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*Correspondence to: Christian U. Riedel; Email: christian.riedel@uni-ulm.de

Introduction

Bifidobacteria are Gram-positive microorganisms with a high-GC content that belong to the Actinobacteria phylum. Many bifidobacteria are commensals of the intestinal tract of humans and animals, and some strains were shown to have beneficial effects on the health status of their hosts. The health-promoting effects described include the production of vitamins, prevention of diarrhea, reduction of cholesterol levels, treatment of irritable bowel syndrome and inflammatory bowel disease, immunostimulation and cancer prevention (reviewed in refs. 1, 3 and 4). Due to these effects, bifidobacteria have attracted considerable commercial interest and are used in a large number of probiotic formulations. Despite their economic importance, the mechanisms that are responsible for the probiotic effects of bifidobacteria are far from understood.

The genomes of a number of strains of different species have been sequenced and annotated and are publically available. However, the detailed analysis of the probiotic effects of bifidobacteria is hampered by the lack of appropriate tools for their genetic modification. While there has been some progress in the development of expression vectors, the currently available protocols do not yield transformation efficiencies above the threshold required to achieve chromosomal integration of non-replicative vectors by homologous recombination. The gold standard to investigate the role of single genes and their products is site-directed mutagenesis and the subsequent phenotypic analysis of the obtained mutants. So far, no system for bifidobacteria has been described that allows for directed mutagenesis in at least a number of different strains and species. As a
Suicide Vectors for Mutagenesis in Bifidobacteria

In B. longum NCC2705, the bl0033 gene encoding the substrate-binding protein of a fructose-specific ABC-type sugar transporter\(^2\) has been disrupted by a classical approach using a suicide vector. The ABC transporter was shown to confer resistance to infection with Enterobacteria coli O157:H7 in a murine model via increased acetate production.\(^3\) For the targeted disruption of bl0033, two 1-kb fragments flanking the gene were cloned up- and downstream of a spectinomycin resistance cassette into pBluescriptSK(\(+)\), an E. coli cloning vector that is non-replicative in bifidobacteria. After electroporation into B. longum NCC2705, transformants were selected with spectinomycin and disruption of bl0033 was confirmed by PCR.\(^4\) However, the transformation protocol used was described to yield a maximum of 1.6 x 10\(^5\) colony-forming units (cfu) per μg DNA in B. longum strains, which is below what is required to allow for homologous recombination. In fact, in a later publication the authors admitted that the process was indeed very time consuming since it took more than one year to obtain a single clone of the mutant.\(^5\) Accordingly, there are no further reports on mutants generated with this system in B. longum NCC2705 or other bifidobacteria.

### Plasmid Artificial Modification to Increase Transformation Efficiencies

The number of available genome sequences of different strains of various Bifidobacterium species has been increasing over the last decade. In all of the 23 fully sequenced and annotated bifidobacterial genomes,\(^6\) restriction-modification (R-M) systems have been identified.\(^6-8\) Some R-M systems of bacteria have evolved to limit the uptake of foreign DNA, e.g., upon infection with a bacteriophage and typically consist of a DNA methyltransferase (MTase) and a restriction endonuclease (REase).\(^9\) Both enzymes recognize the same DNA motifs. However, while the MTase is responsible for the methylation of these motifs, the REase cleaves any DNA that is not methylated in the pattern specific for the host.\(^10\) It is thus not surprising that R-M systems are one of the problems for genetic modification of the more R-M systems a bacterium encodes the more recalcitrant to manipulation it usually is. An approach, which is increasingly used to improve the genetic accessibility of bacteria that are difficult to manipulate, is the methylation of vectors in a pattern specific for the target organisms. This is achieved by either E. coli cloning hosts expressing the respective MTases or by in vitro methylation using recombinant purified MTases.\(^11-14\)

Several bifidobacteria were shown to possess more than one R-M system and the methylation frequencies of the respective strains were successfully expressed in E. coli cloning hosts.\(^13,15\) Using this method termed plasmid artificial modification (PAM), the transformation efficiency of B. adolescentis ATCC15703 was increased from 1–3 x 10\(^5\) cfu/μg DNA for unmethylated DNA to up to 4 x 10\(^5\) cfu/μg when plasmid was isolated from an E. coli TOP10 strain harbouring the two MTases of B. adolescentis ATCC15703.\(^16\) Similarly, transformation efficiencies of B. breve UCC2003, which harbour three R-M systems, was improved from about 1 x 10\(^4\) cfu/μg to about 1 x 10\(^5\) cfu/μg with plasmids isolated from E. coli EC101pNZ-MBbrII-MBbrIII, a strain expressing two of the three MTase genes.\(^17\) The high transformation efficiencies obtained with plasmids isolated from this E. coli cloning host allowed for the successful insertional mutagenesis of apal and galE in B. breve UCC2003 using non-replicative plasmids. Both mutants were confirmed by Southern blot and phenotypic characterization.\(^18\) Since then, a number of other genes were successfully inactivated in B. breve UCC2003 using PAM including cdle, a component of a cellobiose ABC transporter,\(^19\) the stnA gene encoding the ATPase of tight adherence pili,\(^20\) Bbr_0430 encoding the priming glycosylase involved in the synthesis of extracellular polysaccharide\(^21\) and several genes of two-component systems.\(^22\)

PAM has proven successful to increase transformation efficiencies of B. breve UCC2003 above levels required for site-directed recombination with non-replicative vectors. We thus sought to apply this method to generate mutants of B. bifidum S17. This strain is a promising probiotic for the problems for which adheres tightly to various intestinal epithelial cell lines in a process dependent on BopA, a lipoprotein of the cell envelope.\(^23-26\) B. bifidum S17 exhibits potent anti-inflammatory effects by inhibition of LPS-induced NF-κB activation and pro-inflammatory cytokine secretion in cultured intestinal epithelial cells and protects from intestinal inflammation in different models of colitis.\(^27-29\) Analysis of the recently sequenced and annotated genome sequence\(^30\) revealed two putative R-M systems (Fig. 1). Additional MTase genes were identified for which no corresponding REase genes were found (data not shown). Homology to other MTase genes indicates that these genes encode RNA-specific methyltransferases and they were thus excluded from further analysis.

One of the R-M systems shows the typical features of a Type I system with hsdM encoding for the methyltransferase subunit, hsdS for the subunit recognizing the specific sequence motif and hsdR for the endonuclease subunit (Fig. 1A). The genes of the Type I R-M system are inserted into a cluster of genes encoding for enzymes of the arginine biosynthesis pathway (data not shown) and show a markedly lower GC-content (53%) compared to the rest of the chromosome (62.8%). Downstream, a putative inte-grase gene (Ihsf0195) is found indicating that the Type I R-M system was acquired by horizontal gene transfer. The deduced amino acid sequences of HsdM and HsdR show high homology to the respective subunits in other B. bifidum strains (up to 99%) and B. longum strains (over 99%). The homology for the deduced HsdS sequence is less pronounced (79%) to B. bifidum PRL2010 and 52% to B. longum D300A\(^31\) suggesting that the sequence specificity might be different in these strains.

The second R-M system is a putative Type II R-M system with genes encoding...
for an MTase and a corresponding REase (Fig. 1B). Immediately upstream of the Type II MTase gene another putative integrase gene (bbif0709) is located and bbif0709, the MTase and REase genes show a GC-content of 53% again indicating acquisition of the Type II R-M system by horizontal gene transfer. BLAST comparison of the deduced amino acid sequence of the putative Type II REase (bbif0711), using the REBASE database, suggested that the enzyme might be an XhoI isoschizomer. This hypothesis was tested by performing a REase protection assay. In line with this hypothesis chromosomal DNA of B. bifidum was protected from digestion with a commercial XhoI enzyme, i.e., the MTase and REase genes (black arrows) encoding the methyltransferase, sequence recognition and restriction subunit are located in close proximity to a putative integrase gene (gray). (B) Genetic organization and expression of the genes encoding the Type II R-M system of B. bifidum S17 with methyltransferase (bbif0710) and restriction endonuclease genes (bbif0711); black arrows) and the adjacent integrase gene (gray). Expression of all genes was analyzed in RNA samples of bacteria harvested in exponential growth phase by reverse transcription PCR. Negative controls (no reverse transcription, middle) and positive controls (PCR on chromosomal DNA, right bands) were included. Gels were loaded with samples as follows: RT-PCRs in lanes 3, 6 and 9 (3: hsdM, 6: hsdS and 9: hsdR) in (A); 3: bbif0711 and 6: bbif0712 in (B) and corresponding negative (lanes 1, 4, 7) and positive controls (lanes 2, 5 and 8).

Figure 1. (A) Genetic organization and expression of the genes of the Type I R-M system of B. bifidum S17. The hsdM, hsdS and hsdR genes (black arrows) encoding the methyltransferase, sequence recognition and restriction subunit are located in close proximity to a putative integrase gene (gray). (B) Genetic organization and expression of the genes encoding the Type II R-M system of B. bifidum S17 with methyltransferase (bbif0710) and restriction endonuclease genes (bbif0711); black arrows) and the adjacent integrase gene (gray). Expression of all genes was analyzed in RNA samples of bacteria harvested in exponential growth phase by reverse transcription PCR. Negative controls (no reverse transcription, middle) and positive controls (PCR on chromosomal DNA, right bands) were included. Gels were loaded with samples as follows: RT-PCRs in lanes 3, 6 and 9 (3: hsdM, 6: hsdS and 9: hsdR) in (A); 3: bbif0711 and 6: bbif0712 in (B) and corresponding negative (lanes 1, 4, 7) and positive controls (lanes 2, 5 and 8).
An internal fragment of \textit{apuB} was then cloned into pORI19, which is non-replicative due to the lack of \textit{repA} and the resulting vector pORI19-\textit{apuB} was introduced into \textit{B. breve} UCC2003 pTGB019. After successful introduction of both plasmids, growth temperature was shifted to 42°C, which blocks replication of pTGB019 and, as a consequence of the lack of a functional \textit{repA} also of pORI19-derivatives. Presence of the antibiotic selects for integration of \textit{ori} derivatives into the genome of \textit{B. breve} UCC2003 at the desired site, in this case the \textit{apuB} gene and causes gene disruption. Successful disruption of the \textit{apuB} gene was shown by the inability of the mutant to grow on modified Rogosa medium containing starch, amylopectin, glycogen or pullulan as sole carbon source. To date, this method has not been applied for the generation of other mutants in \textit{B. breve} UCC2003 nor has it been used independently by other groups and in other \textit{bifidobacteria}. In fact, we have tried to transform pVE6007 into \textit{B. bifidum} S17 several times and could not obtain any positive clones (data not shown).

Recently, the successful generation of a temperature-sensitive plasmid for deletion of genes in \textit{B. longum} 105-A9 was reported. The authors amplified the \textit{repB} gene of pKKT427 by error-prone PCR, replaced the \textit{repB} in pKKT427 with the PCR product thereby creating a library of clones containing different \textit{repB} mutants. This library was transformed into \textit{B. longum} 105-A and about 3000 clones were screened for growth at 30°C and 42°C. This led to the identification of a single clone containing a temperature-sensitive plasmid. This plasmid was termed pKO403 and subsequently used to create a deletion mutant in the \textit{pyrE} gene of \textit{B. longum} NCC2705, which was obtained earlier by the same group using a classical yet very time-consuming suicide vector strategy. Use of Temperature-Sensitive Plasmids for Mutagenesis

The first report on an insertion mutant of a \textit{bifidobacterial} strain was the disruption of \textit{apuB} in \textit{B. breve} UCC2003 encoding an extracellular type II amylpullulanase. Gene disruption was achieved using an approach initially described for \textit{Lactococcus lactis} but also successfully applied to other Gram-positive organisms including \textit{Listeria monocytogenes}. \textit{B. breve} UCC2003 was first transformed with pTGB019, a derivative of the temperature-sensitive lacticococal plasmid pVE6007 harbouring a functional \textit{repA} gene. A 1 kb
Concluding Remarks

The first report on a mutant in a Bifidobacterium strain was published only 5 y ago.10,11 Since then several groups have proposed different strategies for site-directed mutagenesis in different strains of bifidobacteria. Nevertheless, most strains remain resistant to mutagenesis using PAM. One of the reasons for low transformation efficiencies is that most bifidobacteria possess multiple R-M systems. Further factors leading to low transformation efficiencies have to our knowledge not been investigated systematically. However, one explanation may be differences in cell wall components between bifidobacteria and other Gram-positive organisms. For example, the teichoic acids (TA) of B. adolescentis were shown to have an unusual structure compared with the TA of other Gram-positive organisms.11,12 Moreover, from our own experience efficient lysis of bifidobacteria, e.g., for the preparation of crude extracts, requires lysozyme and mutanolysin. Both enzymes are murin hydrolases cleaving the β(1,4)glycicosidic bond of the N-acetylmuramyl-N-acetylglucosamine backbone of peptidoglycan. However, mutanolysin was shown to have a broader spectrum of activity also cleaving peptidoglycans of group A streptococci, which are resistant to lysozyme treatment.12,13 This indicates that the peptidoglycan of bifidobacteria might have an altered structure resulting in a reduced sensitivity toward the protocols used to prepare competent cells.

By far the most frequently used approach to increase transformation efficiencies is PAM. Using this method a number of mutants have been generated in a B. breve strain.10-11 Improved transformation efficiencies using PAM were independently confirmed for a B. adolescentis strain11 by our own results (Fig. 3). However, in none of these cases PAM was able to increase efficiencies of transformation markedly above those observed with plasmid DNA isolated from the target organism. Thus, while PAM is undoubtedly a valuable method to overcome the R-M barrier in bifidobacteria, levels required for site-directed recombination are only obtained with strains that already show reasonable levels of intrinsic competence or are highly susceptible to the currently available protocols for the generation of competent cells such as B. breve UCC2003. Another drawback of PAM is that it is highly specific for a single strain and the respective E. coli cloning host cannot be used for other strains.

The generation of an insertion mutant in the bld033 gene in B. longum NCC2795 using a suicide vector15 indicates that this strategy is in principal applicable for mutagenesis in bifidobacteria. In consequence, the development of new protocols for the generation of competent cells together with PAM suicide vectors might have potential for targeted mutagenesis for bifidobacteria.

The use of temperature-sensitive replicons for mutagenesis has been successfully used in a wide range of organisms. In particular, the systems based on the temperature-sensitive replicon of pVE6007 and the repA-deficient pORI plasmids was successfully used in a number of Gram-positive bacteria. To date, only one mutant of a B. breve strain has been generated using this system, possibly due to incompatibility of the replicons with the majority of bifidobacteria. More recently, pKO3403, a temperature-sensitive derivative of the E. coli pJOK802 plasmid shuttle vector pKKT427, was successfully applied for the generation of two mutants in B. longum NCC27755.14 The pKKT427 vector is based on the pPTB replicon, which was shown to stably replicate in strains of B. longum, B. breve and B. animalis (reviewed in ref. 7). Thus, at present pKO3403 is the most promising approach for targeted mutagenesis in a wider range of bifidobacteria and might prove more applicable than to improve transformation efficiencies by optimizing protocols and/or PAM. Nevertheless, successful application of pKO3403 for targeted mutagenesis in other strains and species of bifidobacteria needs to be confirmed.

In conclusion, despite their prominent contribution to the intestinal microbiota, the effects on human health and the large economic interest in probiotics, tools for the genetic modification of bifidobacteria are still largely missing. These tools are a fundamental basis for the analysis of the effects of bifidobacteria on the human host and the underlying mechanisms. While methods for targeted mutagenesis are in place for a very limited set of individual strains, no universal system for bifidobacteria is available and might actually prove impossible to achieve. Nevertheless, the development of temperature-sensitive plasmids for a wider range of bifidobacteria is a promising approach and worthwhile to investigate in more detail.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest have been disclosed.

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