Codon-optimized antibiotic resistance gene improves efficiency of transient transformation in *Frankia*

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Frankia is a unique actinobacterium having abilities to fix atmospheric dinitrogen and to establish endosymbiosis with trees, but molecular bases underlying these interesting characteristics are poorly understood because of a lack of stable transformation system. Extremely high GC content of *Frankia* genome (>70%) can be a hindrance to successful transformation. We generated a synthetic gentamicin resistance gene whose codon usage is optimized to *Frankia* (fgm^R) and evaluated its usefulness as a selection marker using a transient transformation system. Success rate of transient transformation and cell growth in selective culture were significantly increased by use of fgm^R instead of a native gentamicin resistance gene, suggesting that codon optimization improved translation efficiency of the marker gene and increased antibiotic resistance. Our result shows that similarity in codon usage pattern is an important factor to be taken into account when exogenous transgenes are expressed in *Frankia* cells.

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1. Introduction

Frankia is a unique actinobacterium having abilities to fix atmospheric dinitrogen (N_2) and to establish endosymbiosis with trees belonging to 8 dicotyledonous families collectively called 'actinorhizal plants' (Benson and Silvester 1993; Huss-Danell 1997; Kucho *et al.* 2010). Symbiosis occurs in root nodule in which *Frankia* fixes N_2 to ammonium and supplies the product to host plants. Owing to the symbiosis, actinorhizal plants grow fast even in oligotrophic lands and improve soil fertility, thus they are often used for afforestation. In 2007, complete genome sequences of three *Frankia* strains with different host ranges were determined (Normand *et al.* 2007), which has opened a new era in *Frankia* research. Omics-approaches making use of those genomic data provide increasing information on candidate genes expected to be involved in symbiotic nitrogen fixation (Alloisio *et al.* 2007; Bagnarol *et al.* 2007; Mastronunzio *et al.* 2008; Mastronunzio *et al.* 2009).

Unfortunately, however, it is at present difficult to define the function of a *Frankia* gene because stable transformation of *Frankia* is not feasible in spite of numerous trials (Mullin and An 1990; Simonet *et al.* 1990; Cournoyer and Normand 1992; Benson and Silvester 1993; Myers and Tisa 2003). Slow growth (especially on solid media) and high GC-content (>70%) of the bacterium can be a part of reasons for those failures. Recently, we introduced a plasmid DNA carrying a fusion maker gene consisting of a tetracycline resistance gene (tet^R) and a promoter of the strain's translation initiation factor 3 gene ($P_{infC\text{-}1}$) by electroporation (Kucho *et al.* 2009). Although the plasmid DNA did not have a replication origin functioning in *Frankia*, it transiently supported growth of *Frankia* cells in selective media because degraded fragments of the plasmid DNA containing the marker gene were still retained in cytoplasm even after several times of cell divisions. Gentamicin resistance gene (gm^R) whose codon usage is less similar to *Frankia* genes did not transform *Frankia* cells as effectively as tet^R did. This result suggests that codon usage

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similarity is an important factor for successful transformation of *Frankia*.

In the present study, we used an artificial gentamicin resistance gene whose codon usage was optimized to the *Frankia* sp. strain CcI3 to elucidate the effects of codon usage similarity of marker genes on transient transformation.

2. Methods

2.1 *Bacterial strains and culture conditions*

Frankia sp. strain CcI3, which establishes symbiosis with *Casuarina* species (Zhang *et al.* 1984), was cultivated in a liquid BAP-T medium (Kucho *et al.* 2009) at 28°C with stirring. Before inoculation, hyphae were homogenized by forcing them to pass through a 21G needle. *Escherichia coli* strain DH5α was used as a host for DNA cloning and grown in LB medium (Ausubel *et al.* 1990) at 37°C.

2.2 *Generation of transformation constructs*

The codon-optimized gm^R gene (fgm^R) was designed and synthesized by Codon Devices (Cambridge, MA, USA) based on sequence of the gentamicin acethyl-transferase-3-I gene (Becker *et al.* 1995). Codon adaptation index (CAI) (Sharp and Li 1987) of the marker genes was calculated by the cai function of the European molecular biology open software suite (EMBOSS, *http://bips.u-strasbg.fr/EMBOSS/*) using the ribosomal protein genes of the strain CcI3 as references. A coding sequence of fgm^R was cloned into a *Hpa*I-*Not*I site of pPTl1 (Kucho *et al.* 2009) which locates between a promoter region of the translation initiation factor 3 gene (Francci3_3182) ($P_{infC\text{-}1}$) and a transcription terminator of the *serC* gene (Francci3_0082) (T_{serC}) of the strain CcI3. The fusion gene ($P_{infC\text{-}1}$::fgm^R::T_{serC}) was excised by *Xba*I and inserted into an *Xba*I site of pTSF1 (Kucho *et al.* 2009) to generate pKKF9. Plasmid DNA used for transformation was purified by the HiSpeed Plasmid Midi Kit (Qiagen, Tokyo, Japan).

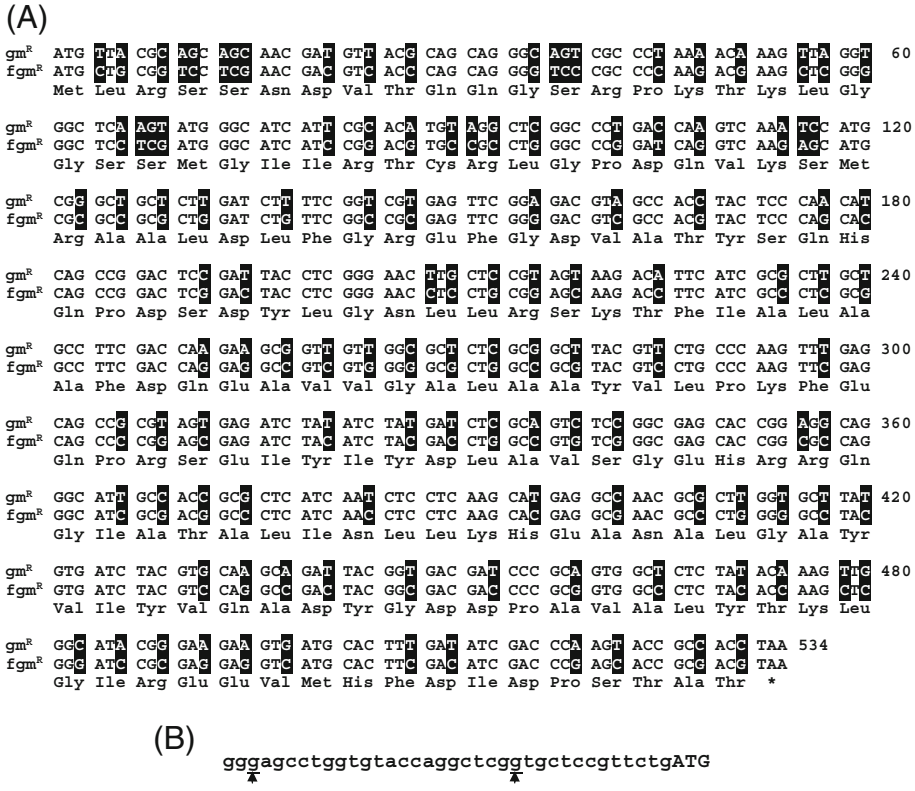

Figure 1. (**A**) Nucleotide sequences of native (gm^R) and codon-optimized (fgm^R) gentamicin resistance genes. Nucleotides altered to optimize codon usage are highlighted. Amino acids encoded by each codon are shown under the nucleotide sequences. An asterisk indicates a stop codon. (**B**) Upstream sequence of start codon of gm^R and fgm^R genes. The start codon ATG is capitalized. Major transcription start sites determined in Kucho *et al.* (2009) are indicated by arrow heads.

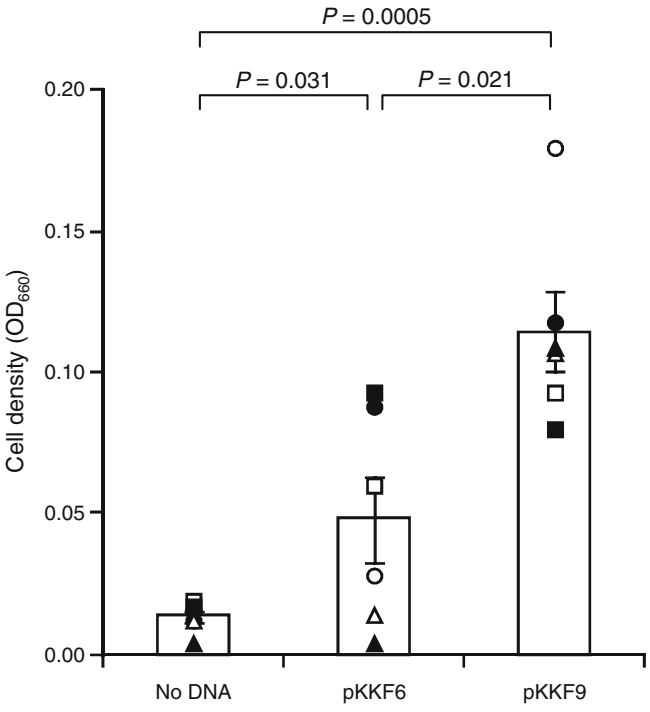

Figure 2. Growth of antibiotic resistant cells in selective media containing gentamicin. Cell density was measured as OD_{660} of fragmented hyphae. We inoculated electroporated cells at an initial OD_{660} of 0.03 and cultured them for 2 to 6 weeks. An average of six independent experiments is shown by white bar with standard error. Data from a same series of experiment are represented by an identical symbol. *P*-values for paired *t*-test are shown above the graph.

2.3 *Transient transformation of Frankia*

We transiently transformed *Frankia* cells according to the procedure described in Kucho *et al.* (2009). Briefly, strain CcI3 was grown to mid- or late-log phase in BAP-T medium. Cells were collected by centrifugation, washed with sterilized distilled water and then resuspended in 10% glycerol. The cells were homogenized by forced passage through a 21G needle, mixed with 10 µg of construct DNA and transferred to an electroporation cuvette with a 2 mm gap (BM, Tokyo, Japan). An electric pulse was delivered by the Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA) with the following settings: capacitance = 25 µF, resistance = 800 Ω, and voltage = 2 kV (10 kV cm^{-1}). Antibiotic resistant cells were selected in 140 mL of BAP-T liquid medium containing 75 µg m^{-1} gentamicin (Nacalai tesque, Kyoto, Japan) with stirring for 2 to 6 weeks at 28°C. Cell density of the culture was evaluated by optical density of homogenized hyphae at 660 nm (OD_{660}) according to the method described in Kucho *et al.* (2009).

2.4 *Southern blot analysis*

Before total DNA was extracted, *Frankia* cell pellet was washed 9 times with approximately 10-times volume of sterilized distilled water to remove construct DNA outside the cells. Total DNA was prepared by acetyltrimethylammonium bromide (CTAB) method with slight modifications as described in Kucho *et al.* (2009). Total DNA (0.5 µg) was digested by *Pvu*II and electrophoresed in 0.8% agarose gel. DNA was blotted to the IMMOBILON-NY+ nylon membrane (Millipore, Billerica, MA, USA) using an alkali transfer buffer (0.4 N NaOH). A flanking sequence of the marker gene was used as a probe (Kucho *et al.* 2009). Labeling and hybridization were performed using the AlkPhos direct labelling and detection system (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions, except that hybridization was performed at 60°C. The hybridization signal was detected with the LAS-1000 image analyser (Fujifilm, Tokyo, Japan).

3. Results and discussion

GC-rich *Frankia* genomes rarely use AT-rich synonymous codons (Sen *et al.* 2008). Native gm^R gene dominantly uses

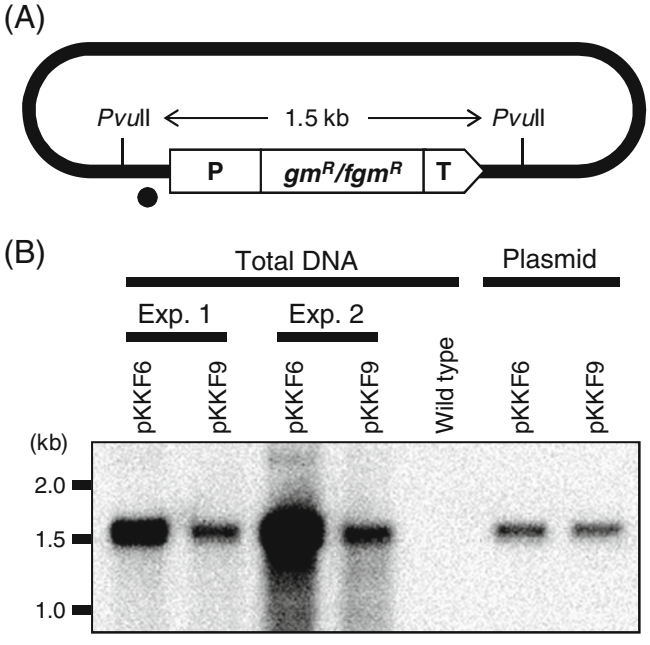

Figure 3. Southern blot analysis. (**A**) Positions of restriction sites and a probe (closed circle) in transformation construct DNA are indicated. P, promoter region of the translation initiation factor 3 gene; T, transcription terminator of the *serC* gene. (**B**) Total DNAs of transient transformants from two independent experiments (Exp. 1 and Exp. 2) and wild-type strain CcI3 were analysed by Southern blot. *Pvu*II-digested plasmid DNAs of pKKF6 and pKKF9 were electrophoresed in the same gel as controls.

such rare codons, which resulted in low CAI value (0.325). Therefore, we altered entire nucleotide sequence of gm^R gene so that those rare codons were replaced to synonymous codons dominantly used by strain CcI3. The resulting artificial gene fgm^R has an identical amino acid sequence with native gm^R gene but 107 of 178 codons were changed to the most or second most frequent synonymous codons in strain CcI3 genome (figure 1A). As the consequence, CAI of fgm^R was much higher (0.825) than that of native gm^R.

We introduced plasmid DNAs pKKF6 (Kucho *et al.* 2009) and pKKF9, each of which carries gm^R or fgm^R fused with a promoter $P_{infC\text{-}1}$, into strain CcI3 cells by electroporation and transient transformation was evaluated by cell density (OD_{660}) of selective liquid culture. Among six trials, three experiments for gm^R (pKKF6) and 6 experiments for fgm^R (pKKF9) showed apparently higher growth than negative control cultures electroporated without construct DNA (figure 2). In addition, cell density of the cultures was significantly higher in experiments using the fgm^R construct (pKKF9) (0.115±0.014, average±standard error) than in those using the gm^R construct (pKKF6) (0.048±0.016) (paired *t*-test *P*-value=0.021). These results indicate that codon optimization improved efficiency of transient transformation.

Southern blot analysis detected bands derived from intact marker genes in DNA extracted from the antibiotic resistant cells (figure 3), confirming introduction of plasmid DNA in strain CcI3 cells. Southern blot analysis of cells exposed to plasmid but not electroporated estimated that approximately 15% of the signal was derived from plasmid adhered on cell surface (data not shown). Unexpectedly, signal strength of the bands was varied depending on construct DNA; it was higher in cells transformed by gm^R construct (pKKF6) than those transformed by fgm^R construct (pKKF9). This may be because fgm^R contains nucleotide sequences susceptible to nuclease in *Frankia* cells.

There are three possible reasons why fgm^R transformed *Frankia* more efficiently than gm^R did: (i) stability of the marker gene was increased, (ii) transcription efficiency was increased, and (iii) translation efficiency was increased. The first possibility can be excluded because less DNA molecules of fgm^R were present in CcI3 cells than that of gm^R (figure 3). The second possibility is also negligible because both marker genes were transcribed from an identical promoter. The third possibility is the most probable case. It is reported that iso-accepting tRNAs corresponding to synonymous codons that are rarely used are less expressed and translation efficiency of genes carrying such rare codons is low (Ikemura 1981). This would be the case of gm^R; and fgm^R, which contains no rare codon, would be translated more efficiently, thereby conferred higher antibiotic resistance. Indeed enhancement of translation by codon usage optimization is also reported in other organisms with biased base composition (Fuhrmann *et al.* 1999; Vervoort *et al.* 2000; Matsuo *et al.* 2006). The gm^R gene contains two TTA codons, which is especially rare in *Frankia*, near the translation initiation codon. Optimization of only the two codons could be enough to enhance the efficiency of expression. Typical ribosome-binding sequence (AGGAGG) is not found in upstream sequence of translation initiation codon (figure 1B). Optimization of the sequence can further enhance the translation efficiency.

It is also possible that translation was enhanced by relaxation of secondary structure of mRNA because tightly folded mRNA prevents translation initiation and reduces protein synthesis (Kudla *et al.* 2009). We calculated minimum free energy for predicted secondary structures of mRNA and found that mRNA of fgm^R was more tightly folded (−206 kcal/mol) than that of gm^R (−153 kcal/mol), indicating that mRNA structure did not contribute to the improvement of transformation efficiency.

In conclusion, the present study showed that codon usage frequency is an important factor to be taken into account when exogenous transgenes are expressed in *Frankia* cells. Codon optimization is an easy and effective method to develop antibiotic resistance and reporter genes functioning in *Frankia*. Such genes will largely contribute to establish stable transformation system in *Frankia*.

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