Transformation of *Aggregatibacter actinomycetemcomitans* with Green Fluorescent Protein

Tomomi Hashizume-Takizawa, Noriko Shinozaki-Kuwahara, and Tomoko Kurita-Ochiai

Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba, 271-8587, Japan

Article History

Received 7 May 2015

Accepted 24 August 2015

Keywords :

green fluorescent protein,
A. actinomycetemcomitans,
leukotoxin

Abstract

Green fluorescent protein (GFP) is one of the most useful biological markers for monitoring gene expression and the localization of particular cells. We constructed a GFP-expressing *Aggregatibacter actinomycetemcomitans* transformant by transposon insertion. This anaerobic, oral, gram-negative bacterium is thought to be the major causative agent in localized aggressive periodontitis and possesses the gene coding for leukotoxin (*ltx*). As this gene is stably expressed, the region extending from the *ltx* promoter to the initiation codon was amplified using PCR and temporarily cloned into a small *Escherichia coli* plasmid, pResKm. The DNA fragment containing the GFP gene (*gfp*) ORF was excised from the commercially available plasmid pAcGFP1-C1, purified by gel electrophoresis, and inserted downstream of the *ltx* initiation codon in the correct reading frame. This gene fusion was further cloned into the *NotI* site of the commercially available shuttle plasmid, pUTmini-Tn5, which contains the gene coding for the transposase, and then introduced into the chromosome of *A. actinomycetemcomitans* strain HK1651 by electroporation. Transformants were isolated by screening for the appropriate antibiotic resistance. FACS analysis revealed that the final *A. actinomycetemcomitans*-GFP integrant exhibited slightly more intense fluorescence than wild-type *A. actinomycetemcomitans*. These data demonstrate that transposon-mediated chromosomal integration is a useful approach for constructing mutant *A. actinomycetemcomitans* strains.

Introduction

Aggregatibacter actinomycetemcomitans is a fastidious, facultatively anaerobic, non-motile, non-spore-forming, small, oral, gram-negative bacterium thought to be one of the major causative agents of localized aggressive periodontitis (1-3).

Green fluorescent protein (GFP) is widely used for monitoring the expression of particular genes and localizing living cells. GFP emits bright visible fluorescence when expressed in a recombinant manner, and no substrate is required (4, 5). The *gfp* gene has been expressed in many bacteria (6, 7). Therefore, construction of a GFP-expressing strain of *A. actinomycetemcomitans* would enable the analysis of the dynamics of this species in experimental

animals.

A promoter recognized by host cells must be located upstream of *gfp* to ensure adequate transcription. In *A. actinomycetemcomitans* SUNY465, promoters from *Escherichia coli* function poorly compared with the natural sequences (5). The nucleotide sequence of the leukotoxin gene (*ltx*) of *A. actinomycetemcomitans* is available, and the promoter thereof constitutively and efficiently transcribes the gene (8, 9). Therefore, efficient recombinant gene expression would be expected were *gfp* to be fused downstream from the *ltx* promoter from *A. actinomycetemcomitans*.

Several vectors, including plasmids and transposons, can be used to construct recombinant bacteria. Plasmids in high copy number and maintained via selection can yield high levels of transcripts and good amounts of recombinant protein. However, host cell growth can be compromised,

Correspondence to :

Tomomi Hashizume-Takizawa

E-mail : hashizume.tomomi@nihon-u.ac.jp

triggering plasmid instability. If transposons are used, only single copies of the desired target gene are integrated into the host chromosome, affording stable maintenance of the foreign gene in the absence of any antibiotic selection (10).

In this study, we used a transposon to introduce the *gfp* gene into the *A. actinomycetemcomitans* chromosome under the control of the *A. actinomycetemcomitans ltx* promoter. This transformation will facilitate more extensive analyses of the biology of *A. actinomycetemcomitans*.

Materials and Methods

Bacterial strains

Aggregatibacter actinomycetemcomitans strain HK1651 (ATCC 700685) was grown in Todd-Hewitt broth (THB; BD Difco, Franklin Lakes, NJ) supplemented with 1% yeast extract (BD Difco) and 5% fetal bovine serum (Biofill Australia Pty Ltd, Victoria, Australia) at 37°C and 5% CO₂. Recombinant *A. actinomycetemcomitans*-GFP was grown in the same medium containing 100 µg/ml streptomycin and 50 µg/ml spectinomycin. *Escherichia coli* DH5 α was grown aerobically in LB broth supplemented with ampicillin (100 µg/ml) when appropriate, at 37°C.

Plasmids

The plasmid pResKm was kindly provided by Dr. Teruaki Shiroza (Nihon University) (11). The plasmid pAcGFP1-C1, a source of *gfp* from *Aequorea coerulescens*, was purchased from Takara Bio Clontech (Shiga, Japan). The transposon plasmid, pUTmini-Tn5 Sm/Sp, was purchased from Biomedal, S.L. (Seville, Spain).

Primers

The following oligonucleotides were designed as primer pairs for amplifying the respective regions of DNA: forward A (*ltx*), 5'-CTTACGGATCCAAACCTGATAACAGT-3'; reverse A (*ltx*), 5'-TTCTCCATGGTAAATCTCCTT-3'; forward B (*gfp*), 5'-ATCACATGAAGCAGCAGCACTT-3'; reverse B (*gfp*), 5'-ACTTGTACAGCTCATCCAT-3'; forward C (*16s*), 5'-AGAGTTTGATCCTGGCTCAG-3'; and reverse C (*16s*), 5'-CACTTAAAGGTCCGCCTACGTGCC-3'.

In primer set A, the underlined sequences denote the mismatches introduced to create the restriction enzyme recognition sequences for *Bam*HI (GGATCC) and *Nco*I (CCATGG).

DNA manipulation

Chromosomal DNA from *A. actinomycetemcomitans* HK1651 and recombinant *A. actinomycetemcomitans*-GFP was purified using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). Plasmid DNA was isolated using Nucleospin Plasmid Quick Pure (Takara Bio). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs Japan, Inc. (Tokyo, Japan). Restriction fragments were extracted from agarose gels using a Nucleo Spin Extract II (Macherey-nagel GmbH & Co. KG, Düren, Germany). Transformation of *E. coli* was carried out via a standard CaCl₂ method or electroporation using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) set at 2,500 V, 200 Ω , and 25 µF (12). Transformation of *A. actinomycetemcomitans* was performed as follows. Cells were harvested by centrifugation in the early logarithmic phase (3 h). The resulting pellets were washed in ice-cold electroporation buffer (15% glycerol, 272 mM sucrose, 2.43 mM K₂HPO₄, and 0.57 mM KH₂PO₄; pH 5.5) and resuspended in fresh electroporation buffer to a final optical density at 600 nm of 6.0. Electroporation was performed as described above. The bacteria were then immediately transferred into 1.0 ml of broth for *A. actinomycetemcomitans* without antibiotics and incubated for 5 h, after which the cells were plated on agar medium containing appropriate antibiotics (12). All of the recombination protocols were approved by the Nihon University Biosafety Committee for Gene Recombination (2010MD002).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from *A. actinomycetemcomitans* HK1651 and recombinant *A. actinomycetemcomitans*-GFP using a PureLink RNA kit (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's instructions. Residual genomic DNA was removed by DNase I treatment. cDNA was synthesized using a Prime-Script RT reagent kit (Perfect Real Time) (Takara Bio), without reverse transcriptase for detection of residual DNA contamination. Quantitative real-time RT-PCR was performed using a TP800 Thermal Cycler Dice Real Time System (Takara Bio) and SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio) according to the manufacturer's instructions. Primer set B (described above) was used for amplification of *gfp*, and primer set C (described above) was used for amplification of *A. actinomycetemcomitans 16S*. The PCR

program comprised an initial denaturation step (94°C for 10 min), followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The level of GFP mRNA expression was normalized to that of 16S. Each gene was examined in triplicate.

Flow cytometry

The expression of GFP in *A. actinomycetemcomitans* was analyzed by flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA).

Results

Construction of the shuttle vector containing the *ltx* promoter and *gfp* fusion

Construction of a shuttle vector containing the *ltx* promoter and *gfp* fusion (pUTmini-Tn5-*ltx p, gfp*) and capable of replicating in *E. coli* and *A. actinomycetemcomitans* is outlined in Figure 1. A commercially available plasmid, pAcGFP1-C1, was employed as the source of the gene encoding GFP. Because the 3'-end of the gene encoding GFP contained a multiple cloning site (MCS), which was unnecessary for the present study, pAcGFP1-C1 was first digested with both *Bgl*II and *Bam*HI and recircularized to simply eliminate the MCS and give pAcGFP1-C1 Δ MCS (Fig. 1A).

To scissor the 0.8-kb portion of the *gfp* fragment from pAcGFP1-C1 Δ MCS, the vector was doubly digested with both *Nhe*I and *Bcl*II, and then the fragments were separated by agarose gel electrophoreses. The 0.8-kb band was cut out from the agarose gel, and the *gfp* fragment was purified. This fragment was ligated with a *Xba*I and *Bcl*II digest of the small plasmid pResKm. As the restriction products of both *Nhe*I and *Xba*I possess the same 5'-CTAG-3' extension, the portion of the *gfp* fragment was cloned into the plasmid to give pResKm-*gfp* (Fig. 1B).

Next, an approximately 0.5-kb 5' portion of the DNA region encompassing the *ltx* promoter and the ribosomal binding site (aggaga: SD sequence) (9) was amplified by PCR using the *A. actinomycetemcomitans* chromosome and primer set A (Fig. 1C). Each primer included a recognition sequence (either GGATCC or CCATGG; see Materials and Methods) corresponding to the *Bam*HI and *Nco*I restriction sites, respectively. Furthermore, as the restriction products of both *Bam*HI and *Bgl*II possessed the same 5'-GATC-3' extension, the PCR product was digested with both *Bam*HI and *Nco*I and inserted into *Bgl*II/*Nco*I-digested pResKm-*gfp* in the correct reading frame to give pResKm-*ltx p, gfp* (Fig.

1D).

The commercially available *E. coli*-*A. actinomycetemcomitans* shuttle plasmid, pUTmini-Tn5, retains the unique *Not*I site (Fig. 1E). The constructed *ltx p, gfp* gene fragment (1.3 kb) was isolated from pResKm-*ltx p, gfp* (Fig. 1D) by digestion with the 8-base cutting enzyme *Not*I, purified by gel electrophoresis, and cloned into the unique *Not*I site of pUTmini-Tn5 to generate pUTmini-Tn5-*ltx p, gfp* (Fig. 1F). This plasmid was electrotransformed into *E. coli* DH5 α .

Transformants were screened on agar plates supplemented with ampicillin, and the correct gene organization of the final construct was confirmed by agarose gel electrophoresis. Digestion of pUTmini-Tn5-*ltx p, gfp* (8.7kb) with both *Sph*I and *Nco*I produced two bands, 1130 and 7600 bases. The smaller fragment included part of the inserted gene encoding AcGFP1. The larger fragment contained the rest of pUTmini-Tn5-*ltx p, gfp* (Fig. 2). In addition, the nucleotide sequences of the cloned fragments were analyzed to confirm the presence of the intended insert.

Transformation of *A. actinomycetemcomitans* with the pUTmini-Tn5-*ltx p, gfp* construct

As pUTmini-Tn5 possesses the RP4oriT origin of transfer sequence responsible for the efficient conjugal transfer of the plasmid from *E. coli* to other gram-negative bacteria, including *A. actinomycetemcomitans*, we first attempted to transform *A. actinomycetemcomitans* with pUTmini-Tn5-*ltx p, gfp* by conjugation. However, transformation of *A. actinomycetemcomitans* HK1651 by this method was unsuccessful. Therefore, we introduced pUTmini-Tn5-*ltx p, gfp* into *A. actinomycetemcomitans* HK1651 chromosome via simple electroporation. Because pUTmini-Tn5-*ltx p, gfp* possesses genes that convey streptomycin and spectinomycin resistance, the *A. actinomycetemcomitans* HK1651 transformants could be screened on agar plates supplemented with streptomycin and spectinomycin, and *A. actinomycetemcomitans* transformants resistant to these two antibiotics were isolated using this approach. PCR experiments utilizing the purified chromosome isolated from the integrants with *gfp*-specific primers clearly indicated that the target region was integrated into the host chromosome (Fig. 3A). Furthermore, quantitative real-time RT-PCR analysis utilizing total RNA isolated from the integrants using *gfp*-specific primers clearly showed that expression of GFP mRNA in the final integrants was up-regulated, whereas no expression of GFP mRNA was

Fig. 1 Construction of pUTmini-Tn5-*ltx p, gfp*. For detailed construction procedures, see text. (A) Structure of pAcGFP1-C1, the commercially available source of *gfp*. Bold line with arrowhead denotes the approximate size (0.8 kb) and direction of *gfp*. The MCS structure present at the 3'-end of the *gfp* gene was eliminated by digestion with both *Bgl*II and *Bam*HI and recircularization to give pAcGFP1-C1 ΔMCS. (B) Structure of the small plasmid pResKm, which is composed of Km^r, p15A ori, and an MCS. The *gfp* fragment was excised by digestion with both *Nhe*I and *Bcl*I and gel-purified. This fragment was introduced into the *Xba*I-*Bcl*I digest of pResKm to give pResKm-*gfp*. (C) Structure around the 5'-region of the *ltx* gene. Dark gray bar shows the DNA fragment amplified by PCR using primer set A. (D) Structure of the pResKm derivative, pResKm-*ltx p, gfp*, which contains the *ltx-gfp* gene fusion. These two genes were connected in the correct reading frame via the *Nco*I site. (E) Structure of the commercially available shuttle plasmid, pUTmini-Tn5, composed of Am^r, Sp^r, Sm^r, the RP4oriT origin of transfer sequence, and transposase coding region with a *Not*I site in the ORF. (F) Structure of the final construct, pUTmini-Tn5-*ltx p, gfp*. As the fused *ltx-gfp* gene is flanked by a *Not*I 8-base cutting site, the target fragment was gel-purified following restriction cleavage and introduced into the *Not*I site of the shuttle plasmid to give the final construct, pUTmini-Tn5-*ltx p, gfp*. (G) After integration into the *A. actinomycetemcomitans* chromosome, Am^r and the transposase coding region in pUTmini-Tn5 were eliminated.

Fig. 2 Restriction analysis of the gene organization in pUTmini-Tn5-*ltx p, gfp*. pUTmini-Tn5-*ltx p, gfp* was digested with both *Sph*I and *Nco*I and analyzed by gel electrophoresis. The locations of the *Sph*I and *Nco*I sites in pUTmini-Tn5-*ltx p, gfp* are indicated in Figure 1F. Lane 1, pUTmini-Tn5-*ltx p, gfp*. M indicates the size marker.

Fig. 3 PCR identification of the *gfp* gene region in the final transformant, and flow cytometric analysis of the final transformant. (A) Agarose gel electrophoresis of PCR products of the *gfp* gene region in the chromosome. Lane 1, wild-type *A. actinomycetemcomitans*. Lane 2, recombinant *A. actinomycetemcomitans*-GFP. Lane 3, pAc-GFP1-C1 (positive control). M indicates the size marker. (B) Quantitative real-time RT-PCR analysis of GFP mRNA expression in *A. actinomycetemcomitans*-GFP and wild-type *A. actinomycetemcomitans*. n.d., not detectable. (C) FACS analysis of FL1 fluorescence intensity of recombinant *A. actinomycetemcomitans*-GFP and wild-type *A. actinomycetemcomitans*.

detected in wild-type *A. actinomycetemcomitans* cells (Fig. 3B). When we performed real-time RT-PCR analysis utilizing cDNA synthesized without reverse transcriptase,

no amplification of the *16S* or *gfp* genes was observed. These data indicated that the GFP mRNA expressed by the final integrants was not derived from genomic DNA.

In addition, *A. actinomycetemcomitans* transformants were also confirmed by ampicillin susceptibility, because authentic transposition results in elimination of the ampicillin-resistance gene in the pUTmini-Tn5 delivery system. All isolated transformants were susceptible to ampicillin (data not shown).

Characterization of *A. actinomycetemcomitans*-GFP

Flow cytometric analysis revealed a slight increase in the intensity of green fluorescence in recombinant *A. actinomycetemcomitans*-GFP compared with wild-type *A. actinomycetemcomitans* (Fig. 3C).

Discussion

In this study, we constructed a recombinant *A. actinomycetemcomitans* strain in which GFP could be expressed under the control of the native *A. actinomycetemcomitans* leukotoxin gene promoter. Several previous studies have reported the expression of GFP in *A. actinomycetemcomitans* transformants by plasmid-mediated transformation (5, 13). In contrast to these previously reported transformants, the chromosomally integrated heterogeneous gene is stably maintained in a single copy in host cells (10). Therefore, we attempted to introduce the *gfp* gene into the *A. actinomycetemcomitans* chromosome by transposon-mediated transformation.

Most commercially available GFP plasmids, including pAcGFP1-C1, are designed to express *gfp* exclusively in eukaryotic cells. This means that no SD sequence for *A. actinomycetemcomitans* exists in the upstream region of the ORF encoding GFP. On the other hand, the *A. actinomycetemcomitans* strain HK1651 (JP2 clone) *ltx* nucleotide sequence has been reported, and this gene is constitutively expressed at a high level via the promoter (9). Taking advantage of this, we designed PCR primers to amplify an approximately 0.5-kb region encompassing the *ltx* promoter region and the 5' portion of the *ltx* ORF, in which the *Nco*I recognition sequence CCATGG (corresponding to the initiation codon) was included in the reverse primer. This allowed us to fuse both the *ltx* promoter and *gfp* in the correct reading frame. Because the initiation codon was preceded by the native SD sequence, the gene fusion was expressed in the *A. actinomycetemcomitans* host cells.

Because pUTmini-Tn5 carries the RP4oriT sequence responsible for the efficient conjugal transfer of the plasmid from *E. coli* to other gram-negative bacteria, it seemed

possible that the recipient *A. actinomycetemcomitans* strain could be transformed via mating with donor *E. coli*. However, this attempt was unsuccessful because both bacteria are resistant to the same antibiotics. It is also unlikely that conjugation between *A. actinomycetemcomitans* and *E. coli* would have occurred. A previous study demonstrated that transformation of *A. actinomycetemcomitans* could be accomplished by optimizing various electroporation conditions, including the growth stage of the host cells, buffer pH, and incubation period for post-electroporation expression (12). In the present study, we isolated *A. actinomycetemcomitans* transformants by adjusting the mildly acidic pH in the buffer system and prolonging the expression period relative to the generally accepted conditions for transforming *E. coli* cells (14).

In our experiments, GFP expression by recombinant *A. actinomycetemcomitans*-GFP was slightly increased compared with wild-type *A. actinomycetemcomitans*. It has been shown that GFP requires molecular oxygen to catalyze the cyclization of amino acid residues to form the active fluorophore structure (15). Furthermore, another study demonstrated that the low oxygen conditions characteristic of tumors lead to reduced GFP levels (16). Taken together, these data indicate that it is highly likely that in anaerobic bacteria such as *A. actinomycetemcomitans*-GFP, the intensity of GFP fluorescence is affected by the availability of molecular oxygen in the culture medium.

In summary, we integrated the *gfp* gene into the *A. actinomycetemcomitans* chromosome using a transposon insertion system. Our results demonstrate that this system is useful for the recombination of *A. actinomycetemcomitans*. However, the final transformant, *A. actinomycetemcomitans*-GFP, exhibited relatively low fluorescence intensity, suggesting that the expression of GFP in *A. actinomycetemcomitans* may be restricted by the availability of oxygen.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (24592845) from the Japan Society for the Promotion of Science and by the Tsuchiya Culture Promotion Foundation.

References

1. Henderson B, JM Ward, D Ready: *Aggregatibacter (Actinobacillus) actinomycetemcomitans*: a triple A* periodon-

- topathogen? Periodontol 2000, 54(1): 78-105, 2010.
2. Slots J, M Ting: *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. Periodontol 2000, 20: 82-121, 1999.
 3. Zambon JJ: *Actinobacillus actinomycetemcomitans* in human periodontal disease. J Clin Periodontol, 12(1): 1-20, 1985.
 4. Prasher DC, VK Eckenrode, WW Ward, FG Prendergast, MJ Cormier: Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene, 111(2): 229-233, 1992.
 5. Lippmann JE, EH Froeliger, PM Fives-Taylor: Use of the *Actinobacillus actinomycetemcomitans* leukotoxin promoter to drive expression of the green fluorescent protein in an oral pathogen. Oral Microbiol Immunol, 14(5): 321-325, 1999.
 6. Park PG, MH Cho, GE Rhie, H Jeong, H Youn, KJ Hong: GFP-tagged *E. coli* shows bacterial distribution in mouse organs: pathogen tracking using fluorescence signal. Clin Exp Vaccine Res, 1(1): 83-87, 2012.
 7. Wei M, Z Kong, L Zhong, L Qiu, Y Li, L Zhao, X Li, W Zhong: Construction of a native promoter-containing transposon vector for the stable monitoring of the denitrifying bacterium *Pseudomonas stutzeri* LYS-86 by chromosomal-integrated gfp. Plasmid, 68(1): 61-68, 2012.
 8. Brogan JM, ET Lally, K Poulsen, M Kilian, DR Demuth: Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. Infect Immun, 62(2): 501-508, 1994.
 9. Lally ET, EE Golub, IR Kieba, NS Taichman, J Rosenbloom, JC Rosenbloom, CW Gibson, DR Demuth: Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins. J Biol Chem, 264(26): 15451-15456, 1989.
 10. Herrero M, V de Lorenzo, KN Timmis: Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol, 172(11): 6557-6567, 1990.
 11. Shiroza T, HK Kuramitsu: Construction of a model secretion system for oral streptococci. Infect Immun, 61(9): 3745-3755, 1993.
 12. Sreenivasan PK, DJ LeBlanc, LN Lee, P Fives-Taylor: Transformation of *Actinobacillus actinomycetemcomitans* by electroporation, utilizing constructed shuttle plasmids. Infect Immun, 59(12): 4621-4627, 1991.
 13. Teughels W, I Sliepen, S De Keersmaecker, M Quirynen, J Lippmann, M Pauwels, P Fives-Taylor: Influence of genetic background on transformation and expression of Green Fluorescent Protein in *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol, 20(5): 274-281, 2005.
 14. Shi X, T Karkut, M Alting-Mees, M Chamankhah, SM Hemmingsen, DD Hegedus: Enhancing *Escherichia coli* electrotransformation competency by invoking physiological adaptations to stress and modifying membrane integrity. Anal Biochem, 320(1): 152-155, 2003.
 15. Heim R, DC Prasher, RY Tsien: Wavelength mutations and posttranslational autooxidation of green fluorescent protein. Proc Natl Acad Sci U S A, 91(26): 12501-12504, 1994.
 16. Coralli C, M Cemazar, C Kanthou, GM Tozer, GU Dachs: Limitations of the reporter green fluorescent protein under simulated tumor conditions. Cancer Res, 61(12): 4784-4790, 2001.