

Technological Advances in Bifidobacterial Molecular Genetics: Application to Functional Genomics and Medical Treatments

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Received November 21, 2011; Accepted January 17, 2012

Bifidobacteria are well known as beneficial intestinal bacteria that exert health-promoting effects in humans. In addition to physiological and immunological investigations, molecular genetic technologies have been developed and have recently started to be applied to clarify the molecular bases of host-*Bifidobacterium* interactions. These technologies include transformation technologies and *Escherichia coli*-*Bifidobacterium* shuttle vectors that enable heterologous gene expression. In this context, a plasmid artificial modification method that protects the introduced plasmid from the restriction system in host bifidobacteria has recently been developed to increase transformation efficiency. On the other hand, targeted gene inactivation systems, which are vital for functional genomics, seemed far from being practically applicable in bifidobacteria. However, remarkable progress in this technology has recently been achieved, enabling functional genomics in bifidobacteria. Integrated use of these molecular genetic technologies with omics-based analyses will surely boost characterization of the molecular basis underlying beneficial effects of bifidobacteria. Applications of recombinant bifidobacteria to medical treatments have also progressed.

Key words: *Bifidobacterium*; molecular genetics; cryptic plasmid; heterologous gene expression; targeted gene inactivation; functional genomics; enzyme/prodrug therapy

INTRODUCTION

Bifidobacteria were firstly described by Tissier in 1900, and their health-promoting effects, such as prevention of diarrhea, alleviation of constipation, resistance to microbial infection, possible roles in cancer prevention and in a treatment of inflammatory bowel disease, have been described [1, 2]. In Japan, many researchers had participated in investigation of the health-promoting functions and intestinal ecology of bifidobacteria under the leadership of Mitsuoka and made tremendous contributions to bifidobacterial research [3, 4]. Development of recombinant DNA techniques in 1970s and their wide application to bacterial genetics from the 1980s to 1990s drove molecular genetics in lactic acid bacteria and bifidobacteria. In addition, starting from the late of

1990s, genome sequencing of these bacteria has been conducted, and tremendous information about their genomes is currently available. In this review, we would like to focus on the contribution of Japanese researchers to the molecular genetic research in bifidobacteria, especially in regard to works using bifidobacterial cryptic plasmids including not only basic research but also medical applications. In addition, genomics and omics-based research are briefly addressed. We also would like to mention the recent development of gene manipulation tools and their application to functional genomics in bifidobacteria.

BIFIDOBACTERIAL CRYPTIC PLASMIDS AND SHUTTLE VECTORS

Bifidobacterial cryptic plasmids were first identified and investigated in the 1980's [5–8]. As one of the early works, Iwata and Morishita investigated the presence of the plasmids in forty-two strains of *Bifidobacterium breve* [8]. In contrast to another work in which no cryptic plasmids were found among investigated *B. breve* strains

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[5], approximately 40% (18 strains) of the *B. breve* strains were revealed to possess the plasmids. These strains were distinguished into five different plasmid profiles according to the electrophoretic migration patterns of plasmid DNA. Identification of *B. breve* plasmids was a promising finding for development of *Bifidobacterium* shuttle vectors, although we had to wait for identification of pNBb1 from *B. breve* ATCC 15698 [9], from which several shuttle vectors were developed [10]. These vectors were basically composed of an *E. coli* plasmid region (from pACYC177 or pUC118), erythromycin-resistance gene and pNBb1 region for plasmid replication in bifidobacteria. Interestingly, plasmid propagation in Dam and Dcm methylase-negative *E. coli* strains affected the transformation efficiencies of *Bifidobacterium* strains [10]. Generally, a lack of methylation of plasmid DNA is expected to decrease transformation efficiency, whereas in this case, the effects varied from strain to strain: the shuttle vector pBEΔ5 from the methylase-negative *E. coli* showed higher transformation efficiency in some *Bifidobacterium* strains but showed lower efficiency in other strains. These differences might not only have been due to the lack of plasmid DNA methylation in *E. coli* but might also have been due to the difference in host restriction systems among the *Bifidobacterium* strains used. The improved vector pBEΔ4* was successfully used for genetic analysis of the *rpsL* gene encoding ribosomal protein S12 to clarify its role in streptomycin resistance in *B. breve* strain Yakult [11].

Beginning in the late 1990's, Kano and his colleagues developed several *E. coli*-*Bifidobacterium* shuttle vectors [12–14]. These vectors were based on a cryptic plasmid pTB6 (3.6-kbp) in *Bifidobacterium longum* BK51 [12]. The plasmid pTB6 was predicted to replicate in the rolling-circle replication manner based on the structural similarity to a known *B. longum* plasmid, pKJ36 [15]. In combination with the highly transformable strain *B. longum* 105-A, pTB6-based shuttle vectors showed relatively high transformation efficiency. For instance, pBLES100 (9.1-kbp) and pDSO44Sp (4.3-kbp) showed transformation efficiencies of 1.6×10^4 and 1.3×10^6 cfu/ μ g DNA, respectively [12, 13]. In addition, pKKT427 (3.9-kbp), the smallest derivative of pTB6-derived shuttle vectors, showed a transformation efficiency of 1.0×10^6 to 3.0×10^6 cfu/ μ g DNA [14]. Development of these vectors contributed to further molecular genetic research in bifidobacteria as mentioned below.

HETEROLOGOUS GENE EXPRESSION IN BIFIDOBACTERIA AND ITS USE FOR MEDICAL TREATMENT

Kano and his colleagues also identified the *hup* gene, encoding the histone-like protein HU, in *B. longum* ATCC 15707 [16]. Expression of *hup* from its native upstream regulatory region restored the growth of Mu phage in integration host factor-deficient *E. coli*, which indicated that the native regulatory region of *hup* works in both *E. coli* and *B. longum* [16]. It was also indicated that the expression level of native *hup* in *B. longum* was high and comparable to that of *E. coli*, which suggested that the regulatory region of *hup* is useful for gene expression in *B. longum* [17]. These observations led to attempts to express an exogenous gene in *B. longum* using the regulatory region of *hup*. These works were conducted by Taniguchi and his colleagues with Kano.

The first example of heterologous expression using the *hup*-regulatory region was *E. coli* cytosine deaminase (CD) [18]. The promoter region and 5' region of *hup* ORF was fused in-frame to the CD gene and cloned into the shuttle vector pBLES100. CD was successfully expressed in *B. longum* 105-A, which was confirmed by the detection of CD by CD-specific antibodies and by quantification of the conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) [18]. This research was aimed at establishing an enzyme/prodrug therapy for solid tumors using 5-FU as an anticancer agent. Administration of a large dose of 5-FU into the whole body is necessary to achieve a sufficient local concentration of 5-FU around a tumor, although 5-FU is toxic to normal growing cells and has various side effects. In contrast, 5-FC is less toxic than 5-FU. Specific localization and growth of *Bifidobacterium bifidum* were also observed in tumors after intravenous administration in mice [19]. This phenomenon is due to the low oxygen partial pressure inside of solid tumors, which is a preferable environment for anaerobic bifidobacteria. These observations and successful expression of CD in *B. longum* 105-A led to establishment of the concept of using recombinant *B. longum* cells to achieve an effective local concentration of 5-FU around tumors by local conversion of low-toxic 5-FC to 5-FU.

Based on these findings, Taniguchi *et al.* began a series of studies related to this concept. Localized growth of wild-type and recombinant *B. longum* 105-A harboring pBLES100 was demonstrated in transplanted cancer tumors generated by inoculation of cultured cancer cells such as B16-F10 melanoma cells and Lewis lung cancer cells in mice [20]. Localization of these strains was also verified in chemically-induced

mammary tumors in rats [21]. Subsequently, *B. longum* 105-A harboring pBLES100-S-eCD that expressed recombinant CD was used in a trial enzyme/prodrug therapy for autochthonous mammary tumors in rats [22]. Selective localization of *B. longum* in the tumor and tumor-specific conversion of 5-FC to 5-FU by the recombinant *B. longum* were observed. As a consequence of the tumor-specific conversion, suppression of tumor growth by the introduction of recombinant *B. longum* via intravenous injection was observed. Surprisingly, antigenic tests of recombinant *B. longum* in guinea pigs showed that the recombinant *B. longum* has little antigenicity, with the level being as low as that of saline. Further improvement of CD activity [23] and expression of CD in another species, *B. breve* [24], were also achieved.

The success of the enzyme/prodrug therapy for solid tumors with the CD-expressing *B. longum* has opened the way to use of recombinant bifidobacteria for medical treatments. Currently reported studies aiming at medical treatments using recombinant bifidobacteria are listed in Table 1. These include not only cancer therapies [25–27] but also the production of recombinant human hormone [28], therapeutic proteins [29, 30], and protective antigens for infectious diseases [31, 32]. As indicated in Table 1, the *hup* regulatory region has become the first choice for the expression of heterologous genes in bifidobacteria [18, 22–24, 29–31], and *B. longum* 105-A has been frequently used for these studies because it has a relatively high transformation efficiency [12]. It is remarkable that recombinant *B. longum* 105-A expressing *Salmonella* flagellin on the cell surface was used as an oral vaccine that successfully protected mice from a challenge with *Salmonella* Typhimurium [32]. In this work, the antigen was successfully expressed with the aid of a bifidobacterial *gltA* gene encoding the galacto-*N*-biose/lacto-*N*-biose I-binding protein, GL-BP, a component of the ATP-binding cassette (ABC) transporter for these disaccharides. Because GL-BP was thought to be anchored to the cell membrane by the N-terminal lipid modification and the C-terminal of this protein had structural flexibility [33], in-frame fusion of the flagellin gene to the 3'-terminus of *gltA* allowed surface expression of *Salmonella* flagellin [32].

Because of their harmless and less antigenic nature in humans, recombinant bifidobacteria will be widely used in various fields of medical treatment.

HETEROLOGOUS GENE EXPRESSION IN BIFIDOBACTERIA FOR FUNCTIONAL CHARACTERIZATION OF BIFIDOBACTERIAL GENES FOR DEGRADATION OF MUCIN GLYCOPROTEINS AND HUMAN MILK OLIGOSACCHARIDES

Yamamoto, Kitaoka and their colleagues have reported various kinds of novel bifidobacterial glycosidases and related proteins for utilization and metabolism of sugar chains of host mucin glycoproteins and human milk oligosaccharides (HMOs) [reviewed in 34 and 35]. These works revealed the importance of the ability to degrade sugar chains of mucin for bifidobacterial colonization in the human large intestine and HMOs for establishment of dominance of several *Bifidobacterium* species in the large intestine of breast-fed infants.

Among their works, heterologous gene expression in bifidobacteria was used to characterize the physiological roles of the three glycosidases in *Bifidobacterium bifidum* JCM 1254, namely, 1,2- α -L-fucosidase (AfcA), 1,3–1,4- α -L-fucosidase (AfcB) [36] and *exo*- α -sialidase (SiaBb2) [37]. *B. longum* 105-A was used as an expression host because it has no endogenous α -L-fucosidase and *exo*- α -sialidase activities. Modified shuttle vectors derived from pBLES100 [12] were used for the introduction of genes encoding these enzymes to *B. longum* 105-A. Fucose was revealed to be liberated from substrate 2'-fucosyllactose by AfcA and from substrates 3-fucosyllactose and lacto-*N*-fucopentaose II by AfcB, respectively. These results indicated that AfcA and AfcB are essential for the utilization of fucosylated HMOs [36]. SiaBb2 was revealed to degrade sialyloligosaccharides such as 3'-sialyllactose and 6'-sialyllactose, indicating its critical importance in the utilization of sialylated oligosaccharides in HMOs [37].

GENOMICS- AND OMICS-BASED STUDIES

Since the first description of the complete genome sequence of *B. longum* NCC2705 in 2002 [38], many genomic sequences have been determined in bifidobacteria. Although the detailed characteristics of genomes in each species and information from comparative genomics are beyond our scope [reviewed in 39–41], it should be noted that Japanese researchers also contributed to the progress in this field. For example, the genomes of the following strains have been completely sequenced: *Bifidobacterium adolescentis* ATCC 15703 (Suzuki *et al.*, GenBank accession No. AP009256), *B. longum* subsp. *longum* JCM 1217^T and *B. longum* subsp. *infantis* 157F [42] and commercially used

Table 1. Heterologous gene expression in bifidobacteria aiming at medical treatments

Objective	Host bifidobacteria	Recombinant protein	Source of heterologous gene	Regulatory region used for expression*	Reference
Enzyme/prodrug tumor therapy	<i>Bifidobacterium longum</i> 105-A	Cytosine deaminase	<i>Escherichia coli</i>	<i>B. longum hup</i>	18, 22, 23
Enzyme/prodrug tumor therapy	<i>Bifidobacterium breve</i> 1-53-8w	Cytosine deaminase	<i>Escherichia coli</i>	<i>B. longum hup</i>	24
Cancer gene therapy	<i>Bifidobacterium longum</i>	Endostatin	Human	λ phage PR-PL	25
Cancer gene therapy	<i>Bifidobacterium longum</i>	Granulocyte colony-stimulating factor (GCSF)	Human	λ phage PR-PL	26
Cancer gene therapy	<i>Bifidobacterium longum</i>	Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Human	λ phage PR-PL	27
Obesity therapy	<i>Bifidobacterium longum</i> NCC2705	Oxyntomodulin	Human	<i>E. coli araC-P_{BAD}</i>	28
Therapy for autoimmune mediated illness	<i>Bifidobacterium longum</i> ATCC 15707	Interleukin-10 (IL-10)	Human	<i>B. longum hup</i>	29
Heterologous expression and secretion	<i>Bifidobacterium breve</i> UCC2003	Basic fibroblast growth factor (FGF-2)	Human	<i>B. longum hup</i> and <i>B. longum gap</i>	30
Mucosal immunization	<i>Bifidobacterium animalis</i> ATCC 27536	Flagellin	<i>Salmonella</i> Typhimurium	<i>B. longum hup</i>	31
Vaccination to <i>Salmonella</i> infection	<i>Bifidobacterium longum</i> 105-A	Flagellin	<i>Salmonella</i> Typhimurium	<i>B. longum gltA</i>	32

* Refer to each reference for detailed descriptions.

strains *B. breve* strain Yakult [43] and *B. longum* BB536 [44]. Here, we would like to describe examples of genomics- and omics-based studies.

Based on the genomic sequence of *B. adolescentis* ATCC 15703, a tiling array was prepared and used for a comparative genomic hybridization (CGH) analysis among *B. adolescentis* strains to investigate the intraspecies diversity [45]. This CGH analysis revealed that the intraspecies genomic differences were point mutations and gene deletions. Representative absent genes in the genomes of tested strains fell into the cluster of orthologous group (COG) category M (cell wall/membrane/envelope biogenesis) and category G (carbohydrate transport and metabolism), suggesting the involvement of these genes in the host-bifidobacterial specificity.

A similar intraspecies comparison using polymorphism analysis of ORFs encoding putative cell-surface adhesins in 42 strains of *B. longum* subsp. *longum* revealed that an ORF named BL0675 encoding putative fimbrial subunit protein had a highly variable sequence in each strain [46]. These sequences were classified into five variant types based on the sequence polymorphisms. The detection frequency of these variant types in human feces was also investigated by a quantitative PCR method using specific primers for each variant type. It was inferred that BL0675 might contribute to host-specific colonization as a fimbrial protein with glycoprotein-binding ability [46].

Screening of candidate genes involved in mucosal cross talk was recently reported by Ishikawa *et al.* [47]. It was hypothesized that the beneficial effects of bifidobacteria are attributed to induction of the genes involved in mucosal cross talk in the gastrointestinal tract. As the first step to verify their hypothesis, candidate genes of *B. breve* strain Yakult (BbrY) implicated in mucosal cross talk were screened. BbrY was administered to germ-free mice, and the contents of the cecum on days 3 or 28 after administration were used for the BbrY transcriptome analyses. Tripartite comparison of BbrY transcriptomes among transcriptomes in the cecal contents and those in *in vitro* m-ILS cultures was conducted by microarray experiments. Thereafter, 93 candidate genes up-regulated in the mouse intestine were identified through clustering analyses [47]. These candidate genes might include genes responding only to the intestinal environment, such as to a lack of nutrition and osmotic pressure, which may not be involved in the mucosal cross talk. Therefore, BbrY transcriptomes in *in vitro* fecal cultures, which mimic nutritional conditions in the

intestine, were compared with those in m-ILS cultures by microarrays to identify the genes responding only to the intestinal environment. Genes upregulated in both the mouse intestine and the fecal cultures were excluded from the candidate genes because these genes were assumed not to be involved in the mucosal cross talk. Consequently, 45 genes were classified as upregulated specifically in the mouse intestine. Most of those genes were involved in sugar transport (e.g., ABC transporters and the phosphotransferase system) and sugar liberation (e.g., glycosidases). These results suggest that BbrY liberates sugar from mucin or residual dietary components and then incorporates them into the cells to maintain energy homeostasis. Free sugars might also be liberated from sugar chains on the epithelium by glycosidases. In addition, genes for acetate production via pyruvate were also upregulated. As acetate is an important energy source for epithelial cells, the supply of acetate through the fermentation of liberated sugars also seems important cross talk that is beneficial to their hosts.

TECHNOLOGICAL DEVELOPMENT FOR FUNCTIONAL GENOMICS

Plasmid artificial modification method

Despite the development of numerous *E. coli*-*Bifidobacterium* shuttle vectors [reviewed in 48 and 49], the generally low transformation efficiency, with a median of up to 10^3 cfu/ μ g DNA for the efficiency of developed host-vector systems [49], has prevented the progress of functional gene analysis. Several methodological trials for improvement of transformation efficiency have been conducted such as addition of high-osmotic solutes into the washing buffer for preparation of competent cells and optimization of electroporation conditions. These improvements are valuable but do not seem to be crucial for the strains with very low transformation efficiency. Suzuki and colleagues made the first breakthrough concerning this issue [14]. They hypothesized that the low transformation efficiency in these strains is mainly due to the degradation of foreign DNA by the bifidobacterial restriction system. This hypothesis seemed in agreement with the other observations indicating that a shuttle vector purified from already transformed bifidobacterial strains produced a higher transformation efficiency than the same vector prepared from *E. coli* [50, 51]. Therefore, putative type II DNA methyltransferase genes in the genome of *B. adolescentis* ATCC 15703 were expressed in *E. coli*, and shuttle vector pKKT427 was modified in

this *E. coli* by these methyltransferases. After the modification, this vector produced elevated transformation efficiency (10^5 cfu/ μ g DNA). It was five orders of magnitude higher than that achieved by the same vector prepared from the parental *E. coli* host. This approach was called plasmid artificial modification (PAM). The PAM approach was also effective in *Lactococcus lactis* IO-1 as a transformation host. A similar strategy was introduced to improve the transformation efficiency in *B. breve* UCC2003, which allowed vector insertion into the chromosomal genes [52]. Although the PAM approach requires genomic information to identify methylase genes of a given bifidobacterial strain, increasing availability of genomic information of many bifidobacterial strains will surely facilitate the application of this approach to various bifidobacterial strains.

Targeted gene inactivation by homologous recombination

Targeted gene inactivation had long been impossible in bifidobacteria, although the technique is essential for functional gene analysis. Currently, at least three different strategies are available for targeted gene inactivation in bifidobacteria, as shown in Figure 1. Here, we would like to describe the fundamental concept and characteristics of each strategy.

a) Single-crossover plasmid insertion

This strategy is achieved by plasmid insertion into a target gene via a single crossover (Fig. 1A). As the first step, an internal DNA fragment of the target gene is cloned into the targeting vector. This vector is basically nonreplicative in the host *Bifidobacterium* strain. After introduction of the targeting vector into the host, homologous recombination can take place between the cloned fragment and chromosomal target gene (a first crossover). This ultimately results in splitting of the target gene by the inserted vector (Fig. 1A). The first example of the application of this strategy in bifidobacteria was the inactivation of the *apuB* gene encoding amylopullulanase in *B. breve* UCC2003 [53]. In this case, a combination of the plasmids allowing conditional replication of the nonreplicative gene targeting vector pORI19 was used to increase the chance of homologous recombination. Although the plasmid insertion mutant of *apuB* was successfully constructed, this method seemed laborious and time-consuming. Therefore, this method was improved and simplified by the approach for escaping from the barrier of the bifidobacterial DNA restriction system [52]. Modification of pORI19 in *E. coli* expressing methylase

genes from the *B. breve* UCC2003 genome resulted in an increase in transformation efficiency, leading to insertion of pORI19 into the target genes in *B. breve* UCC2003 without using the conditional replication strategy described above. Consequently, plasmid insertion inactivation became available by a one-round transformation of the targeting plasmid. This simplified method was also used for inactivation of the gene encoding cellobextrin-binding protein CldE [54] and ATPase for assembly of type IVb tight adherence (Tad) pili in *B. breve* UCC2003 [55].

b) Double-crossover gene disruption

This strategy is achieved by double crossover (Fig. 1B). The mutated allele of the target gene, which is generated by the introduction of a marker gene (e.g., antibiotic resistance gene) into the target allele, is cloned into a nonreplicative targeting vector. Introduction of the targeting vector with mutated allele into the host cell results in integration of the vector into the target locus by homologous recombination (a first crossover). The first crossover can take place at either of the homologous regions flanking the marker gene (Fig. 1B, region X or Y). Subsequently, if the second crossover occurs between the homologous region in the vector and a chromosome in a different region than that used in the first crossover, replacement of the wild-type allele in the chromosome with the mutated one having a marker gene will result (allelic exchange), generating the desired gene disruptant. This strategy was first applied in bifidobacteria for inactivation of an ABC-type carbohydrate transporter gene, BL0033, in *B. longum* subsp. *longum* NCC2705 [42].

c) Double-crossover markerless gene deletion

Double crossover was applied to introduction of markerless gene deletion in the bifidobacterial chromosome (Fig. 1C). The strategy is a patented method by Arigoni and Delley [56]. The flow of the strategy is basically the same as that described for double-crossover gene disruption, except that there are several differences. An *E. coli* plasmid harboring an antibiotic resistance gene is used as a targeting vector by cloning of adjacent regions of the target gene. The targeting vector is introduced into a bifidobacterial strain, and antibiotic selection of the transformants results in identification of the chromosomal integrants of the targeting vector. After subculturing the integrant for 100 generations without antibiotic selection, the second-crossover recombinants appear in the subcultured first-crossover integrants. Antibiotic-sensitive strains that are expected to lose the integrated vector from the chromosome by the second crossover can be identified

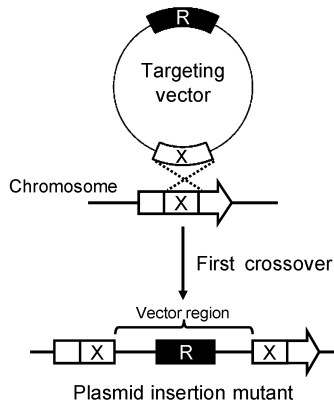
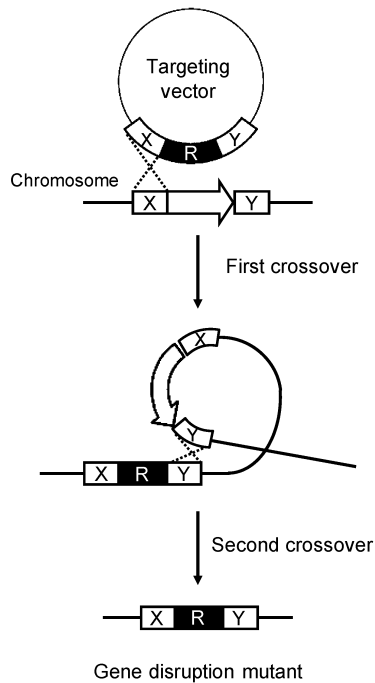
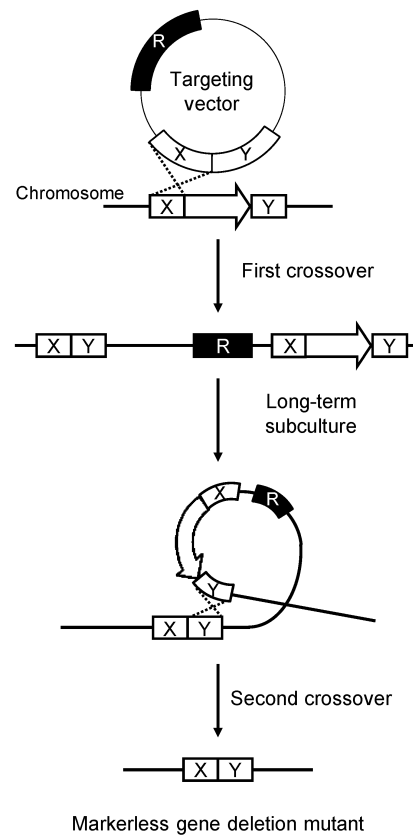
A. Single-crossover plasmid insertion**B. Double-crossover gene disruption****C. Double-crossover markerless gene deletion**

Fig. 1. Schematic representation of the targeted gene inactivation methods currently available in bifidobacteria. Target genes for inactivation are indicated as open arrows. Antibiotic resistance genes are represented as filled squares marked with the letter R. In B and C, theoretically, two types of target gene alleles can be generated in both the first crossover and second crossover. However, for simplicity, only the scheme yielding the desired mutant is described. (A) Single-crossover plasmid insertion. A nonreplicative targeting vector harboring the internal region of the target gene (represented as open squares marked with the letter X) is used for the first crossover. Homologous recombination between the internal region in the vector and the chromosomal target gene results in splitting of the target gene. (B) Double-crossover gene disruption. A nonreplicative targeting vector harboring the 5' and 3' regions of the target gene (represented as open squares marked with the letters X and Y) between which an antibiotic resistance gene is inserted is used for the first crossover. The first crossover occurred between the homologous regions (in this case X), resulting in insertion of the targeting vector into the target gene. If the second crossover occurs between homologous regions different from those used in the first-crossover recombination (in this case Y), the desired gene disruption mutant will be generated. (C) Double-crossover markerless gene deletion. The procedure is similar to that indicated in B. However, a nonreplicative targeting vector that harbors 5' and 3' regions of the target gene (represented as open squares marked with the letters X and Y) is used for the first crossover. The second crossover can occur during long-term subculture (e.g., 100 generations) of the first-crossover integrants. A markerless gene deletion mutant can be generated when the second crossover occurs as described in B.

by the replica plating method. Actually, when the strategy was used for deletion of the *tetW* gene that confers tetracycline resistance in *Bifidobacterium animalis* subsp. *lactis* NCC 2818, 163 out of 750 colonies (21%) were identified as second-crossover recombinants by replica plating of the first-crossover integrants subcultured for 100 generations. In addition, two *tetW*-deletion strains were successfully identified among the 135 second-crossover recombinants by colony PCR [56].

For introduction of targeted gene inactivation to a given *Bifidobacterium* strain, several points should be taken into consideration. Among the strategies mentioned above, strategy a, single-crossover plasmid insertion, is the easiest because it requires only one round of transformation procedure. However, it should be noted that the mutated allele in the plasmid-integrated mutant is principally unstable, because the inserted plasmid might be excised by a subsequent crossover that can occur between the homologous regions left in the target allele. Both strategy b, double-crossover gene disruption, and strategy c, double-crossover markerless gene deletion, can introduce stable mutation into the target gene because no homologous region is left in the target locus after the double crossover. Time-consuming screening of the double-crossover mutant is necessary unless a counter selection marker (e.g., *Bacillus subtilis* *sacB*, which induces lethality in sucrose-containing medium in other bacteria) or conditional replication plasmid (e.g., thermosensitive replication plasmid) is applied. The first two strategies leave the vector DNA (strategy a) or a marker gene (strategy b) in the mutated gene allele. Maintenance of these exogenous DNA regions occasionally affects the gene expression of surrounding genes of the target locus, leading to confusing results in phenotype analysis of the mutant. It also restricts the introduction of multiple mutations in the same strain due to the limited availability of the antibiotic resistance genes in bifidobacteria. Strategy c can overcome these problems. Because time-consuming and rather inefficient procedures are required for isolation of the double-crossover recombinants and objective gene deletion mutants in this strategy, we have developed an improved method for the strategy c. It was achieved by facilitation of a second-crossover step and efficient elimination of the excised plasmid from the cells. Facilitation of a second-crossover step is accomplished using plasmid replication protein RepA, and efficient elimination of the excised plasmid is accomplished using plasmid incompatibility [Hirayama *et al.*, manuscript in preparation, presented at the Tenth

Symposium on Lactic Acid Bacteria (Poster F027), Egmond aan Zee, The Netherlands, August 28 to September 1, 2011].

In any of the above strategies, high transformation efficiency is required to achieve the first crossover because nonreplicative or conditionally replicative vectors are employed. Therefore, improvement of the transformation efficiency by the PAM method [14] will contribute to the application of these inactivation strategies to bifidobacterial strains, especially with low transformation efficiencies.

FUNCTIONAL GENOMICS IN BIFIDOBACTERIA

Development of tools for functional gene analysis and introduction of genomics- and omics-based approaches in addition to the basic biochemical and physiological analyses now leads researchers to conduct integrated, functional genomics in bifidobacteria. Two outstanding reports recently published clearly indicated the importance of targeted gene inactivation in clarification of the function of the candidate genes involved in the beneficial effects of bifidobacteria on hosts.

Fukuda *et al.* [42] revealed the mechanism of bifidobacteria in protection of mice from enterohemorrhagic *E. coli* O157:H7 (*E. coli* O157) infection. A significantly low concentration of Shiga toxin 2 (Stx2), a crucial factor in the lethal infection of *E. coli* O157 strain, was observed in the serum of mice in which the protective *B. longum* strain was administered prior to infection with *E. coli* O157 as compared with that detected in the serum of mice administered a nonprotective *Bifidobacterium* strain. Integrated omics approaches revealed that the amounts of acetate produced from carbohydrate fermentation by the *Bifidobacterium* strains positively correlated with the resistance of mice to the *E. coli* O157 infection. Then comparative genomics of both protective and nonprotective *Bifidobacterium* strains were conducted, and it was revealed that two loci encoding ABC-type sugar transporters were specific to the preventive strains. Of these, BL0033–BL0036 were suggested to be involved in preventing the mice from the mortal effect of *E. coli* O157. A gene knockout mutant of the BL0033 was constructed by inactivation strategy b, double-crossover gene disruption. The knockout mutant was not able to protect mice from the lethal effect of *E. coli* O157 infection due to a reduced concentration of acetate in fecal samples. These results strongly indicated that increased production of acetate by the protective *Bifidobacterium* strain contributes to inhibition of the translocation of Stx2 from inside of the gut to the blood,

resulting in protection of the mice from the mortal effect of *E. coli* O157 infection.

O'Connell Motherway *et al.* [55] described an important host-colonization factor in *B. breve* UCC2003. This factor is the type IVb tight adherence (Tad) pili that are known to be involved in colonization and host pathogenicity in pathogenic bacteria. The *tad* locus was conserved not only in the *B. breve* UCC2003 genome but also in *B. breve* strains tested and other known sequenced bifidobacterial genomes. *In vivo* transcriptome analysis of *B. breve* UCC2003 was performed in a murine colonization model, and the expression of a type IVb Tad pilus-encoding gene cluster (*tad*₂₀₀₃: Bbr_0132–Bbr_0138) was upregulated in the mouse intestine compared with that under *in vitro* culture conditions. To establish whether *tad*₂₀₀₃ was involved in colonization of *B. breve* UCC2003, a *tadA* gene (Bbr_0133, encoding ATPase) mutant was constructed by inactivation strategy a, single-crossover plasmid insertion. Wild-type and mutant strains could colonize in conventional mice at similar levels in the beginning (after 3 days), whereas the colonization level of the *tadA* mutant was 70-fold lower than that of the wild-type strain at 8 days after administration. Interestingly, both strains maintained colonization at a similar level for three weeks after administration in germ-free mice probably because no competing microbiota were present in the monoassociated mice. Subsequent transfer of monoassociated mice to a conventional area resulted in shedding of *tadA* mutant 7 days after transfer, and the *tadA* mutant became undetectable in feces two weeks after transfer in contrast to continued detection of the wild-type strain two weeks after transfer. These results demonstrated that the *tad*₂₀₀₃ locus is crucially important for colonization and persistence of *B. breve* UCC2003 as part of the competing gastrointestinal tract microbiota.

CONCLUSION

In addition to the genomic sequences, molecular genetic tools such as shuttle vectors, gene expression systems and targeted gene inactivation systems are now available in bifidobacteria. Tools that are currently unavailable, such as random mutagenesis using mobile genetic elements including endogenous insertion sequences, should be developed in the future. Refinement of target gene inactivation tools using thermosensitive plasmids or counter selection markers is also necessary. Future studies of bifidobacteria using these molecular genetic tools will be mainly focused on the molecular basis of host-microbe interactions that exert beneficial effects on the host, which have been

largely unexplored. It is expected that development of various genetic tools for bifidobacteria will boost these study area as well as the application of recombinant bifidobacteria to various kinds of medical treatments.

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