Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 426–430
BBRC
www.elsevier.com/locate/ybbrc

Development of a new "GFP hop-on assay" system for insertion sequence transposition in *Bacillus subtilis* 168 using IS*4Bsu1* from *B. subtilis* (*natto*)

Kiwamu Takahashi [a], Taku Chibazakura [a], Yasuhiko Sekine [b], Hirofumi Yoshikawa [a,*]

[a] *Department of Bioscience, Tokyo University of Agriculture, Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan*
[b] *Department of Life Science, College of Science, Rikkyo (St. Paul's) University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan*

Received 23 January 2007
Available online 8 February 2007

Abstract

While most studies involving transposition have focused on analyzing the detailed mechanisms of transposition, the cellular conditions under which transposition occurs remain to be elucidated. In *Escherichia coli*, papillation assay is a powerful tool for transpositional analysis and the isolation of mutants affecting transposition. On the other hand, while our assay system based on the *E. coli* papillation assay can detect transpositional events in *Bacillus subtilis* 168, it is not suitable for quantitating transposition frequency because blue papillae on the transposant colonies of *B. subtilis* are not countable. We succeeded in developing a new "GFP hop-on assay" system that facilitates quantitative detection of the transposition of the FACS-optimized GFP mutant gene. Our assay system is a step forward in understanding the cellular conditions under which transposition occurs.

Keywords: *Bacillus subtilis*; Insertion sequence; IS*4Bsu1*; Transposition; Papillation assay; Green fluorescent protein (GFP); Fluorescence-activated cell sorting (FACS)

Various insertion sequences (IS) have been identified in bacteria and the detailed mechanisms of their transposition have been studied [1,2]. A papillation assay that facilitates the detection of independent transposition events as blue papillae on bacterial colonies proved to be a powerful tool for transpositional analysis of *Escherichia coli* [3]. The plasmid for papillation assay carries a transposon containing a truncated *lacZ* gene that lacks transcription and translation start signals, consequently, β-galactosidase is not directly expressed at the original location in the plasmid. Transposition of the *lacZ* gene into a chromosomal gene X in the correct orientation and reading frame would lead to the expression of a fusion β-galactosidase, resulting in the emergence of blue papillae.

Bacillus subtilis (*natto*), used for producing a traditional fermented Japanese food item called "natto", is closely related to *Bacillus subtilis* Marburg 168 [4], which is the best-characterized gram-positive bacterium and whose genome has been sequenced. The IS found in natto strains is a member of the IS*4* family; designated IS*4Bsu1*, it is 1406 bp in length, and it has imperfect 18-bp terminal inverted repeats (IRs). It also contains an open reading frame that encodes a 374-amino acid transposase and generates a 9-bp duplication of the target site during insertion [5]. We developed a transposition assay that uses *B. subtilis* 168 and modified IS*4Bsu1*. We found that the target sequences of IS*4Bsu1* were not conserved although they are AT-rich, and that the transposition frequency increased under high temperature and competence-developing conditions (Takahashi et al., unpublished data).

The transposition activity of transposons is mediated by various host factors. Histone-like proteins (or DNA chap-

* Corresponding author. Fax: +81 3 5477 2668.
E-mail address: hiyoshik@nodai.ac.jp (H. Yoshikawa).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.01.179

erones) such as HU and integration host factor (IHF) function in the transposition reactions of some bacterial transposons [6,7]. While a nucleoid-associated protein, H-NS, is required for IS*1* transposition, other histone-like proteins are not [8], indicating that a particular host factor is not essential for the transposition of all transposons. In this context, it is interesting to isolate the mutants that affect the transposition process in *B. subtilis*. While papillation assay is useful for analyzing differences in transposition frequency among different bacterial clones and for isolating such mutants in *E. coli* [9,10], it is not suitable for quantitative analyses of *B. subtilis* because blue papillae on *B. subtilis* colonies are not countable.

In many prokaryotic and eukaryotic organisms, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been used as a marker for gene expression and as a tag for studying protein localization since its photoluminescence requires no cofactors [11–13]. The expression of GFP can be monitored by fluorescence-activated cell sorting (FACS) using the 488-nm line of an argon laser and a FACS-optimized mutant of GFP, whose peak excitation wavelength is 488 nm, has been cloned [14].

To facilitate quantitative analysis of transpositional events in *B. subtilis*, we developed a transposition assay that uses the FACS-optimized GFP mutant. Our "GFP hop-on assay" facilitates the detection of transposition-dependent GFP expression in a single cell and the direct measurement of transposition frequency.

Materials and methods

Bacterial strains, media, plasmids, and antibiotics. *Bacillus subtilis* Marburg 168 (*trpC2*) and *E. coli* DH10B [F^- *mcrA Δ(mrr hsdRMS mcrBC)* Φ80*dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara leu)7697 galU galK l*$^-$ *rpsL endA1 nupG*] were grown in Luria–Bertani (LB) medium at 37 °C. The shuttle vector pDG148 [15] (Amp^R for *E. coli* and Km^R for *B. subtilis*) was used for pQG1 and pQG1Δtnp (see below). When necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added at a final concentration of 40 μg/ml. The antibiotics ampicillin and kanamycin were used at the concentration of 50 and 5 μg/ml, respectively.

Construction of plasmids. The 1.2-kb *Sal*I–*Sph*I PCR-generated fragment (fragment 1, using primers P1 and P2, Table 1) that harbors the transposase (*tnp*) gene of IS*4Bsu1* containing the SD sequence of the *B. subtilis rpsJ* gene was cloned into pDG148 to construct pDG-tnp. To construct pQG1 for the GFP hop-on assay, a 2.1-kb PCR fragment (fragment 4) containing the "GFP-hopper" and the *lac*I gene was cloned into *Sph*I–*Bam*HI sites of pDG-tnp. In the construction process, we first amplified 2 primary PCR-generated fragments. One was a PCR product (fragment 2) containing a 800-bp open reading frame without the initiation codon of the *gfpmut2* gene [14], which encodes the FACS-optimized GFP mutant and is flanked by 40-bp left- and right IR (IRL and IRR, respectively) of IS*4Bsu1* (derived from a PCR product with primers P3 and P4). The other was a 1.3-kb fragment of the *lacI* gene (fragment 3, primers P5 and P6). Primers P4 and P5 were designed to have 5′ add-on sequences to overlap with each other. Fragment 4 (primers P3 and P6) was amplified using the 2 primary PCR products and a recombinant PCR method [16]. The templates for generating fragments 1, 2, and 3 were *B. subtilis* (*natto*) RIK7102 [17] genomic DNA, pKEN2-GFPmut2 [14], and pDG148, respectively. The control was the *tnp* gene-deleted plasmid pQG1Δtnp. PrimeSTAR DNA polymerase (TaKaRa, Japan) was used in all PCR. Plasmids pQP1 and pQP0 for papillation assay were constructed in the same manner as pQG1 (see Fig. 1); they carry the *lacZ*- instead of the *gfpmut2* gene and lack the *lacI* gene.

Detection of GFP-hopper transposition. GFP hop-on assay was performed as follows: Plasmid pQG1 or pQG1Δtnp was introduced into the *B. subtilis* 168 strain, several transformant colonies were pooled and inoculated into 5 ml LB medium containing 5 μg/ml kanamycin with or without 1 mM isoproryl-β-D-thiogalactopyranoside (IPTG), and grown for 16 h at 37 °C. Harvested cells were then resuspended in FACSFlow buffer (BD Biosciences, USA) at $OD_{600} = 0.3$. Flow cytometry was on a FACSCalibur cytometer using CELLQUEST software (BD Biosciences). GFP fluorescence was detected with a FL1 detector; its intensity was expressed as an arbitrary logarithmic value (FL1-H) within the range of 10^0–10^4. The transposition-frequency was estimated as the ratio of the number of events within region M1 ($>2.5 \times 10^1$ FL1-H) or M2 ($>10^2$ FL1-H) to the number of total events.

Results and discussion

Deficiency in the papillation assay for B. subtilis 168

We previously developed an assay system similar to the *E. coli* papillation assay (Takahashi, et al. unpublished data) in *B. subtilis* 168 using modified IS*4Bsu1* from *B. subtilis* (*natto*). Transpositions of the *lacZ* marker could be detected as blue colonies (Fig. 1B). However, different from the phenotypes of *E. coli* transposants, no independent, dot-shaped, countable blue papillae (for examples, see [9,10]) were discernible on the transposant colonies of *B. subtilis* 168. Rather, blue zone exhibited a fan-shape (Fig. 1C) or were spread throughout the colony (Fig. 1D). Consequently, the papillation-like assay system using *B. subtilis* 168 was able to detect transpositional events, however, it is not suitable for quantitating transposition frequency.

Construction of the GFP hop-on assay

The deficiency in the papillation assay for *B. subtilis* 168 led us to develop an assay that allows the quantitative

Table 1
Primers used in this study

Primer	Sequence (5′–3′)
P1	*GGGCGTCGACGCGAAAAGGAGGGAAAAGC*ATGGATAAGTTTACACG
P2	*GGGCGCATGC*GCTTTCTTGTTTTTGCTCATGAG
P3	*GGCGCATGCCACTAGTGTTGCATAAATTTTGACTGAATTTTCTGCTTTC*TTATTTGTATAGTTCATCC
P4	*TCGTTTCCACCGACACTAGTGTCGCATAAAAATAATTCAACGATTTATAAAAA*GAGTAAAGGAGAAGAAC
P5	*TGCGACACTAGTG*TCGGTGGAAACGAGGTC
P6	*GGC*GGATCCTAACTCACATTAATTGCGTTGCGC

Additional sequences not corresponding to the sequences of relevant genes are italicized; restriction sites are underlined.