Improvement of bacterial transformation efficiency using plasmid artificial modification

Kazumasa Yasui1, Yasunobu Kano2, Kaori Tanaka3, Kunitomo Watanabe3, Mariko Shimizu-Kadota4,5, Hirofumi Yoshikawa5 and Tohru Suzuki1,*

1The United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, 2Department of Molecular Genetics, Kyoto Pharmaceutical University, 1 Shichono-cho, Misasagi, Yamashina-ku, Kyoto 607-8412, 3Division of Anaerobe Research, Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, 4Department of Environmental Science, Musashino University, Shinmachi Nisshitoko-shi, Tokyo 202-8585 and 5Department of Bioscience, Tokyo University of Agriculture, Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

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ABSTRACT

We have developed a method to improve the transformation efficiency in genome-sequenced bacteria, using ‘Plasmid Artificial Modification’ (PAM), using the host’s own restriction system. In this method, a shuttle vector was pre-methylated in Escherichia coli cells, which carry all the putative genes encoding the DNA modification enzymes of the target microorganism, before electroporation was performed. In the case of Bifidobacterium adolescentis ATCC15703 and pKKT427 (3.9 kb E. coli-Bifidobacterium shuttle vector), introducing two Type II DNA methyltransferase genes lead to an enhancement in the transformation efficiency by five orders of magnitude. This concept was also applicable to a Type I restriction system. In the case of Lactococcus lactis IO-1, by using PAM with a putative Type I methyltransferase system, hsdMS1, the transformation efficiency was improved by a factor of seven over that without PAM.

INTRODUCTION

Recently, vast amounts of sequence information concerning bacterial genomes have become available. Currently, 670 whole-genome bacterial sequences have been published and over 1900 projects are in progress. However, much of the data has been used inefficiently in molecular biological studies since reverse genetic tools, such as convenient shuttle vectors, an efficient transformation method, gene knockout and random mutagenesis techniques, etc., have not been available. Accordingly, we have been working towards developing simple methods that would establish transformation techniques for bacteria for which the genome sequence is available.

It is well known that most bacteria carry a specific restriction modification (R–M) system which acts as a barrier against the invasion of foreign DNA by infected phages or conjugative plasmids, etc. (1). The restriction enzymes recognize a specific 4 bp–8 bp DNA sequence and cleave the DNA, but do not recognize the same sequence when modified by the sequence-specific DNA methylase (2). This prevents the degradation of the host’s own DNA by the restriction enzyme. According to REBASE (3), 88% of bacterial genomes carry R–M systems and 43% carry four or more R–M systems. These multiple R–M systems, acting to prevent the incorporation of foreign DNA, make it difficult to apply reverse genetics techniques. To predict the gene-encoding modification enzyme from the genome sequence information is not difficult, since it is usually located in the region flanking that encoding the restriction enzyme, also the specific motifs of the DNA methylase have been well studied (3). It was conjectured that if all, or at least some, of modification enzymes were to be expressed in Escherichia coli, then a plasmid prepared in the E. coli would be modified as if it was replicated in the target bacterium. Thus, it would elude cleavage by restriction enzymes during the transformation of the target bacterium and greatly improve the efficiency. We term this approach ‘Plasmid Artificial Modification’ (PAM, Figure 1).

Bifidobacterium adolescentis is one of the dominant commensal bacteria of the adult human large intestine. We have recently analysed the whole-genome sequence of the strain B. adolescentis ATCC15703 (DDBJ/EMBL/Genbank Accession# AP009256). However, it was impossible to perform reverse genetic experiments using standard methodology because the transformation efficiency was at an extremely low level (1–3 × 10⁶ CFU/μg

*To whom correspondence should be addressed. Tel: +81 58 293 2996; Fax: +81 58 293 2992; Email: suzuki@gifu-u.ac.jp

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Therefore, we used this strain to demonstrate the experimental approach using PAM.

MATERIALS AND METHODS

Vectors and bacterial strains

pBAD33 (4) was used as low copy number cloning vector. A *Bifidobacterium-E. coli* shuttle vector, pKKT427 (Figure 3), was modified from a pBRASTA101 replicon (5). The pKKT427, 3.9 kb vector, carried a spectinomycin resistance gene, a multi-cloning site and two replication origins including repB from *B. longum* and ColEI ori (Figure 3). The bacterial strains, *B. adolescentis* ATCC15703 was obtained from the American Type Culture Collection. An *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA, USA) (Table 1) was used as a host for cloning and methyltransferase expression.

Culture and transformation conditions for *Bifidobacterium*

*Bifidobacterium adolescentis* ATCC15703 was grown anaerobically at 37°C in MRS medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 0.02% L-cysteine (Nacalai Tesque, Kyoto, Japan) and 0.34% L-ascorbic acid sodium salt (Nacalai Tesque). Spectinomycin hydrochloride (Wako, Osaka, Japan) was added to an MRS agar plate at 150 μg/ml for the transformation experiment. *Escherichia coli* transformants were grown in LB medium supplemented with 150 μg/ml of spectinomycin and/or 20 μg/ml of chloramphenicol. The electro- poration of *B. adolescentis* ATCC15703 was performed as described by Matsumura et al. (6).

Cloning of the methyltransferase genes

The putative methyltransferase genes of *B. adolescentis* ATCC15703 were chosen on the basis of a BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and REBASE (3) (http://rebase.neb.com/rebase/rebase.html). Genomic DNA of *Bifidobacterium* was extracted and purified (7), and was used as a template in PCR using KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan). PCR primers (Table 2) were designed using IMC (In Silico Biology, Inc., Yokohama, Kanagawa, Japan)
Table 1. Bacterial strains and plasmids

| Bacterial strains | Genotype and Properties |
|-------------------|-------------------------|
| Escherichia. coli TOP 10 | F−, mcrA, mcrBC, 80lacZ M15 lacY74, recA1, araD139, araB7697, galU, galK, rpsL(Str8), endA1, mupG |
| BL21 (DE3) | F−, ompT, hsdS(rB−, mB−), dcm, gal, lac (DE3) |
| Bifidobacterium adolescentis ATCC 15703 | Type strain, isolated from adult human feces |
| Lactococcus lactis IO-1 | Isolated from household wastewater |
| Lactococcus lactis IL1403 | Isolated from cheese starter |

Table 2. Primer sequence

| Primer | Sequence |
|--------|----------|
| PMT1-F | 5'-ggcggcgaattc-ATGAGCAAGGAATCAAAGT-3' |
| PMT1-R | 5'-gactggaggaattc-CTACCGTTTCAATCGTTG-3' |
| PMT2-F | 5'-ggcggcgaattc-ATGATAAATAACCGGGAGTA-3' |
| PMT2-R | 5'-gacaacgggtttgTCATTCTCGTCTACCA-3' |
| OMT-F | 5'-ACAACGATTCGAAAAGATGATAAATAACCGGGAG-3' |
| OMT-R | 5'-ACTCCCGGTTTATTTACATCGTTTCAATC-3' |

Initiation and stop codons are underlined. Lower case shows the parts corresponding to vector cleavage sites.

and Primer 3 (8). The PCR products were ligated to pBAD33 using an In-Fusion Dry-Down PCR cloning kit (Clontech, Mountain View, CA, USA) (9).

Plasmid DNA preparations from transformed B. adolescentis ATCC15703

A plasmid preparation from Bifidobacterium was obtained based on the alkaline-SDS method (7), using the lytic enzyme, mutanolysin. A 15 ml Bifidobacterial culture transformant was centrifuged and the cell pellet was suspended in 15 ml of 0.9% NaCl, recentrifuged and then resuspended in 100 l of TE-glucose [50 mM glucose, 10 mM EDTA, 25 mM Tris–HCl (pH 8.0)]. The suspension (100 l) was treated with 25 l of 25 mg/ml lysozyme, 5 l of 10 U/ml mutanolysin (Sigma-Aldrich, St. Louis, MO, USA) and 1 l of 10 mg/ml RNase A (Roche Diagnostics, Basel, Switzerland) at 37°C for 30 min. To this, 200 l of an alkaline-SDS solution (0.2 N NaOH, 1% SDS) was added and the mixture was incubated for 10 min, following which it was neutralized by adding 100 l of 3 M potassium acetate (pH 4.8) and then centrifuged at 10 000 g for 15 min at 4°C. The supernatant was treated with the same volume of phenol–chloroform–isoamylalcohol (25:24:1). The upper layer was collected and to this, 2.5 times its volume of ethanol was added following which the mixture was subjected to further centrifugation. The pellet was rinsed with 70% ethanol, dried and then dissolved in 50 l of TE buffer.

Transformation of Lactococcus lactis IO-1

The culture and transformation conditions for L. lactis IO-1 were as described previously (10). The plasmid pGKV11 was used as a shuttle vector. For cloning of hsdMI and hsdSI of L. lactis IO-1, E. coli BL21(DE3) and pETNH were used as a host-vector system (11), where the cloned gene was tightly repressed in the absence of the inducer.

RESULTS AND DISCUSSION

Two putative R–M clusters were found in the annotated genome of B. adolescentis ATCC15703 (Figure 2A). Each cluster contained one putative gene-encoding DNA methyltransferase, BAD_1233 (M. Sau3AI homologue) and BAD_1283 (M. Kpn2KI homologue). These two genes have been amplified and introduced into pBAD33 (4), a low copy number vector. Three plasmids, carrying BAD_1233 and/or BAD_1283 were constructed and designated pPAM1233, pPAM1283 and pPAM1233–1283 (Figure 2B). There are no reported cryptic plasmids in B. adolescentis, accordingly a B. longum–E. coli shuttle vector pKKT427 (Figure 3) was used in the transformation experiments, compacted to 3.9 kb, and this gave a high transformation efficiency of the shuttle vector for B. longum 105-A (1–3 × 10^6 CFU/µg DNA, Figure 4) (5). The plasmid, pKKT427, was introduced into PAM hosts E. coli TOP10 and its recombinants carrying pPAM1233, pPAM1283 or pPAM1233–1283 (Table 1). A colony resistant to the antibiotics spectinomycin and chloramphenicol was selected, the vector, pKKT427 was then extracted and introduced into B. adolescentis ATCC15703 by electroporation (12). It was then spread onto a MRS agar plate and cultured at 37°C under anaerobic conditions (5,6).
The shuttle vector pKKT427 acted as a replicon in the target cells, as confirmed by plasmid extraction (Figure 5). The transformation efficiency without the PAM plasmid was $1–3 \times 10^6$ CFU/µg DNA. The CFU with a single gene PAM (pBAD1233 or pBAD1283) was around $10^5$ CFU/µg DNA (Table 3). Dual gene PAM (pBAD1233–1283), carrying both methylase BAD_1233 and BAD_1283, yielded $10^5$ CFU/µg DNA (Table 3, Figure 2). Construction of pPAM plasmids. (A) The B. adolescentis ATCC15703 genome includes two R-M clusters, BAD_1227–1234 and BAD_1279–1284. Red boxes show putative restriction genes. The blue boxes (BAD_1233 and BAD_1283) show putative methyltransferase genes. (B) The putative methyltransferase genes were amplified by PCR using primers as listed in Table 2. The PCR products were joined by in vitro homologous recombination to plasmid vector pBAD33, which had been cleaved by HincII, using the In-Fusion Dry-Down PCR cloning kit (Clontech) to obtain pPAM plasmids. Overlap extension PCR was used for BAD_1233–1283. The pPAM1233–1283 plasmid was a constructed operon of BAD_1233 and BAD_1283. In the first PCR, the coding region of BAD_1233, which was added to the downstream 19 bases from the 5'-end of BAD_1283 was amplified. BAD_1283 was obtained in the same manner 20 bases from the 3'-end of BAD_1233. In the second PCR, the first PCR products were used as a DNA template and PMT1-F and PMT2-R primers were used. The amplified DNA fragment was ligated to the same vector and the plasmid pPAM1233–1283 then obtained.
Figure 4. The transformation efficiency with pPAM1233–1283 was higher than that with pPAM1233 or pPAM1283. It is postulated that the recognition sites of the two methyltransferases, encoded by BAD_1233 and BAD_1283, were different and act synergistically.

A combination of two or the putative modification enzymes yielded a synergistic effect. When pKKT427 was extracted from transformed B. adolescentis ATCC15703 and introduced into the same cells, the efficiency was almost the same, $6 \times 10^4$ CFU/μg DNA, as for PAM with pBAD1233–1283 (Table 3). When the plasmid pKKT427 was prepared from B. longum 105A, the transformation efficiency into B. adolescentis ATCC15703 was also improved by a factor of $10^3$ compared with that from E. coli TOP10 without PAM plasmid, which was almost the same as that with pPAM1233 or 1283 (Table 3). It should be noted that the R–M system of B. longum 105A was not determined, while that of B. longum NCC2705 (13) carried a BAD_1233 orthologue (78% identical to BAD_1233). It appears that 105-A carried a BAD_1233-like modification enzyme, which also improved the transformation efficiency of B. adolescentis by a factor of $10^3$.

These experiments clearly demonstrate that the PAM concept (Figure 1B) is an effective approach for constructing a transformation system for a bacterium for which the whole-genome sequence is known. A reverse sequence procedure was also tried (Figure 1C) and this also improved the efficiency.

Next, we applied this method to another bacterium, L. lactis IO-1 (14), which is capable to utilizing xylose and produce lactic acid efficiently. This strain has the potential to utilize biomass for lactic acid production; however, the transformation efficiency was too low to produce using the gene knockout technique. For this strain, the shuttle plasmid, pGKV11 (10), prepared from L. lactis IO-1(pGKV11), showed a 26-fold higher efficiency than that of E. coli BL21(DE3) harbouring pGKV11 (Table 4), suggesting that the strain IO-1 has R–M system(s). Primers were constructed based on the sequences
of L. lactis IL1403 hsdMS (15) and used to amplify putative hsdMS genes using the IO-1 genomic DNA as a template. Then, we constructed a PAM plasmid, which is carrying a methyltransferase subunit gene, designated hsdS1 and its specificity subunit gene, hsdI1 of the IO-1 strain, and designated pETMS1. The transformation efficiency was successfully improved by a factor of seven under the induced conditions (in the presence of IPTG), the transformation efficiency using pGKV11 increased by a factor of 7 compared with pETNH, which is an empty vector of the PAM plasmid pETMS1.

Table 3. Transformation efficiency of the shuttle vector pKKT427 into B. adolescentis ATCC15703

| Modification host | PAM plasmid | Transformation efficiency (CFU/µg DNA) |
|-------------------|-------------|----------------------------------------|
| E. coli TOP10     | –           | 1 – 3 x 10⁶                            |
| pPAM1233          |             | 4 – 6 x 10⁴                            |
| pPAM1283          |             | 1 – 2 x 10⁴                            |
| pPAM1233-1283     |             | 9 x 10⁶ – 4 x 10⁵                      |
| B. adolescentis   | –           | 6 – 9 x 10⁶                            |
| ATCC15703         |             |                                        |
| ATCC15703-2       |             |                                        |
| B. longum 105-A   | –           | 6 x 10³ – 8 x 10³                      |

In summary, a new method for constructing a transformation system for bacteria has been developed. With this system it is feasible to increase efficiency to > 10⁵ CFU/µg DNA for B. adolescentis ATCC15703, at which point it becomes relatively easy to set up other molecular tools, such as site-directed mutagenesis, etc. Using the L. lactis IO-1 strain, we also demonstrated that this system is applicable not only to a Type II R–E system but also to a Type I multi-subunit R–E system. This simple but powerful method may be generally applicable for other bacterial strains, which carry R–M systems. It could potentially promote post-genomic research into bacterial molecular biology.

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