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Analysis and characterization of eight estradiol inducible genes and a strong promoter from the steroid degrading marine bacterial strain S19-1

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ABSTRACT

Buttiauxella strain S19-1 is a new marine bacterium, isolated from the Baltic Sea, which can degrade steroids. In this report, a meta-genomic approach was used to isolate estradiol inducible genes from S19-1. *Sal*I-fragments from the chromosomal DNA of S19-1 were ligated into plasmid pKEGFP2 bearing an EGFP gene as the reporter system. All resulting plasmids harboring *Sal*I-fragments were transformed into *Escherichia coli* HB101 to measure the relative fluorescent units (RFU). *E. coli* cells showing higher RFU after estradiol induction than those without estradiol induction, were selected and the respective plasmids were sequenced. Sequences of 8 positive plasmids were analyzed and aligned by BLAST. Among the predicted genes we found similarities to the major facilitator superfamily, glycerol dehydratase activator, formate acetyltransferase activating enzyme, histidinol-phosphate/aromatic aminotransferase, ABC-transporter, transcriptional regulator nadR, lipoate-protein ligase A, and alcohol phosphatidyl-transferase. Interestingly, one of the *E. coli* cell clones (containing plasmid p302) showed up in green color by normal light microscopy, which indicated that a strong promoter was present in this plasmid. Sequencing and deletion-mutagenesis revealed that the putative promoter comprises a 108 bp DNA fragment within p302, from which the putative −10 and −35 regions are TTTGAT and TTGGTT, respectively. The promoter might be used to construct S19-1 mutants in which steroid degradation occurs at high levels.

1. Introduction

Contaminated soil and water by hormonally active agents represent a potential health risk to humans and all kinds of animals [1,2]. Hormonally active agents are mainly steroids and include estrogens, gestagens and androgens. Elimination of these stable compounds from the contaminated environment is an absolute requirement for the sustainable development of the biosphere [3]. 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 [4–7]. Although a series of bacterial strains have been reported to degrade estradiol, the degradation pathway of estradiol in these microorganisms still remains unclear [8–10].

Studies on steroid contaminations started in the 1990s, in which estrogens and other steroids were detected in sea water, lakes and rivers in different areas [2,11–13]. These steroidal estrogens have especially been shown to be the main contributors to the estrogenic activity observed in aquatic systems contaminated with sewage treatment effluents [13]. Most of polluted rivers by estrogens finally flow into the ocean, and also cause contaminations in the marine environment, a problem which gets more and more attention.

In 2008, selective agar plates containing steroids were used for isolating bacteria from the Baltic Sea at Kiel, Germany, which can degrade steroids [14]. Twelve bacterial isolates capable of degrading 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 ranging from 0.6% to 5.1% and at a temperature range from 5 to 27 °C [15]. This suggests that S19-1 might be used for the clearance of different contaminated oceans and other aquatic environments in the future.

Although it has been demonstrated that marine strain S19-1 can use estradiol and other steroids as the carbon and energy source [15], the degradation pathway of estradiol in this strain remains unclear. To get a starting point for studying estradiol metabolism in S19-1, we focused on the isolation and characterization of estradiol-inducible genes and relevant regulatory elements from S19-1 by a metagenomic approach.

In general, the expression of most catabolic enzymes involved in the degradation of aromatics and steroids is inducible by the substrates and/or metabolites of the catabolic pathway [16]. Also, expression of the related regulatory elements is dependent on

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the presence of inducer molecules [17,18]. As previously reported by Uchiyama et al., a substrate-induced gene-expression screening (SIGEX) method was developed for the isolation of catabolic genes from environmental metagenome libraries [19]. This method was then improved by measuring relative fluorescence units (RFU) instead of fluorescence-activated cell sorting (FACS) by our group [20]. Two estradiol-inducible genes were successfully isolated from another steroid-degrading marine bacterial strain H5 by this method [21].

In the present work, fluorescence microplate assay (FMA) established in our lab, which is also a substrate-induced gene-expression screening method, was used for isolating estradiol-inducible genes and their regulatory elements from the steroid-degrading bacterium strain S19-1. Not only catabolic enzymes and relevant regulatory elements, but also inducible promoters can be obtained by this method. Accordingly, plasmid p302 containing an estradiol-inducible fragment from S19-1 was shortened by different restriction enzymes and PCR amplification until a 108 bp promoter sequence was obtained. The putative −10 region and −35 region of this promoter were predicted. Furthermore, the activity of this new promoter was also compared to that of the *lac* promoter.

2. Materials and methods

2.1. Bacterial strains and plasmids

Host strain *Escherichia coli* HB101 (Promega, Amersham, Germany) was used for gene cloning and other experiments. Plasmid pK18 containing the kanamycin resistance gene was a gift from Ciba Pharmaceuticals, Inc., Department of Biotechnology, Basel, Switzerland [22]. Plasmid pKEGFP-2 containing an EGFP gene was used as vector for isolating estradiol-inducible genes. Plasmid pK3α-4.6-EGFP3 was constructed by our group [20] and served as positive control.

2.2. Growth media and growth conditions

E. coli HB101 cells were grown in Standard I Nutrient broth medium (SIN) obtained from Merck (25 g/l, containing 15.0 g of peptones, 3.0 g of yeast extract, 6.0 g of sodium chloride and 1.0 g of D (+)-glucose), and incubated at 37 °C in a shaker at 180 rpm. If necessary, 30 μg/ml kanamycin was added into the medium.

2.3. 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. TOPO TA cloning kit was from Invitrogen. QIAGEN Plasmid Midi Kit was from QIAGEN. Steroids including estradiol were purchased from Sigma. Ethanol, phenol and chloroform were of analytical grade and obtained from Roth.

2.4. Isolation of chromosomal DNA of 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. Two ml of overnight culture of S19-1 cells was harvested by centrifugation at 13,000 rpm for 20 s and then resuspended in 1 ml distilled water at room temperature for 30 min containing 100 μg lysozyme. Cells were lysed by freezing (−20 °C, 30 min) and thawing (at room temperature, 30 min) 3 times. DNA was then recovered from the lysate by phenol/chloroform extraction, followed by ethanol precipitation. The DNA was suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) and stored at 4 °C. The purified chromosomal DNA was used for subsequent construction of a meta-genomic library and other experiments.

2.5. Construction of a genomic library of SalI fragments from chromosomal DNA

Strain S19-1 chromosomal DNA was digested with restriction enzymes *Sal*I, *Pst*I, *Xba*I and *Bam*HI. The reaction mixtures were extracted by the phenol/chloroform method and resulting fragments were cloned into pKEGFP-2 (Fig. 1). The ligation mixtures were transformed into chemically competent *E. coli* HB101 which was prepared with 0.1 M of $CaCl_2$. After transformation, the cell mixture was 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 chromosomal DNA fragments from S19-1 were used for fluorescence microplate assay. The results revealed that more estradiol inducible colonies were found when the chromosomal DNA had been digested with *Sal*I (Fig. 1).

2.6. Fluorescence microplate assay

Fluorescence microplate assay (FMA) was performed as previously described [20]. The plasmids containing the *Sal*I fragments from chromosomal DNA of S19-1 were transformed into *E. coli* HB101 and 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 *Sal*I fragments of strain S19-1 chromosomal DNA, were adjusted to OD_{595} nm = 0.1 (using distilled water for dilution) for RFU measurement. To each well of 96-well Costar black plates was added 4 fM estradiol or 10 μl distilled water (as control) together with 90 μl of *E. coli* HB101 cells (OD_{595} 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 shown as relative fluorescence units (RFU). The RFU values are given as "RFU-IAP" (RFU Increased Amount Percentage) which is defined as (RFU-IAP) = $(RFU_{sample} - RFU_{water})/RFU_{water}^{*}$ 100%.

2.7. Genomic organization of estradiol-inducible fragments and sequence analyses

Estradiol inducible plasmids harboring a chromosomal *Sal*I fragment from S19-1 were selected for sequencing. The size of normal 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). Multiple sequence alignments were made according to the CLUSTAL W method [23].

2.8. Construction of deletion mutants of plasmid p302

Since the pellet of cells containing plasmid p302 became green, there should be a strong promoter present in the *Sal*I fragment of this plasmid. To locate this novel promoter in plasmid p302, a series of deletion mutants of plasmid p302 were constructed by using

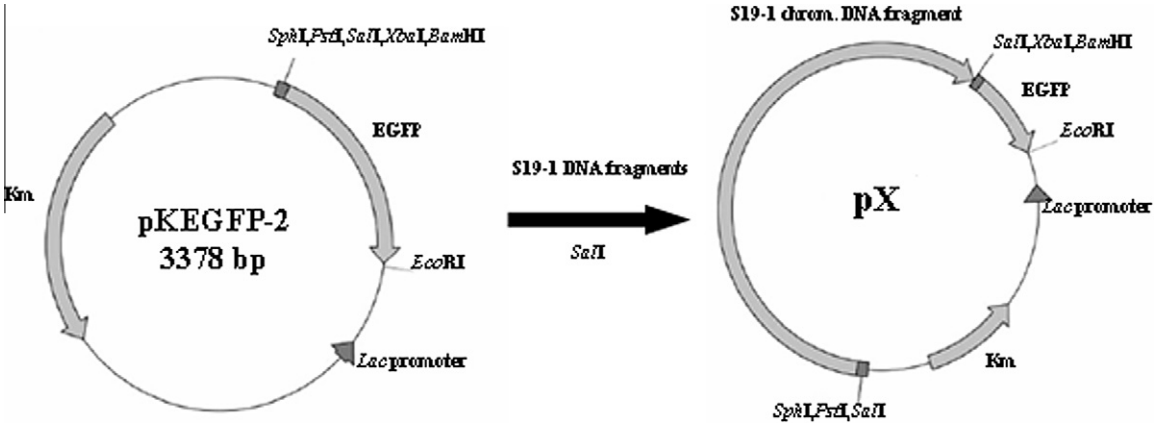

Fig. 1. Schematic representation of plasmids used for isolating estradiol-inducible genes from S19-1. Chromosomal DNA of S19-1 was digested with *Sal*I and then cloned into plasmid pKEGFP-2 which contained the gene encoding enhanced green fluorescent protein (EGFP) as a reporter. The resulting plasmids, here generally indicated as pX, were transformed into *E. coli* HB101 cells, which were then induced by estradiol for detecting estradiol-inducible plasmids.

a variety of appropriate restriction enzymes, such as *Bam*HI, *Pst*I, *Eco*RV, and *Bgl*II (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′-AAGCTTGTTATTTATTAATTA-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.

The PCR reactions were carried out in a final volume of 50 μl in small PCR tubes in an Eppendorf thermal cycler. The thermal profile involved 25 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s, with a final elongation at 72 °C for 5 min. The PCR product was identified by 0.8% agarose gel electrophoresis.

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, EGFP gene was obtained by digestion of pKEGFP-2 with *Eco*RI and then the

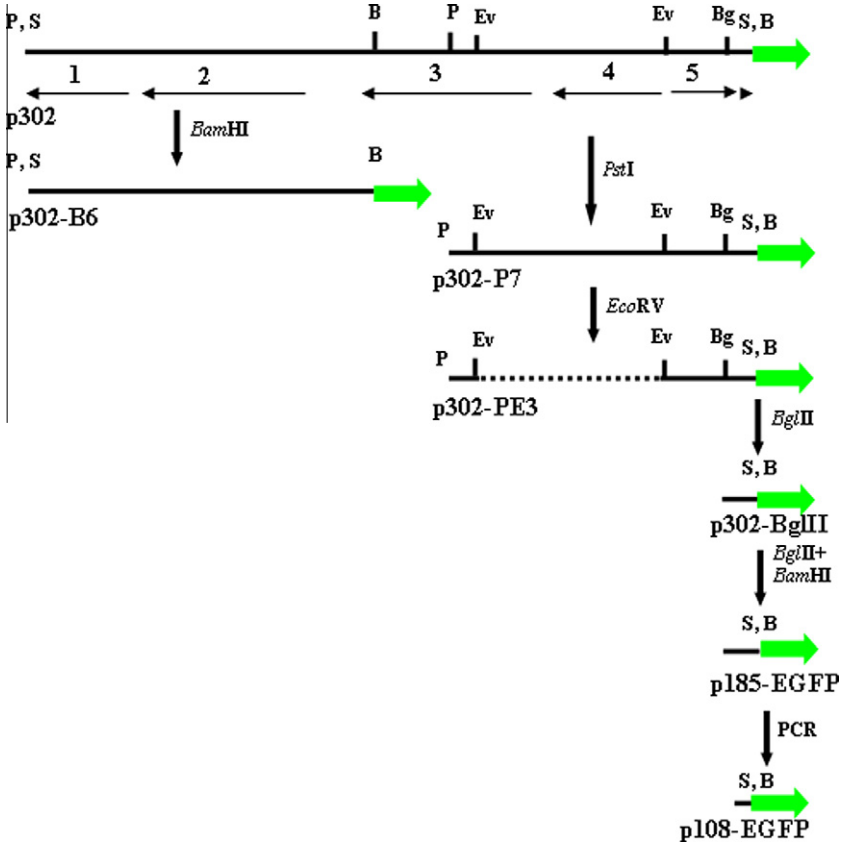

Fig. 2. Construction of deletion mutants of plasmid p302. A variety of restriction enzymes were used to construct the derivatives of plasmid p302. P: *Pst*I; S: *Sal*I; B: *Bam*HI; Ev: *Eco*RV; Bg: *Bgl*II. Genes of p302 code for: 1, L-serine dehydratase; 2, serine transporter SdaC; 3, decarboxylase; 4, 7-cyano-7-deazaguanine reductase; 5, suppressor of SecY dominance family protein. Green arrow indicates the EGFP gene.

resulting *Eco*RI fragment was ligated into pK18 to yield a 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 *Sal*I fragments from the chromosomal DNA of S19-1 was constructed by using a meta-genomic method. All of the resulting plasmids were transformed into *E. coli* HB101 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 these 37 estradiol-inducible plasmids was about 4–7%, which was similar to the positive control with pK3α-4.6-EGFP3. The induction efficiency of other plasmids containing chromosomal DNA of S19-1 was less than 1%, which was similar to the 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 were 6.2%, 7.3%, 5.7%, 6.1%, 5.6%, and 5.9%, respectively. This indicates that genes involved in estradiol metabolism may be present in these plasmids. Thus, the inserts of 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 were above 3.0 kb. Smaller fragments were constructed by a series of deletion mutants to facilitate sequencing. Each fragment was sequenced at least three times to get the consensus sequence. The obtained sequences were used for homology search by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and to locate the putative genes in combination with analysis by the DNASTAR program. Genetic organization of the genes present in the 6 plasmids is shown in Fig. 3. Detailed information thereon and amino acid sequence identities to orthologs in closely related bacterial strains are given in Table 1. The putative genes code for transcriptional regulators including FimZ (fimbrial expression regulator), LuxR (Lux operon repressor), GntR (Gluconate operon repressor) and nadR (nicotinamide ribonucleotide regulator), substrate transporters comprising ABC transporter and major facilitator superfamily protein, catabolic enzymes involved in tricarboxylic acid cycle, and so on. To analyze if these putative genes are involved in estradiol metabolism, the deduced amino acid sequences were aligned with their orthologs in other related bacteria which have been previously characterized. Since many members of the GntR-type transcriptional regulator family are involved in the metabolism of aromatic compounds and because estradiol contains an aromatic A ring, the putative gene *GntRx*, encoding a GntR transcriptional regulator in plasmid p5, was selected for subsequent analysis.

3.3. GntRx may be involved in estradiol metabolism in S19-1

GntRx is located in plasmid p5 and is adjacent to genes encoding phosphomethyl-pyridine kinase and PfkB kinase (Table 1). The open reading frame of *GntRx* consists of 744 bp and translates into a protein of 247 amino acids with a predicted molecular mass of 28 kDa. Although *GntRx* shows 81% amino acid sequence identity with its ortholog in *Citrobacter* (Table 1), the amino acid sequence identity between *GntRx* and other *GntRs* involved in the metabolism of aromatic compounds is 9.7–16.1%. While multiple sequence alignment of *GntRx* and these *GntRs* showed that the N-terminal DNA binding domain is highly conserved, especially for Arg65, Ala67, Leu71 and Gly84, the C-terminal coinducer binding region is less conserved (Fig. 4). This alignment further confirmed that *GntRx* is a member of the GntR-type transcriptional regulator family.

3.4. Induction of plasmid p302 and its derivatives by estradiol

To analyze the estradiol inducing effect of plasmid p302, two derivatives of this plasmid were prepared. Plasmid p302-P7 was obtained by digestion of p302 with *Pst*I and then self-ligated (Fig. 2). Plasmid p302-PE3 was constructed by digestion of p302-P7 with *Eco*RV and then self-ligated. After transformation of these three plasmids into *E. coli* HB101, the overnight cultures were induced by estradiol and RFU were determined. As a result, plasmids p302, p302-P7 and p302-PE3 turned out to be inducible by estradiol (Fig. 5), in comparison to the negative and positive control vectors pKEGFP-2 and pK3α-4.6-EGFP3, respectively. The RFU-IAP

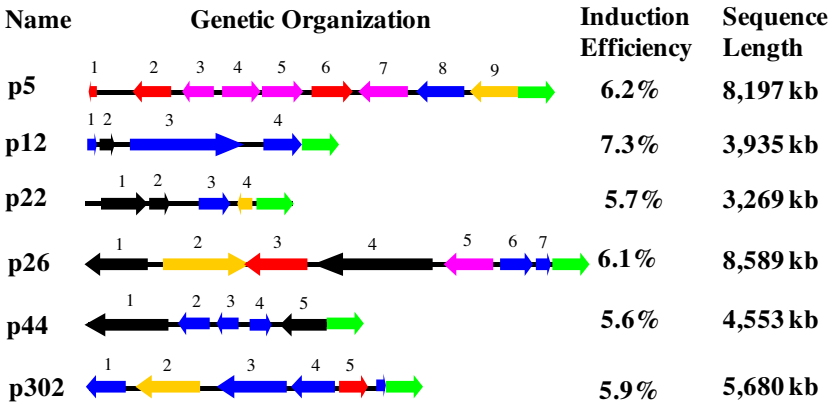

Fig. 3. Genomic organization of estradiol-inducible plasmids. The recombinant plasmids containing the *Sal*I fragments from S19-1 chromosomal DNA were identified to be induced by estradiol after transformation into *E. coli* HB101 by using fluorescence microplate assay. Induction efficiency of these plasmids was calculated by RFU-IAP. The whole sequence of the insert fragments of these plasmids was determined and the putative genes were predicted by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Arrows indicate genes encoding EGFP (green), catabolic enzymes (blue), transcriptional regulators (red), transporter proteins (yellow), kinases (pink) and others or unknowns (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

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	*Citrobacter*	72
p5-2	256	Response regulator receiver protein	YP_003611795.1	240	*Enterobacter*	31
p5-3	200	Transcriptional regulator LuxR	ZP_06353324.1	202	*Citrobacter*	46
p5-4	256	Hydroxyethyl-thiazole kinase	YP_004829381.1	256	*Enterobacter*	71
p5-5	268	Phosphomethyl-pyridine kinase	ZP_08498807.1	266	*Enterobacter*	81
p5-6	248	GntR transcriptional regulator	YP_003365775.1	248	*Citrobacter*	82
p5-7	312	PfkB kinase	ZP_05968388.1	317	*Enterobacter*	69
p5-8	306	ADP-ribosylglyco-hydrolase	YP_003941144.1	335	*Enterobacter*	73
p5-9	305	Major facilitator superfamily protein	YP_003365772.1	423	*Ciotrobacter*	86
p12-1	67	Aldehyde dehydrogenase	YP_003941287.1	461	*Enterobacter*	68
p12-2	99	Hypothetical protein	ZP_03069838.1	99	*E. coli*	82
p12-3	723	Formate C-acetyltransferase	ZP_04558545.1	765	*Citrobacter*	49
p12-4	256	Pyruvate formate-lyase 3 activating enzyme	NP_756398.1	305	*E. coli*	80
p22-1	290	Copper resistance protein	YP_001177131.1	290	*Enterobacter*	50
p22-2	112	Hypothetical protein	YP_003611966.1	112	*Enterobacter*	79
p22-3	192	Serine acetyltransferase	ZP_07950303.1	198	*Enterobacter*	40
p22-4	104	Transport-associated protein	ZP_07950651.1	104	*Enterobacter*	71
p26-1	399	Lytic murein transly cosylase	YP_001439403.1	644	*Cronobacter*	80
p26-2	543	ABC transporter	YP_001175290.1	555	*Enterobacter*	96
p26-3	410	Transcriptional regulator nadR	YP_004592319.1	410	*Enterobacter*	87
p26-4	461	DNA repair protein	YP_003943269.1	461	*Enterobacter*	94
p26-5	322	Phophoserine phosphatase	YP_003611312.1	322	*Enterobacter*	84
p26-6	217	Lipoate protein ligaseA	ZP_04559767.1	213	*Citrobacter*	78
p26-7	94	Lipoate protein ligaseB	ZP_04559766.1	338	*Citrobacter*	99
p44-1	516	Type VI secretion system Rhs/Vgr-family protein	YP_003366347.1	692	*Citrobacter*	69
p44-2	206	CDP-alcohol phosphatidy-ltransferase	ZP_04561712.1	207	*Citrobacter*	84
p44-3	134	Decarboxylase	YP_003364885.1	199	*Citrobacter*	65
p44-4	140	NTP pyrophospho-hydrolase	YP_001453352.1	140	*Citrobacter*	69
p44-5	295	DNA topoisomerase III	ZP_04561707.1	660	*Citrobacter*	78
p302-1	262	L-Serine dehydratase	YP_003614613.1	455	*Enterobacter*	84
p302-2	432	Serine transporter SdaC	ZP_06355175.1	429	*Citrobacter*	69
p302-3	454	Decarboxylase	YP_003366359.1	454	*Citrobacter*	89
p302-4	280	7-Cyano-7-deazaguanine reductase	YP_001177962.1	280	*Enterobacter*	79
p302-5	182	Suppressor of SecY dominance family protein	YP_003940482.1	181	*Enterobacter*	63

values of p302 and p302-P7 were higher than that of plasmid p302-PE3, especially for plasmid p302. This suggests that the *Eco*RV fragment of p302-P7 contains important estradiol-responsive elements.

3.5. Location of a novel promoter in plasmid p302

To locate the position of the novel promoter in plasmid p302, a series of further deletion mutants of plasmid p302 were constructed (Fig. 2). Plasmid p302-B6 was constructed by digestion of p302 with *Bam*HI and then self-ligated. Plasmid p302-BglII was prepared by deletion of the *Bgl*II fragment from plasmid p302-PE3 and then self-ligated, such that the *Bgl*II-*Pst*I fragment of the empty vector pK18 was also deleted. To test if the latter deletion 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 to yield p185-EGFP6. Finally, the last 108 bp of the 185 bp *Bgl*II–*Bam*HI fragment of p185-EGFP6 together with the EGFP gene were cloned into pK18 by PCR to get plasmid p108-EGFP1 (Fig. 2).

After transformation of these plasmids into *E. coli* HB101, the pellets of cells harboring plasmids p302, p302-P7, p302-PE3, p302-BglII, p185-EGFP6 and p108-EGFP1 became green, but not those with p302-B6. To test the activity of the novel promoter, RFU of cells containing the relevant plasmids was measured. As shown in Fig. 6, the RFU values of cells with p302, p320-P7, p302-PE3, p302-BglII, p185-EGFP6, and p108-EGFP1 were significantly higher than those with p302-B6 or the negative control vector pKEGFP-2. Taken together, the novel strong promoter locates in the 108-bp fragment adjacent to the EGFP gene of p302.

3.6. Comparison of the activity of the novel promoter with the lac promoter

To compare the activity between the novel promoter and the *lac* promoter, plasmids p185-EGFP5, p185-EGFP6, pKEGFP-2, and pKEGFP3 were constructed. As shown in Fig. 7, the *lac* promoter in plasmid pKEGFP3 has the same direction as the EGFP gene, but in pEGFP-2, the direction of the *lac* promoter is opposite to that of the EGFP gene. Plasmids p185-EGFP5 and p185-EGFP6 contain the novel 185 bp promoter in different directions. Whereas it is in the same direction as EGFP in p185-EGFP6, it is in the opposite direction to EGFP in p185-EGFP5. 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 values of cells with plasmids p185-EGFP5 and pKEGFP-2 were very low, largely because the promoters cannot perform their function to promote the transcription of the EGFP gene. In contrast, the RFU value with p185-EGFP6 was very high. This indicates a significant activity of the novel 185 bp promoter on EGFP transcription, while it is not as strong as the *lac* promoter in pKEGFP3. However, like the *lac* promoter the novel promoter was able to make the pellets of the bacteria green which makes it interesting for further research with regard to its relevance in this natural marine bacterium.

3.7. Sequence analysis of the novel promoter

To get more detailed information, the novel promoter was sequenced. In the resulting 108 bp promoter sequence (Fig. 8), the putative −10 region and −35 region were predicted based on the consensus promoter sequence in prokaryotes (−10 elements:

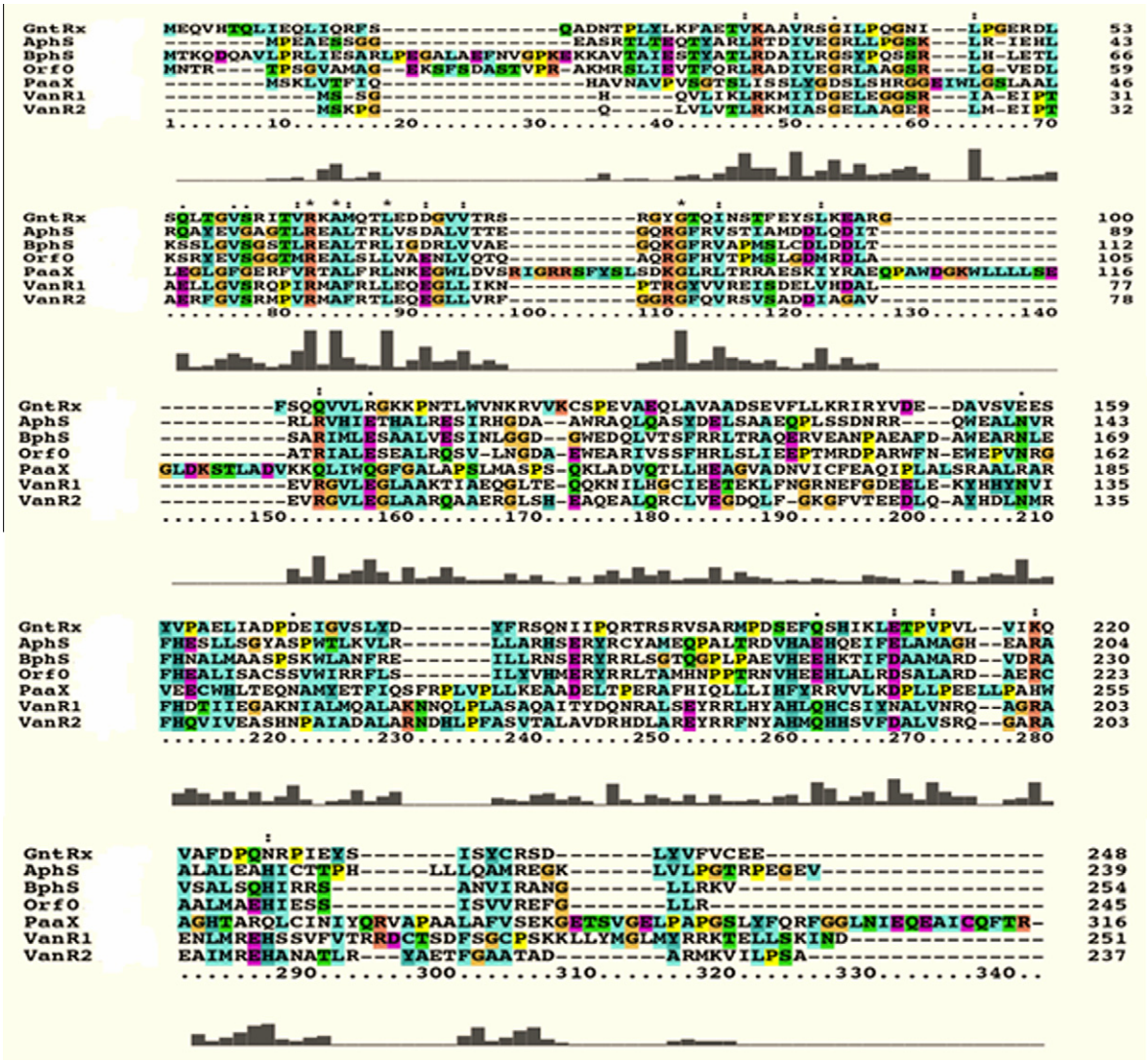

Fig. 4. Multiple alignment of amino acid sequences of *GntRx* and other GntRs. "*" indicates amino acids identical among all the proteins; ":" and "." indicate amino acids of high and low similarity, respectively. Swissprot or PDB accession numbers are: AphS from *Comamonas* (BAA89295); BphS from *Pseudomonas*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

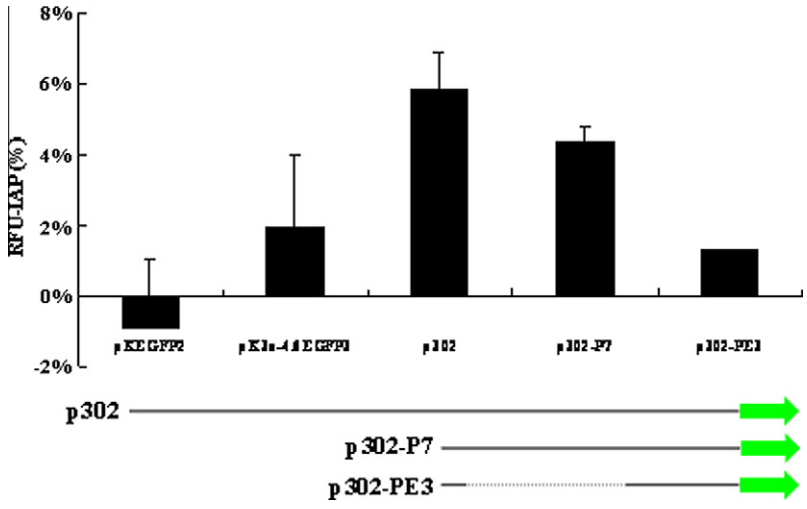

Fig. 5. Induction of p302 and its derivatives by estradiol. Before measuring the RFU, the bacterial cells harboring these plasmids were incubated with 4 fM estradiol for 60 min at room temperature. Plasmids pKEGFP-2 and pK3α-4.6EGFP3 were used as the negative and positive control, respectively. Each sample had 4 replicates. Green arrow indicates the EGFP gene. Bars represent the average and standard deviation of at least three independent experiments.

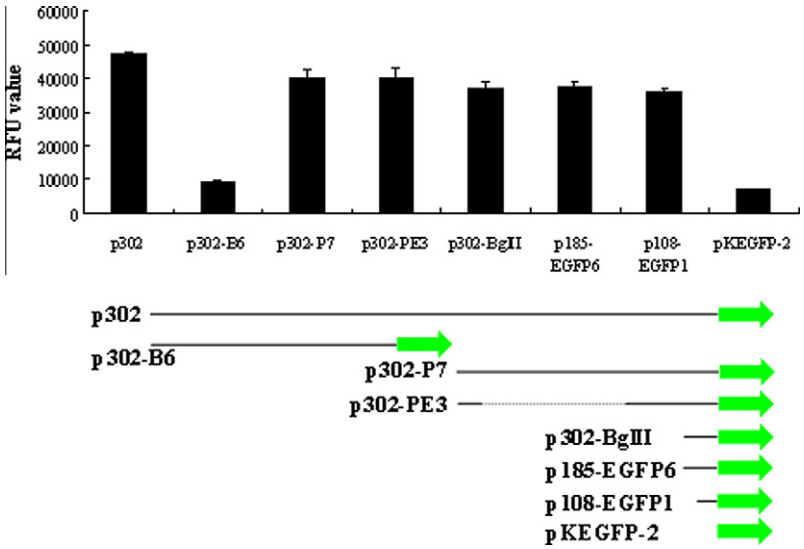

Fig. 6. Localization of the novel promoter in plasmid p302. The optical density of the overnight cultures containing the relevant plasmid was measured at 595, and 100 μl of cells (OD = 0.1) was placed to black microplates to determine the RFU. Each sample had 6 replicates. Green arrow indicates the EGFP gene. Bars represent the average and standard deviation of at least three independent experiments.

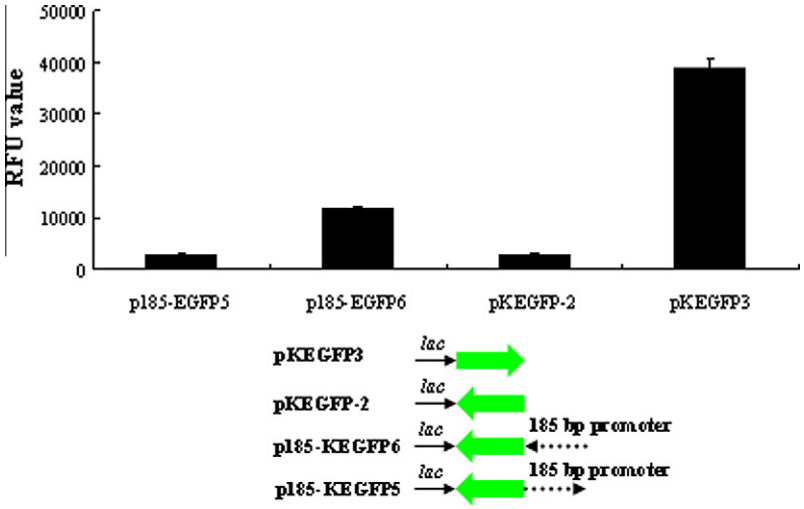

Fig. 7. Activity determination of the novel 185 bp promoter. Overnight cultures containing the indicated four plasmids were used for measuring the RFU. The results revealed a considerable activity of the novel promoter from the marine strain S19-1, although the *lac* promoter was stronger in activating EGFP gene expression in *E. coli* HB101. Green arrow indicates the EGFP gene. Bars represent the average and standard deviation of at least three independent experiments.

GTTATTTATTAATTAGTTGTGTTGATTTC***TTGTCG***
-35
CTTTTGAAGAAAGGAAACG***TAAGAT***TGGTTGT
-10
GAGTGCCTTGCGAGATGAACGAAATAGAGGATT

CTATGTCCGTCGAC

Fig. 8. Sequence of the novel strong promoter from S19-1. The putative −10 region (TAAGAT) and −35 region (TTGTCG) are indicated in italic bold.

TATAAT; −35 elements: TTGACA). The −10 region and −35 region in this promoter were proposed to be TAAGAT and TTGTCG, respectively, and the distance between these two elements was determined to be 19 bp. These promoter elements should be verified in future studies by point mutations and DNase I footprinting.(see Fig. 9).

4. Discussion

4.1. Estradiol inducible genes in S19-1

Strain S19-1 is a new isolated marine bacterium which can use estradiol as carbon and energy source, but little is known about the genes involved in this catabolic pathway. To get an overview of estradiol degradation in this bacterium, a substrate-induced gene-expression screening method was used for isolating estradiol-inducible genes. This method was first developed by Taku Uchiyama for isolating target genes from an environmental metagenome [19]. It is based on the knowledge that catabolic gene expression is generally induced by substrates and metabolites of catabolic enzymes and, in many cases, is controlled by regulatory elements situated proximate to catabolic genes. According to the findings from Taku Uchiyama, a large number of substrate-inducible fragments were obtained by this method. This is probably because the pathway was not only induced by the substrate estradiol

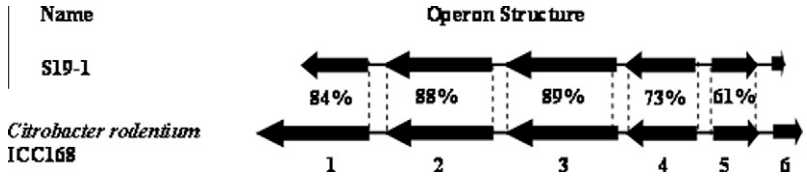

Fig. 9. Prediction of the genes controlled by the novel promoter. The genetic organization in plasmid p302 from S19-1 is the same as that of their orthologs in *Citrobacter rodentium* ICC168. The amino acid sequence identity between the orthologs in these two bacteria is also indicated. Genes of p302 code for: 1, L-serine dehydratase; 2, serine transporter SdaC; 3, decarboxylase; 4,7-cyano-7-deazaguanine reductase; 5, suppressor of SecY dominance family protein; 6, miscellaneous small RNA.

itself for the corresponding catabolic enzymes, but also by similar pathway intermediates or some structural analogs of the natural effector (gratuitous inducer) for bacteria [24]. After that, this method was improved by our group by measuring RFU instead of fluorescence-activated cell sorting in 2010 [21].

Six fragments from S19-1 chromosomal DNA which were induced by estradiol were sequenced and analyzed. Transcriptional regulators, kinases, transport-associated proteins and catabolic enzymes which are involved in the tricarboxylic acid cycle were found in the plasmid inserts from S19-1. In plasmid p5, the putative genes are organized as follows: transcriptional regulator FimZ, response regulator receiver protein, transcriptional regulator LuxR (some members of this family are involved in the steroid catabolic pathway in *C. testosteroni*) [25], 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 phosphorylate acceptors with an alcohol or phosphate group [26], respectively. GntR family transcriptional regulator was found to be involved in the degradation of some aromatic compounds, such as vanillate [27] and biphenyls [28]. The Pfkb kinase superfamily comprises kinases that accept a wide variety of substrates, including carbohydrates and aromatic small molecules, all are phosphorylated at a hydroxyl group. 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 [29].

According to analysis of the six estradiol-inducible fragments, plasmid p5, which contains the regulators LuxR and GntR, might include an estradiol-inducible DNA fragment. In addition, since many members of the GntR-type transcriptional regulator family are involved in the degradation of aromatic compounds, and because estradiol contains an aromatic A ring, *GntRx* from plasmid p5 was selected for further investigation. "Gnt" was first reported by BÄchi and Kornberg who studied genes involved in the uptake and catabolism of gluconate by *E. coli* [30]. The GntR regulator family was named after the first member identified, the *Bacillus subtilis* repressor of the gluconate operon [31]. Later, the GntR family became one of the most abundant groups of HTH (helix-turn-helix) bacterial metabolite-responsive transcription factors which contained >8500 members in the Pfam database as of January 2009 [32]. A striking characteristic feature of regulators belonging to this family is that they possess a conserved N-terminal DNA binding domain and a diverse C-terminal domain involved in the effector binding and/or oligomerization. Since the physiological role of these regulators is critically dependent upon effector binding and operator sites, four subfamilies FadR (Fatty acid metabolism regulator), HutC (Histidine utilization repressor), MocR (Probable rhizopine catabolism regulator), and YtrA were analyzed and classified by Rigali [33]. Subfamily FadR which is named after the fatty acid degradation regulator of the same name includes the genes AphS (Regulator of genes involved in phenol utilization), BphS (Repressor of genes involved in biphenyl degradation) and VanR (Vanillate demethylase synthesis repressor) which were found in the regulation of aromatic compound degradation by bacteria [24,33,34].

A multiple alignment of *GntRx* with other GntR family members involved in the degradation of aromatic compounds indicates that the conserved N-terminal DNA binding domain is present in this GntR-like gene (Fig. 4). This suggests that *GntRx* might be involved in the biodegradation pathway of estradiol in S19-1. Until now, it is not clear what intermediates derive from this pathway. These should be determined in future studies by gas chromatography, nuclear magnetic resonance or other methods.

4.2. Induction of EGFP in plasmid p302 and its derivatives by estradiol

Plasmids p302, p302-P7, and p302-PE3 were found inducible by estradiol, where the induction efficiency of the latter was reduced compared to the first two plasmids. As shown in Fig. 2, the *Eco*RV fragment of p302-P7 was deleted in p302-PE3 and this fragment may affect estradiol induction. Sequence analyses revealed that this 1.5 kb *Eco*RV fragment consists of part of the decarboxylase gene and the gene encoding 7-cyano-7-deazaguanine reductase. These two genes are divergently transcribed such that their intergenic region may be involved in estradiol induction. Until now, little is known about the role of the decarboxylase and the 7-cyano-7-deazaguanine genes in this bacterium. In addition, plasmid p302-B6 containing the *Pst*I fragment of p302 can be also induced by estradiol (data not shown). This may in part be the reason why the induction efficiency of p302 is higher than that of p302-P7, which is the *Pst*I deletion fragment of p302 (Fig. 2).

4.3. Identification of a novel promoter in S19-1

The lac promoter is a strong promoter for the lactose operon which is required for transport and metabolism of lactose in *E. coli* and some other bacteria. In the present study, the activity of the novel promoter from S19-1 is lower than that of the *lac* promoter, in which the −10 and −35 elements are TATAAT and TTGACA, respectively, and the distance between these two elements is 18 bp. The −10 and −35 elements from the novel promoter in S19-1 are TAAGAT and TTGTCG, respectively, and their distance is 19 bp. The differences between these two promoters may cause a different affinity of the RNA polymerase to the target sites and result in different transcription efficiencies. On the other hand, the novel promoter was obtained from the marine bacterium S19-1, such that its activity may be affected in a heterologous expression system. Sequence analyses and the genetic organization of genes revealed that strain S19-1 is closely related to *Citrobacter rodentium* ICC168 Fig. 9. In *C. rodentium* ICC168, one of the ortholog genes controlled by this novel promoter is a miscellaneous small RNA, which serves a variety of functions, including enzyme-like catalysis or serving as switch for turning genes on and off.

Taken together, a variety of estradiol-inducible genes have been found in S19-1 by a metagenomic approach. In addition, a novel promoter from this marine bacterium was identified and

characterized. Yet, we do not know the target genes which are controlled by this promoter in the marine bacterium S19-1.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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