Expression of green fluorescent protein in *Bacillus brevis* under the control of a novel constitutive promoter F1 and insertion mutagenesis of F1 in *Escherichia coli* DH5α

Yunpeng Chen a,b, Jian Yan a, Mingjie Yang a, Jingwen Wang a, Daleng Shen a,*

a Institute of Genetics, State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, 220 Handan Rd, Shanghai 200433, PR China
b Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Institute of Biodiversity Science, School of Life Sciences, Fudan University, 220 Handan Rd, Shanghai 200433, PR China

Received 1 June 2003; received in revised form 18 October 2003; accepted 19 October 2003
First published online 13 November 2003

Abstract

The constitutive expression vector pHY300-F1gfp was constructed to test the function of a promoter, F1, cloned from the rice epiphyte *Bacillus brevis* strain DX01. The DX01 cells harboring the plasmid pHY300-F1gfp were shown to produce bright green fluorescence. The results were confirmed by Western blot analysis and fluorescence-activated cell sorting. Expression of the F1 promoter was constitutive. To improve the activity of F1, insertion mutagenesis of F1 based on in vitro transposition reaction was performed. Seven mutants with enhanced transcription activity in *Escherichia coli* DH5α were obtained. The enhanced promoters showed similar high activities in *B. brevis* strain DX01.

© 2003 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: *Bacillus brevis*; Green fluorescent protein; Insertion mutagenesis

1. Introduction

Recent studies show that *Bacillus brevis*, a Gram-positive plant epiphyte, could produce antifungal antibiotics [16,19] and suppress wheat root pathogens [26]. However, this bacterium is not widely used in practice because its resistance spectrum is limited. Theoretically, introduction of foreign resistance genes (such as *Raphanus sativus* antifungal protein gene (RS-AFP) [23], *Bacillus thuringiensis* δ-endotoxin gene) into *B. brevis* can improve and develop its resistance. If such genetically modified *B. brevis* strains were applied for biocontrol as an effective component of biological pesticides or fungicides, environmental contamination resulting from traditional chemicals could be avoided or alleviated.

In the past decades, expression vectors in *Bacillus* have been constructed mostly for *Bacillus subtilis* [28]. Their promoters were usually inducible, such as the xylose-inducible promoter Pxyl [17]. To our knowledge, there has not been any study on *B. brevis* genetically engineered with a constitutive promoter.

Since the *gfp* cDNA from *Aequorea victoria* was cloned in 1992 [21], the green fluorescence protein (GFP) has attracted considerable attention as a marker/reporter system [4,13]. GFP provides a convenient tool to image pathogenic bacteria [8,9]. GFP expression does not adversely affect bacterial survival. Errampalli et al. [7] reviewed the applications of GFP as a molecular marker in environmental microorganisms.

Transposable elements are useful tools for insertional mutagenesis and have many potential applications. Garraway et al. [10] developed two transposons, TyK and TyK’GFP+, which can be introduced into target DNAs by Ty1-mediated transposition in vitro. Merkulov et al. [20] reported two new artificial transposons used to generate expression libraries of GFP fusions.

The promoter pCP01 (GenBank accession number AF294434) was cloned from *B. brevis* in our laboratory and its structure was analyzed preliminarily [3]. In this study, a 474-bp fragment (designated F1) derived from...
promoter pCP01 (site −309 to site +165) was fused with the gfp-S65T promoterless gene in the Escherichia coli-B. subtilis shuttle vector pHY300PLK [14,15] to construct the constitutive expression vector pHY300-F1gfp for monitoring gfp-S65T gene expression in B. brevis strain DX01. Following the confirmation of the transcriptional function of F1, MuA transposase-mediated insertion mutagenesis of F1 was performed.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Strains B. brevis DX01 [2], E. coli DH5α, TG1 were from our collection. Competent cells of E. coli were transformed according to the classical CaCl₂ chemical transformation method. Plasmid DNA was introduced into B. brevis DX01 by electroporation transformation [2]. All the bacterial strains were maintained on LB medium at 37°C. Plasmid pGEM-T was from Promega, the E. coli-B. subtilis shuttle vector pHY300PLK from Takara. Plasmid pSG1164 [17] harboring the gfp-S65T gene [6] was a generous gift from Peter J. Lewis. The promoter-probe vector ECE7 [1] containing a promoterless chloramphenicol (Cam) acetyltransferase (CAT) gene and pUC118 were from our collection.

2.2. Proteins and reagents

Restriction endonucleases and T4 DNA ligase were from Takara, Taq plus DNA polymerase and polymerase chain reaction (PCR) primers from Sangon (China). All enzymes were used under the reaction conditions recommended by the supplier. The MGS® kit (Mutation Generation System) was from Finnzymes (Espoo, Finland), which was designed for inserting 15 bp randomly into target DNA. The kit contains a single purified enzyme, MuA transposase, and a 1254-bp artificial transposon Entraceposon (M1-Cam8). The CAT ELISA kit was from Roche (Germany).

2.3. DNA isolation and manipulation

General microbiological techniques were performed by standard methods [22]. Total and plasmid DNAs of B. brevis DX01 were extracted according to the protocol described by Wang [27].

2.4. Subcloning of functional fragment F1

Using the total DNA of B. brevis DX01 as template, a 474-bp fragment F1 was amplified by PCR. The forward primer was 5’-CGGGATCCCTGCCAACATTTCTTCCAA-3’, which contained a BamHI site (underlined); the reverse primer was 5’-TCCCTGCAGctcTTTCTGCGACCTT-

CATCAC-3’, which contained a PstI site (underlined) and a consensus Gram-positive ribosome binding site (lower-case letters). The PCR was performed with the following procedure: 3 min at 94°C, 35 cycles of 1 min at 94°C, 45 s at 60°C and 1 min at 72°C (with a final elongation step at 72°C for 4 min). Amplified products were purified and sequenced to verify that no misincorporation of nucleotides had occurred during the PCR process.

2.5. Construction of GFP constitutive expression vectors pUC118-F1gfp and pHY300-F1gfp

The GFP expression vector pUC118-F1gfp was constructed, in which the gfp-S65T promoterless gene was fused to the fragment F1 in a correct orientation. A 6-bp interval separated the two elements. The PCR fragment F1 was digested with BamHI and PstI (474 bp) and cloned into plasmid pUC118 leading to pUC118-F1. A 717-bp EcoRI-XbaI fragment containing the gfp-S65T promoterless gene from plasmid pSG1164 was inserted into pUC118 (pUC118-gfp). The pUC118-F1gfp was obtained by cloning a 717-bp PstI fragment from pUC118-gfp into the PstI site of plasmid pUC118-F1. The 1.2-kb BamHI-HindIII fragment of pUC118-F1gfp was cloned in the E. coli-B. subtilis vector pHY300PLK.

2.6. Expression of GFP in E. coli DH5α and B. brevis DX01

The cell populations of E. coli DH5α::pUC118-F1gfp and B. brevis DX01::pHY300-F1gfp were cultured until the OD600 reached 0.6–0.8 for DH5α, or 0.9–1.0 for DX01. Cultures were then cooled at 4°C for several hours, and centrifuged at 4000×g for 15 min, the pellet was rinsed twice and resuspended in phosphate-buffered saline solution. Bacterial suspension (10 μl) was visualized under a fluorescent microscope (Olympus BH-2, Japan) to detect gfp expression. The excitation filter was 485 nm and photographs were taken with a CCD camera (Nikon E950).

2.7. Western blotting analysis

Western blotting was conducted according to the protocol recommended by the manufacturer. Lysate (1.5 ml containing 25–75 μg of proteins) from GFP-expressing bacteria grown overnight was electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the gel was blotted to a Nylon membrane (Amersham Pharmacia Biotech, UK) using semi-dry electrophoresis transfer (Bio-Rad, USA). The blot was probed with rabbit anti-GFP antiserum (Clontech, #8367-2) diluted 1:100, followed by secondary goat anti-rabbit antibody conjugated with alkaline phosphatase (Southern Biotechnology Associates, USA). The signal was then detected by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Roche, USA).
2.8. Random mutagenesis of F1 based on the bacteriophage Mu in vitro transposition reaction

In order to improve the transcriptional activity of F1, insertion mutagenesis based on the transposition reaction of MuA transposase was performed. Plasmid pUC118-F1 was used as target DNA. Standard reaction mixtures (20 μl) contained 350 ng of target pUC118-F1 DNA, 0.22 μg MuA transposase, 100 ng Entranceposon (M1-CamR), 25 mM Tris–HCl (pH 8.0), 100 μg ml⁻¹ bovine serum albumin, 15% (w/v) glycerol, 0.05% (w/v) Triton X-100, 126 mM NaCl and 10 mM MgCl₂. The components were mixed gently, and reaction was carried out for 1 h at 30°C. 5 μl transposition reaction product was used to transform E. coli DH5α. Mutant plasmids resulting from a single transposition in F1 were identified by PCR with the primer pair specific for F1. The 1733-bp BamHI-PstI fragment of mutated plasmids were cloned into pUC118. The 1254-bp Entranceposon (M1-CamR) was removed by NsiI digestion and self-ligation, resulting in a 15-bp insertion in F1. The insertion clones were designated pUC118-FmX (X indicates different insertions), and the corresponding mutants of F1 were designated FmX.

2.9. Assessment of FmX activity in E. coli DH5α

In order to test the activity of FmX, the expression vector ECE7-FmX, containing the Cam promoterless gene, was used. The 489-bp fragments of FmX were amplified by PCR with primers 5′-CCGGAATTCTGCA-CAATTCTTCGAA-3′ and 5′-CCGGAATTCCCTCCC-TTCTGCAAGCTTCCATCAC-3′, containing an EcoRI site (underlined). The PCR fragments were cloned upstream of the Cam gene and the plasmids were referred to as ECE7-FmX. Each harbored a 15-bp insertion as demonstrated by DNA sequencing.

The activity of the FmX was assessed by determining the minimum inhibitory concentration (MIC) of Cam and the CAT synthesis in the E. coli DH5α transformants. The MIC of Cam was determined by the tube dilution test method [18]. The quantitation of CAT was performed by colorimetric enzyme immunoassay according to the supplier’s protocol.

2.10. Assessment of the activity of the enhanced promoters in B. brevis strain DX01

The expression vectors pHY300-FmX’gAFP were constructed to test the activities of several enhanced promoters in B. brevis strain DX01. A 1.2-kb fragment of F1-gfp containing a BamHI and a SalI restriction site was amplified from pUC118-F1gfp with PCR, in which the terminator codon of the gfp-S65T gene was mutated. Subsequently, this product was cloned into the BamHI-SalI site of plasmid pUC118-F1gfp leading to pUC118-F1’gfp. A 274-bp SalI-HindIII fragment containing the anti-fungal protein gene (Rs-AFP) derived from radish by reverse transcription PCR was fused to the 3′ end of the mutated gfp-S65T gene of pUC118-F1’gfp resulting in pHY300-FmX’gAFP. Promoter F1 of pUC118-F1’gAFP was replaced by cloning the different enhanced promoter FmX into the BamHI-PstI site to obtain pUC118-FmX’gAFP. The 1.47-kb BamHI-HindIII fragment of pUC118-FmX’gAFP was inserted into the E. coli-B. subtilis vector pHY300PLK. The resulted pHY300-FmX’gAFP was transformed to B. brevis. GFP-expressing cells of B. brevis were sorted by fluorescence-activated cell sorting (FACS) analysis.

3. Results

3.1. Expression of GFP in E. coli DH5α and B. brevis strain DX01

The green fluorescence could be detected under fluorescence microscopy with an excitation wavelength of 485 nm for E. coli DH5α cells harboring plasmid pUC118-F1gfp (Fig. 1a), as well as for B. brevis DX01 carrying plasmid pHY300-F1gfp (b).

Fig. 1. Detection of gfp-S65T expression in E. coli DH5α cells harboring plasmid pUC118-F1gfp (a) and in B. brevis DX01 cells carrying plasmid pHY300-F1gfp (b).
pHY300-F1gfp (Fig. 1b). No background fluorescence was observed with the control strains E. coli DH5α and B. brevis::pHY300PLK. GFP expression was effectively driven by F1 promoter activity in both species. The transcription from F1 remained constitutive although it was 280 bp shorter than in pCP01.

GFP-expressing cells were also sorted by FACS analysis. As expected, no fluorescence was detected in untransformed E. coli DH5α and B. brevis DX01, whereas a strong fluorescence was measured for DH5α::pUC118-F1gfp (MFI = 30.42, Fig. 2a) and DX01::pHY300-F1gfp (MFI = 60.68, Fig. 2b). The fluorescence intensity of cell populations of DX01::pHY300-F1gfp was two-fold higher than that of DH5α::pUC118-F1gfp, which implied that the GFP expression vector pHY300-F1gfp was highly efficient in B. brevis DX01. Though F1 showed no host cell specificity, it displayed higher transcriptional activity in B. brevis DX01 than in E. coli DH5α.

3.2. Western blotting analysis of GFP expression in B. brevis strain DX01

Expression of GFP protein was verified by Western blotting analysis using GFP polyclonal antibody. A 27-kDa protein was detected in B. brevis DX01::pHY300-F1gfp (Fig. 3). The result matched previously published data [8,11].

3.3. Analysis of insertion clones based on the transposition reactions

Since F1 was confirmed to be an effective promoter, transposon-mediated mutagenesis on it was performed to

---

Table 1
Activities of promoter FmX generated in the transposition reaction

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Transposition site</th>
<th>Duplicated target site (5’-3’)</th>
<th>MIC of Cam (µg ml⁻¹)</th>
<th>CAT concentration (pg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1(CK)</td>
<td>–</td>
<td>–</td>
<td>250</td>
<td>880</td>
</tr>
<tr>
<td>Fm-1</td>
<td>–270→–266</td>
<td>CAGAA</td>
<td>150</td>
<td>856</td>
</tr>
<tr>
<td>Fm-2</td>
<td>–170→–166</td>
<td>TCTCA</td>
<td>550</td>
<td>887</td>
</tr>
<tr>
<td>Fm-3</td>
<td>–118→–114</td>
<td>GGACG</td>
<td>900</td>
<td>923</td>
</tr>
<tr>
<td>Fm-4</td>
<td>–115→–111</td>
<td>CGAAT</td>
<td>550</td>
<td>916</td>
</tr>
<tr>
<td>Fm-5</td>
<td>–105→–101</td>
<td>GAAGG</td>
<td>300</td>
<td>884</td>
</tr>
<tr>
<td>Fm-6</td>
<td>–94→–90</td>
<td>ATCAA</td>
<td>60</td>
<td>866</td>
</tr>
<tr>
<td>Fm-7</td>
<td>–90→–86</td>
<td>ACAGG</td>
<td>550</td>
<td>877</td>
</tr>
<tr>
<td>Fm-8</td>
<td>–59→–55</td>
<td>AAAGA</td>
<td>550</td>
<td>917</td>
</tr>
<tr>
<td>Fm-9</td>
<td>–25→–21</td>
<td>CTAGA</td>
<td>250</td>
<td>895</td>
</tr>
<tr>
<td>Fm-10</td>
<td>+66→+70</td>
<td>TCTCA</td>
<td>900</td>
<td>930</td>
</tr>
<tr>
<td>Fm-11</td>
<td>+128→+132</td>
<td>TCCAT</td>
<td>150</td>
<td>810</td>
</tr>
<tr>
<td>Fm-12</td>
<td>+131→+135</td>
<td>ATTAGA</td>
<td>150</td>
<td>850</td>
</tr>
<tr>
<td>Fm-13</td>
<td>+136→+140</td>
<td>TGAGA</td>
<td>250</td>
<td>905</td>
</tr>
</tbody>
</table>

*aMutant, mutagenesis of promoter F1.
screen mutants with enhanced transcriptional activities. In this study, 227 insertion clones were screened from an insertion library, 13 mutants were identified and sequenced. Then their MIC of Cam and CAT synthesis were assayed (Table 1). All E. coli DH5α cells harboring plasmid ECE7-FmX displayed resistance to Cam of 60–900 μg ml⁻¹. The concentration of CAT ranged from 0.81 to 0.93 ng ml⁻¹.

The results of sequencing revealed that the insertions were distributed relatively evenly. The insertions resulted in different effects. There were seven positive-effect sites in the sequence of F1. Insertions in the sites −118 to −114 and +66–+70 resulted in the highest MIC values for E. coli DH5α::ECE7-Fm3 and for E. coli DH5α::ECE7-Fm10, respectively (Table 1). Their MICs were 2.6 times higher than that of E. coli DH5α::ECE7-F1. Four negative-effect sites were also detected. The MIC of E. coli DH5α::ECE7-Fm6 was the lowest among all the mutants. It was about one quarter of that in control.

The positive insertions were found to distribute mainly upstream of F1. Remarkably, the insertions in the sites −94 to −90 and −90 to −86 led to diametrically opposing effects, i.e. the MIC of E. coli DH5α::ECE7-Fm6 decreased sharply whereas the MIC of E. coli DH5α::ECE7-Fm7 increased significantly in comparison with that of the control. In general, the MIC of Cam was positively related to CAT activity in E. coli DH5α cells (Table 1).

3.4. GFP expression in B. brevis strain DX01 under the control of enhanced promoters

It was demonstrated that the enhanced promoters showed similar high activities in B. brevis by FACS analysis. Compared to F1-gfp activity (MFI = 33.85, Fig. 4d), for instance, Fm-10 (MFI = 67.72, Fig. 4b) and Fm-2 (MFI = 53.92, Fig. 4c) showed obviously higher activities.

4. Discussion

pHY300PLK is a high copy number plasmid with 14 unique cloning sites [16], so that it is very convenient for the construction of a GFP expression system to detect activity of promoters. The expression of gfp-S65T in B. brevis harboring pHY300PLK-F1gfp showed that frag-
ment F1 has strong transcriptional activity. Though F1 was subcloned from promoter pCP01, and was much shorter than the latter, it is still an effective promoter. It seems that F1 is the essential part of pCP01. Our study revealed that F1 is a very useful promoter in the genetic engineering of *B. brevis* DX01. If foreign resistance genes are fused to vector pHY300-F1gfp, and then introduced into strain DX01, the resulting genetically modified bacteria could be applied in biocontrol. There has been considerable application of GFP as a bacterial marker in microbial ecology recently due to its advantage in situ non-destructive monitoring [24, 25].

The expression vector pHY300-F1gfp can be used in the monitoring of genetically modified *B. brevis* DX01. GFP provides a marker system for detection and enumeration of marked DX01 cells in field studies.

Insertional mutagenesis based on transposition reaction is an effective approach in the investigation of promoter activity. Charng et al. [5] constructed a PR-1a::Ae transposase fusion which drives higher transposition. Haapa et al. [12] described an efficient and accurate integration of mini-Mu transposons in vitro for functional genetic analysis and molecular biology applications. In this study, we adopted a similar method to obtain a series of mutants with enhanced promoter activities. As far as we know, this is the first report on random insertion mutagenesis of a promoter in *B. brevis* DX01. The mutated promoters Fm-3 and Fm-10 could be used to construct highly efficient constitutive expression vectors to facilitate the expression of other foreign resistance genes in *B. brevis* DX01.

Some mutations in promoter F1 result in an important variation in the MIC of Cam and a slight effect on CAT concentration, Fm-2 and Fm-7 for example. This may be due to the fact that the mutations in different regions of the promoter F1 affected the strength of the resulting mutants’ activities, then that had influence on the growth rate of the cells and led to interference with the results of the assessment of CAT concentration.

In conclusion, we demonstrate that mutants with enhanced transcription activities derived from the insertion mutagenesis of promoter F1 based on in vitro transposition reaction have similar high activities in *B. brevis* strain DX01. The demonstration of the utility of the promoter F1 and its enhanced mutants in the construction of GFP expression vectors used for *E. coli* and *B. brevis* strain DX01 reveals that these promoters are all constitutive; they are novel and can be directly used for studying high level expression of the gene of interest.

Acknowledgements

The authors thank Dr. Peter J. Lewis for providing plasmid pSG1164. This study was financed by the McKnight Foundation and the Shanghai Commission of Science and Technology (Grant KBH1322093).

References

richia coli* and *Bacillus subtilis*. II. Plasmid pHY300PLK, a multi-
mid-determined resistance to erythromycin and lincomycin in *Esche-


