Identification of a new steroid degrading bacterial strain H5 from the Baltic Sea and isolation of two estradiol inducible genes

Yingying Sang^{a,b}, Guangming Xiong^a, Edmund Maser^{a,*}

^a Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunswiker Strasse 10, 24105 Kiel, Germany

^b State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, PR China

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ABSTRACT

The presence of steroid hormones in the aquatic environment is potentially threatening the population dynamics of all kinds of sea animals and public health. Environmental estrogens in water have been reported to be associated with abnormal sexual development and abnormal feminizing responses in some animals. New approaches for the bioremediation of steroid hormones from the environment are therefore urgently sought. We have previously isolated a steroid degrading bacterial strain (H5) from the Baltic Sea, at Kiel, Germany. In the present investigation, 16S rRNA analysis showed that marine strain H5 belongs to the genus *Vibrio*, family *Vibrionaceae* and class *Gamma-Proteobacteria*. To enable identification of steroid inducible genes from bacterial strain H5, a library was constructed of H5 chromosomal DNA fragments cloned into a fluorescent reporter (pKEGFP-2). A reporter plasmid pK3 α -4.6-EGFP3 containing the estrogen-inducible gene 3 α -hydroxysteroid dehydrogenase/carbonyl reductase (3 α -HSD/CR) from *Comamonas testosteroni* (*C. testosteroni*) was created as a positive control. Steroid induction could be detected by a microplate fluorescence reader, when the plasmids were transformed into *Escherichia coli* (*E. coli*) HB101 cells. With our meta-genomic pKEGFP-2 approach, we identified two estradiol-inducible genes from marine strain H5, which are obviously involved in steroid degradation. Sequencing of the pKEGFP-2 inserts and data base research at NCBI revealed that one gene corresponds to 3-ketosteroid- Δ -1-dehydrogenase from several *Mycobacterium* strains, while the other showed high similarity to carboxylesterase in *Sebadella termitidis* and *Brachyspira murdochii*. Both 3-ketosteroid- Δ -1-dehydrogenase and carboxylesterase are one of the first enzymes in steroid degradation. In addition, we identified a strain H5 specific DNA sequence of 480 bp which allows sensitive PCR detection and quantification of strain H5 bacteria in "unknown" seawater samples. Currently, the exact characterization and systematic classification of the marine steroid degrading bacterial strain H5 is envisaged, which might be used for the bioremediation of steroid contaminations in seawater.

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1. Introduction

Natural and synthetic steroid hormones are constantly excreted into the environment from human and animal sources. Some decades ago, several findings suggested that hormonally active agents interfere with sexual development and reproduction and may cause adverse health effects to all kinds of life [1–9]. In environmental studies, estrogens and other steroids were detected in seawater, lakes and rivers in different areas [10–14].

Since steroids act on the molecular level by binding to and activating their cognate intracellular steroid receptors [15], even trace concentrations of steroids in the environment may impact normal cell physiology, a fact which is also of relevance regarding the safety

and health of humans [1]. Endocrine disrupting activity has also been reported from a variety of polycyclic aromatic hydrocarbons (PAH) [16] which mimic the action of steroid hormones in the body and which have also been found in highly contaminated areas.

For example, in 1990 in Israel 100–500 pM concentrations of estrogens were found in treated sewage water used for irrigation with a peak maximum of 1260 pM in summer [11]. The same investigation detected testosterone in concentrations ranging from 200 to 1000 pM [11]. Furthermore, in China a wide range of total PAH concentrations was detected, ranging from 1304 to 3369 μ g/kg in soil collected from different wastewater-irrigated sites in Tianjing, and ranging from 2687 to 4916 μ g/kg in soil collected from different wastewater-irrigated sites in Beijing [16]. In addition, the total estrogen levels in the Xili and Shenzhen Reservoirs were in a range of with 3–11 ng/l with a mean value of 7 ng/l, where that in four rivers in Shenzhen were found to be 47–90 ng/l and 60.25 ng/l, respectively. In the seawater near the sewage discharge points they were 260–300 ng/l and 287 ng/l, respectively [13].

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

Many scientists and policy makers have attached great importance to this problem and new systems for the bioremediation of steroid hormones and other endocrine disrupting chemicals are urgently needed. Based on our long experience with the soil bacterium *Comamonas testosteroni* (*C. testosteroni*) which is able to catabolize a variety of steroids and PAH [17–20], we now seek to identify and characterize new marine bacterial strains that might be used for the bioremediation of steroids in seawater.

By using 3 α -hydroxysteroid dehydrogenase/carbonyl reductase (3 α -HSD/CR) [17], which is a key enzyme in adaptive steroid degradation in *C. testosteroni*, as an indicator system, we have recently isolated a new marine bacterium (strain H5) from the Baltic Sea at Kiel, Germany [21]. This strain was shown to degrade steroids like testosterone, estradiol and cholesterol, and could be best grown in Standard I Nutrient broth medium (SIN medium) supplemented with 1.6–4.1% NaCl [21].

In the present study, we aimed at the systematic identification and characterization of this marine bacterial strain. By Gram-staining and 16S rRNA analysis we provide evidence that strain H5 belongs to the genus *Vibrio*, family *Vibrionaceae* and class *Gamma-Proteobacteria*. Moreover, by employing a new meta-genomic system we successfully isolated two estradiol-inducible genes from the strain H5 genome. A bioinformatic approach led us to identify the genes to code for 3-ketosteroid- Δ -1-dehydrogenase and carboxylesterase which indeed both have a function in steroid catabolism. In addition, a chromosomal H5 specific DNA sequence was identified that allows for the identification and quantification of marine bacterium strain H5 in the water body with high sensitivity.

2. Materials and methods

2.1. Bacterial strains and plasmids

Host strain *Escherichia coli* (*E. coli*) HB101 (Amersham, Germany), *C. testosteroni* ATCC 11996 (Deutsche Sammlung für Mikroorganismen) and marine bacterium strain H5 (isolated from the Baltic Sea at Kiel, Germany) [21] were used for gene cloning and gene expression. The pCR2.1-TOPO kit (Invitrogen, Karlsruhe, Germany) was applied for cloning of PCR fragments. Plasmid pK18 containing the kanamycin resistance gene was a gift from Ciba Pharmaceuticals, Inc., Department of Biotechnology, Basel, Switzerland [22]. Plasmid pGEM-EGFP containing the enhanced green fluorescence protein (EGFP) gene was a gift from Peking University, China. The preparation of further recombinant plasmids was carried out following standard techniques according to Sambrook and Russel [23]. Details regarding the construction of reporter plasmids for steroid determination and plasmids for meta-genomic identification of estradiol-inducible genes are given in the respective sections of Section 3.

2.2. Growth media and growth conditions

Bacterial cells were grown in a shaker (180 rpm) in Standard I Nutrient broth medium (SIN medium) (Merck, Darmstadt) (*E. coli* and *C. testosteroni*) or in Standard I Nutrient broth medium plus with 1.5% NaCl (marine strain H5). Cultivation temperatures were 37 °C for *E. coli*, 27 °C for *C. testosteroni*, and room temperature for marine strain H5. Growth media contained 60 μ g/ml ampicillin and 30 μ g/ml kanamycin.

2.3. Restriction enzymes and other reagents

Restriction enzymes were obtained from Boehringer Mannheim, Biolabs, MBI and Amersham, and used according to the manufacturers' instructions. Ampicillin and kanamycin

were from AGS, Heidelberg. All other chemicals were of analytical grade and obtained from commercial suppliers.

2.4. Chromosomal DNA isolation

Five hundred μ l of an overnight culture of H5 cells was mixed with 50 μ l SDS (10%) at room temperature for 2 min. Then, 300 μ l phenol and 300 μ l chloroform were used twice to extract the chromosomal DNA of H5. After DNA precipitation with 1 ml ethanol and 25 μ l NaCl (5 M), the DNA was centrifuged and washed in 70% ethanol three times. Finally, the DNA was resuspended in 100 μ l TE buffer. The purified DNA was used as template for PCR reactions and other experiments (cf. Section 3).

2.5. Polymerase chain reaction (PCR)

The PCR reactions were carried out in a volume of 50 μ l in small PCR tubes in an Eppendorf thermal cycler. The reaction mixture consisted of 1 μ l or 2 μ l template DNA (50 ng), 1 μ l of primers (10 pmol/l), ultra pure water, 1 U/ μ l Taq polymerase, 10 mM of dNTPs and 25 mM MgCl₂. The thermal cycler (94 °C for 2 min $\times$ 1 cycle; 94 °C for 30 s, 45 °C for 30 s, 72 °C for 30 s $\times$ 25 cycles; 72 °C for 2 min $\times$ 1 cycle, and finally 4 °C) heated and cooled the reaction tubes to achieve the temperatures required at each step of the reaction. PCR reaction products were separated and identified by 0.8% agarose gel electrophoresis.

2.6. 16S rRNA sequence analysis of the bacterium H5

The bacterial strain H5 was grown in SIN medium supplemented with 1.5% NaCl at 27 °C overnight. After chromosomal DNA isolation, 2 μ l of the DNA suspension was routinely used for PCR. 16S rRNA genes of bacterial strain H5 were amplified by PCR with primers 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-GGT TAC CTT GTT ACG ACTT-3'. The PCR reaction products (1400 bp) corresponding to 16S rRNA of strain H5 were cloned into vector pCR2.1-TOPO (Invitrogen) to yield plasmid pTOPO-H5-16S.

2.7. Determination of the H5 titer by colony formation

Strain H5 was cultured in liquid SIN medium supplemented with 1.5% NaCl and adjusted to OD_{595 nm} = 1.0 with the same medium. To determine the bacterial titer, the H5 liquid culture was diluted to OD_{595 nm} = 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹ with SIN medium containing 1.5% NaCl. From each dilution, 300 μ l were spread on an agar plate with SIN medium (1.5% NaCl) and incubated at room temperature overnight.

2.8. Fluorescence microplate assay

H5 chromosomal DNA was isolated and digested with restriction enzymes as indicated in Section 3. The DNA fragments were cloned into reporter plasmid pKEGFP-2. GENios Pro Fluorescence Microplate Reader from Tecan, Austria GmbH was applied for fluorescence determination, at excitation: 485 nm and emission: 535 nm [24]. The stock concentration of estradiol was 266 fg/l, which was 10-fold diluted with water to a final concentration of 26.6 fg/l. To each well of 96-well black plates was added 10 μ l of 26.6 fg/l estradiol or 10 μ l of water (as control) together with 90 μ l of *E. coli* HB101 cells (OD_{595 nm} = 0.1) harbouring different plasmids, such as pKEGFP-2, pK3 α -4.6-EGFP3 or plasmids containing fragments of strain H5 DNA (for details cf. Section 3). Two or three replicate wells per assay were run for each sample. After 60 min of incubation at room temperature, the inducible genes were detected by EGFP expression shown as RFU (relative fluorescence units). The RFU values are given as "RFU-IAP"

Fig. 1. Gram-staining of the steroid degrading bacterium H5 from the Baltic Sea. Microscopic inspection (3000 $\times$) of H5 revealed this bacterium to be Gram-negative with a bacilliform shape.

(RFU Increased Amount Percentage) which is defined as $(\text{RFU}_{\text{sample}} - \text{RFU}_{\text{water}}) / \text{RFU}_{\text{water}} \times 100\%$.

2.9. Basic Local Alignment Search Tool (BLAST) for 16S rRNA analysis and gene annotation

For both analysis of strain H5 16S rRNA and identification of the two estradiol inducible genes found in marine strain H5 with our meta-genomic system, the Basic Local Alignment Search Tool (BLAST) (via website <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was applied.

DNASTAR Lagergene EditSeq was used to translate the DNA sequences corresponding to estrogen-induced genes in the microplate assay. The resulting amino acid sequences were used to query the protein database at the National Center for Biotechnology Information (NCBI) using the blastp algorithm. Sequences were annotated based on similarity to previously characterized proteins.

3. Results

3.1. Gram-staining of the bacterium H5

As shown in Fig. 1, bacterial strain H5 which was isolated from the Baltic Sea at Kiel, Germany, is a Gram-negative bacterium [21] with a bacilliform shape.

3.2. 16S rRNA sequence analysis of the bacterium H5

Sequencing of plasmid pTOPO-H5-16S revealed H5 belonging to the genus *Vibrio*, family *Vibrionaceae*, and class *Gamma-Proteobacteria* (Fig. 2). The 16S rRNA sequence of strain H5 (GenBank accession number: HQ663851) showed 99% identity with the 16S rRNA sequence of both *Vibrio porteresiae* strain MSSRF31 (GenBank accession number: EF488080) and *V. porteresiae* strain CAIM 662 (GenBank accession number: HM749744).

3.3. Specific identification in seawater and titer determination of strain H5

3.3.1. Determination of the H5 titer by colony formation

Strain H5 was cultured in liquid SIN medium and spread on an agar plate with SIN medium (1.5% NaCl) and incubated at room temperature overnight. The number of colonies obtained corresponded to the concentration of the diluted H5 bacteria and could be determined in the range between 4×10^7 and 5×10^7 cells/ml, when bacterial H5 culture was at $\text{OD}_{595 \text{ nm}} = 1.0$ (Table 1).

Table 1

Determination of the bacterial strain H5 titer.

Dilution times	The number of colonies	Titer (cells/ml) ($\text{OD}_{595 \text{ nm}} = 1.0$)
10^{-5}	120	4×10^7
10^{-6}	15	5×10^7
10^{-7}	0	–

300 μl of diluted H5 bacteria were spread on an agar plate and incubated at room temperature overnight. The titer of strain H5 (cells/ml) corresponded to the number of colonies obtained on the agar plate. When the respective dilution times were considered, the titer of strain H5 ($\text{OD}_{595 \text{ nm}} = 1.0$) ranged from 4×10^7 cells/ml to 5×10^7 cells/ml.

3.3.2. Specific identification and titer determination of strain H5 in seawater by PCR

To specifically identify and quantify H5 bacteria in seawater samples via PCR, an H5 specific DNA template was needed. 3,17 β -Hydroxysteroid dehydrogenase (3,17 β -HSD) is known as an important enzyme involved in bacterial steroid degradation [25]. According to the sequence of the 3,17 β -HSD gene in *C. testosteronei*, two primers P3-17L: 5'-CAT ATG ACA AAT CGT TTG CAG-3' and P3-17R: 5'-GGA TCC ATC TAT AGC CCC ATG-3' were used to identify a possible 3,17 β -HSD orthologous gene in the H5 chromosomal DNA that could be used as the above mentioned template. Unfortunately, we did not find the specific 3,17 β HSD PCR fragment, obviously because the 3,17 β -HSD gene is either not present in H5 or has quite different sequences that do not match the two primers used. However, a non-specific band with a molecular weight different from the anticipated 3,17 β -HSD gene was found. This DNA fragment was cloned into pCR2.1-TOPO to yield plasmid pTHM7 and, after sequencing, shown that it indeed did not represent the 3,17 β -HSD gene. To test, if this insert in pTHM7 could be used as template for specific H5 identification and titer determination via PCR, two primers were prepared according to the pTHM7 sequence. The primers for pTHM7 were pML: 5'-GGCGACTGGCATCATAT-3' and pMR: 5'-GGAGCGTTAGTGGCTGT-3'. A PCR product of 480 bp from pTHM7 was obtained which turned out to be specific for bacterial strain H5 identification in seawater samples when tested with a variety of other bacteria as templates, e.g. *E. coli* HB101, *E. coli* BL21, marine bacterium strain S19-1, and *C. testosteronei* (Fig. 3a). Marine strain S19-1 is another steroid degrading bacterium that has been isolated from the Baltic Sea in our lab. Plasmid pTHM7 containing the "M7" fragment from H5 chromosomal DNA served as the positive control. As shown in Fig. 3a, only the bacterial strain H5 from seawater (lane 5) and the positive control pTHM7 (lane 6) yielded the specific 480 bp PCR fragment upon agarose gel electrophoresis.

The "M7" chromosomal H5 DNA fragment could also be used for strain H5 titer determination in seawater samples. As shown in Fig. 3b, the 480 bp fragment could be clearly found in lane 4 when the bacterial H5 culture was diluted into a concentration corresponding to an $\text{OD}_{595 \text{ nm}} = 10^{-3}$ (approximately 5×10^4 cells/ml or 50 cells/ μl). This means that only fifty bacterial H5 cells also could be detected by this PCR approach when 1 μl of the diluted solution was applied as template in the PCR reaction. Hence, primers pML and pMR which were prepared according to the sequence of the pTHM7 insert from the "M7" chromosomal H5 DNA fragment can be used to determine the concentration of the marine bacterium H5 quickly, specifically and with high sensitivity in the water body.

3.4. A new meta-genomic system for steroid inducible gene isolation from the marine bacterium H5

3.4.1. Construction of the meta-genomic reporter plasmid pKEGFP-2 and plasmid pK3 α -4.6-EGFP3 as positive control

Initially, the enhanced green fluorescence protein (EGFP) gene was amplified by PCR from plasmid pGEM-EGFP and then cloned into vector pK18 between its BamHI and EcoRI sites to yield

Fig. 2. Phylogenetic analysis of the 16S rRNA sequence of the bacterium H5 from the Baltic Sea. According to its 16S-rRNA sequence, the bacterium H5 belongs to the genus *Vibrio*, family *Vibrionaceae*, and class *Gamma-Proteobacteria*. The GenBank accession number for the 16S rRNA gene sequences of strain H5 is HQ663851. All other 16S rRNA sequences involved in this tree were obtained from the GenBank at the National Center for Biotechnology Information. The phylogenetic tree was constructed by the neighbour-joining method using the CLUSTAL W program. The branch lengths represent the evolutionary distances between two consecutive nodes. The bar means 1 nucleotide substitution per 100 nt. Bootstrap analysis was performed by generating 1000 reiterations. Bootstrap values are indicated for each node.

the kanamycin-resistance reporter plasmid pKEGFP-2 (Fig. 4). 3α -HSD/CR in *C. testosteronei* is a steroid-inducible key enzyme in the degradation of steroid compounds [19,20,26–29] and was used in our laboratory to establish a new biosensor system for steroid detection in the environment [24,30]. As shown in Fig. 4, a 4.610 kb DNA fragment containing the 3α -HSD/CR gene together with its

regulatory region from the *C. testosteronei* chromosomal DNA was cloned into the reporter plasmid pKEGFP-2 at its BamHI and Sall sites to yield plasmid pK3 α -4.6-EGFP3. Plasmid pK3 α -4.6-EGFP3 was transformed into *E. coli* HB101 to yield a biosensor system for steroid-regulated genes [24,30]. Steroid-dependent induction of the 3α -HSD/CR gene should lead to an enhanced

Fig. 3. Identification of the bacterial strain H5 by PCR. Two primers were designed to detect a specific template fragment of the chromosomal DNA from H5, and to determine the titer of H5 bacteria in different samples. For comparison and specificity determination, some other bacterial strains (*E. coli*, *C. testosteronei*, S19-1) were included in this experiment. The 480 bp PCR fragments obtained were separated on a 0.8% agarose gel and stained by ethidium bromide. (a) Lane 1: *E. coli* HB101; lane 2: *E. coli* BL21, lane 3: S19-1 (a formerly identified marine bacterium from the Baltic Sea); lane 4: *C. testosterone*; lane 5: strain H5 of the present study; lane 6: plasmid pTHM7 harbouring the H5 template sequence; M: DNA marker. (b) M: DNA marker, lane 1: plasmid pTHM7 harbouring the H5 template sequence; lanes 2–8: different dilutions of H5 ($OD_{595\text{nm}}$) that were used for the PCR reaction; lane 2: $OD = 1.0$; lane 3: $OD = 10^{-2}$; lane 4: $OD = 10^{-3}$; lane 5: $OD = 10^{-4}$; lane 6: $OD = 10^{-6}$; lane 7: $OD = 10^{-8}$ and lane 8: $OD = 10^{-10}$. The dilution of H5 corresponded well with the intensity of the specific band at 480 bp.

Fig. 4. A meta-genomic system for the detection of steroid inducible genes in H5. The EGFP gene was cloned into vector pK18 to yield the reporter plasmid pKEGFP-2. As a positive control, a 4.6 kb DNA fragment containing the 3α -HSD/CR gene from *C. testosteronei* was cloned upstream of the EGFP gene to yield plasmid pK3 α -4.6-EGFP3. To identify steroid inducible genes, H5 chromosomal DNA was digested with XbaI and cloned into the reporter plasmid pKEGFP-2. The different H5 DNA fragments are indicated as H5X. Steroid inducible genes could be detected with this system due to the resulting increase in fluorescence (RFU = relative fluorescence units). For details see Section 2.

expression of the EGFP gene. Therefore, plasmid pK3 α -4.6-EGFP3 was used as a positive control to select estradiol inducible genes in strain H5. Steroid-mediated gene induction was recorded via the corresponding increase in fluorescence (relative fluorescence units = RFU).

For meta-genomic identification of hitherto unknown steroid inducible genes from the marine bacterium H5, plasmid pKEGFP-2 (Fig. 4) was used. Digested chromosomal DNA fragments of the marine strain H5 were ligated into the multiple cloning site (MCS) upstream of the EGFP gene in pKEGFP-2. Steroid responsive DNA fragments from the H5 chromosome could now be identified via EGFP induction and the corresponding increase in RFU (Fig. 4).

3.4.2. Estradiol inducible gene identification with meta-genomic plasmid pKEGFP-2

To obtain DNA fragments for the meta-genomic approach, chromosomal DNA was isolated from overnight cultures of H5. The DNA was digested with XbaI and the DNA fragments obtained were cloned into plasmid pKEGFP-2 at the MCS to yield several hundred meta-genomic plasmids. The ligated plasmids were transformed into *E. coli* HB101, spread on a kanamycin containing agar plate and cultured overnight. Single colonies obtained were inoculated in kanamycin containing liquid medium and cultured overnight again. Fluorescence detection was performed in 96-well black plates containing 10 μ l of an estradiol solution. Considering the quenching effect of fluorescence [24] and other influence factors, the estradiol solution was adjusted to a concentration of 26.6 fg/l. After an incubation period of 60 min at room temperature RFU were measured. By this meta-genomic approach, two *E. coli* HB101 colonies were identified that harbour plasmids with estradiol-inducible DNA fragments from H5, pKEGFP-H5-38 and pKEGFP-H5-24.

3.4.2.1. Identification of 3-ketosteroid- Δ -1-dehydrogenase in pKEGFP-H5-38. Restriction analysis of pKEGFP-H5-38 (Fig. 5) revealed an insert of 6.0 kb which contained multiple genes. Therefore, pKEGFP-H5-38 was additionally digested with SphI to yield the new plasmid pKEGFP-H5-38S2 with an insert size of 1.3 kb (Fig. 5). Fluorescence microplate assays with the various plasmids transformed in *E. coli* HB101 revealed a strong increase in RFU

Fig. 5. Identification of a first steroid inducible DNA fragment from H5. Out of several hundred plasmids obtained from our meta-genomic approach, plasmid pKEGFP-H5-38, containing a chromosomal XbaI DNA fragment from H5 of about 6.0 kb, was expressed in *E. coli* HB101 and shown to harbour an estradiol inducible gene. Restriction with SphI yielded a smaller fragment of about 1.3 kb in pKEGFP-H5-38S2, which was also estradiol inducible. pK3 α -4.6-EGFP3 served as the positive control, empty vector pKEGFP-2 was the negative control. Upper panel: relative fluorescence (RFU) signals; lower panel: restriction sites; $n = 3$.

with the positive control pK3 α -4.6-EGFP3 and a very weak signal with the negative control (empty plasmid) pKEGFP-2 upon estradiol induction (Fig. 5). Because pKEGFP-H5-38 and pKEGFP-H5-38S2 yielded nearly identical fluorescence, we inferred that the estradiol-inducible gene was present within the 1.3 kb insert of pKEGFP-H5-38S2 (Fig. 5).

After sequencing of the insert in pKEGFP-H5-38S2, a new gene comprising 801 bp and 266 amino acids (aa) was identified, which started 107 bp downstream the 5' end of the 1.3 kb insert. Analysis with the DNASTAR Lagergene EditSeq software program and blasting in protein databases at NCBI resulted in the identification of a conserved domain within the gene insert of pKEGFP-H5-38S2 that relates to aa 10–43 of the enzyme 3-ketosteroid- Δ 1-dehydrogenase of *Mycobacterium* species (*Mycobacterium gilvum* PYR-GCK, *Nocardia farcinica* IFM 10152, *Mycobacterium smegmatis* str. MC2 155). The Bit Scores of two of these related *Mycobacterium* gene domains (PRK12837 and PRK12844) are 38.65 and 36.66, respectively, and their e-values are 1.27×10^{-03} and 5.43×10^{-03} . The corresponding gene sequence of strain H5 is shown in Fig. 6.

3.4.2.2. Identification of carboxylesterase in plasmid pKEGFP-H5-24. The second estradiol inducible plasmid was pKEGFP-H5-24 that contained a 9.0 kb insert. Plasmid pKEGFP-H5-24 was digested with Sall, ClaI, and HincII. After ligation, plasmids pKEGFP-H5-24S1, pKEGFP-H5-24C2 and pKEGFP-H5-24H2 were obtained, respectively (Fig. 7). *E. coli* HB101 cells harbouring the plasmids were incubated with estradiol, resulting in an increase of RFU with pKEGFP-H5-24, pKEGFP-H5-24S1, pKEGFP-H5-24C2 and pK3 α -4.6-EGFP3 (as the positive control) (Fig. 7). No increase in RFU was found with pKEGFP-H5-24H2 and pKEGFP-2 (negative control). Based on these results, the short 584 bp insert of pKEGFP-H5-24C2 was sequenced.

After sequencing the insert of pKEGFP-H5-24C2, an estradiol-inducible gene was identified. According to an analysis with the DNASTAR Lagergene EditSeq software program and querying the protein databases at NCBI, it turned out that this gene has high similarity with the enzyme carboxylesterase in *Sebadella termitidis* ATCC 33386 (GenBank accession number: YP_003307168) and *Pantoea* sp. aB (GenBank accession number: ZP_07379392). In both cases the identity was more than 40%, and the e-values were 5×10^{-24} and 9×10^{-24} , respectively.

The gene coding region was identified between 88 and 513 bp on the 584 bp fragment (Fig. 8). Upstream of the ATG start codon, the promoter sequences (–10 and –35) could be identified, together with the SD region for ribosome binding. Downstream of the stop codon of the carboxylesterase gene, there is a 71 bp spacing until the start of the EGFP gene. The fact that no other promoter sequence and ribosome binding site could be found between the genes for carboxylesterase and EGFP indicates that estradiol inducibility of EGFP is mediated via the 88 upstream promoter sequence of carboxylesterase. This is further evidence for the finding that estradiol induces carboxylesterase expression.

4. Discussion

The data presented in this report indicate that the newly identified marine bacterium H5 belongs to genus *Vibrio*, family *Vibrionaceae*, and class *Gamma-Proteobacteria*. The taxonomic relationship was documented by phenotypic similarities as Gram-staining and the 16S rRNA sequence of bacterium H5. A specific chromosomal DNA sequence in H5 was found that allowed for quick and specific identification and titer determination of strain H5 in “unknown” water samples by PCR. In our experiments, the specificity of strain H5 detection was proven by comparing with bacteria *E. coli* HB101, *E. coli* BL21, marine bacterium strain S19-1 (which is

another steroid degrading bacterium that has been isolated from the Baltic Sea in our lab) and *C. testosteronei* ATCC 11996. However, because there are many more bacterial species in natural aquatic systems, the specificity of this method for marine strain H5 must be further checked in the future. On the other hand, due to the relatively small size of the 480 bp PCR fragment, DNA isolates without cells might be sufficient to detect the presence of strain H5, respectively, its chromosomal DNA or fragments thereof, in “unknown” seawater samples.

To further substantiate that strain H5 endows the machinery necessary for steroid degradation, a meta-genomic approach was performed to identify steroid-responsive genes within the H5 genome. The first step was to prepare a biosensor system for steroid-dependent gene induction. We initially cloned the EGFP gene from plasmid pGEM-EGFP into plasmid pK18 to obtain plasmid pKEGFP-2. Then, the steroid inducible gene 3 α -HSD/CR from *C. testosteronei* was introduced into plasmid pKEGFP-2 to yield plasmid pK3 α -4.6-EGFP3 which served as the positive control. With this method, steroid-mediated gene induction could be determined as a result of an increased EGFP expression and could be measured as a result of an increase in fluorescence (RFU). Next, plasmid pKEGFP-2 which was also suitable for meta-genomic studies, was used as a reporter system for the identification and isolation of steroid inducible genes from the bacterium H5. We made use of the fact that plasmid pKEGFP-2 contained two multiple cloning sites (MCS) both upstream and downstream the fluorescence reporter gene (EGFP), which was convenient for sequencing and other further research to identify the steroid responsive fragments from strain H5 chromosomal DNA.

Finally, strain H5 chromosomal DNA was digested and the fragments cloned into the meta-genomic reporter plasmid pKEGFP-2. After steroid-responsive gene induction through estradiol exposure and subsequent fluorescence measurement, two positive clones, pKEGFP-H5-24 and pKEGFP-H5-38, were identified. After sequencing and BLAST identification at NCBI, it turned out that plasmid pKEGFP-H5-24C2 harbours a gene coding for carboxylesterase, while pKEGFP-38S2 was shown to contain a fragment with high identity to 3-ketosteroid- Δ 1-dehydrogenase.

Although not catalyzing the breakdown of the steroid nucleus directly, carboxylesterase is an important enzyme in the steroid degradation pathway. Normally, steroids like estrogens are excreted in their conjugated form. However, conjugated estrogens cannot be digested by bacteria directly, but must undergo a preceding deconjugation step first. Carboxylesterase is an enzyme catalyzing the deconjugation of a variety of estrogen-esters to form free estrogens which then could be degraded by bacteria [31]. Therefore, the gene coding for carboxylesterase which we isolated in our studies might be an important factor for steroid degradation in the marine bacterial strain H5.

For example, in Stonecutters Island sewage treatment work (STW) in Hong Kong, which applies the technique of “Chemically Enhanced Primary Treatment (CEPT)” for wastewater cleaning, e.g. without biological treatment, a removal of estrogens does not occur [13]. A similar phenomenon was found in one STW in Italy [32]. In other words, biological treatment of wastewater, involving the action of steroid degrading bacteria, is required to remove estrogens and other steroids from wastewater [31]. As mentioned above, most of the steroids are excreted from animal and human sources in their conjugated form which requires deconjugation by enzymes like carboxylesterase to make the steroid nucleus available for bacterial degradation upon biological treatment of wastewater.

The other estradiol-inducible gene we have isolated codes for 3-ketosteroid- Δ 1-dehydrogenase, an enzyme which is directly involved in the breakdown of the steroid nucleus. Moreover, this enzyme is one of the first to initiate steroid degradation. As shown in Fig. 9, testosterone, for example, first undergoes

AAGACTAGCGTGACTGTATGGACACCATTATCACCACACGAGATACCGCGTGTGCGA
 -35 -10
 TTAACCCAGTGATTGGTGAAGAAGAGCTCTATTCTCTCCAGCTGCC
 SD
 ATGCCAGAAGTGACCAAAACAGTGCTTGTGCTGGCGGCGGTCCTGGCGGAATGCAAGCT
 Start
 GCTCTAAGTGCAGCCAAACGCCATCATAACGTCATTCTTTGTGAAGCAACGAATAAGTTGG
 GTGGTCAAATTAAGTGTGAAGCCCATGTAGACTTTAAAAAGAACTACTATGGCTTCTCAA
 ATGGTTGATTACCAAATCGGCAAATACGACAACATAGAAGTTCGCCTCAATACCAAGGTC
 GATAACGCATTGGTGAAGCGATCAATCCGGATTCACTGATCTGCGCTATTGGCGCAACAC
 CGATTCAACCCTAAGATTCTGGCATCGATGATCCTAGAGTGGTGTACTGCACTGAATTAGC
 GAAAGATGTTCTGAAGTCGGTGAAAAGGTCGTGATCATCGGGGCGGCTCTTGTGGGCTC
 AGAATCTGCTATTCACTTTAAGCGTGAACATAAACAGGTCACCTTAATTGAAGCTCGCGAT
 GATTTTGCGGTTGACTCCAAATGTGTTCCACAAGATGGGGCTAAATATCGAATTGAGAGAAG
 GCATTGATGTACATCTTGGCACCAAGTGAAGCGATCACTGCCGAAGGTGTGGTTGCGGT
 TGACCAACAAGGTCAACGCCACGCTCTTCCCGCAGATACTATCTTGCTCAGTGGGTATGA
 GATCTCGCGAAGATGAGCGCGAAGCTTTGCGCCAAAGCATTCTCGATTTACCCCGATTGGT
 GACTGTGTCGCTCCAGGAAAAGTCGCTGCCGCGAGTCCATCATGGCCACTACGCAGCAATGG
 ATCTCTAA
 Stop

Fig. 6. The insert in pKEGFP-H5-38S2 was identified as being the gene for 3-ketosteroid- Δ^1 -dehydrogenase. Since pKEGFP-H5-38S2 contains only 1.3 kb but shows high estradiol inducibility (Fig. 5), the corresponding SphI-XbaI insert was sequenced and found to exhibit high similarity to 3-ketosteroid- Δ^1 -dehydrogenase of some *Mycobacterium* species. In the sequence indicated are the –10 and –35 hypothetical promoter sequences, the ribosome binding site (SD), the ATG start codon (in italics), and the stop codon. The GenBank accession number for 3-ketosteroid- Δ^1 -dehydrogenase gene sequence of strain H5 is HQ663850.

dehydrogenation of the 17 β -hydroxyl group to androst-4-ene-3,17-dione. This step is immediately followed by dehydrogenation of the A-ring of androst-4-ene-3,17-dione to androsta-1,4-diene-3,17-dione, which is catalyzed by 3-ketosteroid- Δ^1 -dehydrogenase. Androsta-1,4-diene-3,17-dione then undergoes 9 α -hydroxylation to produce 9 α -hydroxy-1,4-androstadiene-3,17-dione in which non-enzymatic B-ring opening occurs to yield 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione. The latter is hydroxylated at the C-4 position to produce an A-ring catechole which is cleaved between C-4 and C-5 through a *meta*-cleavage reaction. Further degradation of the steroid nucleus to CO₂ and H₂O is described in [33].

In conclusion, we have isolated a new bacterial strain from the marine environment, which contains genes associated with steroid degradation. By Gram-staining and 16S-rRNA analysis, we have identified this strain as belonging to the genus *Vibrio*, family *Vibrionaceae* and class *Gamma-Proteobacteria*. Following a meta-genomic approach, two estradiol inducible genes were identified that are involved in steroid catabolism. These molecular data indicate that strain H5 is likely to be able to degrade steroids. In the future, knock-out studies of the two genes will be performed to infer their physiological relevance in the steroid catabolic pathway. Furthermore, the applicability of marine strain H5 in the bioremediation of environmental steroid contaminations is envisaged.

Fig. 7. Identification of a second steroid inducible DNA fragment from H5. Out of several hundred plasmids obtained from our meta-genomic approach, plasmid pKEGFP-H5-24, containing a chromosomal XbaI DNA fragment from H5 of about 9.0 kb, was expressed in *E. coli* HB101 and shown to harbour an estradiol inducible gene. Restriction with different restriction enzymes yielded three smaller fragments that were tested for estradiol inducibility. From these, the 0.584 kb SalI–ClaI fragment (pKEGFP-H5-24C2) and to a lesser extent, the 4.7 kb SalI–XbaI fragment (pKEGFP-H5-24S1) were shown to be estradiol inducible. The 1.5 kb HincII–XbaI fragment (pKEGFP-H5-24H2) did not respond to estradiol induction. pK3α-4.6-EGFP3 served as the positive control, empty vector pKEGFP-2 was the negative control. Upper panel: relative fluorescence (RFU) signals; lower panel: restriction sites; *n* = 3.

GCTGACCATGCTTAAGGCGAAATTTGCTGCCCCATTATGCTGGCAAAATCAACTAC
 Sall -35 -10
GATCAGATCCAATTATTACTGGACCTGCCATGACGCGCATCGGGTCATTCCACGGCGTTT
 SD Start
TCATTCCGTTATTAGACTCCAGCGTCAATATGCCCATGTTTGCAATGCGTTTAAACGG
AAGGTGCGCAGCAGCTCGTCACGATTGGAAAAACACATTGCTGCGTTTATTCGTCAGG
GTAACCGTCAAATGCGATTGATGTGGAATGGAAACCGTGAATAAAACACGGCCACCA
ACGGTGAGTCACTTTATGTTTTCGATGCGAATACGAAAAACAGCGTGCTGTATCGCACTG
ACCAAGCCTATCAAACGAAAGATATTATTGAGTTGATGGATCAGGATTATCGCCTGAGCG
AGGAAGATAAGAGCAAGTTAATCCATTCCGTGTTAAACGGACGCTGGTTCAGTCAGCAAC
TTGATGAACATTACCACTCACCTTCGCTGTGGTAAAGGCATTCTTGTAAATAACAACC
 Stop
GAGCAACCTGCCAGCGCTCTCTTTTCCAATCGTTTCATGGGAAT(CGAT)A
 (ClaI)

Fig. 8. The insert in pKEGFP-H5-24C2 was identified as being the gene for carboxylesterase. Since pKEGFP-H5-24C2 contains only 0.584 kb but shows high estradiol inducibility (Fig. 7), the corresponding Sall–ClaI insert was sequenced and found to exhibit high similarity to carboxylesterase of *Sebadella termitidis* ATCC 33386 and *Brachyspira murdochii* DSM 12563. In the sequence indicated are the –10 and –35 hypothetical promoter sequences, the ribosome binding site (SD), the ATG start codon (in italics), and the stop codon. The GenBank accession number for carboxylesterase gene sequence of strain H5 is HQ663852.

Fig. 9. Function of 3-ketosteroid-delta-1-dehydrogenase in testosterone degradation in *C. testosteroni*. 3-Ketosteroid-delta-1-dehydrogenase, as being identified in H5 as the insert in pKEGFP-H5-38S2, catalyzes the conversion of androst-4-ene-3,17-dione to androsta-1,4-diene-3,17-dione which is known to be one of the first steps in steroid degradation in *C. testosteroni*.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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