betrug im Durchschnitt 4-5%, bei sechs Plasmiden wurde eine Zunahme von 6-7% beobachtet. Eine BLAST-Suche mit diesen sechs Konstrukten nach deren Sequenzierung ergab, dass es sich um Transkriptionsregulatorproteine, Transporter, Kinasen und katabole Enzyme handelte.

Nicht nur katabol wirkende Enzyme und Regulatorelemente, auch induzierbare Promotoren konnten durch diese Methode gefunden werden. Unerwarteterweise wies ein Zellpellet mit dem Estradiol-induzierbaren Plasmid p302 eine grüne Farbe auf. Dies ließ vermuten, dass ein sehr starker Promotor in diesem Fragment enthalten sein könnte. Da sich ein starker Promotor in einem neu identifizierten marinen Bakterienstamm für unsere weiteren Arbeiten als sehr nützlich erweisen könnte, wurde eine Anzahl von Deletionsmutanten von p302 zur weiteren Charakterisierung hergestellt. Letztendlich wurde der Promotor in der Nähe des EGFP-Gens lokalisiert und eine 108 bp große Sequenz mit putativer -10 und -35 Region identifiziert.

## General introduction

Steroid hormones consisting of progestagens, glucocorticoids, mineralocorticoids, androgens and estrogens are powerful signal molecules that regulate a host of organismal functions. Among them, estrogens are responsible for the development of female secondary sex characteristics. Because of their acceleration of metabolism, they are often used as additives of animal feed. In addition, estrogens are often used as contraceptives, especially synthetic estrogens. Thus, large amounts of estrogens are constantly excreted into the environment from humans and animals. When these stable compounds make their way into the environment they can cause endocrine disruption to living organisms in the aquatic environment. Since the elimination of environmental pollutants from the contaminated environment is an absolute requirement for sustainable development of biosphere, researches concerning detection of estrogen levels in the environment and degradation of these relatively inert substrates have become a worldwide topic for the last 30 years.

### 1. Biosynthesis and Chemical Characteristics of Estrogens

Estrogens are a group of steroid compounds named for their importance in the estrous cycle of humans and other animals. Three major naturally occurring estrogens are estrone (E1), estradiol (E2) and estriol (E3), which are synthesized from pregnenolone. The synthesis of estrone and estradiol in humans and animals is shown in Fig. 1 (Berg *et al.*, 2006). Cholesterol is the precursor of the five major classes of steroid hormones, which contain 21 or fewer carbon atoms but cholesterol contains 27. Thus, the first step of estrogens synthesis is the removal of a six-carbon unit from the side chain of cholesterol to yield pregnenolone. After oxidation of the 3-hydroxyl group to a keto group and the isomerization of the  $\Delta^5$  double bond to  $\Delta^4$  double bond of pregnenolone, progesterone is synthesized. Androgens and estrogens are synthesized from progesterone, which is transformed into androstenedione (an androgen) by hydroxylation at C-17 and the removal of the side chain consisting of C-20 and C-21.

Testosterone (another androgen) is formed by reduction of the 17-keto group of the 17-keto group of androstenedione. Estrone is derived from androstenedione, whereas estradiol is formed from testosterone by the loss of the C-19 angular methyl group and the formation of an aromatic ring.

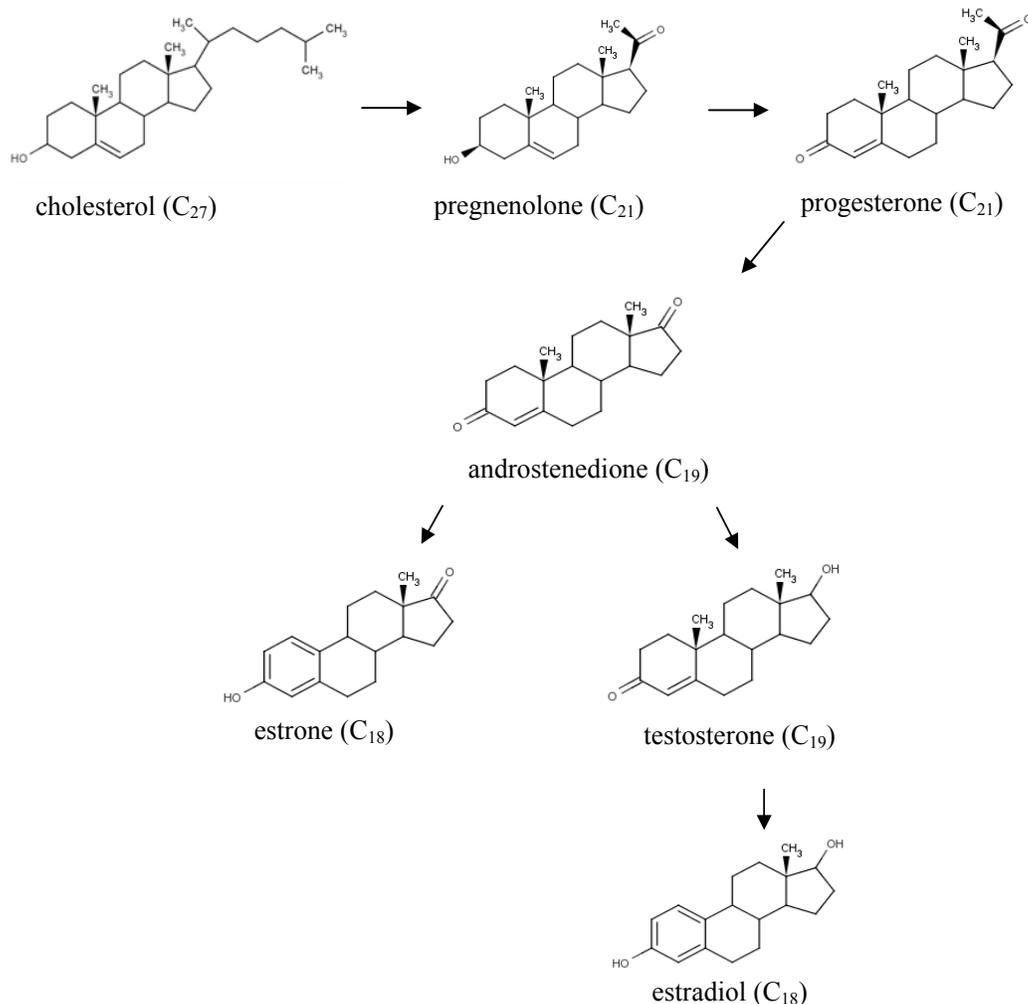


Fig. 1. Biosynthetic pathway of estrone and estradiol from cholesterol.

Estriol (E3) is produced in significant amounts during pregnancy. The human placenta produces pregnenolone from circulating cholesterol. In the fetal adrenal gland, pregnenolone is converted into dehydroepiandrosterone, a C-19 steroid, and then subsequently sulfonated to dehydroepiandrosterone sulfate which is converted to 16-OH dehydroepiandrosterone sulfate in the fetal liver. The placenta converts 16-OH

dehydroepiandrosterone sulfate to estriol. The structure of estriol is shown in Fig. 2. In humans and animals, estrogens are synthesized mainly in the ovary but also, in other organs such as the testis, brain, hypothalamus, adipose tissue, and placenta.

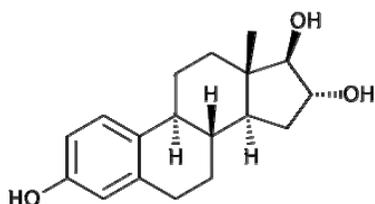


Fig. 2. Structural formula of estriol.

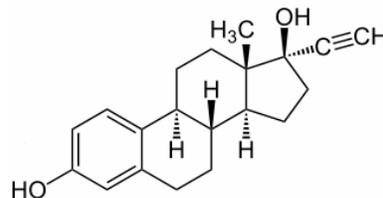


Fig. 3. Structural formula of 17 $\alpha$ -ethinylestradiol.

17 $\alpha$ -ethinylestradiol (EE2) is the first orally active semi-synthetic steroidal estrogen, which was synthesized in 1938 by Hans Herloff Inhoffen and Walter Hohlweg at Schering AG in Berlin and mainly used for contraception purpose (Djerassi, 2006). EE2 is released into the environment causing strong estrogenic effects as a xenoestrogen from the urine and feces of people who take it as a medication. The structure of EE2 is shown in Fig. 3.

The estrogen hormones are chemically very stable in the environment because of the aromatic ring. They are lipophilic and poorly soluble in water and can cause endocrine disruption in fish after exposure to them (Jobling *et al.*, 1998; Shore, 1993). The major concern of estrogens for environmental contamination is 17 $\beta$ -estradiol, which is considered about 50 times more potent than estrone and 6 times than estriol (Hernandez-Raquet, 2010). Although EE2 got the highest estrogenic activity, concentration of 17 $\beta$ -estradiol is much higher than EE2 in the environment. Thus, 17 $\beta$ -estradiol was chosen in the present research. Detailed characteristics of natural and synthetic estrogens are shown in Table 1.

Table 1 Properties of natural and synthetic estrogens (Hernandez-Raquet, 2010)

compound	molecular weight (g/mol)	water solubility (mg/L)	vapor pressure (mm Hg)	Log Kow	Log Kd	relative estrogenic activity
estrone (E1)	270.4	13	$2.3 \times 10^{-10}$	3.43	2.44-2.72	2.54
17 $\beta$ -estradiol (E2 $\beta$ )	272.4	13	$2.3 \times 10^{-10}$	3.94	2.68-2.83	100
17 $\alpha$ -estradiol (E2 $\alpha$ )	272.4	3.9	NA	3.57	NA	0.26
estriol (E3)	288.4	13	$6.7 \times 10^{-15}$	2.81	NA	17.6
17 $\alpha$ -ethinylestradiol (EE2)	296.4	4.8	$4.5 \times 10^{-11}$	4.15	2.65-2.86	246

NA: not available.

## 2. Origins of Estrogen Contamination in the Environment

Estrogens in the environment are derived from human beings and livestock. Estrogen excretion by humans varies as a function of their sex, physiological and developmental state, which most of estrogens were excreted from human via urine (95%) (D'Ascenzo *et al.*, 2003). It has been reported that a group of 73 women, which was selected to represent a typical cross section of the female inhabitants of a Roman condominium, excreted 106, 14 and 32  $\mu\text{g/day}$  of E3, E2 and E1, respectively (D'Ascenzo *et al.*, 2003). Another experiment showed that estrogen excreted from women at a reproductive age (15-59 years old) varies from 5-31  $\mu\text{g/day}$  for E1 and 3-19  $\mu\text{g/day}$  for E2. Except during these periods, a woman estrogen excretion is similar to that of a man, with levels about 4-12  $\mu\text{g/day}$  for E1 and 1.5-7  $\mu\text{g/day}$  for E2 (Hernandez-Raquet, 2010). For therapeutic purposes, the daily synthetic hormone intake (EE2) is around 20-60  $\mu\text{g}$  for contraception and about 10  $\mu\text{g}$  to control menopausal disorders; from such ingested dose, around 30-90% is excreted in urine and feces. The total amount of estrogens discharged by humans into the environment has been estimated at some 4.4 kg/year/million inhabitants (Hernandez-Raquet, 2010). Given the Earth's population this year (7.0 billion inhabitants), this amounts to 30.8 tons of estrogens/year.

The combined farm animal population in the world is considerably larger than the human being, implying a possibly important contribution to the environmental load of

steroid hormones entering water. Johnson reported comparisons on the amount of steroidal hormones produced by the different livestock, the information of which was gathered on the structure of the UK farm animal populations, and the amount of hormones excreted by animals at each of their life stage. The results showed that the combined farm animal population generates around four times more estrogens than the human population in the UK (Johnson *et al.*, 2006).

In livestock, the excretion of estrogens varies as a function of animal species, sex, age, circadian cycle, and reproductive state. Although a lot of researches had been done for obtaining the data, an accurate estimate of total estrogen input from animals into the environment is difficult to make, because data concerning estrogen levels in animal excretion are biased. Shore and Shemesh reported male chicken produced 14  $\mu\text{g}$  of estrogens/kg dry matter from slurries of chicken manure and 533  $\mu\text{g}$  for laying hens (Shore and Shemesh, 2003). Johnson reported 1.8 mg/day of estrogens for dairy cow (Johnson *et al.*, 2006). Lange reported 884, 242 and 48  $\mu\text{g}/\text{day}$  of estrogens for cattle, pigs and sheep (Lang *et al.*, 2002). In fact, most of the information derives from female and mature animals in relation to hormonal cycle for reproduction purposes, which does not represent the whole picture.

Information on the contribution of nondomestic animals to the environmental estrogen load was few reported. Shore and Shemsh reported fields in which wild turkeys were present for several months showed high levels of estradiol in soil (Shore and Shemesh, 2003). Water in fish aquariums reached equilibrium values ranging from 3.5 to 15 ng/L of 17 $\beta$ -estradiol, and fish ponds studied over a four-year period had equilibrium concentrations of 5 to 7 ng/L estrogens (Shore and Shemesh, 2003). In a pond with 150 wild birds, the concentration of estrogens was between 2-5 ng/L over a six-month period. Estrogens were also found in dry feces with concentrations between species from 100 ng/g in the elephant to 17  $\mu\text{g}/\text{g}$  in the musk ox (Shore and Shemesh, 2003).

### **3. Methods for Detecting Estrogens in the Environment**

Natural (estradiol, estrone, estriol) and synthetic estrogens (17 $\alpha$ -ethinylestradiol) are constantly excreted into the environment from human and animal sources through discharge from sewage treatment plants (STP) and manure disposal units. For the last years, several methods have been developed for detecting them in the environment. High pressure liquid chromatography (HPLC), liquid chromatography coupled with fluorescence measurement or mass spectrometry (MS), or gas chromatography coupled with mass spectrometry (GC/MS) are classical analytical techniques for estrogens based on structural information (Ying *et al.*, 2002; Rodriguez-Mozaz *et al.*, 2004). Due to some disadvantages of these methods in time consuming and complex sample preparation, other methods based on antibody and receptor assays have been developed these years, such as surface plasmon resonance sensing (SPR), electrochemical immunosensor, effect-oriented assays, the estrogen screen (E-Screen), yeast estrogen screen (YES), enzyme-linked receptor assay (ELRA), bipartite recombinant yeast system (BRYS), reflectometric interference spectroscopy (RIFS) (Jiang *et al.*, 2009; Soto *et al.*, 1995; Seifert *et al.*, 1998; Maser and Xiong, 2010).

In 2010, a new method, *Comamonas testosteroni* steroid sensor (COSS), for detecting steroids in the environment, was established by our group, which is based on a bacterial steroid-inducible gene *hsdA* encoding 3 $\alpha$ -hydroxysteroid dehydrogenase/carbonyl reductase (3 $\alpha$ -HSD/CR) and its regulatory region (Maser and Xiong, 2010). *C. testosteroni* ATCC 11996 is a Gram-negative bacterium which can use steroids as carbon and energy source (Möbus and Maser, 1998; Xiong and Maser, 2001). It has been demonstrated that 3 $\alpha$ -HSD/CR is one of the first enzymes in the degradation of steroid compounds in *C. testosteroni*, which catalyzes the interconversion of hydroxy- and oxo-groups at position 3 of the steroid ring structure (Maser *et al.*, 2000). The expression of 3 $\alpha$ -HSD/CR is only at basal levels in the absence of steroids, but can be significantly induced by steroids like testosterone which can bind to and inactivate two repressors (Xiong and Maser, 2001; Xiong *et al.*, 2003). After elucidating the complex regulation mechanism, the 3 $\alpha$ -HSD/CR gene *hsdA* was replaced by a green fluorescence protein (GFP) gene which could detect the fluorescence directly by

TECAN GENios Pro machine. Relative fluorescence units of bacteria harboring this steroid inducible fragment increased after incubation with estrogens for 30 min. This is the latest new bioassay for detecting estrogens from the environment. Except its high background, it has a lot of advantages, such as speed, easy to perform; cost-effectiveness; 96 well-plate high-throughput, sensitivity. Even 0.027 pg/mL estradiol could also be detected by this method (Maser and Xiong, 2010).

#### **4. Estrogen Concentrations Detected in the Environment**

##### **4.1. Estrogen Levels Detected in Sewage Treatment Plants (STP) and Animal Manure**

Sources of estrogen pollution are mainly from sewage effluent of human, domestic animals in the form of runoff from cattle pasture, fishpond effluent, fields fertilized with chicken manure and effluent from crops and barn. So concentrations of estrogens in urban sewage treatment plants and animal waste effluents from different countries were analyzed.

In raw sewage, the reported estrogen levels vary between a few and a hundred nanograms per liter depending on the source. For example, concentrations of estrogens from two STP in Germany were analyzed with 27-77 ng/L of E1, 12-19 ng/L of E2 and 2-10 ng/L of EE2 (Ternes *et al.*, 2002; Andersen H. *et al.*, 2003). Such high concentrations of estrogens could cause severe damage to human beings and animals. Normally, it would be treated by different methods before releasing into the environment. The classic removal procedures in an urban STP include primary treatment, biological secondary treatment and sludge handling (Joss *et al.*, 2004). During primary treatment, part of estrogens will be removed from the liquid phase by sorption to the organic solid fraction. Estrogens are mainly eliminated by biological secondary treatment. After treatment, an average of 88% of E2 and 74% of E1 would appear to be removed (Johnson *et al.*, 2000).

The concentration of estrogens in animal waste effluents is even much higher than

that in STP, which ranges from hundreds to thousands nanograms per liter. In slurries from dairy farms, values of about 600-1600  $\mu\text{g}$  estrogen/kg total solids have also been reported. And 800-1300  $\mu\text{g}$  of estrogen/kg dry matter (dm) manure were found from pregnant cows. Chicken manure contains up to 533  $\mu\text{g}$  estrogen/kg dm (Shore and Shemesh, 2003).

#### **4.2. Estrogen Levels Detected in Natural Environment**

It has been reported that large amounts of estrogens are released into the environment. Some of them stayed in the soil, some reached the oceans with river flows. In general, concentrations of estrogens in surface water were generally below 10 ng/L (Shore and Shemesh, 2003). In one study, 17 $\beta$ -estradiol was detected in 6 out of 117 samples in a concentration range between 0.8 and 29 ng/L with a median concentration of 1.7 ng/L. Estrone was found in 8 surface waters with a median concentration of 2.3 ng/L, and estriol was detectable in 7 samples with a median concentration of 3.0 ng/L (Shore and Shemesh, 2003). But an extensive reconnaissance study by the USGS (U. S. Geological Survey) reported very high levels of estrogens in 10-20% of the 139 stream studies. Specifically, the following maximum and median values in ng/L were obtained: estrone 117, 27; 17 $\beta$ -estradiol 93, 9; 17 $\alpha$ -estradiol 74, 30; estriol 51, 19 (Kolpin *et al.*, 2002). A maximum reported value of <0.5 ng/L of EE2 from drinking water in Germany was reported by Webb in 2003 (Webb *et al.*, 2003). Concentrations of estrogens from ocean sediments were measured by Braga, in which estrone was 0.16-1.17 ng/g, 17 $\beta$ -estradiol was 0.22-2.48 ng/g and 17 $\alpha$ -ethinnylestradiol was <0.05-0.5 ng/g (Braga *et al.*, 2005).

Springs and wells providing drinking water for human beings were also analyzed in different countries. The usual levels are under 1 ng/L. In an old survey of 64 wells in southern Germany in 1977, levels of <0.1-0.9 ng estradiol/L with an average of 0.2 ng/L were found (Shore and Shemesh, 2003). In a survey of wells receiving contaminated water, it was found that six wells receiving water from rocky strata had detectable estrogen in the range of 1.7-5 ng/L. However, estrogens were undetectable

(<0.5 ng/L) in six wells that received water filtered through sandy strata (Shore and Shemesh, 2003). Detailed information of estrogen concentrations from different places in different countries is summarized below (Table 2).

Table 2 Concentrations (ng/L) of estrogens in surface water

country	E1	E2	E3	EE2	ES	References
Coastal/estuarine and rivers The Netherlands	0.1-3.4	0.3-5.5	NM	0.1-4.3	NM	Belfroid <i>et al.</i> , 1999
Scheldt estuary, Belgium	0.37-10	<LOD*	NM	<LOD*	NM	Noppe <i>et al.</i> , 2007
Baltic Sea, Germany	0.1-0.53	<LOD*	NM	<LOD-17.2*	NM	Beck <i>et al.</i> , 2005
Danube, Nau, Blau Rivers, Germany	0.7	1.2	NM	0.8	NM	Kuch <i>et al.</i> , 2001
Paris seven Rivers, Fance	0.8-3.9	0.8-3.6	NM	0.6-3.5	NM	Cargouet <i>et al.</i> , 2004
Jalle D'Eysines Rivers, France	<LOD-30*	<LOD-1.5*	NM	<LOD-2	NM	Labadie, 2005
Yundang Lagoon, China	<LOD-5.34*	<LOD-1.56*	NM	<LOD-0.43*	NM	Zhang <i>et al.</i> , 2011
Marine waters in HongKong, China	NM	0.72-4.18	NM	NM	NM	Gracia <i>et al.</i> , 2008
Marine waters in Shenzhen, China	NM	NM	NM	NM	0.1-18	Lee <i>et al.</i> , 2006
Dasha river, China	NM	NM	NM	NM	50-400	Lee <i>et al.</i> , 2006
Yongding New River, China	0.64-20.2	<LOD-13.6*	NM	<LOD-12.0*	NM	Lei <i>et al.</i> , 2009
Tama River, Japan	8.7-19.7	4.2-5.3	NM	<LOD*	NM	Kawaguchi <i>et al.</i> , 2004
Kanda River, Japan	NM	32	5.5	NM	NM	Majima <i>et al.</i> , 2002
Tiber River, Italy	1.5	0.11	NM	0.04	NM	Baronti <i>et al.</i> , 2000
Lower Jordan River, Israel	1.2-3.2	4.3-9.4	0.5->40	0.7->40	NM	Cohen <i>et al.</i> , 2006
Elkhorn River, USA	0.9-8.3	0.084-3.2	NM	NM	NM	Soto <i>et al.</i> , 2004
Sado river, Portugal	10	18	NM	18	NM	Ribeiro <i>et al.</i> , 2009a
Mondego river, Portugal	<5.0	<3.0	NM	<12.0	NM	Ribeiro <i>et al.</i> , 2009b

\* NM, not measured; LOD, limit of quantification; ES, total concentrations of estrogens.

## 5. Possible Impacts of Estrogens to Living Organisms on the Earth

Substantial quantities of estrogens are constantly excreted into the environment from human beings and animals. They can interact with physiological systems and cause alterations in development, growth and reproduction of living organisms, such as fish, molluscs, barnacle, even plants. Since fishes are the most common species inhabiting waters and they are very easy to be affected by estrogens in the aquatic environment, the negative effect targeting to fishes has attracted considerable attention of the public. The effect to fish ranges from subtle changes in the physiology and sexual behavior to permanently altered sexual differentiation and impairment of fertility. Estrogen contamination in the environment may also represent a human health risk, such as

increasing incidence of cancer in hormonally sensitive tissues to women, precocious puberty to children and a decreasing reproductive fitness of men (Daston *et al.*, 1997; Juberg, 2000; Partsch and Sippell, 2001).

### **5.1. Endocrine Disruption of Fish Caused by Exposure to Estrogens**

Estrogen contamination often causes developmental problems of fish in an aquatic environment, such as intersex. Experiments with rainbow trout, which was placed in cages in the effluent from sewage treatment works, were conducted by Purdom (Purdom *et al.*, 1994). Concentrations of vitellogenin in the plasma of the male fish, which is a protein synthesized by the liver of oviparous fish in response to estradiol stimulation and then form the yolk, were measured after one to three weeks exposure. Results obtained from fifteen sewage treatment works, which were distributed throughout England, showed that vitellogenin concentration in the plasma increased 500-100,000 fold depending on site. Laboratory studies on the potency of EE2 demonstrated that levels as low as 1 to 10 ng/L could generate the response shown by the caged fish and that positive responses may arise at 0.1 to 0.5 ng/L (Purdom *et al.*, 1994).

Zebrafish was also exposed to 17 $\alpha$ -ethinylestradiol for observing development effects by Andersen (Andersen L. *et al.*, 2003). Results showed that all exposure periods significantly induced vitellogenin synthesis and affected the sex differentiation leading to development of ovo-testis or complete feminization of the exposed fish depending on exposure period. Complete sex reversal was obtained in groups exposed from 20 to 60 days post hatch (Andersen L. *et al.*, 2003).

Scholz and Gutzeit reported that juvenile male medaka (*Oryzias latipes*) were sex reversed and had developed an ovary after exposure to 100 ng/L 17 $\alpha$ -ethinylestradiol. Reduced production of eggs was reflected in a significantly reduced gonadal weight at 10 ng/L 17 $\alpha$ -ethinylestradiol in females. Aromatase in gonads, which is normally only expressed in ovaries, was detectable in testis of males exposed to 10 ng/L

17 $\alpha$ -ethinylestradiol (Scholz and Gutzeit, 2000). Nash reported that life-long exposure of breeding populations (n=12) of zebrafish (*Danio rerio*) over multiple generations in the F1 generation to 5 ng/L of environmental EE2 caused a 56% reduction in fecundity and complete population failure with no fertilization (Nash *et al.*, 2004).

A total of 60-100 adult roach was collected from each location of 8 river sites in UK by Jobling for sexual disruption research in wild fish. They found that all of the fish collected from these sites appeared to be either male or female. However, histological examination of the gonads revealed that a surprisingly large proportion of the males were in fact intersex at all sites. The proportion of intersex males at downstream sites from sewage treatment works ranged from 16% to 100%; at upstream sites ranged from 11.7% to 44.4%; and at the control sites ranged from 4% to 18.1%. This result suggested that the incidence of intersexuality in roach from the rivers in U.K. is considerably higher than expected (Jobling *et al.*, 1998).

In 2002, an assessment of the gonad of male fish in three species carried out in coastal waters in the Baltic Sea, Germany, was performed by Gercken and Sordyl (Gercken and Sordyl, 2002). Histopathology of the testis revealed the presence of intersexuality in specimens of all three species, where the intersex condition was defined by the simultaneous presence of primary oocytes within the apparently normal testis tissue (Gercken and Sordyl, 2002). In 2003 and 2004, surface water samples were collected at five locations in the Baltic Sea in Germany by Beck. The estradiol equivalents of these samples were conducted by screening for estrogenic activity method. Results showed that estradiol equivalents were in the range of 0.01 to 0.82 ng/L (Beck *et al.*, 2005). Later then, the main contributors to the overall estrogenic activity were determined by liquid chromatography-tandem mass spectrometry analysis, which turned out to be mainly synthetic and natural estrogens (Beck *et al.*, 2006).

Kidd reported that the concentrations of estrogens observed in freshwater can impact

the sustainability of wild fish population (Kidd *et al.*, 2007). A 7-year, whole-lake experiment at the experimental lakes area in northwestern, Canada, was conducted and showed that chronic exposure of fathead minnow (*Pimephales promelas*) to low concentrations (5-6 ng/L) of EE2 led to feminization of males through the production of vitellogenin, impacts on gonadal development as evidenced by intersex in males and altered oogenesis in females, and ultimately, a near extinction of this species from the lake. Overall, the concentrations of estrogens observed in freshwaters can impact the sustainability of wild fish populations (Kidd *et al.*, 2007).

### **5.2. DNA Damage Caused by Exposure to 17 $\beta$ -estradiol**

An experiment was conducted by Atienzar to evaluate the effects of 17 $\beta$ -estradiol on the genomic DNA of barnacle larvae by using the random amplified polymorphic DNA assay (Atienzar *et al.*, 2002). The results showed that DNA extracted from larval barnacles exposed to 17 $\beta$ -estradiol produced random amplified polymorphic DNA profiles that differed from the control patterns. 62.5% of all primers used in that study showed obvious changes in random amplified polymorphic DNA profiles when compared to the control pattern (Atienzar *et al.*, 2002).

### **5.3. Impacts of Growth and Development of Plants and Sex Determination of Flowers by Estrogens**

Geuns reported that estrone could stimulate the growth of isolated pea embryos grown on a solid culture medium containing salts and 4% sucrose. The root growth was enhanced by 30, 46, and 66% after 1, 2, and 3 weeks, respectively. 17 $\beta$ -estradiol could induce flowering in the plant *Salvia splendens*. Flower buds of *Melandrium dioecum* could also be induced to develop in a female by applying estrogens to the roots before the flowering (Geuns, 1978).

## **6. Methods for Removing Estrogens from the Environment**

Since estrogens can cause endocrine disruption of living organisms, especially to the aquatic organisms when they make their way into the environment, it's very important

to clean up these stable pollutants from the contaminated environment. Until now, there are several ways including physical-chemical interaction and bioremediation that are used to remove the environmental estrogens. Since the vapor pressure of estrogens is very low (as mentioned in Table 1), estrogen elimination via volatilization is likely to be negligible under natural conditions of temperature and pressure (Shore and Shemesh, 2003).

But estrogens are susceptible to photodegradation, because estrogens show maximum absorbance peaks in the ultraviolet area of the electromagnetic spectrum (Coleman *et al.*, 2004). Photodegradation can potentially occur both in the presence of the ultraviolet portion of solar radiation and ultraviolet radiation used in wastewater treatment plants and half-lives were reported in the order of 10 days for 17 $\beta$ -estradiol under ideal conditions (Liu and Liu, 2004). Irmak reported decomposition and complete degradation of 17 $\beta$ -estradiol in aqueous medium by using ozone (O<sub>3</sub>) only and ozone/UV advanced oxidation techniques (Irmak *et al.*, 2005). The results showed that the time needed for conversion of E2 (0.1 mmol) were 55, 75, and 90 min for the applied ozone dosages of  $15.78 \times 10^{-3}$ ,  $12.25 \times 10^{-3}$ , and  $9.78 \times 10^{-3}$  mmol/min, respectively. Roughly equal amounts of ozone ( $0.889 \pm 0.027$  mmol) are consumed for the conversion of 0.1 mmol of 17 $\beta$ -estradiol. And the O<sub>3</sub>/E2 ratio for oxidation of E2 by that process is 8.89. For applied ozone/UV advanced oxidation techniques, O<sub>3</sub> consumption was decreased by 22.5% in converting the same amount of E2 and also the time to convert the same amount of E2 was considerably decreased (Irmak *et al.*, 2005).

Another most important way for the complete removal of estrogens from the environment is biodegradation which is conducted by bacteria under aerobic and anaerobic conditions and by algae. The ability to degrade E2 with the production of E1 as the main metabolite has been observed in algal cultures of *Chlorella vulgaris* incubated in both light and dark conditions (Lai *et al.*, 2002). According to Lai's reports, in the light, 50% estradiol was further metabolized to an unknown product.

The concentration of estradiol in the culture medium fell rapidly over the first 3 h of incubation, and this decline was more pronounced in the light than the dark. However, the subsequent change in the estradiol concentration was more rapid in the dark and was accompanied by an increase in the concentration of estrone in the medium solution (Lai *et al.*, 2002). This indicates that the alga is able to interconvert estrone and estradiol. But *Chlorella vulgaris* prefers to convert estradiol into estrone. Under light conditions, about 50% of estradiol was metabolized into an unknown product and some of the estradiol was transformed to estrone, while under dark conditions biotransformation to estrone which accumulated in *Chlorella vulgaris* was the major pathway (Lai *et al.*, 2002).

Some experiments on biodegradation of 17 $\beta$ -estradiol (E2) and 17 $\alpha$ -ethinylestradiol (EE2) under anaerobic conditions were performed by Czajka and Londry (Czajka and Londry, 2006). Cultures were established using lake water and sediment under methanogenic, sulfate-, iron-, and nitrate-reducing conditions. Results showed that EE2 could not be degraded under anaerobic conditions, while E2 was transformed to estrone (E1). The oxidation of E2 to E1 was not inhibited by E1. Under some conditions, reversible interconversion of E2 and E1 was also observed in alga *Chlorella vulgaris*. Although E2 could be readily transformed to E1 under anaerobic conditions, the complete degradation of estrogens under these conditions was minimal, suggesting that they would accumulate in anaerobic environments (Czajka and Londry, 2006).

### **7. Bacteria can Degrade Estrogens under Aerobic Conditions**

Estrogens of human and animal are being delivered into the environment over thousands of years, especially to an increasing extent due to growing population and more intensive farming. Some organisms have acquired pathways to make use of these compounds as growth substrates. Since soils polluted by animal manure and STP for collecting urban wastewater contain most of animal estrogens, so bacteria which can degrade estrogens were mainly isolated from those places.

Bacteria which could degrade estrogens were successfully isolated from different origins, such as activated sludge, aquifer sand, manure and freshwater. Early in 1965, Stumm-Zollinger and Fair reported that microorganisms, which can use estrogens as their sole source of carbon, were found in soils and in wastewaters (Stumm-Zollinger and Faire, 1965). In 2002, a  $17\beta$ -estradiol degrading bacterium *Novoslingobium* was isolated from activated sludge in a sewage treatment plant in Japan (Fujii *et al.*, 2002). Later then, four strains of *Rhodococcus* which specifically degrade estrogens include E1, E2, E3 and EE2 were successfully isolated by using enrichment culture of activated sludge from wastewater treatment plants. They could completely and rapidly degrade estrogens (Yoshimoto *et al.*, 2004). A denitrifying bacterium *Denitratisoma oestradiolicum* AcBE2-1 was also isolated from activated sludge of a municipal wastewater treatment plant using  $17\beta$ -estradiol as sole source of carbon and energy. E2 was oxidized completely to carbon dioxide and water in this strain by reduction of nitrate to a mixture of dinitrogen monoxide and dinitrogen, with the intermediate accumulation of nitrite (Fahrbach *et al.*, 2006). In China, a  $17\alpha$ -ethinylestradiol degrading bacterium *Sphingobacterium* sp. JCR5 was isolated by Ren from the activated sludge of the wastewater treatment plant of an oral contraceptives producing factory. This bacterium could metabolize up to 87% of EE2 added (30 mg/L) within 10 d at 30°C, as well as other estrogens like E1, E2 and E3 (Ren *et al.*, 2007).

Since estrogen excretion into the ocean is a severe environmental issue, it's urgent to obtain marine bacterial isolates for bioremediation of estrogens in the ocean. In 2008, a total of six samples of sea water were collected by our group from different places of the Baltic Sea around Kiel city, which were the suspected sites of steroid contaminated water from the city. 12 steroid-degrading strains of bacteria were successfully isolated (Xiong *et al.*, 2009), including strains S19-1 and H5. Bacteria involved in estrogen degradation belong to different genera of varied classes including  $\alpha$ -Proteobacteria,  $\beta$ -Proteobacteria,  $\gamma$ -Proteobacteria and *Actinobacter*. Some bacteria could degrade all of the estrogens, but some only could degrade one or

two estrogens. Detailed information of some bacteria which can degrade estrogens is listed in Table 3.

Although so many estrogen degrading bacteria exist in the environment, the removal efficiency remains low. Sometimes we need to add nutrients into the aquatic environment to help microorganisms grow better to degrade estrogens more efficient, especially used in STP or some severe polluted places (Watanabe, 2001). The degradation ability of these estrogen-degrading bacteria could also be improved by genomic change, such as constructing mutant strains by incorporating activators to enhance the expression some key enzymes which are in charge of estrogen degradation. This is also the problem what we are trying to solve.

Table 3 Bacteria involved in the degradation of estrogens

Bacteria	Classification	Compounds degraded	Origin	Reference
<i>Novosphingobium</i> ARI-1	$\alpha$ -proteobacteria Sphingomonadales	E1,E2,EE2	Activated sludge	Fujii <i>et al.</i> , 2002
<i>Aminobacter</i> sp. C4	$\alpha$ -proteobacteria Rhizobiales	E1,E2	Activated sludge	Yu <i>et al.</i> , 2007
<i>Phyllobacterium myrsinacearum</i>	$\alpha$ -proteobacteria Rhizobiales	E1,E2,E3, EE2	NA	Pauwels <i>et al.</i> , 2008
<i>Nitrosomonas europaeae</i> NCIMB	$\beta$ -proteobacteria	E1,E2,EE2	Activated sludge	Shi <i>et al.</i> , 2004
<i>Ralstonia picketti</i>	$\beta$ -proteobacteria Burkholderiales	E1,E2	Activated sludge mixed culture	Weber <i>et al.</i> , 2005
<i>Buttiauxella</i> S19-1	$\gamma$ -proteobacteria Enterobacteriales	E2	Sea water	Zhang T. <i>et al.</i> , 2010
<i>Vibrio</i> H5	$\gamma$ -proteobacteria Enterobacteriales	E2	Sea water	Sang <i>et al.</i> , 2011
<i>Pseudomonas</i> sp.	$\gamma$ -proteobacteria Pseudomonadales	E1,E2,E3, EE2	Activated sludge	Pauwels <i>et al.</i> , 2008
<i>Actinetobacter</i> sp.	$\gamma$ -proteobacteria Pseudomonadales	E1,E2,E3, EE2	Compost	Pauwels <i>et al.</i> , 2008
<i>Steroidobacter denitrificans</i>	$\gamma$ -proteobacteria Xanthomonadales	E2	Anoxic digested sludge	Fahrbach <i>et al.</i> , 2006
<i>Rhodococcus rhodochrous</i> DSM	Actinobacteria	E1,E2,E3	NA	Geize <i>et al.</i> , 2004
<i>Nocardia</i> sp. E110	Actinobacteria	E2	NA	Coombe <i>et al.</i> , 1966
<i>Sphingobacterium</i> sp. JCR5	Actinobacteria	E1,E2,E3, EE2	Activated sludge	Ren <i>et al.</i> , 2007
<i>Flavobacterium</i> sp.	Bacteroidetes	E2	Activated sludge	Yu <i>et al.</i> , 2007
<i>Bacillus subtilis</i>	Firmicutes	E1,E2	Activated sludge	Jiang <i>et al.</i> , 2010

NA: not available.

## 8. Biodegradation Pathway of 17 $\beta$ -estradiol by Bacteria under Aerobic Conditions

The degradation pathway of 17 $\beta$ -estradiol is very complicated and remains greatly unclear, although a lot of researches have been carried out. Fate and degradation pathway of 17 $\beta$ -estradiol in the aquatic environment are not yet fully understood. Few data on the degradation products of E2 in the environment are available in the open literature. In 1966, Coombe reported that incubation of *Nocardia* sp. with E1 resulted in the oxidative fission between C-4 and C-5 in the aromatic ring, leading to the formation of a dicarboxylic acid intermediate (Coombe *et al.*, 1966). But further degradation pathways were not elucidated, as the organism did not oxidize the proposed intermediates.

In 2002, Lee and Liu reported another biodegradation pathway of E2 by bacteria (Lee and Liu, 2002). 17 $\beta$ -estradiol was generally oxidized by bacteria at the C-17 of ring D, leading to the transformation of E2 to E1. During the early stage of E2 degradation by the culture, a new metabolite lactone was confirmed later by mass spectrometry. Aromatic A ring of E2 was not the most favored site for the initial biodegradation attack (Lee and Liu, 2002). The proposed degradation pathway of 17 $\beta$ -estradiol by bacteria under aerobic conditions is shown in Fig. 4.

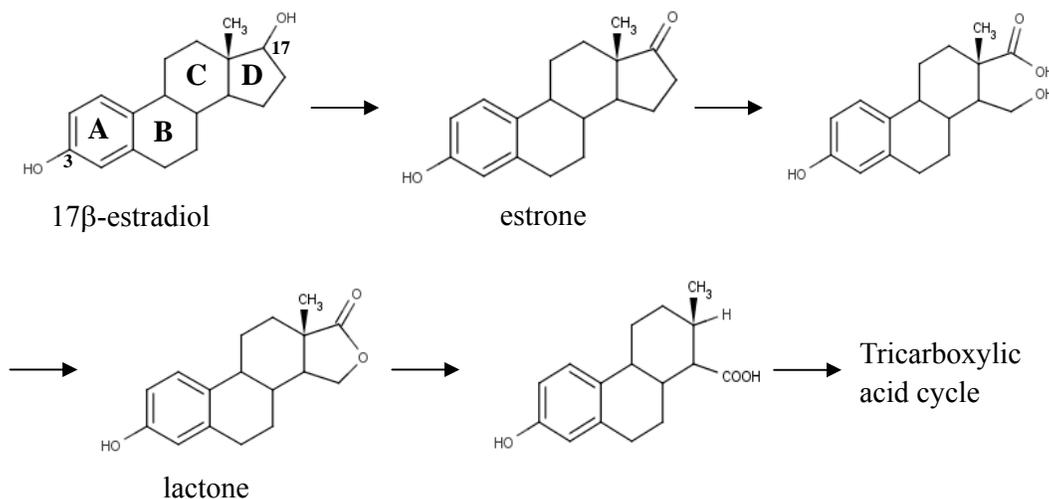


Fig. 4. Proposed degradation pathway of 17 $\beta$ -estradiol by bacteria (Lee and Liu, 2002).

However, E2 is not systematically degraded with E1 as an intermediate metabolite. Ternes showed that estrone was quickly degraded under aerobic conditions, and no other intermediate metabolites were detected (Ternes *et al.*, 1999). What is more, two other aerobic strains (*Rhodococcus zopfii* and *Novosphingobium spp.*) were also able to use E2 as the sole carbon source without E1 accumulation (Geize *et al.*, 2004). This suggested that other metabolic pathways may exist (Fahrbach *et al.*, 2006; Fujii *et al.*, 2002; Yoshimoto *et al.*, 2004). Therefore, it's important to elucidate the degradation pathway of estrogens by bacteria. In the present study, we try to get an overview of estradiol metabolism in strain S19-1 via isolating estradiol-inducible genes by using a fluorescent microplate assay.

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# **Characterization of the Steroid Degrading Bacterium S19-1 from the Baltic Sea at Kiel, Germany**

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**Chemico-Biological Interactions 191 (2011) 83-88.**

## Abstract

Steroid contamination of sea water is an ever growing problem and impacts population dynamics of all kinds of sea animals. We have long experience with the soil bacterium *Comamonas testosteroni* ATCC11996 which is able to catabolize a variety of steroids and polycyclic aromatic hydrocarbons, and which might be used in the bioremediation of contaminated places. In *Comamonas testosteroni* about 20 enzymes which are involved in steroid degradation could be induced by 0.5 mM testosterone. In this work, we isolated a bacterial strain, S19-1, from the Baltic Sea at Kiel (Germany) which degrades steroids and is able to use steroids as carbon source. S19-1 was characterized as being Gram-negative, and 16S rRNA analysis showed that it belongs to the genus *Buttiauxella* of the *Enterobacteriaceae* family. Strain S19-1 could be best grown in SIN medium supplemented with 0.6-5.1% NaCl at 20°C. As a carbon source, testosterone, estradiol or cholesterol in minimal medium could be used by S19-1. Moreover, S19-1 could grow up in medium with 50 µg/ml erythromycin. Plasmids pK18 and other plasmids were successfully transformed into S19-1 by chemical transformation (0.1 M CaCl<sub>2</sub>) and shown to replicate in the cells. Currently, work is ongoing to find the estradiol inducible genes.

Keywords: Steroid degradation; Steroid bioremediation; Marine bacteria; S19-1; 16S rRNA; *Buttiauxella*

## 1. Introduction

Large numbers and large quantities of hormonally active agents are constantly being released into the environment and contaminated soil and water represent a potential health risk for humans. Continuous exposure to such compounds can pose a threat to the development and reproduction not only to humans but to all kind of animals (Colborn *et al.*, 1993). Studies on steroid contaminations started to appear in the 1990s, in which estrogens and other steroids were detected in sea water, lakes and rivers in different areas (Barel-Cohen *et al.*, 2006; Shore *et al.*, 1993; Soto *et al.*, 2004). For example, steroidal estrogens have especially been shown to be the main contributors to the estrogenic activity observed in aquatic systems contaminated with sewage treatment work effluents. Moreover, estrogens and testosterone were observed in drinking water (Shore *et al.*, 1993). The interest in discovering how bacteria are dealing with hazardous environmental pollutants has driven a large research community and has resulted in important biochemical, genetic, and physiological knowledge about the degradation capacities of microorganisms (Fahrbach *et al.*, 2006; Fujii *et al.*, 2003; Marsheck *et al.*, 1972; Yoshinoto *et al.*, 2004).

Steroids can be degraded by certain bacterial species of different genera (Fahrbach *et al.*, 2006; Fujii *et al.*, 2003; Marsheck *et al.*, 1972; Yoshinoto *et al.*, 2004). But only few of them were isolated from sea and cultured and characterized on a biochemical and molecular level in laboratories. For example, *Comamonas testosteroni* ATCC11996 has long been studied in our previous investigations (Möbus *et al.*, 1997; Möbus and Maser, 1998; Oppermann and Maser, 1996; Oppermann *et al.*, 1993; Xiong and Maser, 2001; Xiong *et al.*, 2003; Oppermann *et al.*, 1996; Göhler *et al.*, 2008). *C. testosteroni* is a Gram-negative proteobacterium found in soil and fresh water. It is able to grow on steroids or aromatic hydrocarbons as sole carbon and energy source and might be used for the bioremediation of contaminated areas. However, *C. testosteroni* can not be used to clear contaminated marine areas because

it does not grow on high NaCl concentrations.

On the other hand, we used an important enzyme of the steroid degradation machinery from *C. testosteroni*, 3 $\alpha$ -hydroxysteroid dehydrogenase/carbonyl reductase (3 $\alpha$ -HSD/CR), to identify marine steroid degrading bacteria (Xiong *et al.*, 2009). The enzyme 3 $\alpha$ -HSD/CR from *C. testosteroni* is a member of SDR superfamily and catalyzes the dehydrogenation of hydroxyl-groups at position 3 of a variety of steroids, thereby initiating the complete breakdown of the steroid skeleton. Importantly, the expression of the 3 $\alpha$ -HSD/CR gene can be induced in the presence of testosterone and a variety of other steroids (Möbus and Maser, 1998; Oppermann and Maser, 1996; Xiong and Maser, 2001; Xiong *et al.*, 2003).

In order to isolate bacteria from the marine environment that could degrade steroid compounds, selective agar plates containing steroids were used and 12 bacterial isolates were successfully isolated from the Baltic Sea at Kiel, Germany (Xiong *et al.*, 2009). Marine bacterium S19-1 is one of these isolated strains and was further characterized in the present work. Analysis of its 16S rRNA sequence suggested that strain S19-1 belongs to the genus *Buttiauxella* of the *Enterobacteriaceae* family. Its capability of steroid degradation was determined by HPLC and S19-1 growth could be enhanced by addition of different steroids to the culture media. In the future, S19-1 might be used to clear environmental steroids from sea water areas, especially those contaminated with estrogens.

## **2. Materials and Methods**

### **2.1 Bacterial Strains and Plasmids**

Unless otherwise stated, *E. coli* HB101 and *C. testosteroni* ATCC 11996 (Deutsche Sammlung für Mikroorganismen) cells were grown in a shaker (180 rpm) in Standard I Nutrient broth medium (SIN, Merck, Darmstadt) at 37°C or 27°C, respectively. In this study, a series of plasmids were used. Plasmid pK18 containing the kanamycin resistance gene was a gift from Ciba Pharmaceuticals, Inc., Department of Biotechnology (Basel, Switzerland) (Pridmore, 1987). Plasmid pBBRIMCS-2 containing kanamycin resistance gene was a gift from Peterson (Kovach *et al.*, 1995). Plasmids pBR322 and pUC18 containing the ampicillin resistance gene was obtained from Invitrogen. Cloning of PCR fragments was carried out in pCR2.1-TOPO (Invitrogen, Invitrogen Corporation Attn: EvoQuest™ Laboratory Services 1600 Faraday Avenue Carlsbad, CA 92008 USA).

### **2.2 Growth Conditions of S19-1**

Marine bacterial strain S19-1 was cultured in SIN medium with NaCl concentrations ranging from 0.6 to 5.1% overnight at different temperatures (5°C, 12°C, 20°C, 27°C and 37°C), respectively. The bacterial concentration was determined at an optical density of OD<sub>595</sub> in a 1:10 dilution.

### **2.3 Gram Staining**

Gram-staining of S19-1 was carried out as the following procedure. S19-1 was cultured in SIN medium containing 2.1% NaCl for 18 h at 20°C. A suspension of 20 µl fresh bacterial culture was placed onto a microscope slide and fixed at room temperature. The slide was washed off with water after flooding with crystal violet for 90 sec. The complexes of crystal violet and iodine (CV-I) were washed off with water after flooding the slide with Gram's iodine for 3 min. The slide was then decolorized it with ethanol until the solvent flowed colorlessly from the slide and was washed off with water. Safranin was used to recolor the bacteria for 1 min. Subsequently,

safranin was washed off with water and the slide was dried at 50°C. Stained bacteria were inspected with a light microscope (Bresser, BGV A3, Germany) and use of the 100× oil-immersion objective.

#### **2.4 Isolation of Chromosomal DNA and Amplification of 16S rRNA**

Chromosomal DNA was purified by standard procedures (Sambrook and Russel, 2001) from bacterial cells harvested from SIN medium after growth for 24 h at 20°C. Subsequently, the bacterial cells were suspended in distilled water containing 0.1 mg/ml lysozyme and lysed by freezing (-20°C, 30 min) and thawing (room temperature, 30 min) 3 times. DNA was then recovered from the lysate by phenol/chloroform extraction, followed by ethanol precipitation. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to store at 4°C.

To determine the classification of strain S19-1, 16S rRNA was amplified by PCR using specific primers (gift from Professor R. Schmitz-Streit, Institute of General Microbiology, Kiel University): forward primer, 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer, 5'-GGT TAC CTT GTT ACG ACT T-3'. The thermal profile involved 25 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec, with a final polymerization at 72°C for 5 min. PCR product of 16S rRNA was cloned into TA cloning vector pCR2.1-TOPO and the recombinant plasmid was used for insert sequencing (MWG).

#### **2.5 Phylogenetic Analysis**

The obtained 16S rRNA sequence of S19-1 was edited with DNASTAR (version 5.01) and then compared with all known sequence data in the GenBank databases using the BLAST algorithm (Altschul *et al.*, 1990). The following reference sequences were retrieved from the NCBI database: *Novosphingobium tardaugens* sp. nov (AB070237); *Buttiauxella* sp. (AJ293683); *Buttiauxella agrestis* (AJ293684); *Buttiauxella noackia* (AJ233405); *Comamonas testosteroni* ATCC11996 (M11224) and *Denitratisoma oestradiolicum* AcBE2-1 (AY879297).

Multiple sequence alignments were performed by using the program CLUSTAL W (version 1.83) (Thompson *et al.*, 1994). The phylogenetic tree of 16S rRNA sequences of S19-1 and other bacteria was subjected to maximum-likelihood analysis using the PHYLIP package version 3.61 (Felsenstein, 1993). Bootstrap re-sampling with 1000 replicates using the Seqboot, DNAdist and Neighbour programmes provided confidence limits for the constructed phylogenies, and consensus trees were generated with the Consense programme. Phylogenetic trees were generated using the Drawtree and Drawgram programmes (Phylip), and bootstrap values were visualised using Treeview (Page, 1996).

## **2.6 Estradiol Degradation**

A suspension of 100 µl bacterial S19-1 cells was added into the 2 ml SIN medium containing 0.25 mM estradiol. Samples of 200 µl were immediately pipetted out to store at -20°C as non-cultured samples. Other samples of 200 µl were collected at different time points (5h, 9h, 12h, 24h and 48h) after incubation. All samples were extracted 3 times with equal volumes of ethyl acetate and then evaporated in vacuum. The precipitate was dissolved in 50 µl running buffer (acetornitril: H<sub>2</sub>O-50: 50) and subjected to HPLC.

HPLC (Waters system, Milford, MA, USA) analysis was carried out using a system which contains a 515 HPLC pump, 717 plus autosampler and a 2487 dual absorbance detector. The apparatus was interfaced to a PC compatible computer. Chromatographic analysis of estradiol was achieved on a RP-18 (5 µm, Merck, Darmstadt, Germany) column. The mobile phase for HPLC analysis was acetornitril: H<sub>2</sub>O (50: 50) with a flow rate of 1.0 ml/min. In each case, 5 µl samples were injected into the HPLC system. Detection was performed at two wavelengths of 210 nm and 225 nm at room temperature. Under the chromatographic conditions estradiol can be determined with retention time of estradiol at 7 min.

## 2.7 Antibiotic Resistance of S19-1

S19-1 cells were cultured overnight at 20°C in SIN medium containing the following antibiotics: 100 µg/ml ampicillin, 30 µg/ml kanamycin, 25 µg/ml chloramphenicol, 300 µg/ml carbenicillin, 50 µg/ml erythromycin, 50 µg/ml streptomycin, respectively. The overnight culture was 1:10 diluted with distilled water prior to OD<sub>595</sub> determination.

## 2.8 Plasmids Extraction and Transformation

An amount of 1 ml fresh bacterial S19-1 culture was extracted after centrifugation at 13,000 rpm for 20 sec with standard method (Sambrook and Russel, 2001). The bacterial cells were suspended in 100 µl buffer P1 (50 mM Tris-Cl, 10 mM EDTA and 100 µg/ml RNase A, pH 8.0) and lysed with 100 µl buffer P2 (200 mM NaOH and 1% SDS (w/v)) and 100 µl buffer P3 (3.0 M potassium acetate, pH 5.5). The mixtures were centrifuged at 13,000 rpm for 20 sec. The supernatant was then precipitated by 2 volumes of ethanol and was centrifuged at 13,000 rpm for 7 min. The pellet was dissolved in 20 µl H<sub>2</sub>O and subjected to agarose gel electrophoresis.

To test which plasmids could replicate in S19-1, the cells were spread on the SIN plates with antibiotics after transformation with plasmids pK18, pBBR1MCS-2, pBR322, pUC18, pCR2.1-TOPO by chemical transformation (0.1 M CaCl<sub>2</sub>), respectively (Sambrook and Russel, 2001). After incubation at 20°C overnight, colonies were selected from these plates to culture in SIN medium containing different antibiotics. Plasmids isolated from these bacteria were then transformed into *E. coli* HB101 and incubated at 37°C overnight. Plasmids were extracted and applied to agarose gel electrophoresis.

## 2.9 Protein Extraction

The overnight culture of S19-1 was harvested by centrifugation at 13,000 rpm for 20 sec. The resulting pellet was suspended in 200 µl buffer B (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris-Cl, pH 8.0). The suspension was lysed by freezing (-20°C, 30 min) and

thawing (room temperature, 30 min) 3 times. Finally, the samples were centrifuged at 13,000 rpm for 20 min. The supernatant was used for protein determination.

### **2.10 Protein Determination**

Protein concentration was measured by the method of Bradford with Roti-Quant solution (Roth) using bovine serum albumin as a standard (Bradford, 1976).

### 3. Results

#### 3.1 Growth of S19-1 in the Presence of Different NaCl Concentrations and at Low Temperature

As a novel marine bacterial strain, isolated from the Baltic Sea, the optimal growth conditions of S19-1 could be different from that of soil bacteria, such as *C. testosteroni*, especially regarding temperatures and NaCl concentrations. Normally, sea water contains 2-5% NaCl and temperatures of sea water are usually lower than 20°C. Therefore marine strain S19-1 and *C. testosteroni* ATCC11996 (the later for comparison) were incubated in SIN medium containing different concentrations of NaCl and were cultured at different temperatures, respectively. As shown in Fig. 1, the growth of *C. testosteroni* significantly decreased when the medium containing 2.1% NaCl, while strain S19-1 could grow very well in the medium with NaCl concentrations ranging from 0.6 to 4.1%, especially with 2.1% NaCl. Regarding the optimum temperature, S19-1 could best grow at 20°C as shown in Fig. 2. Accordingly, further experiments with strain S19-1 were performed in SIN medium containing 2.1% NaCl and at 20°C.

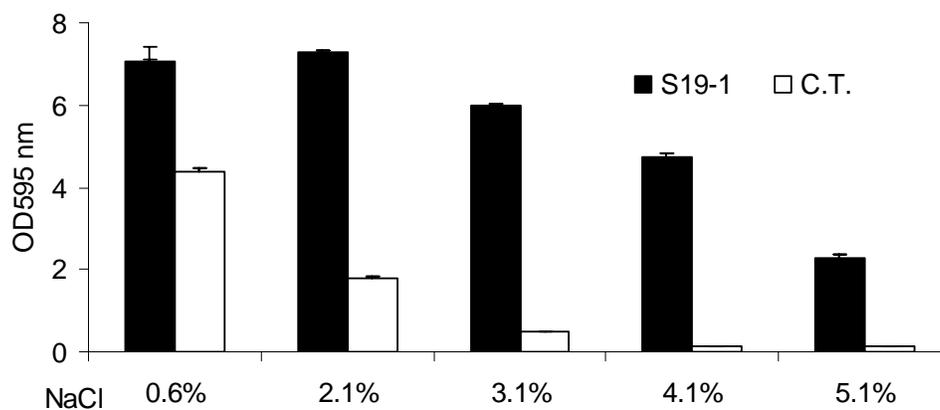


Fig. 1. Salt tolerance of marine strain S19-1. Strain S19-1 and *C. testosteroni* were cultured in SIN medium with NaCl concentrations ranging from 0.6 to 5.1% overnight at 20°C and 27°C, respectively. The samples were 1:10 diluted with distilled water prior to OD<sub>595</sub> determination. Whereas S19-1 could grow up well with 0.6-4.1% NaCl, *C. testosteroni* grew up only with 0.6% NaCl, and to a lesser extent with 2.1% NaCl.

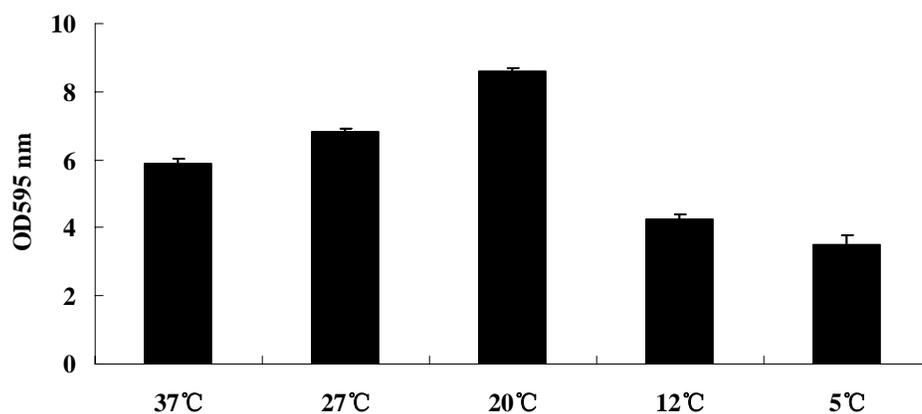


Fig. 2. Culture temperature for strain S19-1. Strain S19-1 was cultured overnight at 5°C, 12°C, 20°C, 27°C and 37°C in SIN medium (with 2.1% NaCl), respectively. Bacterial concentration was determined at OD<sub>595</sub>. As the result, 20°C is the best temperature for S19-1 growth.

### 3.2 S19-1 is a Gram-negative Bacterium

Strain S19-1 isolated from the Baltic Sea and cultured on SIN plates appeared as smooth, round, *E. coli* like colonies. For further characterization of strain S19-1, a Gam-staining was performed which revealed that S19-1 is a Gam-negative, round shaped bacterium (Fig. 3).

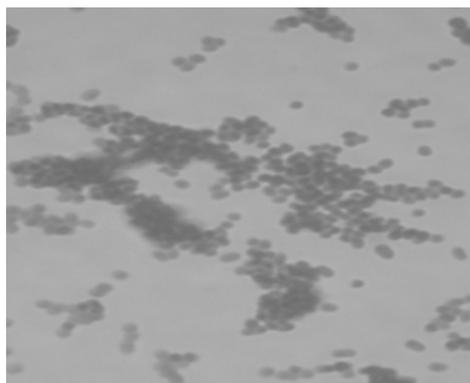


Fig. 3. Gram-staining of strain S19-1. Strain S19-1 was cultured in SIN medium with 2.1% NaCl for 18 h at 20°C and the fresh bacteria culture was used for Gram-staining. The picture was captured under light microscopic inspection (100x oil-immersion objective).

### 3.3 S19-1 Belongs to the Genus *Buttiauxella* of the Family *Enterobacteriaceae*

To infer the taxonomy of S19-1, 16S rRNA analysis was performed and a phylogenetic tree of the novel isolate and related organisms was constructed (Fig. 4). The bioinformatics approach revealed that marine strain S19-1 is closely related to *Buttiauxella* sp. of the family *Enterobacteriaceae*. The 16S rRNA sequence of S19-1 showed 99.3% identity with *Buttiauxella agrestis*, 99.7% with *Buttiauxella noackia*, 99.2% with *Buttiauxella* sp., 81.7% with *Comamonas testosteroni* ATCC11996, 81.2% with *Denitratisoma oestradiolicum* AcBE2-1, 80.2% with *Novosphingobium tardaugens* sp. nov. Among genus *Buttiauxella*, the percent identity was above 99.2%. As shown in Fig. 4, S19-1 and other *Buttiauxella* species form a cluster. This indicates that S19-1 belongs to the genus *Buttiauxella* of the family *Enterobacteriaceae*.

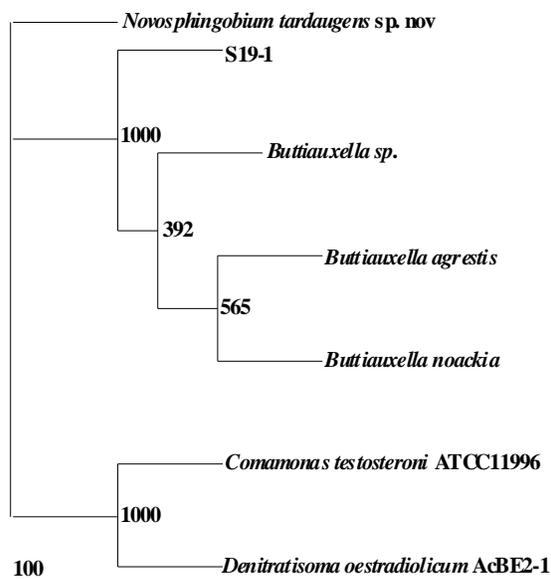


Fig. 4. Phylogenetic analysis of strain S19-1. Phylogenetic tree of the 16S rRNA sequences of S19-1 and other taxa. The tree was rooted via a neighbor-joining method, and numbers within the dendrogram indicate the occurrence of the branching order in 1000 bootstrapped trees. *Novosphingobium* sp. nov. was utilized as an outgroup. The GenBank accession number of the 16S rRNA of S19-1 is HQ662633.

### 3.4 S19-1 can Degrade Estradiol

Strain S19-1 was successfully isolated from the Baltic Sea by using a selective medium which contained steroids (Xiong *et al.*, 2009). To further test the ability of S19-1 to degrade estradiol, 0.25 mM estradiol was added into SIN medium (containing 2.1% NaCl) and incubated with S19-1 at 20°C. The estradiol remaining in the cell cultures collected at different time points (0h, 5h, 9h, 12h, 24h, 48h) was extracted by ethyl acetate and then detected by using HPLC. As shown in Fig. 5, at 0 h (without incubation with S19-1), estradiol in the medium was measured as about 1700 of area value of estradiol. It was degraded fast over the first 5 h of incubation with S19-1, which the area value of estradiol was about 700. After incubation for 48 h, most of estradiol was degraded, which the area value of estradiol were about 300. 80% of estradiol supplemented in the medium was degraded by strain S19-1 after 48 h incubation. This clearly shows that marine strain S19-1 is able to degrade estradiol. The HPLC charts of estradiol detected under UV absorbance at two wavelengths (210 nm and 225 nm) were shown in Fig. 6.

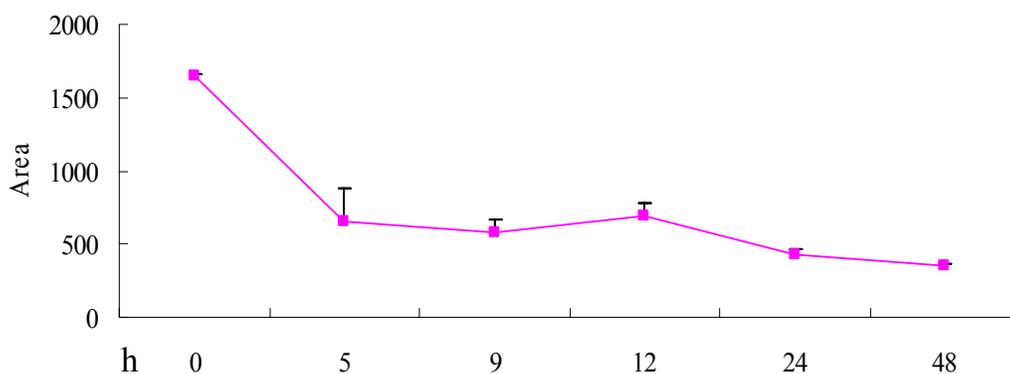


Fig. 5. Degradation of estradiol in strain S19-1. Strain S19-1 was incubated with 0.25 mM estradiol at room temperature. The estradiol remaining in the cell cultures collected at different time points (0h, 5h, 9h, 12h, 24h and 48h) was extracted by ethyl acetate and then detected by using HPLC. The results showed that 80% of estradiol added in the medium was degraded by strain S19-1 after 48 h incubation.

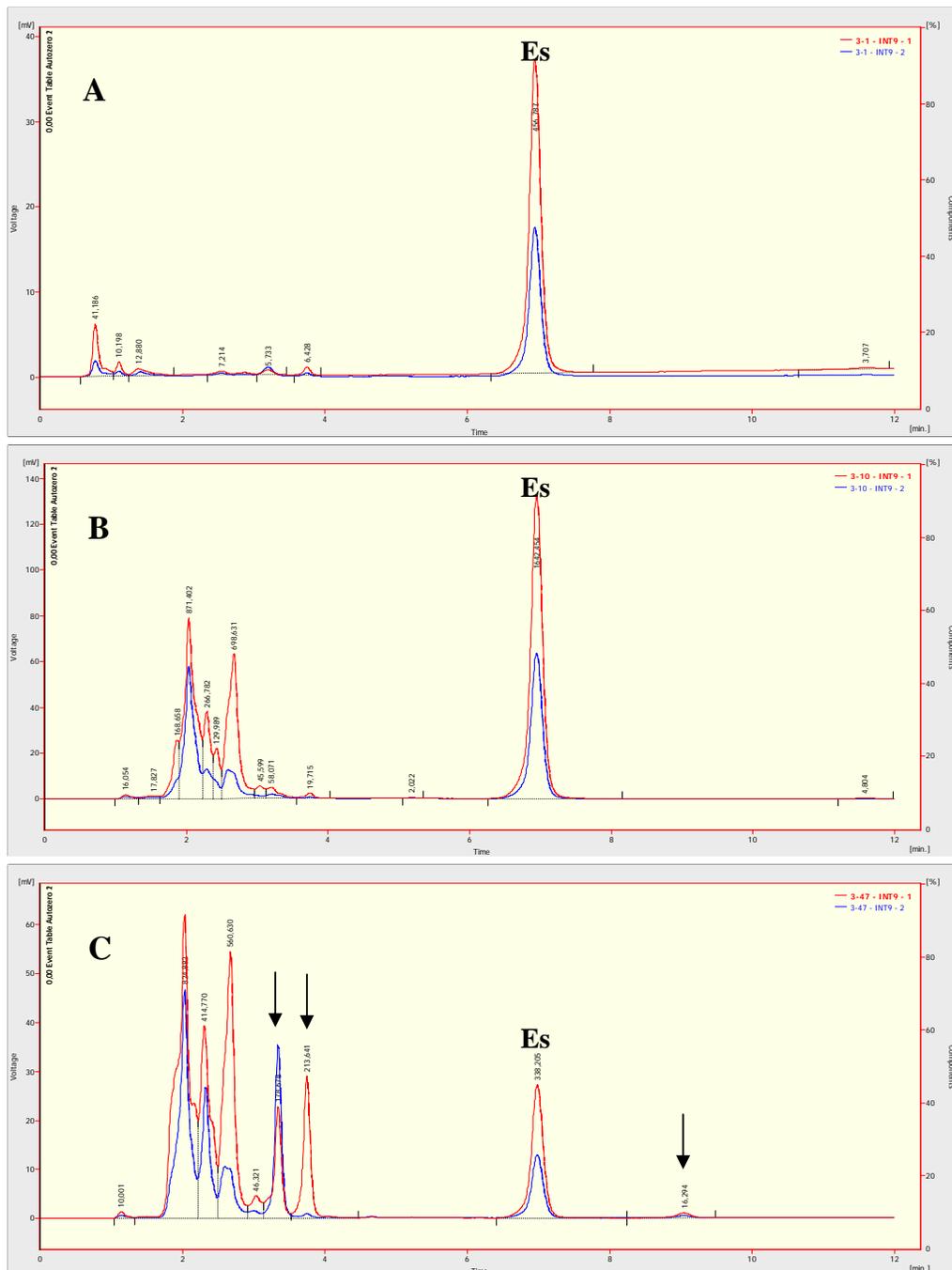


Fig. 6. HPLC charts of estradiol. Red line and blue line represent signals of estradiol detected under UV absorbance at two wavelenths (210 nm and 225 nm). The retention time of estradiol is about 7 minutes and acetonitrile: H<sub>2</sub>O (50:50) was used as the running buffer. Es: estradiol. A: 0.1 mM Estradiol without bacteria incubation. B: 0.25 mM Estradiol before incubation; C: Estradiol after 48 h incubation with S19-1.

### 3.5 Antibiotic Resistance of S19-1

To characterize its antibiotic resistance, S19-1 was incubated in the presence of a variety of antibiotics. Compared to the culture without antibiotics, S19-1 was shown to be sensitive to all antibiotics except to 50 µg/ml erythromycin (Fig. 6).

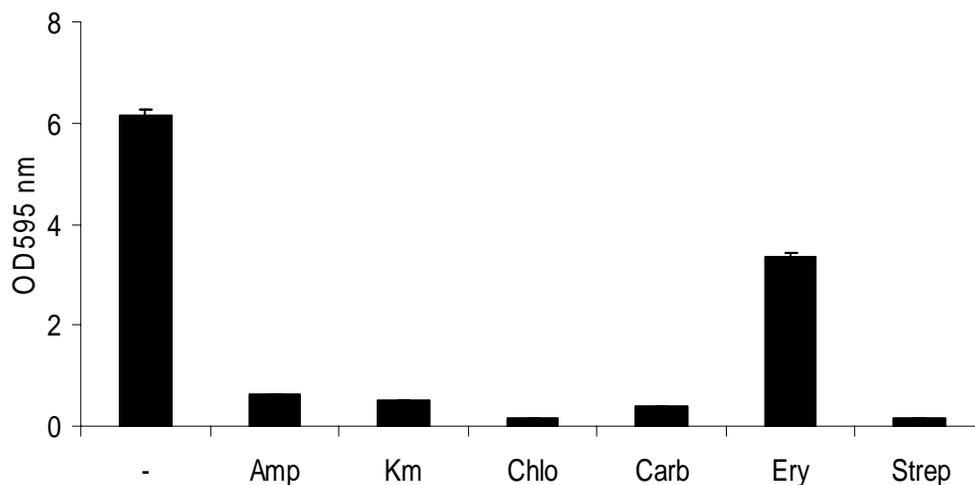


Fig. 6. Antibiotic resistance of strain S19-1. Strain S19-1 was cultured overnight at 20°C in SIN medium with 2.1% NaCl and several antibiotics (-: none; ampicillin (Amp): 100 µg/ml; kanamycin (Km): 30 µg/ml; chloramphenicol (Chlo): 25 µg/ml; carbenicillin (Carb): 300 µg/ml; erythromycin (Ery): 50 µg/ml and streptomycin (Strep): 50 µg/ml). The optical density of bacterial cells was measured at 595 nm. Compared to the control (“-”), S19-1 is sensitive to all antibiotics tested except to erythromycin.

### 3.6 Plasmids Transformation into S19-1

In addition to their chromosomal DNA, bacteria may harbor genes coding for important physiological functions on plasmids, which might also be the case with the steroid degrading machinery in marine strain S19-1. To understand whether S19-1 contains plasmids or not, plasmid extraction was applied according to Sambrook and Russel (Sambrook and Russel, 2001). Since no plasmid could be extracted by this method, S19-1 does probably not contain any plasmid.

On the other hand, it is high interest to transform strain S19-1 with plasmids and to

introduce new genes into the bacterium to obtain steroid inducible genes or to prepare knock-out strains. In our lab, plasmids pK18, pBBRIMCS-2, pBR322, pUC18 and pCR2.1-TOPO are most commonly used to study the degradation pathway of steroids in bacteria, and were here tested for S19-1 transformation. The results revealed that all of the plasmids tested can be transformed into S19-1 by chemical transformation (0.1 M CaCl<sub>2</sub>) and replicate within this bacterium as revealed by plasmid isolation, thereby providing possibilities for further investigations with S19-1.

### 3.7 Growth of Strain S19-1 in the Presence of Different Steroids

Since S19-1 has been shown to degrade estradiol (described in section 3.4), it might use steroids as carbon and energy source and therefore should grow better in the presence of steroids. S19-1 was cultured overnight at 20°C in minimal medium supplemented with various steroids in different concentrations. OD<sub>595</sub> analysis revealed that, indeed, S19-1 bacteria could grow much better in the presence of testosterone, estradiol and cholesterol, when compared to negative control (Fig. 7). In addition, the amount of total bacterial protein in the presence of steroids increased (Fig. 8). Accordingly, this novel marine bacterial strain S19-1 can use different steroids as carbon source.

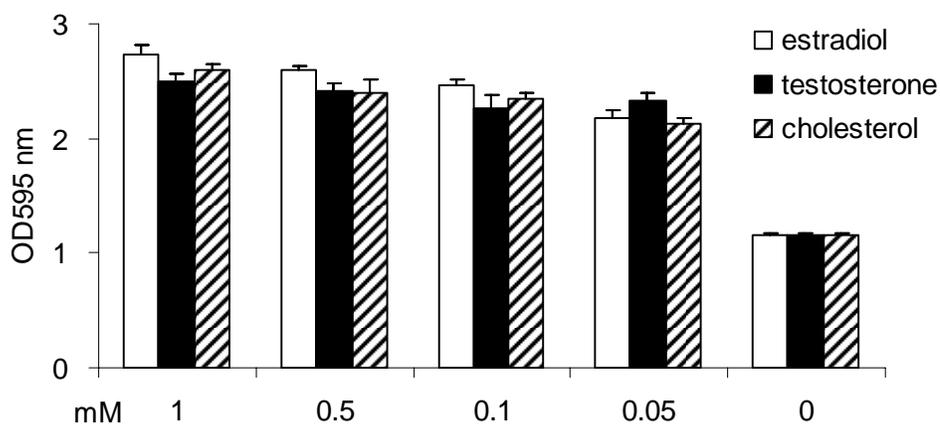


Fig. 7. Steroids as substrates for S19-1. Strain S19-1 was cultured overnight at 20°C in minimal medium containing 2.1% NaCl and supplemented with various steroids in different concentrations. Bacterial growth was determined at OD<sub>595</sub>.

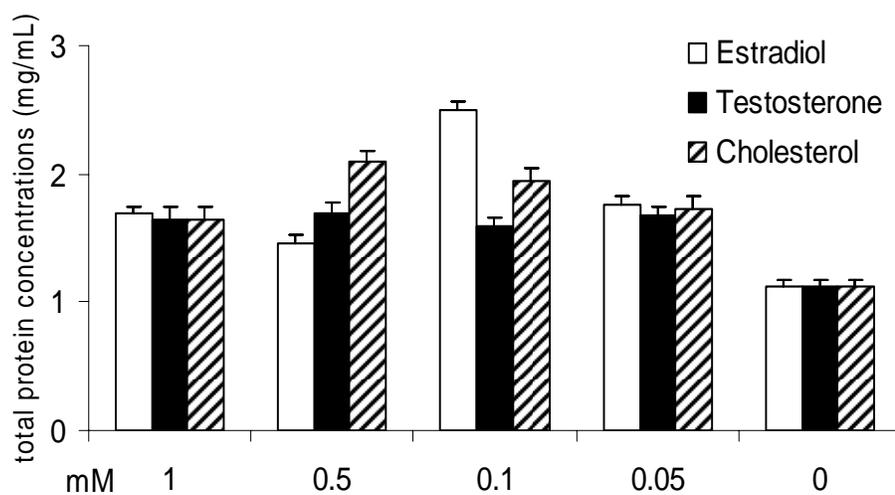


Fig. 8. Steroids as substrates for S19-1. Strain S19-1 was cultured overnight at 20°C in minimal medium containing 2.1% NaCl and supplemented with various steroids in different concentrations. Total protein extracted from the bacterial cultures was assayed by the Bradford method.

## 4. Discussion

Natural and synthetic hormones are constantly excreted into the environment over thousands of years, especially to an increasing extent due to growing populations and more intensive farming. These increasing steroidal hormones in the environment might pose serious risks to human and wildlife health (Cloborn *et al.*, 1993). In the past 10 years, many research papers covering the many different aspects of steroid hormone contaminations in the environment have been published (Colbor *et al.*, 1993; Barel-Cohen *et al.*, 2006; Shore *et al.*, 1993; Soto *et al.*, 1993; Fahrbach *et al.*, 2006; Fujii *et al.*, 2003; Marsheck *et al.*, 1972). Several methods have been applied to treat these contaminations, and microbial biodegradation is the most popular one. There are lots of microorganisms and other organisms in the environment which could degrade steroid hormones. Many bacteria have been isolated, such as *Comamonas testosteroni* ATCC11996 which can use testosterone as the sole carbon and energy source (Xiong *et al.*, 2009), *Denitratisoma oestradiolicum* AcBE2-1 which is a 17 $\beta$ -oestradiol degrading, denitrifying beta-proteobacterium (Fahrbach *et al.*, 2006), *Novosphingobium tardaugens* sp. nov which can degrade estrogens (Fujii *et al.*, 2003). But most of them were isolated from soil or fresh water. As to the increasing contamination of sea water by steroids, marine bacteria are needed to clean the contaminated ocean. In our lab, 12 strains were successfully isolated from the Baltic Sea around Kiel (Germany) in which we suspected the presence of steroid contaminated water from the city (Xiong *et al.*, 2009). S19-1 is one of them and was studied in more detail in the present work.

According to the phylogenetic analysis, marine bacterium strain S19-1 belongs to the genus *Buttiauxella*. The genus *Buttiauxella* was created by Ferragut *et al.* in 1981 as being a small, rod-shaped Gam-negative bacterium (Ferragut *et al.*, 1981), which have been isolated mostly from water and soil (Gavini *et al.*, 1983). Lately, six new species (isolated from soil, snails, and human sputum) were added to the genus *Buttiauxella*

(Müller *et al.*, 1996). Analysis of 16S rRNA reveals a very high degree of phylogenetic relatedness among the type strains of the seven *Buttiauxella* species (99.1-99.7% similarity) (Spröer *et al.*, 1999). So far, no reports showed that genus *Buttiauxella* has pathogenicity to animals or humans. Interestingly, this is the first report providing evidence for genus *Buttiauxella* to degrade steroids.

Strain S19-1 turned out to be a Gam-negative bacterium that could best be grown at 20°C in SIN medium containing 2.1% NaCl. S19-1 was found to be sensitive to antibiotics ampicillin, kanamycin, chloramphenicol carbenicillin or streptomycin, but was resistant against erythromycin. Interestingly, S19-1 could grow much better in minimal medium supplemented with testosterone, estradiol or cholesterol. The capability of marine strain S19-1 to use steroids as carbon source was further supported by the fact that the total amount of bacterial protein increased when the bacteria were grown on steroids. In addition, after 48 h of incubation with 0.25 mM estradiol, 80% of estradiol was degraded. And three significantly different peaks (as shown in Fig. 6C as black arrows) showed up in the HPLC charts of estradiol after 48 h incubation with S19-1. They might be intermediates of estradiol. Other experiments need to be done for further confirmation. Combined, marine strain S19-1 might be used in the bioremediation of steroid contaminated sea water. Molecular work on the genes related to steroid degradation together with mechanistic studies on the corresponding enzymes is currently in progress.

## **Acknowledgements**

The “State-Sponsored Scholarship Program for Graduate Students” funded by Chinese Scholarship Council is acknowledged for financial support. This work was supported by grants from the Deutsche Forschungsgemeinschaft (MA 1704/4-1; MA 1704/4-2) and the excellence Cluster “The Future Ocean”.

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# **Isolation of Estradiol-inducible Genes and a Novel Promoter from S19-1 by Meta-genomic Methods**

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## Abstract

Endocrine disruption of aquatic life caused by environmental estrogens has led to an increasing interest in the bioremediation of these stable compounds. In previous investigation, strain S19-1 belonging to the genus *Buttiauxella*, which was isolated from the Baltic Sea at Kiel, Germany, was characterized to be able to degrade steroids including testosterone, estradiol and cholesterol. To analyze the estradiol degradation pathway in this marine isolate, a meta-genomic approach was combined with a fluorescence microplate assay to isolate genes involved in estradiol metabolism. The chromosomal DNA of strain S19-1 was first digested with restriction enzyme *SalI* and the resulting *SalI* fragments were cloned into pKEGFP-2. *E. coli* cells harboring the recombinant plasmids were induced by 4 fmole estradiol and relative fluorescence units (RFU) of bacteria were measured. Compared to the negative control pKEGFP-2, 37 plasmids out of the meta-genomic library containing 323 plasmids could be induced by estradiol. Relative fluorescence units-increased amount percentage (RFU-IAP) of 6 plasmids from these 37 plasmids was about 6-7%. This is higher than the other 31 inducible plasmids, which was about 4-5%. So the insert fragments of these 6 plasmids were sequenced and then analyzed by BLAST search. Sequence analysis showed that the inserts of these plasmids comprise transcriptional regulators, substrate transporters, kinases and catabolic enzymes. Inserts of plasmid p5 containing members of LuxR and GntR family might be involved in the degradation of estradiol in S19-1.

Not only catabolic enzymes and relevant regulatory elements, but also inducible promoters could be obtained by this method. Among the genomic library of 323 colonies containing the *SalI* fragments of chromosomal DNA of S19-1, bacteria containing one of the estradiol-inducible plasmids, plasmid p302, became green after incubation overnight. This suggested that a strong promoter may exist in this plasmid. Since a strong promoter from a new marine bacteria may be very useful in our future

work, different restriction enzymes and PCR amplification was used to short the insert of plasmid p302, until a 108-bp fragment containing the promoter was obtained. The putative -10 region (TAAGAT) and -35 region (TTGTCG) of this promoter were predicted. Furthermore, the activity of this new promoter was also compared to that of the *lac* promoter.

Keywords: S19-1; estradiol-inducible genes; fluorescence microplate assay; promoter

## 1. Introduction

Contaminated soil and water by hormonally active agents represent a potential health risk to humans and all kinds of animals (Colborn *et al.*, 1993; Sumpter and Johnson, 2005). The hormonally active agents mainly are steroids which include estrogens and testosterone. Estradiol is the most active one among them. As estimated by the United States Census Bureau for 2009, 29.5 tons of estrogens were released into the environment every year and cause harm (Hernandez-Raquet, 2010). Thus, the elimination of these stable compounds from contaminated environments is an absolute requirement for the sustainable development of the biosphere. It has been demonstrated that biologic processes play an important role in the removal of these pollutants. For example, *Comamonas testosteroni* (*C. testosteroni*) is known for its ability to degrade steroids such as testosterone and the degradation pathway of testosterone has been extensively studied (Horinouchi *et al.*, 2001; Horinouchi *et al.*, 2003a; Horinouchi *et al.*, 2003b; Horinouchi *et al.*, 2010). Although a series of bacterial strains have been reported to degrade estradiol, the degradation pathway of estradiol in these microorganisms remains unclear (Fahrbach *et al.*, 2006; Fujii *et al.*, 2002; Yoshimoto *et al.*, 2004). Thus the interest in discovering how bacteria degrade estrogens has driven a large amount of relevant researches.

Most of polluted rivers by estrogens finally flow into the ocean, and also cause contaminations in there. Recently, contamination and treatment of the marine environment attracted more and more attention. In 2008, selective agar plates containing steroids were used for isolating bacteria which can degrade steroids from the Baltic Sea at Kiel, Germany (Xiong, *et al.*, 2009). 12 bacterial isolates, which are able to degrade steroids, were successfully obtained. Marine bacterium S19-1 is one of those isolated strains and was characterized to grow in a very wide environment with concentrations of NaCl from 0.6% to 5.1% and at a temperature ranging from 5°C to 27°C (Zhang *et al.*, 2011). This suggests that S19-1 might be used in the

clearance of contaminated oceans and other aquatic environments in the future.

To get an overview of estradiol metabolism in S19-1, we focus on the isolation and characterization of estradiol-inducible genes and relevant regulatory elements from S19-1 in the present work. In general, most catabolic enzymes involved in the degradation of aromatics and steroids can be induced by the substrates and/or metabolites of the catabolic pathway. And in many cases, relative regulatory elements situate proximate to catabolic genes which are involved in the degradation of aromatic compounds. This mechanism was used to develop a substrate-inducible gene-expression screening by Uchiyama for isolation of catabolic genes from environmental metagenome (Uchiyama *et al.*, 2005). And this method was improved by measuring relative fluorescence units (RFU) instead of fluorescence-activated cell sorting, which was used by Uchiyama, by our group last year. It was named fluorescence microplate assay (FMA) (Sang *et al.*, 2001a). Two estradiol-inducible genes were successfully isolated from another steroid-degrading bacterium strain H5 by FMA (Sang *et al.*, 2011b). So this method was chosen for isolating estradiol-inducible genes and their regulatory elements from steroid-degrading bacterium strain S19-1 in the present study.

37 plasmids from a meta-genomic library containing 323 colonies could be induced by estradiol using this method. Six of them were sequenced and analyzed. Sequence analysis showed that the inserts of these plasmids comprise transcriptional regulators, substrate transporters, kinases and catabolic enzymes. Among the genomic library of 323 colonies containing the *SalI* fragments of chromosomal DNA of S19-1, bacteria containing one of the estradiol-inducible plasmids, plasmid p302, became green after incubation overnight. This suggested a strong promoter might exist in this fragment. In the present work, different restriction enzymes and PCR amplification was used to short the insert of plasmid p302, until a 108-bp fragment containing the promoter was obtained.

## **2. Materials and Methods**

### **2.1 Bacterial Strains, Plasmids and Growth Conditions**

Host strains *E. coli* HB101 (Promega, Amersham, Germany) and marine bacterial strain S19-1 (Zhang *et al.*, 2011) were used for gene cloning and gene expression. Subcloning of fragments was carried out in plasmid pK18 containing the kanamycin resistance gene (a gift from Ciba Pharmaceuticals, Inc., Department of Biotechnology, Basel, Switzerland) (Pridmore, 1987). Plasmid pCR2.1-TOPO (Invitrogen) was served for TA cloning of PCR product and sequencing. Plasmids pKEGFP-2 and pK3 $\alpha$ -4.6-EGFP3 were constructed by our group for isolating the estradiol-inducible genes from bacteria (Sang *et al.*, 2011a).

*E. coli* HB101 cells were grown in a shaker (180 rpm) in Standard I Nutrient broth medium (SIN medium) obtained from Merck, Darmstadt (25 g/liter, 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) or in SIN medium containing 2.1% NaCl for growth of S19-1. Cultivation temperatures were 37°C for *E. coli* and room temperature for S19-1. Growth media contained 100  $\mu$ g/ml ampicillin and/or 30  $\mu$ g/ml kanamycin if necessary.

### **2.3 Restriction Enzymes, Kits and Other Reagents**

Restriction enzymes were from New England Biolabs. Shrimp alkaline phosphatase was from USB. T4 DNA ligase and Taq DNA polymerase were from Fermentas. RNase A was from Boehringer (Mannheim). TOPO TA cloning kit was from Invitrogen (Invitrogen, Invitrogen Corporation Attn: EvoQuest™ Laboratory Services 1600 Faraday Avenue Carlsbad, CA 92008 USA). QIAGEN Plasmid Midi Kit was from QIAGEN (QIAGEN GmbH, Germany). Estradiol was purchased from Sigma. All other chemicals were of analytical grade and obtained from commercial suppliers.

### **2.4 Isolation of Chromosomal DNA from S19-1**

To construct the meta-genomic library of colonies containing estradiol-inducible

genes, the chromosomal DNA of S19-1 was isolated by phenol/chloroform extraction. 2 ml of overnight culture of S19-1 cells was harvested by centrifugation at 13,000 rpm for 20 sec and then resuspended in distilled water containing 0.1 mg/ml lysozyme. Bacteria were lysed by freezing (-20°C, 30 min) and thawing (at room temperature, 30 min) 3 times. DNA was then recovered from the lysate by phenol/chloroform extraction, followed by ethanol precipitation. The DNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C. The purified chromosomal DNA was used for subsequent construction of the meta-genomic library and other experiments.

### **2.5 Construction of the Meta-genomic Library of *Sa*I Fragments from S19-1 Chromosomal DNA**

As shown in Fig. 1, strain S19-1 chromosomal DNA was digested with restriction enzyme *Sa*I. The reaction mixtures were extracted by phenol/chloroform method and then the resulting fragments were cloned into pKEGFP-2 which had been digested with *Sa*I. The ligation mixtures were transformed into chemically competent *E. coli* HB101 which was prepared with 0.1 M of CaCl<sub>2</sub>. After transformation, the cell mixtures were plated on SIN agar plates containing 30 µg/ml kanamycin. After 10 min, the plate was inverted and incubated at 37°C overnight. All of the colonies were picked up and incubated to isolate plasmids for confirmation. Only the plasmids harboring the chromosomal DNA fragment of S19-1 were used for the fluorescence microplate assay.

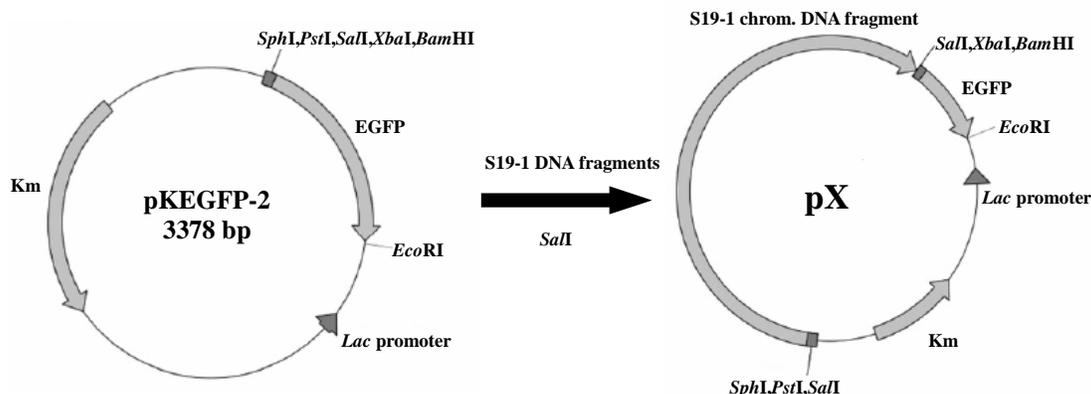


Fig. 1. Schematic representation of plasmids used for isolating estradiol-inducible genes from S19-1. Chromosomal DNA of S19-1 was digested with *SalI* and then cloned into plasmid pKEGFP-2 which contains the EGFP gene as a reporter. The resulting plasmids, which are indicated as pX, were transformed into *E. coli* HB101 cells and then induced by estradiol for detecting estradiol-inducible genes.

## 2.6 Fluorescence Microplate Assay

Fluorescence Microplate Assay (FMA) was performed as previously described (Sang *et al.*, 2011a). The plasmids containing the *SalI* fragment from chromosomal DNA of S19-1 were transformed into *E. coli* HB101 and cells harboring the plasmid were incubated at 37°C overnight in the medium containing 30 µg/ml kanamycin. In addition, plasmids pKEGFP-2 and pK3α-4.6-EGFP3 were used as negative or positive control in FMA. These overnight cultures harboring different plasmids, such as pKEGFP-2, pK3α-4.6-EGFP3 or plasmids containing *SalI* fragments of strain S19-1 chromosomal DNA, were adjusted into  $OD_{595\text{ nm}} = 0.1$  (using distilled water for dilution) for RFU measurement. Each well of 96-well Costar black plates was added 4 fmole estradiol or 10 µl distilled water (as control) together with 90 µl of *E. coli* HB101 cells ( $OD_{595\text{ nm}} = 0.1$ ) harboring different plasmids. Three replicate wells per assay were run for each sample. After incubation for 1 h, the inducible genes were detected by EGFP expression and shown as relative fluorescence units (RFU). The RFU values are given as “RFU-IAP” (RFU Increased Amount Percentage) which is defined as  $(RFU_{\text{sample}} - RFU_{\text{water}}) / RFU_{\text{water}} * 100\%$ .

## 2.7 Sequence Analysis

Estradiol inducible plasmids harboring chromosomal *SalI* fragments of S19-1 were selected for sequencing. The average size of digested chromosomal DNA fragments is above 3.0 kb. To facilitate sequencing, restriction enzymes were used to digest them into small fragments about 1.0 kb. Sequencing of these plasmids was performed by MWG (Germany). The obtained sequences were edited and analyzed by DNASTAR. The deduced amino acid sequences were used to query the protein database at the National Center for Biotechnology Information (NCBI) using BLAST. Sequences were annotated based on similarity to previously characterized proteins (<http://www.ncbi.nlm.nih.gov/BLAST>).

## 2.8 Construction of Deletion Mutants of Plasmid p302

Since the pellet of *E. coli* HB101 containing plasmid p302 became green, there should be a strong promoter present in the *SalI* fragment of this plasmid. To locate this novel promoter in plasmid p302, a series of deletion mutants of plasmid p302 were constructed by using a variety of appropriate restriction enzymes, such as *Bam*HI, *Pst*I, *Eco*RV and *Bg*III (Fig. 2).

After determination of the promoter in a 185-bp region which is adjacent to the EGFP gene, PCR was used to construct a shorter 108-bp fragment together with the EGFP gene using specific primers: forward primer 5'-AAGCTTGTATTATTAATTA-3' (*Hind*III) and reverse primer 5'-CTGCAGTTACTTGTACAGCTC-3' (*Pst*I). The resulting PCR product was digested with *Hind*III and *Pst*I and then cloned into pK18 (Pridmore, 1987).

The PCR reactions were carried out in a final volume of 50  $\mu$ l in small PCR tubes in an Eppendorf thermal cycler. The thermal profile involved 25 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec, with a final polymerization at 72°C for 5 min. The PCR product was analyzed by agarose gel electrophoresis.

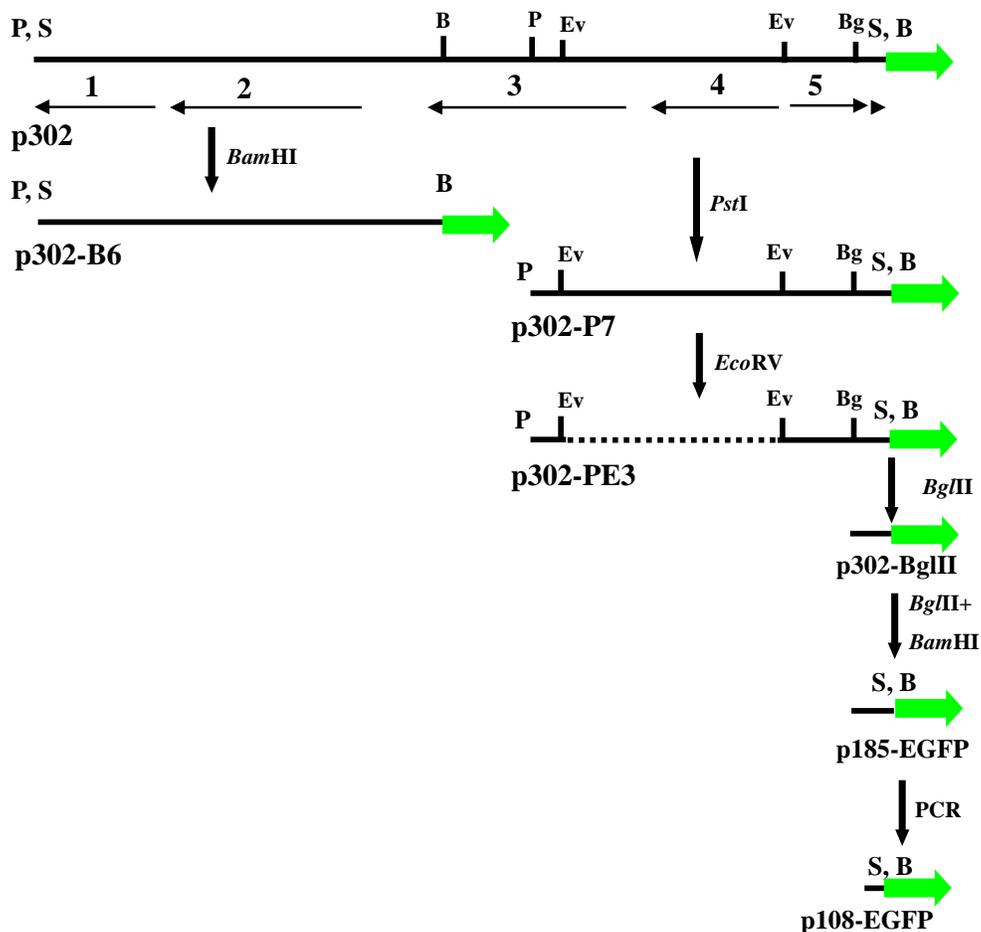


Fig. 2. Construction of deletion mutants of plasmid p302. A variety of restriction enzymes were used to construct the derivatives of plasmid p302. P: *PstI*; S: *SalI*; B: *BamHI*; Ev: *EcoRV*; Bg: *BglII*. Genes of p302 code for: 1, L-serine dehydratase; 2, serine transporter; 3, decarboxylase; 4, 7-cyano-7- deazaguanine; 5, suppressor of SecY dominance family protein. Green arrows indicate the EGFP gene.

In plasmid pKEGFP-2, the direction of the EGFP gene is opposite to the *lac* promoter. To make a recombinant pK18 plasmid in which the EGFP gene is under the control of the *lac* promoter, the EGFP gene was obtained by digestion of pKEGFP-2 with *EcoRI* and then the resulting *EcoRI* fragment was ligated into pK18 to yield the plasmid pKEGFP3 in which the direction of the EGFP gene is the same as the *lac* promoter. Both plasmids, pKEGFP-2 and pKEGFP3, were used as control for measuring RFU.

### 3. Results

#### 3.1 Isolation of Estradiol-inducible Genes by Fluorescence Microplate Assay

To isolate estradiol-inducible genes from S19-1, a genomic library of 323 colonies containing the *SalI* fragment from the chromosomal DNA of S19-1 was constructed by using a meta-genomic method. All colonies were used for estradiol-induced gene-expression screening by fluorescence microplate assay. Compared to the negative control pKEGFP-2, 37 colonies from the genomic library could be induced by estradiol which was reflected by the increased RFU value. The RFU-IAP of 37 estradiol-inducible plasmids containing the chromosomal *SalI* fragments of S19-1 is about 4%-7%, which is the same case as the positive control with pK3 $\alpha$ -4.6-EGFP3. The induction efficiency of other plasmids containing chromosomal DNA of S19-1 was less than 1%, which is the same case as negative control with pKEGFP-2. Among the 37 estradiol-inducible plasmids, colonies containing plasmids p5, p12, p22, p26, p44, and p302 showed the highest induction efficiency, the RFU-IAP of these plasmids is 6.2%, 7.3%, 5.7%, 6.1%, 5.6%, and 5.9%, respectively. This indicates that there may be genes involved in estradiol metabolism which are present in these plasmids. Thus, the inserts in these plasmids were completely sequenced.

#### 3.2 Sequence Analysis and Genetic Organization of Estradiol-inducible Plasmids

As shown in Fig. 3, the sequence lengths of the inserts of plasmids p5, p12, p22, p26, p44, and p302 are above 3.0 kb. To facilitate sequencing of these plasmids, a series of deletion mutants were constructed which contain part of the insert of fragment. The obtained sequence was analyzed for homology search by using BLAST program to locate the putative genes, which was also in combination with analysis by using DNASTAR program. Genetic organization of genes present in these 6 plasmids is shown in Fig. 3. The detailed information of these putative genes and the amino acid sequence identity with orthologs in closely related bacterial strains are listed in Table 1. The putative genes in these plasmids code for transcriptional regulators (FimZ, LuxR, GntR and nadR), substrate transporters (ABC transporter and major facilitator

superfamily protein) and catabolic enzymes. To analyze if these putative genes are involved in estradiol metabolism, the deduced amino acid sequences of the relative genes were aligned with their orthologs in other related bacteria which have been previously characterized.

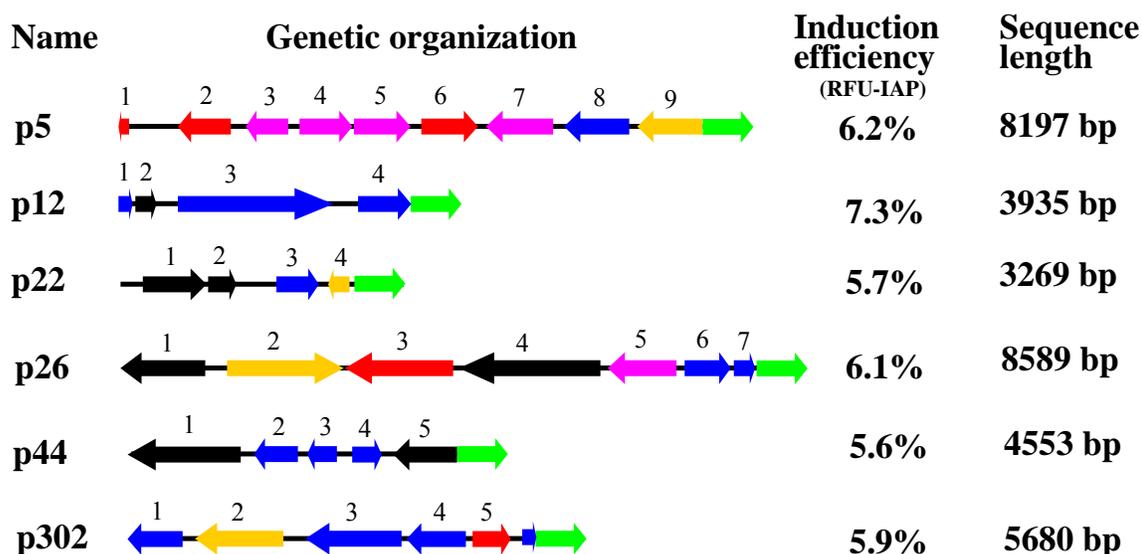


Fig. 3. Genetic organization of estradiol-inducible plasmids. The recombinant plasmids containing the *SaII* fragments from chromosomal DNA of S19-1 were identified to be induced by estradiol after transformation into *E. coli* HB101 by using a fluorescence microplate assay. Induction efficiency of these plasmids was indicated as RFU-IAP. The whole sequence of the insert fragments of these plasmids was sequenced and the putative genes were predicted by using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Arrows indicate gene encoding EGFP (green), catabolic enzyme (blue), transcriptional regulator (red), transporter protein (yellow), kinase (pink) and other or unknown (black).

Table 1 Putative genes in estradiol-inducible plasmids

ORF		Homologous protein (function identified)				
Name	Length (aa)	Name and function	Accession no.	Length (aa)	Host organism	Identity (aa%)
p5-1	50	Transcriptional regulator FimZ	YP_001454166.1	210	<i>Citrobacter</i>	72%
p5-2	256	Response regulator receiver protein	YP_003611795.1	240	<i>Enterobacter</i>	31%
p5-3	200	Transcriptional regulator LuxR	ZP_06353324.1	202	<i>Citrobacter</i>	46%
p5-4	256	Hydroxyethyl-thiazole kinase	YP_004829381.1	256	<i>Enterobacter</i>	71%

p5-5	268	Phosphomethyl- pyridine kinase	ZP_08498807.1	266	<i>Enterobacter</i>	81%
p5-6	248	GntR transcriptional regulator	YP_003365775.1	248	<i>Citrobacter</i>	82%
p5-7	312	PfkB kinase	ZP_05968388.1	317	<i>Enterobacter</i>	69%
p5-8	306	ADP-ribosylglyco- hydrolase	YP_003941144.1	335	<i>Enterobacter</i>	73%
p5-9	305	Major facilitator superfamily protein	YP_003365772.1	423	<i>Citrobacter</i>	86%
p12-1	67	Aldehyde dehydrogenase	YP_003941287.1	461	<i>Enterobacter</i>	68%
p12-2	99	Hypothetical protein	ZP_03069838.1	99	<i>E.coli</i>	82%
p12-3	723	Formate C- acetyltransferase	ZP_04558545.1	765	<i>Citrobacter</i>	49%
p12-4	256	Pyruvate formate- lyase 3 activating enzyme	NP_756398.1	305	<i>E.coli</i>	80%
p22-1	290	Copper resistance protein	YP_001177131.1	290	<i>Enterobacter</i>	50%
p22-2	112	Hypothetical protein	YP_003611966.1	112	<i>Enterobacter</i>	79%
p22-3	192	Serine acetyltransferase	ZP_07950303.1	198	<i>Enterobacter</i>	40%
p22-4	104	Transport- associated protein	ZP_07950651.1	104	<i>Enterobacter</i>	71%
p26-1	399	Lytic murein transly cosylase	YP_001439403.1	644	<i>Cronobacter</i>	80%
p26-2	543	ABC transporter	YP_001175290.1	555	<i>Enterobacter</i>	96%
p26-3	410	Transcriptional regulator nadR	YP_004592319.1	410	<i>Enterobacter</i>	87%
p26-4	461	DNA repair protein	YP_003943269.1	461	<i>Enterobacter</i>	94%
p26-5	322	Phosphoserine phosphatase	YP_003611312.1	322	<i>Enterobacter</i>	84%
p26-6	217	Lipoate protein ligaseA	ZP_04559767.1	213	<i>Citrobacter</i>	78%
p26-7	94	Lipoate protein ligaseB	ZP_04559766.1	338	<i>Citrobacter</i>	99%
p44-1	516	Type VI secretion system Rhs/Vgr- family protein	YP_003366347.1	692	<i>Citrobacter</i>	69%
p44-2	206	CDP-alcohol phosphatidy- ltransferase	ZP_04561712.1	207	<i>Citrobacter</i>	84%
p44-3	134	decarboxylase	YP_003364885.1	199	<i>Citrobacter</i>	65%
p44-4	140	NTP pyrophospho- hydrolase	YP_001453352.1	140	<i>Citrobacter</i>	69%
p44-5	295	DNA topoisomerase III	ZP_04561707.1	660	<i>Citrobacter</i>	78%
p302-1	262	L-serine dehydratase	YP_003614613.1	455	<i>Enterobacter</i>	84%
p302-2	432	Serine transporter SdaC	ZP_06355175.1	429	<i>Citrobacter</i>	69%
p302-3	454	decarboxylase	YP_003366359.1	454	<i>Citrobacter</i>	89%
p302-4	280	7-cyano-7-deazaguanine reductase	YP_001177962.1	280	<i>Enterobacter</i>	79%
p302-5	182	Suppressor of SecY dominance family protein	YP_003940482.1	181	<i>Enterobacter</i>	63%

### 3.3 Location of the Novel Promoter in Plasmid p302

To further locate the position of the novel promoter in plasmid p302, a series of deletion mutants of plasmid p302 were constructed (Fig. 2). Plasmid p302-B6 was constructed by digestion of p302 with *Bam*HI and then self-ligation. Plasmid p302-P7 was constructed by digestion of p302 with *Pst*I and then self-ligation, which was digested by *Eco*RV and self-ligation to obtain plasmid p302-PE3 (Fig. 2). While p302-BglII was prepared by the deletion of *Bgl*II fragment of plasmid p302-PE3 and then self-ligated. So the *Bgl*II-*Pst*I fragment of the empty vector pK18 was also deleted (Fig. 2, *Bgl*II existing in vector pK18 locates 329 bp away from the left of *Pst*I, not shown in the figure). To test if the deletion of the *Bgl*II-*Pst*I fragment of the vector affects the activity of the promoter, p302-BglII was digested by *Bgl*II and *Bam*HI and the resulting *Bgl*II-*Bam*HI fragment was cloned into pKEGFP-2 which had been digested by *Bam*HI, to yield p185-EGFP6 (Fig. 2). Furthermore, the last 108 bp of the 185-bp *Bgl*II-*Bam*HI fragment of p185-EGFP6 together with the EGFP gene were amplified by PCR and then cloned into pK18 to get the plasmid p108-EGFP1 for further determining the position of the promoter.

After transformation of these plasmids into *E. coli* HB101, the pellets of bacteria harboring plasmids p302, p302-P7, p302-PE3, p302-BglII, p185-EGFP6 and p108-EGFP1 became green, but not with p302-B6. To test the activity of the novel promoter in these plasmids, RFU of cells containing the relative plasmid was also measured. As shown in Fig. 4, the RFU of cells with p302, p302-P7, p302-PE3, p302-BglII, p185-EGFP6, and p108-EGFP1 was significantly higher than that with p302-B6 or the negative control vector pKEGFP-2. Taken together, the novel strong promoter exists in the 108-bp fragment, which is adjacent to the EGFP gene of plasmid p302.

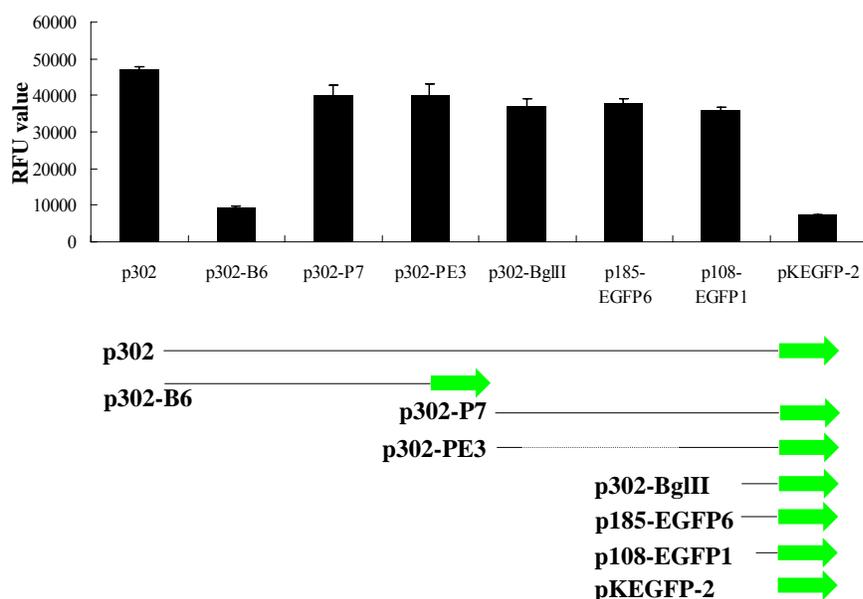


Fig. 4. Location of the novel promoter in plasmid p302. The optical density of the overnight culture containing the relative plasmids was measured at 595 nm, and 100  $\mu$ l of cells ( $OD_{595 \text{ nm}} = 0.1$ ) were placed to the black microplate to detect the RFU. Each sample has 6 replicates. Green arrows indicate the EGFP gene.

### 3.4 Comparison of the Activity of the Novel Promoter with the *lac* Promoter

To compare the promoter activity between the novel promoter and the *lac* promoter, plasmids p185-EGFP5, p185-EGFP6, pKEGFP-2 and pKEGFP3 were constructed. As shown in Fig. 5, the *lac* promoter in plasmid pKEGFP3 has the same direction as the EGFP gene, but in pKEGFP-2, the direction of the *lac* promoter is opposite to that of the EGFP gene. To facilitate the construction of plasmids p185-EGFP5 and p185-EGFP6, the 185-bp promoter was used and the direction of this promoter in p185-EGFP5 is opposite to that of the EGFP gene, the latter being under the control of the 185-bp promoter in p185-EGFP6. In addition, the direction of the *lac* promoter in plasmids p185-EGFP5 and p185-EGFP6 is opposite to the EGFP gene. As expected, the RFU of bacteria with plasmids p185-EGFP5 and pKEGFP-2 was very low (Fig. 5), largely because the promoters cannot perform their function to promote the

transcription of the EGFP gene. Compared to p185-EGFP5, the RFU value with p185-EGFP6 is very high. This indicates the strong activity of the 185-bp promoter on the EGFP transcription, while this novel promoter is not as strong as the *lac* promoter according to the RFU value obtained with p185-EGFP6 and pKEGFP3. Although the activity of the *lac* promoter is 3-fold higher than that of the novel promoter, the latter could make the pellets of bacteria green together with a high RFU value. It seems that the activity of this promoter from natural marine bacterium S19-1 is also strong.

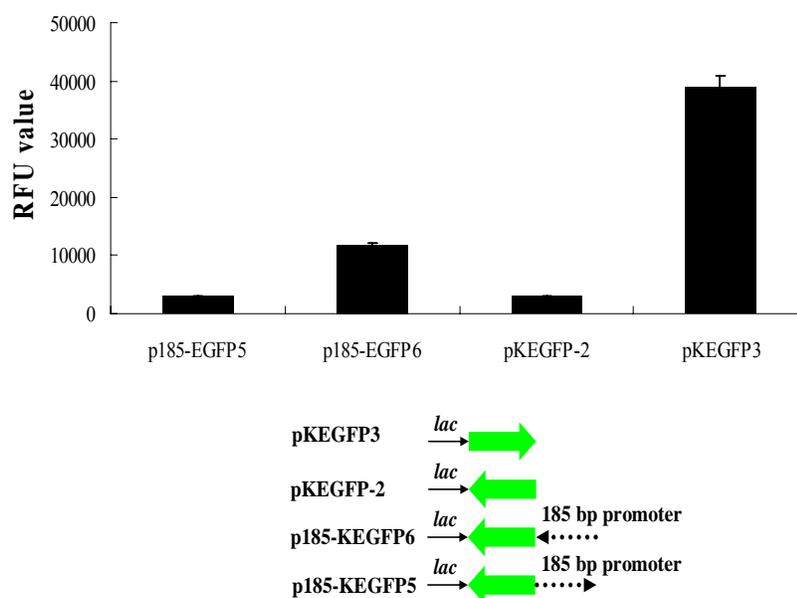


Fig. 5. The activity of the new 185-bp promoter is lower than that of the *lac* promoter. The overnight cultures containing these four plasmids were used for measuring the RFU. Each sample has 6 replicates. The results showed that the *lac* promoter has stronger activity on the EGFP gene expression in *E. coli* HB101 than the new 185-bp promoter from S19-1. Green arrows indicate the EGFP gene.

### 3.5 Analysis of the Sequence of the Novel Promoter

To get more detailed information of this novel promoter, it was sequenced at MWG (Germany). In the 108 bp promoter sequence, the putative -10 region and -35 region were predicted based on the consensus promoter sequence in prokaryotes (-10

elements: T<sub>80</sub>A<sub>95</sub>T<sub>45</sub>A<sub>60</sub>A<sub>50</sub>T<sub>96</sub>; -35 elements: T<sub>82</sub>T<sub>84</sub>G<sub>78</sub>A<sub>65</sub>C<sub>54</sub>A<sub>45</sub>) (Fig. 6). The -10 region and -35 region in this promoter were proposed to be TAAGAT and TTGTCG, respectively, and the distance between these two elements is 19 bp. This finding should be further determined by point mutations.

```

GTTATTTATTAATTAGTTGTGTTGATTCTTGTCG
                                     -35
CTTTTGAAGAAAGGAAACGTAAGATTGGTTGT
                               -10
GAGTGCCTTGCGAGATGAACGAAATAGAGGATT
CTATGTCCGTCGAC

```

Fig. 6. Sequence of the new strong promoter. The putative -10 region (TAAGAT) and -35 region (TTGTCG) of the new promoter from S19-1 are indicated in blue bold.

## 4. Discussion

Strain S19-1 is a new isolated marine bacterium which could use estradiol as carbon and energy source, but little is known about how it degrades estradiol or which genes are involved in this catabolic pathway. To get an overview of estradiol metabolism in this bacterium, a meta-genomic approach was combined with a fluorescence microplate assay to isolate estradiol-inducible genes. This method is based on the knowledge that catabolic gene expression is generally induced by substrates and/or metabolites of catabolic enzymes and, in many cases, is controlled by regulatory elements situated proximate to catabolic genes. In 2005, Uchiyama adopted this substrate-induced method for isolating benzoate catabolic enzymes from environmental metagenome using benzoate as a substrate (Uchiyama *et al.*, 2005). According to their results, a large number of benzoate inducible fragments were obtained by this method (Uchiyama *et al.*, 2005). In this study, a lot of estradiol-inducible fragments were also obtained, from which 10% of chromosomal DNA fragments of S19-1 could be induced by estradiol. This is probably because the pathway is not only induced by the substrate estradiol itself for the corresponding catabolic enzymes, but also by a similar pathway intermediate or some structural analogues of the natural effector (gratuitous inducer) for bacteria (Díaz and Prieto, 2000).

### 4.1 Analysis of estradiol-inducible genes from S19-1 by meta-genomic methods

Six estradiol-inducible fragments from S19-1 were sequenced and analyzed in the present study. Transcriptional regulators, kinases, transport-associated proteins and catabolic enzymes were found in the plasmid inserts from chromosomal DNA of S19-1. In plasmid p5, the putative genes are organized like this: transcriptional regulator FimZ, response regulator receiver protein, transcriptional regulator LuxR, hydroxyethylthiazole kinase, phosphomethylpyridine kinase, transcriptional regulator

GntR, PfkB kinase, ADP-ribosylglycohydrolase, and major facilitator superfamily protein (Fig. 3 and Table 1). Among them, both hydroxyethylthiazole kinase and phosphomethylpyrimidine kinase belong to the family of transferases which transfer phosphorus-containing groups with an alcohol group as acceptor for hydroxyethylthiazole and with a phosphate group as acceptor for phosphomethylpyrimidine kinase (Nosaka *et al.*, 1994), respectively. 6-phosphofructokinase (PfkB) kinase superfamily comprises kinases that accept a wide variety of substrates, including carbohydrates and aromatic small molecules. Major facilitator superfamily (MFS) protein is one of the two largest families of membrane transporters found on earth, which was originally believed to function primarily in the uptake of sugars (Pao *et al.*, 1998). In 2004, Pruneda-Paz reported a steroid-inducible gene *teiR* (testosterone-inducible regulator) in *Comamonas testosteroni* belongs to luxR-type family, which was also found in plasmid p5 (Pruneda-Paz, *et al.*, 2004). Göhler also reported the *teiR*-disrupted mutant strain *C. testosteroni* was completely unable to use testosterone as the sole carbon and energy source (Göhler *et al.*, 2008). A GntR family transcriptional regulator was found to be next to the LuxR gene in plasmid p5 (Talbe 1 and Fig. 2). “Gnt” was first reported by BÄchi and Kornberg. It is involved in the uptake and catabolism of gluconate by *E. coli* (BÄchi and Kornberg, 1975). Later then, some members of this family were identified in the degradation of aromatic compounds in bacteria, such as AphS, BphS, Orf0, PaaX and VanR (Mouz *et al.*, 1999; Arai *et al.*, 1999; Ferrández *et al.*, 1998; Morawski *et al.*, 2000; Teramoto *et al.*, 2001; Watanabe *et al.*, 2000). Since the biodegradation pathway of estradiol in bacteria remains greatly unclear and estradiol is one of those steroids which has an aromatic ring, this fragment from plasmid p5 might be involved in the degradation of estradiol in S19-1. Both LuxR and GntR may be selected out for further confirmation.

#### **4.2 Comparison of the Novel Promoter with the *lac* Promoter**

*Lac* promoter is a strong promoter to promote the operon which is required for the transport and metabolism of lactose in *E. coli* and some other bacteria. In the present

study, the activity of the novel promoter was identified to be lower than the *lac* promoter. The -10 element and -35 element of *lac* promoter are TATGTT and TTTACA and the distance between these two elements is 18 bp. For the novel promoter identified in the present study, the -10 element and -35 element are TAAGAT and TTGTCG and the distance is 19 bp. The difference in these elements between these two promoters may result in the different affinity of RNA polymerase to bind to the target sites. Therefore, the activity of them to promote gene transcription is different. What's more, the novel determined promoter was obtained from the marine bacterium S19-1 and it is not clear if the activity of this promoter is affected when it promotes the gene transcription in a heterologous system.

#### **4.3 Analysis of the Gene Controlled by the Novel Promoter**

In the present study, this new promoter was identified in a 108-bp DNA fragment and the putative -10 and -35 regions were proposed, but we do not get the target genes controlled by this promoter from plasmid p302. Sequence analyses showed that strain S19-1 is closely related to *Citrobacter rodentium* ICC168. As shown in Fig. 7, the genetic organization of genes in plasmid p302 is the same as that in *Citrobacter rodentium* ICC168. The amino acid sequence identity between the orthologs in these two bacteria is very high. In *Citrobacter rodentium* ICC168, the ortholog(s) of the gene downstream from the new promoter is a miscellaneous small RNA, which serves a variety of functions, including some enzyme-like catalysis or serving as switches, turning genes on and off or unknown function. Until now, it's not clear what the target gene controlled by this novel promoter in strain S19-1 is.

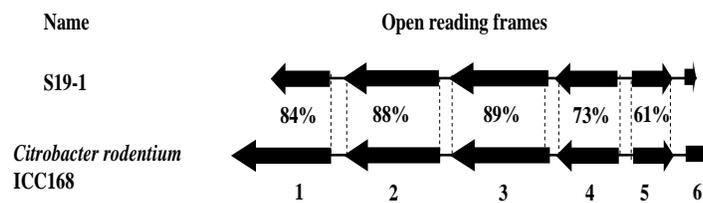


Fig. 7. Prediction of the gene controlled by this new promoter. The genetic organization in plasmid p302 from S19-1 is the same as that of their orthologs in *Citrobacter rodentium* ICC168. The amino acid sequence identity between the orthologs in these two bacteria is also indicated. Genes of the fragment from *Citrobacter rodentium* ICC168 code for: 1, L-serine dehydratase; 2, serine transporter; 3, decarboxylase; 4, 7-cyano-7-deazaguanine; 5, suppressor of SecY dominance family protein; 6, miscellaneous small RNA.

## **Acknowledgements**

The “State-Sponsored Scholarship Program for Graduate Students” funded by Chinese Scholarship Council is acknowledged for financial support. This work was supported by grants from the Deutsche Forschungsgemeinschaft (MA 1704/4-1; MA 1704/4-2) and the excellence Cluster “The Future Ocean”.

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## General discussion

Steroid compounds are very important in the reproduction, growth and development of human beings and animals. Over the past decades, large amounts of these compounds (both natural and synthetic) are excreted into the environment such as soil, river and ocean, which can cause endocrine disruption to living organisms. In particular, the developmental disorder of aquatic organisms and human beings caused by the environmental estrogens has been attracted considerable public concern. Therefore, the elimination of estrogens from the contaminated environment is required for the sustainable development of the biosphere and is extensively studied in many research groups of all over the world. Many researches have demonstrated that microorganisms, especially bacteria, have acquired the capacity to degrade these stable steroid compounds and bioremediation has the potential to clean up these pollutants from the environment. In the present study, we focus on the characterization of an estradiol-degrading bacterium strain S19-1, which is isolated from the Baltic Sea, Germany. To elucidate the catabolic pathway of estradiol in S19-1, fluorescence microplate assay was established to isolate the estradiol-inducible genes from this bacterium. In addition, a strong promoter from this marine isolate S19-1 was characterized.

### 1. Classification and characteristics of marine isolate S19-1

Studies showed that bacteria involved in the estrogen degradation belong to different genera of varied classes including  $\alpha$ -Proteobacteria,  $\beta$ -Proteobacteria,  $\gamma$ -Proteobacteria and *Actinobacter*. Here, strain S19-1 was classified to be a member of genus *Buttiauxella* which belongs to the  $\gamma$ -Proteobacteria. Genus *Buttiauxella* was created by Ferragut in 1981 (Ferragut *et al.*, 1981). Species of this genus have been isolated from soil, snails, and human sputum, which were characterized as Gram-negative and small, rod shaped cells (Ferragut *et al.*, 1981; Gavini *et al.*, 1983). So does strain S19-1. In particular, S19-1 has the ability to use steroids as carbon and energy source.

Although several genera belonging to  $\gamma$ -Proteobacteria were identified to have the capacity to degrade estrogens, such as *Steroidobacter denitrificans* (Fahrbach *et al.*, 2006), *Acinetobacter* sp. (Ke *et al.*, 2007), *Escherichia coli* (Yu *et al.*, 2007), *Pseudomonas* sp. (Pauwels *et al.*, 2008) and *Schewanella baltica* (Ivanov *et al.*, 2010), S19-1 is the first member of the genus *Buttiauxella* which is able to utilize estrogens.

Strain S19-1 was isolated from the Baltic Sea by our group in 2008 (Xiong *et al.*, 2009). Although the optimal temperature for the growth of this bacterium was shown to be 20°C, it is still able to grow at 5°C. This is probably because the temperature of the Baltic Sea water is about 10°C or less and then, growth of S19-1 at low temperature occurs. Strain S19-1 can grow in the medium with very wide concentrations of chloride sodium from 0.6% to 4.1%, even to 5.1% while another steroid-degrading strain *Comamonas testosteroni* only grows well in the medium with 0.6% chloride sodium. This suggests that S19-1 might be useful in the removal of estradiol from the contaminated environment such as ocean. The optical density of S19-1 at 595 nm can reach even 8.0 after 24 h incubation in the medium with 2.1% chloride sodium at 20°C. This value is very high compared to *E. coli* HB101 and *Comamonas testosteroni*, in which the value of OD<sub>595 nm</sub> is less than 5.0. Until now, it is not clear why S19-1 grows so well.

## **2. Substrate-induced Meta-genomic Methods for Obtaining Estradiol**

### **Inducible Genes from S19-1**

Molecular biology has grown very fast in the last 50 years. With the development of new techniques, a lot of methods for obtaining the target genes were established. The most commonly used two screening methods are nucleotide sequence-based screening and function-based screening. When the sequence of a target gene is known or the conserved primers could be obtained by comparing a limited number of catabolic genes from the other similar organisms, PCR or hybridization methods are very useful to obtain the target gene. When we have no idea about the sequence of the target gene,

regarding the difference of the whole gene expression under different conditions, the genes which have the altered expression might be very interesting, so they could be selected for the subsequent research. In this situation, genomics and 2-dimensional gelelectrophoresis could be good choices. But S19-1 is a new isolated bacterium from the Baltic Sea and the degradation pathway of estradiol in bacteria remains unclear, so it is hard to isolate estradiol-catabolic genes from this bacterium by those two methods.

In 2005, Uchiyama *et al.* reported a substrate-induced gene-expression screening method for isolation of benzoate-catabolic genes from environmental metagenome libraries (Uchiyama *et al.*, 2005). The mechanism of this method based on the fact that the expression of catabolic enzymes and their regulatory elements, which are needed for mineralization of the substrate compound, is controlled by the presence of substrate and/or metabolites. In many cases, relative regulatory elements situate proximate to catabolic genes which are involved in the degradation of the substrate compound. These regulatory proteins and regulated promoters are key elements that control the transcription of catabolic operons to assure an adequate feedback when a particular substrate serves as the nutrient source. The transcriptional regulation of aromatic catabolic pathways in bacteria is shown in Fig. 1.

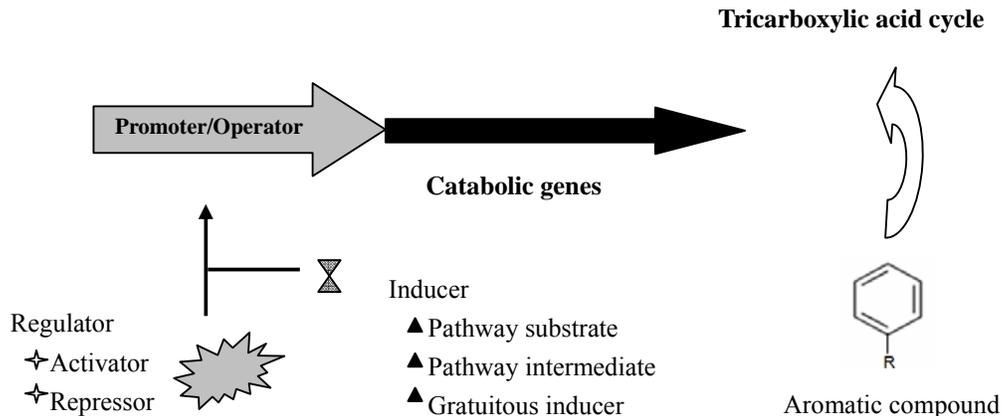


Fig. 1. Schematic representation of transcriptional regulation of aromatic catabolic pathways in bacteria (Díaz and Prieto, 2000). The regulator-promoter couple is the key element of the pathway-specific regulation. The regulator binds to the operator region of the cognate catabolic genes and, in the presence of the inducer molecule, leads to the expression of the catabolic genes encoding the enzymes that transform the aromatic compound into tricarboxylic acid cycle intermediates. The inducer molecule can be the pathway substrate and/or a pathway intermediate; some structural analogues of the natural effector (gratuitous inducer) can also induce the pathway, although they may not themselves be a substrate for the corresponding catabolic enzymes.

In Uchiyama's report, fluorescence activated cell sorting was used to determine which plasmids contain the substrate inducible fragments (Uchiyama *et al.*, 2005). Since it is expensive and time consuming, this method was modified by our group with measuring relative fluorescence units (RFU) instead of fluorescence activated cell sorting. In these two methods, the most commonly used bioreporters GFP and EGFP were used. Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish, *Aequorea victoria*, or from the sea pansy, *Renilla reniformis*. Its role is to transduce the blue chemiluminescence of aequorin into green fluorescent light by energy transfer. Green fluorescent protein is comprised of 238 amino acids. It could be detected with excitation wavelength at 485 nm and emission wavelength at 535 nm (Yang, *et al.*, 1996). The availability of *E. coli* clones expressing GFP has led to extensive mutational analysis of GFP function.

Screens of random and directed point mutations for changes in fluorescent behavior have uncovered a number of informative amino acid substitutions with effects of fluorescence intensity and expression level. The combination of improved fluorescence intensity and higher expression levels yield enhanced GFP (EGFP) variants which provide greater sensitivity (Kain, 1999). EGFP can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function. If this EGFP gene was used as a reporter gene to replace the catabolic gene or locate downstream the catabolic gene, the difference of fluorescence value could be directly detected by using Tecan GENios Pro machine under conditions with or without substrate induction, which is a fully automatic, computer controlled fluorescence instrument produced by TECAN in Austria for measuring samples in a microplate. So the catabolic enzymes and their relevant regulatory elements could be trapped by this method according the difference of fluorescence value. The mechanism of fluorescence microplate assay is shown in Fig. 2.

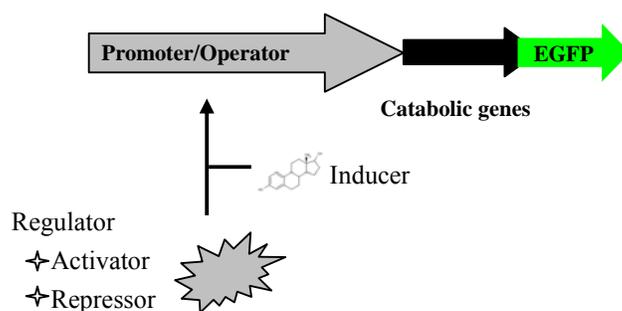


Fig. 2. Mechanism of fluorescence microplate assay. In the absence of inducer, expression of the catabolic genes is inhibited by repressor upon binding to the target operator or can not be enhanced by the inactive activators, and then EGFP expression is at the basal level. In the presence of appropriate inducer, repressors are released from the operator or the activators are activated upon binding of inducer to them. In this case, the expression of both the catabolic genes and the downstream EGFP gene significantly increased.

In our group, EGFP gene was cloned into plasmid pK18 to yield a reporter plasmid

pKEGFP-2 in which the transcriptional direction of EGFP is opposite to the *lac* promoter, so the *lac* promoter in the plasmid pKEGFP-2 can not affect the expression of EGFP. A 4.6 kb DNA fragment containing the steroid-inducible 3 $\alpha$ -HSD/CR gene from *C. testosteroni* (Xiong and Maser, 2001; Xiong, *et al.*, 2003) was cloned into pKEGFP-2 to yield another plasmid pK3 $\alpha$ -4.6-EGFP3 as a positive control. Both plasmids pKEGFP-2 and pK3 $\alpha$ -4.6-EGFP3 were transformed into *E. coli* HB101 and cultured in SIN medium containing 30  $\mu$ g/ml Kanamycin at 37°C overnight. RFU were measured directly by using Tecan GENios Pro machine after both bacteria were incubated with or without estradiol for one hour. The RFU values are given as “RFU-IAP” (RFU Increased Amount Percentage) which is defined as  $(\text{RFU}_{\text{sample}} - \text{RFU}_{\text{water}}) / \text{RFU}_{\text{water}} * 100\%$ . Results showed that the RFU-IAP of bacteria harboring plasmid pK3 $\alpha$ -4.6-EGFP3 was about 4.5% after incubation with estradiol, while there was only less than 1% for negative control plasmid pKEGFP-2. Plasmids containing chromosomal DNA fragments from bacteria were transformed into *E. coli* HB101 and induced by estradiol. Plasmids had almost the same RFU-IAP as positive control pK3 $\alpha$ -4.6-EGFP3 were supposed to have estradiol-inducible fragments. Two estradiol inducible genes were successfully obtained from another steroid-degrading strain H5 by this approach (Sang *et al.*, 2011a; Sang *et al.*, 2011b).

Compared to nucleotide sequence-based screening and function-based screening, substrate-induced gene-expression screening has some advantages. First, it can be used as an approach to obtain novel substrate inducible genes from known or unknown bacteria, even those microorganisms in the environment are resistant to culture. Since strain S19-1 is a new isolated estradiol degrading bacterium from the Baltic Sea and the pathway for estradiol degradation in bacteria remains unclear, this method is especially right for our aim. Second, it is high throughput. Third, not only the catabolic genes, but also relative regulatory elements could be obtained at the same time. After confirmation by other methods, if the inducible genes are indeed involved in the degradation of a substrate, then the research about how regulation of this gene expression could be done since the regulatory elements are also trapped in.

Fourth, when the catabolic genes and the regulatory elements are identified, this fragment could be used as a biosensor system to detect the relevant substrate from the environment.

Although substrate-induced gene-expression screening has so many advantages, this method still has some disadvantages. For example, genes for catabolic enzymes obtained by this method may sometimes be partial when they are situated at the end of a cloned genome fragment, sometimes even only get the promoter region, just like the promoter obtained from plasmid p302 in this research. So other experiments, such as PCR, need to be done for obtaining the whole gene when it is necessary, and it is not easy to get it. But the most important disadvantage is that not all the substrate-inducible genes are specific for degradation of this substrate. In Uchiyama's report, a large number of benzoate-inducible fragments containing open reading frames coding for transcriptional regulators and catabolic enzymes were obtained by this method, where benzoate was used as a substrate. But not all of them were specific for the degradation of benzoate. In this study, there are also a lot of plasmids containing chromosomal DNA fragment of S19-1 could be induced by estradiol. But few genes from those estradiol-inducible plasmids were reported to be specific involved in the degradation of estradiol after analyzing the sequence of these fragments. This is probably because some of these genes are indeed specific in the degradation of estradiol, since the biodegradation pathway of estradiol in bacteria remains unclear; or because not only pathway substrate and pathway intermediates, but unknown gratuitous inducer could also induce expression of catabolic genes (as shown in Fig. 1) (Díaz and Prieto, 2000).

### **3. Obtained Genes might be Involved in Estradiol Metabolism in S19-1**

By using the fluorescence microplate assay, 37 out of 323 colonies from the meta-genomic library were found to be induced by estradiol in the present study. Six

of them which had the highest RFU-IAP were supposed to have estradiol-inducible fragments from S19-1, so the insert fragments of these plasmids were sequenced and analyzed. Transcriptional regulators, kinases, transport-associated proteins and catabolic enzymes were found in these fragments. Formate C-acetyltransferase from plasmid p12 is an enzyme that catalyzes the reversible reaction of transformation of acetyl-CoA and formate into CoA and pyruvate. This enzyme belongs to the family of transferases, specifically those acyltransferases transferring groups other than aminoacyl groups and mainly participating in 3 different metabolic pathways: pyruvate, propanoate and butanoate metabolism (Knappe *et al.*, 1974). Serine acetyltransferase from plasmid p22 is an enzyme that catalyzes the reversible reaction of acetyl-CoA and L-serine into CoA and O-acetyl-L-serine. This enzyme also belongs to the family of transferases, specifically those acyltransferases transferring groups other than aminoacyl groups and mainly participating in cysteine metabolism and sulfur metabolism (Kredich *et al.*, 1966). Decarboxylase was found both in plasmids p44 and p302. So far it is not clear about the exact functions of this decarboxylase and whether this enzyme is involved in the degradation of estradiol. 7-cyano-7-deazaguanine from plasmid p302 catalyzes the NADPH-dependent reduction of 7-cyano-7-deazaguanine (preQ0) to 7-aminomethyl-7-deazaguanine (preQ1) (Lee *et al.*, 2007). To understand if these genes are involved in the degradation of estradiol in S19-1, some more experiments need to be performed for further confirmation.

In plasmid p26, ABC-transporters (ATP-binding cassette transporters) were found, which are named on the sequence and organization of their ATP-binding cassette (ABC) domains, and implicated in the transportation of lipids (Pohl *et al.*, 2005). ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes. They transport a wide variety of substrates across extra- and intra-cellular membranes, including metabolic products, lipids, sterols and drugs (Davidson and Chen, 2004; Pohl *et al.*, 2005). This ABC

transporter might be involved in the uptake of estradiol in S19-1. The adjacent gene of this ABC transporter in plasmid p26 is a transcriptional regulator gene *nadR* encoding nicotinamide mononucleotide adenylyltransferase repressor NadR, which acts as bifunctional kinase and repressor. Nicotinate-nucleotide adenylyltransferase participates in nicotinate and nicotinamide metabolism (Dahmen *et al.*, 1967; Kornberg and Pricer, 1951). It is not clear if these genes are involved in the degradation of estradiol, this should be confirmed by further experiments.

Genes encoding transcriptional regulator FimZ, response regulator receiver protein, transcriptional regulator LuxR, hydroxyethylthiazole kinase, phosphomethylpyridine kinase, transcriptional regulator GntR, PfkB kinase, ADP-ribosylglycohydrolase, and major facilitator superfamily protein were found in plasmid p5. In previous investigations, a LuxR-type transcription factor, TeiR, was identified as a steroid sensor in testosterone metabolism in *C. testosteroni*. It can be tightly controlled at the transcriptional level by the presence of testosterone in the culture medium (Pruneda-Paz *et al.*, 2004). The *teiR*-disrupted mutant strain was completely unable to use testosterone as the sole carbon and energy source (Göhler *et al.*, 2008; Pruneda-Paz *et al.*, 2004). Several members of the GntR-family are also reported to be involved in the degradation of aromatic compounds (Mouz *et al.*, 1999; Arai *et al.*, 1999; Arai *et al.*, 1999; Ferrandez *et al.*, 2000; Morawski *et al.*, 2000; Teramoto *et al.*, 2001; Watanabe *et al.*, 2000), while estradiol has an aromatic A ring. The genes in the estradiol-inducible plasmid p5 might be involved in estradiol metabolism, and one of these genes, *GntRx*, might be chosen to identify the function in the catabolic pathway of estradiol in S19-1 in future. So this *GntRx* was selected out for detailed analysis.

“Gnt” was first reported by BÄchi and Kornberg as belonging to genes involved in the uptake and catabolism of gluconate by *E. coli*. They found that a mutant *E. coli* K12 could not take up and utilize gluconate, and proposed that this phenotype was due to the malfunction of a negative control gene *gntR*, which specified the activity of a gluconate system (BÄchi and Kornberg, 1975). This family proteins share amino

acid sequence similarities in the N-terminal region, which is predicted as a highly conserved helix-turn-helix motif and less conserved effector binding and oligomerization domain (Fig. 3) (Haydon and Guest, 1991).



Fig. 3. Schematic representation of the conserved domains of GntR. GntR family members have a highly conserved N-terminal HTH DNA binding domain and a less conserved effector binding and oligomerization domain (E-b-O domain).

The substrate binding region mainly locates at the C-terminus, which displays high heterogeneity among the GntR family (Tropel and Meer, 2004). GntR members were classified into four subfamilies FadR, HutC, MocR and YtrA by Rigali based on effector binding domain among 270 members (Rigali *et al.*, 2002). Almost all the GntR-like regulators involved in the degradation of aromatic compounds including AphS, BphS and VanR belong to the FadR subfamily, which was named after the fatty acid biosynthesis and degradation regulator. Proteins belonging to the FadR subfamily consist of an all-helical C-terminal domain with six or seven  $\alpha$ -helices. The average C-terminal length of this subfamily is about 150-170 amino acids (Rigali *et al.*, 2002). The similarity between the 6 GntRs (AphS, BphS, Orf0, PaaX, VanR1 and VanR2) involved in the degradation of aromatic compounds varies from 12.2-40.7% (Mouz *et al.*, 1999; Arai *et al.*, 1999; Ferrández *et al.*, 1998; Morawski *et al.*, 2000; Teramoto *et al.*, 2001; Watanabe *et al.*, 2000), while the similarity of amino acid sequences between GntRx and other 6 GntRs is 9.7-16.1%. The results of multiple sequence alignment of GntRx and other six GntRs showed identical residues Arg65, Ala67, Leu71 and Gly84 in the conserved N-terminal region (numbering according to the GntRx amino acid sequence, marked in box) (Fig. 4). This suggested that *GntRx* in plasmid p5 might be involved in the degradation of estradiol in S19-1, but this should be elucidated by further experiments.

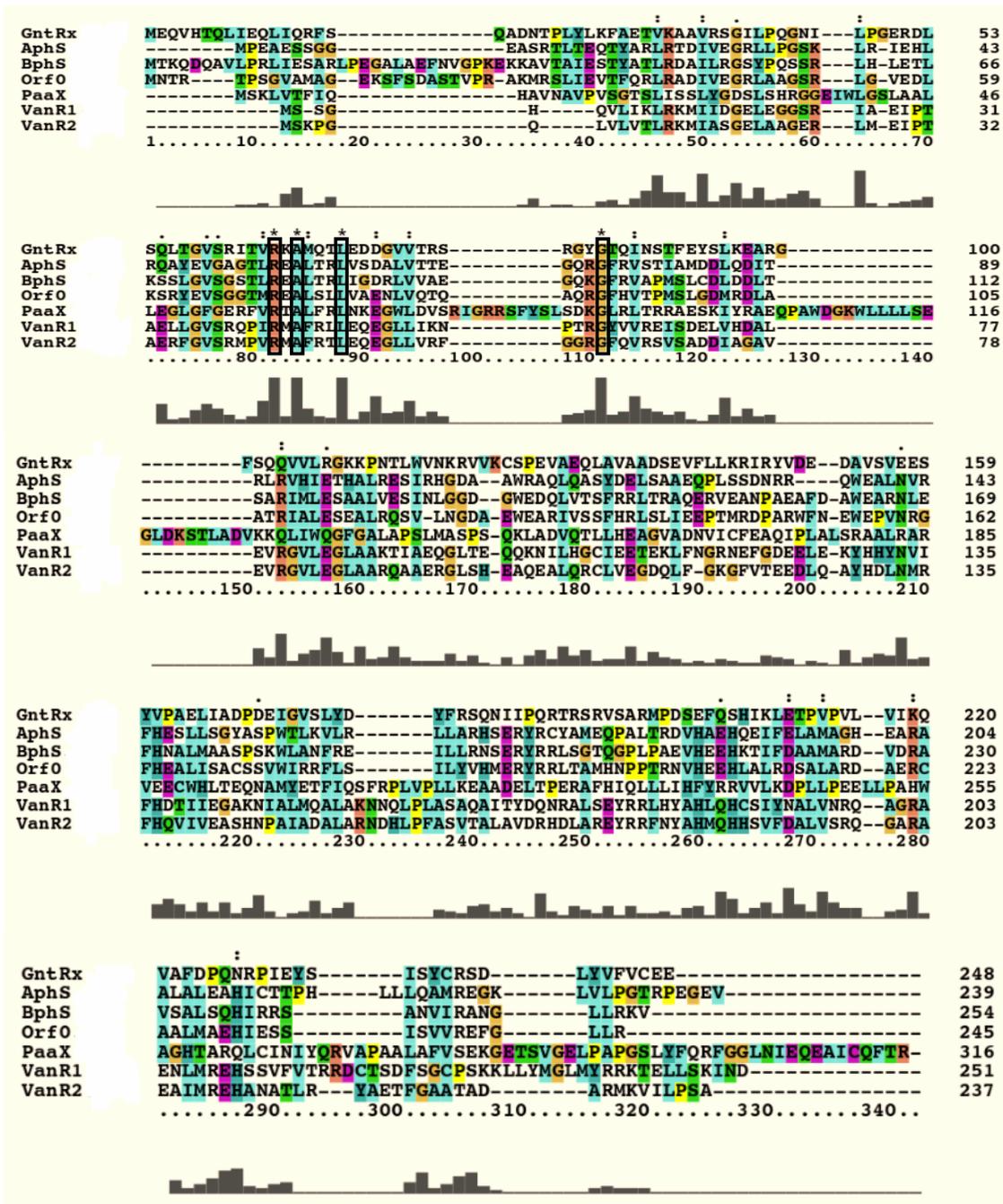


Fig. 4. Multiple alignment of amino acid sequences of GntRx and other GntRs. “\*” indicates amino acids identical among all the proteins which are indicated in box; “/” and “/” indicate amino acids of high and low similarity, respectively. Swissprot or PDB accession numbers are: AphS from *Comamonas* (BAA89295); BphS from *Pseudomonas* (BAB64312); Orf0 from *Pseudomonas* (BAA12882); PaaX from *Escherichia* (CAA66101); VanR1 from *Acinetobacter* (O24839); VanR2 from *Pseudomonas* (AJ252091).

#### **4. Conclusion**

In conclusion, the new isolated marine steroid-degrading strain S19-1 was characterized to be a member of the genus *Buttiauxella* of the *Enterobacteriaceae* family. The optimal growth conditions of strain S19-1 require the presence of NaCl (2.1%) and a temperature of 20°C. To understand the estradiol degradation pathway in this marine bacterium, the meta-genomic approach was used to isolate estradiol-inducible genes from this bacterium in combination with a fluorescence microplate assay. Transcriptional regulators, substrate transporters, kinases and catabolic enzymes were obtained by using this method. More experiments need to be done to determine which genes are indeed involved in the degradation of estradiol in S19-1. A 108-bp promoter sequence was obtained from an estradiol-inducible plasmid p302 by deletion, the putative -10 region (TAAGAT) and -35 region (TTGTCG) of which were also predicted.

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## Acknowledgements

It is a great pleasure for me to thank all the people who made this thesis possible and gave me help in my life.

First, I would like to express my most sincere acknowledgement to Prof. Dr. Edmund Maser for accepting me to study in the Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School, Schleswig-Holstein, Campus Kiel. Thank you for your patient guidance and for correcting mistakes in my Ph.D thesis. Special thanks for helping me improve my English. I will keep all this in my mind.

I would like to thank my co-supervisor Dr. Guangming Xiong for bringing me here and advising my Ph.D study. Thank you for all the advices in the experiments during these three years.

I would like to express my special and deep gratitude to Prof. Dr. Xuming Deng and Prof. Dr. Changchun Tu in China for giving me the chance to study abroad and for your encouragement.

I am deeply grateful to my boyfriend Wenjie for his love and help. I would never have gone so far without you. Warm thanks to all the Chinese friends I have met in Kiel.

Let me thank all the colleagues and staffs in our institute for their friendly help these three years. These include Dr. Hans-Jörg Martin, Frau Kubach, Dr. Andre Göhler, Dr. Claudia Röhl, Dr. Claudia Staab, Dr. Christiane Aschmann, Dr. Bettina Ebert, Dr. Michael Gulden, Dr. Heike Papavlassopoulos, Dr. Hasso Seibert, Dr. Hermann Kruse, Yanyan Han, Yingying Sang, Pu Huang, Christine Lörz, Petra Malatkova, Sabine Seibert, Jan Hintzpeter, Claudia Hirsch, Ellen Klauser, Michael Kisiela, Jochen Stegk, Tereza Hartmanova, Julia Kammann and Katrin Köbsch.

I would like to thank China Scholarship Council for the financial support of Ph.D study and Lianping Huang who works in Education Section, Embassy of the People's Republic of China, Berlin, Germany, for her kindly help through my study in Kiel, Germany.

Last but not least, I would like to thank my family for their love and support. Although I am far away from home, I can always depend on you whenever I need you.

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Zhang T., Xiong G. and Maser E., Characterization of the steroid degrading bacterium S19-1 from the Baltic Sea at Kiel, Germany, *Chem. Biol. Interact.* 191 (2011) 83-88.

Zhang T., Xiong G. and Maser E., Isolation of Estradiol-inducible Genes and a novel promoter from S19-1 by Meta-genomic Methods (to be submitted).

## **Erklärung**

Eidesstattliche Erklärung:

Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation mit dem Titel “Identification of a New Marine Steroid-degrading Bacterium S19-1 and Isolation of Estradiol-inducible Genes and a Novel Promoter from this Bacterium“ selbständig und ohne unerlaubte Hilfe angefertigt habe und dass ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt habe.

Kiel, 2011

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(Tingdi Zhang)