Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003

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Summary

In *silico* analysis of the *Bifidobacterium breve* UCC2003 genome predicted two distinct loci, which encode three different restriction/modification systems, each comprising a modification methylase and a restriction endonuclease. Based on sequence homology and observed protection against restriction we conclude that the first restriction endonuclease, designated BbrI, is an isoschizomer of BbeI, the second, BbrII, is a neoschizomer of Sall, while the third, BbrIII, is an isoschizomer of PstI. Expression of each of the *B. breve* UCC2003 methylase-encoding genes in *B. breve* JCM 7017 established that BbrII and BbrIII are active and restrict incoming DNA. By exploiting knowledge on restriction/modification in *B. breve* UCC2003 we successfully increased the transformation efficiency to a level that allows the reliable generation of mutants by homologous recombination using a non-replicative plasmid.

Introduction

The commensal gut microbiota has long been appreciated for its influence on gut health (reviewed by O’Hara and Shanahan, 2006; Turrioni et al., 2008). Bifidobacteria constitute a specific group of mostly commensal bacteria, which inhabit the gastrointestinal tract (GIT) of mammals, including the human GIT, where they are estimated to represent 3–6% of the adult faecal flora (Ventura et al., 2004; Saxelin et al., 2005; Zoetendal and Vaughan, 2006). The presence of bifidobacteria in the human GIT has been associated with many beneficial health effects, such as the prevention of diarrhoea, amelioration of lactose intolerance and immunomodulation (reviewed by Leahy et al., 2005). Indeed, the health benefits of probiotic bacteria such as bifidobacteria have been shown to extend beyond the GIT (Lenoir-Wijnkoop et al., 2007). These many positive attributes have led to the widespread incorporation of bifidobacteria as live components of commercial health-promoting probiotic foods. Despite these commercial and scientific interests, fundamental knowledge is still scarce regarding the exact molecular mechanisms by which bifidobacteria contribute to host health and well-being. Such scientific knowledge is essential to scientifically explain the purported health benefits, and consequently support the inclusion of such bacteria as probiotics in functional foods.

The genome sequences on *Bifidobacterium longum* subsp. *longum* NCC2705 (Schell et al., 2002), *B. longum* subsp. *longum* DJ010A (Lee et al., 2008), *B. adolescentis* ATCC15703 (Suzuki et al., 2006), *B. adolescentis* L2-32 (Fulton et al., 2007), *B. dentium* ATCC27678 (Sudarshanam et al., 2008) and *B. animalis* subsp. *lactis* HN019 (Collett et al., 2008) have recently become available and have contributed very significantly to advancing our knowledge on bifidobacterial genetics and metabolism. However, the availability of a genome sequence is merely a first step towards a better understanding of a specific probiotic property, and unravelling the molecular mechanisms by which bifidobacteria bring about positive host responses demands the availability of suitable molecular tools. To date, relatively few molecular tools for bifidobacteria have been developed, which explains why the genetics of these microbes is rather poorly understood, certainly when compared with other bacteria of industrial importance.

Available genetic tools for bifidobacteria include bifidobacterial plasmids, which were first reported by Sgorbati and colleagues (1982). In recent years significant effort has focused on identifying and sequencing plasmids from bifidobacteria, and exploiting some of these native bifidobacterial replicons for the creation of *Escherichia coli–Bifidobacterium* shuttle vectors (Lee and O’Sullivan 2006; Alvarez-Martín et al., 2007; Cronin et al., 2007; Sangrador-Vegas and colleagues, 2007). A limitation of
many of these shuttle vectors is the low transformation efficiency of many of the bifidobacteria tested, coupled in some cases with segregational instability (Lee and O’Sullivan, 2006).

The observed differences in transformation efficiency among different strains of bifidobacteria may be attributed, at least in part, to restriction/modification (R–M) systems, which are ubiquitous among prokaryotes and generally comprise of a restriction endonuclease (REase) and cognate methyltransferase (MTase) (Murray, 2002; Tock and Dryden, 2005). R–M systems are believed to serve primarily as defensive instruments that protect prokaryotic cells against invading DNA such as promiscuous plasmids or infecting bacteriophage. R–M systems are classified into four groups (designated type I, II, III and IV) on the basis of their subunit composition, co-factor requirement, recognition sequence structure and the cleavage site relative to the recognition sequence (Roberts et al., 2003). Type I R–M systems consist of three different subunits, HsdM, HsdR and HsdS, that are responsible for modification, restriction and sequence recognition respectively. Type I REases require ATP, MG₂⁺ and AdoMet for activity. In general they interact with two asymmetrical bi-partite recognition sites, translocate the DNA in an ATP hydrolysis-dependent manner and cut the DNA distal to the recognition sites, approximately halfway between two sites (Murray, 2002). Typically, in a type II R–M system the REase recognizes and cleaves within a short (4–8 bp) palindromic DNA sequence. Protection of ‘self’ DNA from restriction occurs by methylation using an MTase, which modifies specific adenosyl or cytosyl residues within the sequence recognized by the corresponding REase (Kobayashi, 2001; Pingoud et al., 2005). Type III R–M systems consist of two subunits, Mod, responsible for DNA recognition and modification, and Res, responsible for DNA cleavage. Active nucleases require ATP and MG₂⁺ for activity and are stimulated by AdoMet. The holoenzyme, composed of two Res and two Mod subunits, interacts with two unmodified asymmetric target sites positioned in inverse orientations with respect to each other and cuts the DNA close to one recognition site (Janscak et al., 2001). Type IV R–M systems are specified by either one of two structural genes encoding proteins with specificities for methylated, hydroxymethylated or glucosyl-hydroxymethylated bases in the target DNA molecule (Roberts et al., 2003).

REase activity in *Bifidobacterium* was first described by Khosaka and colleagues (1982) and to date a total of 23 bifidobacterial proven or putative R–M systems have been identified, as listed on the REBASE website (http://rebase.neb.com/rebase). Bbel, the first bifidobacterial REase to be described, was isolated from *Bifidobacterium breve* YIT4006, recognizing and cleaving the sequence 5′-GGCGC⁻¹C-3′. However, two copies of the Bbel recognition sequence are required for full endonuclease activity (Khosaka et al., 1982). Subsequently Khosaka and colleagues (1983) reported on the identification of the REases BinSI and BinSII from *B. longum* subsp. *infantis* S76e. BinSI is an isoschizomer of EcoRII (recognizing and cleaving the sequence 5′-CCWG-3′), while BinSII exhibits the same restriction specificity as Bbel (5′-GGCGC⁻¹C-3′). BinI was isolated from *B. longum* subsp. *infantis* 659, and recognizes the asymmetric pentanucleotide sequence 5′-GGATCNNNNN’N-3′ (Khosaka and Kiwaki, 1984). Skrypina and colleagues (1988) showed that four out of 12 bifidobacterial strains exhibited REase activity, of which two, Badl from *B. adolescentis* LVA1 and Bbfl from *B. bifidum* LVA3, are isoschizomers of Xhol (5′-C⁻¹TGCAGC-3′), while the REases Bbf7411I from *B. bifidum* 7411 and Bia7920l from *B. lactis* 7920 are neoschizomers of BspMII (5′-T⁻¹CCGGA-3′). Hartke and colleagues (1996) identified two REases from *B. longum* subsp. *longum* BL2: Bbl is an isoschizomer of XhoI (5′-R⁻¹GATCY-3′), while BblI is an isoschizomer of PstI (5′-CTGCA⁻¹G-3′).

In the current study we report on the identification and preliminary characterization of three R–M systems encoded on the genome of *B. breve* UCC2003. Circumventing these R–M systems allowed the development of a reliable method for the creation of gene disruptions in *B. breve* UCC2003.

**Results**

*Sequence, genetic organization and amino acid analysis of the Bbri, Bbril and BbrIle R–M systems from B. breve UCC2003*

Two loci, predicted to encode three different R–M systems, were identified from the annotation of the genome sequence of *B. breve* UCC2003 (S. Leahy. M. O’Connell Motherway, J. Moreno Munoz, G.F. Fitzgerald, D. Higgins and D. van Sinderen, unpubl. results) and designated Bbri, Bbrill and BbrIle (Fig. 1A). The G+C content for each system is 58% which is in agreement with the approximately 60% G+C content for bifidobacteria (Ventura et al., 2007). The first gene of the Bbri R–M system, *bbrilM*, codes for a protein (M.Bbri; 43.2 kDa) with 60% and 53% identity to cytosine-specific MTases from Clavibacter *michiganensis* and *Photorhabdus luminescens* respectively; M.Bbri also contains the six highly conserved motifs characteristic of known 5′-methylcytosine MTases (Kumar et al., 1984) (Fig. 1B). The cytosine-specific MTases from *C. michiganensis* and *P. luminescens* are known to methylate of the sequence 5′-GGCG⁻¹C-3′, which is also the recognition sequence of the Bbri REase identified by Khosaka and colleagues (1982) from *B. breve* YIT4006. The protein product of the second ORF, bbrIle215, exhibits 94% identity to a hypothetical protein encoded by *B. longum* subsp. *longum* NCC2705 (Schell
et al., 2002). The third gene of the BbrI gene cluster, bbrIR, is separated from bbr0215 by remnants of an insertion sequence element. The bbrIR gene encodes a protein (30 kDa) exhibiting low homology (33%) to various type II R–M system restriction subunits and for this reason it is predicted to represent the restriction component of the BbrI R–M system, probably an isoschizomer of BbeI.

The R–M systems BbrII and BbrIII are located adjacent to each other on the genome of UCC2003 (Fig. 1A). The first gene of the BbrII R–M system, bbrIIM, codes for a 349-amino-acid protein (38.8 kDa), exhibiting 47% identity to the HgiDII cytosine-specific MTase (Düsterhöft and Kröger, 1991). As was the case for M.BbrI, the six highly conserved motifs of cytosine-specific MTases are present in M.BbrII (Fig. 1B). The encoded product of the bbrIIR gene is a 695-amino-acid protein (79.4 kDa) exhibiting 40% identity to R.HgiDII, which recognizes the sequence 5′-G↓TCGAC-3′. This is the same recognition sequence as that of SalI; however, M.SalI is a N6-adenosine MTase, while M.BbrII and M.HgiDII are predicted to be cytosine-specific MTases. R.BbrII therefore is assumed to represent a neoschizomer of SalI.
The third identified R–M system on the genome of *B. breve* UCC2003, BbrII, is predicted to encode an isoschizomer of PstI and Boll, the latter representing a REase identified from *B. longum* subsp. *longum* BL2 (Hartke et al., 1996). The first gene, *brbrIM*, encodes a 315-amino-acid protein (36.6 kDa), exhibiting 38% identity to the amino-acid protein (36.3 kDa), which shares 32% identity with M.PstI, an N6-adenosine MTase (Walder, Walder et al., 1984). The four conserved motifs characteristic of N6-adenosine-methyltransferase, CMIs, CMII and CMIII (Timinskas et al., 1995), can be identified in M.BbrII (Fig. 1C). The second gene *brbrlIIIM* encodes a 355-amino-acid protein (36.3 kDa), exhibiting 38% identity to the REase PstI (5′-CTGCA1-G-3′).

**Assessment of R–M activity in *B. breve* UCC2003**

To establish if the identified R–M systems are functional in *B. breve* UCC2003 and whether they affect transformation efficiency of this strain, the transformation frequency of two *E. coli*–bifidobacterial shuttle vectors, pPKCM7 and pAM5 (Table 1), was determined when these plasmids had been isolated either from *B. breve* UCC2003 (DNA protected from R–M) or from *E. coli* JM101 (DNA sensitive to R–M). 200 ng quantities of each of these plasmid DNAs isolated from these two different hosts was used to transform *B. breve* UCC2003 by electroporation. Transformants were selected on RCA supplemented with chloramphenicol (Cm) in case of plasmid pPKCM7, or tetracycline (Tet) in case of plasmid pAM5, and enumerated following anaerobic incubation at 37°C for 48 h (Fig. 2). For each plasmid there was a 500-fold higher transformation efficiency of the plasmid DNA isolated from *B. breve* UCC2003 as compared with the DNA isolated from *E. coli*, thus indicating that one or more of the identified R–M systems encoded by *B. breve* UCC2003 is functional and contributes to the

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**Table 1. Bacterial strains and plasmids used in this study.**

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|--------------------------|---------------------|
| **E. coli strains** |                          |                     |
| EC101             | Cloning host, repA+ km’  | Law (1995)          |
| JM109             | F’ traD36 proAB lacZ M15 recA1 relA1 endA1 thi hsdR17 | Yanisch-Perron et al. (1985) |
| JM101             | supE, thi (lacproAB) (F’ traD36 proAB lacZ M15 | Yin et al. (1997) |
| BM101             | E. coli JM101 with bbrIM and bbrlIIIM integrated in the chromosome and transcribed by an IPTG-inducible lac promoter | This study |
| **B. breve strains** |                          |                     |
| NCFB 2257         | Isolate from infant intestine | NCFB |
| NCFB 2258         | Isolate from infant intestine | NCFB |
| NCFB 8815         | Isolate from nursing stool | NCFB |
| NCFB 11815        | Isolate from infant intestine | NCFB |
| Yakult            | Isolate from infant intestine | Oishi et al. (2008) |
| LMG 13208         | Isolate from infant intestine | UCC |
| JCM 7017          | Isolate from infant intestine | JCM |
| JCM 7019          | Isolate from infant intestine | JCM |
| UCC2003           | Isolate from nursing stool | UCC |
| UCC2003           | Isolate from infant intestine | UCC |
| UCC2003-galA-744  | pORI19-tet-G744 insertion mutant of UCC2003 | This study |
| UCC2003-galA-476  | pORI19-tet-G476 insertion mutant of UCC2003 | This study |
| UCC2003-apuB-939  | pORI19-tet-apuB insertion mutant of UCC2003 | This study |
| **Plasmids**      |                          |                     |
| pNZ8048           | Cm’, nisin-inducible translational fusion vector | de Ruyter et al. (1996) |
| pNZ-M.BbrI        | pNZ8048 derivative containing bbrIM (bbr0216) | This study |
| pNZ-M.BbrII       | pNZ8048 derivative containing bbrlIIIM (bbr1121) | This study |
| pNZ-M.BbrIII      | pNZ8048 derivative containing bbrIM (bbr1119) | This study |
| pNZ-M.BbrII + M.BbrIII | pNZ8048 derivative containing bbrIM and bbrlIIIM | This study |
| pAM5              | pBC1-puc19-Tc’          | Alvarez-Martin et al. (2007) |
| pPKCM7            | pblueCm harbouring rep pCIBA089 | Cronin et al. (2007) |
| pREP4             | Low-copy-number LacI expressing pQE60 companion plasmid | Qiagen |
| pQE60             | AmpR overexpression vector | Qiagen |
| pQE60-M.BbrI + M.BbrIII | pQE60 derivative containing bbrIM and bbrlIIIM transcriptionally fused to IPTG-inducible promoter | This study |
| pKVB2             | To’, Km’ containing internally deleted E. coli gfp gene 11.7 kb | Kiel et al. (1987) |
| pKVB2-M.BbrI-M.BbrIII | pKVB2 derivative containing bbrIM and bbrlIIIM transcriptionally fused to IPTG-inducible promoter | This study |
| pORI19            | Em’, repA’, ori’, cloning vector | Law et al. (1995) |
| pORI19-tet-G744   | Internal 744 bp fragment of galA and tetW cloned in pORI19 | This study |
| pORI19-tet-G476   | Internal 476 bp fragment of galA and tetW cloned in pORI19 | This study |
| pORI19-tet-upB    | Internal 939 bp fragment of apuB and tetW cloned in pORI19 | This study |

JCM, Japan Collection of Microorganisms; NCFB, National Collection of Food Bacteria, Reading, UK; UCC, University College Cork, Cork, Ireland.
efficiency at which plasmids can be introduced in this strain.

**BbrI, BbrII and BbrIII represent three R–M systems**

In order to verify the prediction that M.BbrI, M.BbrII and M.BbrIII represent distinct MTases that protect, based on their similarities to characterized R–M systems, DNA sequences cut by Bbel, SalI and PstI, respectively, genomic DNA of *B. breve* UCC2003 was restricted with these enzymes and analysed by agarose gel electrophoresis. The results obtained showed that *B. breve* UCC2003 genomic DNA is protected from restriction with Bbel and PstI, but not SalI (Fig. 3A).

To establish the precise MTase activity of each of the predicted MTase-encoding genes in *B. breve* UCC2003, *bbrIM, bbrIIIM, bbrIIIM* and their corresponding upstream regions (presumed to contain their native promoters) were amplified by PCR and cloned in pNZ8048 to generate pNZ-M.BbrI, pNZ-M.BbrII and pNZ-M.BbrIII respectively (see Experimental procedures and Table S1). These plasmids, as well as the control plasmid pNZ8048, were introduced into *B. breve* JCM 7017, whose genomic DNA is susceptible to Bbel, SalI and PstI restriction (data not shown). Restriction analysis revealed that genomic DNA of *B. breve* JCM 7017 expressing M.BbrI, M.BbrII or M.BbrIII were protected from restriction with Bbel, SalI or PstI, respectively, while genomic DNA of *B. breve* JCM7017 harbouring pNZ8048 was restricted by all three enzymes (Fig. 3B). Collectively these results demonstrate that *B. breve* UCC2003 encodes three MTases that methylate within the sequences, 5′-GGCGCC-3′ (for M.BbrI), 5′-GTCGAC-3′ (for M.BbrII) and 5′-CTGCAG-3′ (for M.BbrIII).

To establish if the methylase activities associated with the BbrI and BbrIII R–M systems were present in other *B. breve* strains, genomic DNA from nine additional *B. breve* strains was restricted with Bbel or PstI (Table S2). Only for three strains, *B. breve* UCC2004, NCFB 2258 and NCFB 8815, the DNA was protected from restriction with Bbel.

![Insertion mutagenesis in bifidobacteria](Image)

**Fig. 2.** Transformation efficiency of pPKCM7 or pAM5 DNA isolated from *E. coli* (grey bars) or *B. breve* UCC2003 (black bars).

**Fig. 3.** A. Restriction analysis of total DNA from *B. breve* UCC2003. Lane 1, molecular weight marker X (Roche). Lanes 2–6: total *B. breve* UCC2003 DNA restricted with lane 2, BamHI; lane 3, HindIII; lane 4, Bbel; lane 5, SalI; lane 6, PstI.

B. Restriction analysis of total DNA from *B. breve* JCM7017 harbouring pNZ8048, pNZ-M.BbrI, pNZ-M.BbrII or pNZ-M.BbrIII. Lane 1, molecular weight marker X (Roche). Lane 2, JCM7017 harbouring pNZ8048 restricted with Bbel; lane 3, JCM7017 harbouring pNZ-M.BbrI restricted with Bbel; lane 4, JCM7017 harbouring pNZ-M.BbrII restricted with SalI; lane 5, JCM7017 harbouring pNZ-M.BbrIII restricted with Sall; lane 6, JCM7017 harbouring pNZ-M.BbrIII restricted with PstI; lane 7, JCM7017 harbouring pNZ-M.BbrIII restricted with PstI. The restrictions were analysed on a 1% agarose gel followed by staining with ETBR.
addition, DNA from *B. breve* NCFB 8815 was also protected from restriction with PstI. Genomic DNA from the remaining six strains was restricted by these two enzymes. This would indicate that different strains of *B. breve* exhibit quite a variety of different R–M activities.

To determine the individual effect of each R–M system on the transformation frequency of *B. breve* UCC2003, we first introduced plasmid pAM5, which harbours one PstI, two Sall and three Bbel sites, into *B. breve* JCM7017 strains harbouring either pNZ28048, pNZ-M.BbrI, pNZ-M.BbrII or pNZ-M.BbrIII. The methylation of the pAM5 DNA at the appropriate sequence in each of the methylase expressing strains was confirmed by restriction analysis (results not shown) prior to introducing 200 ng of each plasmid preparation into *B. breve* UCC2003 by electroporation. The number of transformants was determined after 48 h of anaerobic incubation at 37°C on RCA with tetracycline selection (Fig. 4). pAM5 DNA isolated from JCM7017 expressing M.BbrIII was effected by the addition of 10 mM IPTG prior to the isolation of plasmid DNA. Plasmid preparations from the remaining six strains was restricted by these two enzymes. This would indicate that different strains of *B. breve* exhibit quite a variety of different R–M activities.

From the data presented above it was clear that all three REases BbrI, BbrII and BbrIII are active in *B. breve* UCC2003. In order to enhance transformation efficiencies of *B. breve* UCC2003 by prior methylation of plasmid DNA, two *E. coli* strains expressing both M.BbrII and M.BbrIII were constructed. In the first, *E. coli* pNZ-M.BbrII-M.BbrIII, two of the bifidobacterial methylases were expressed on plasmid pNZ28048 (see Experimental procedures and Table S1). As expected, chromosomal (and plasmid) DNA from *E. coli* strain EC101 harbouring pNZ-M.BbrII-M.BbrIII is protected from restriction with PstI. The second *E. coli* strain, BM1, harbours *bbrIIIM* and *bbrIIIM* under the control of an IPTG-inducible promoter integrated into the *gllB* gene on the *E. coli* JM101 chromosome (see Experimental procedures). Upon induction with 10 mM IPTG total DNA from *E. coli* BM1 is protected from restriction with PstI (Fig. S1A). However, complete protection from Sall restriction was not observed (results not shown) and this may be due to the lower level expression of *bbrIIIM* from the *E. coli* chromosome as compared with expression from plasmid pNZ-M.BbrII-M.BbrIII. In addition, Sall can restrict hemi-methylated DNA, therefore the observed restriction by Sall may be a reflection of incomplete methylation.

To evaluate the effect of methylation of plasmid DNA on transformation efficiency, pAM5 was introduced into *E. coli* pNZ-M.BbrII-M.BbrIII and *E. coli* BM1 by electroporation. Expression of M.BbrII and M.BbrIII in BM1 harbouring pAM5 was effected by the addition of 10 mM IPTG prior to the isolation of plasmid DNA. Plasmid preparations of *E. coli* harbouring pNZ-M.BbrII-M.BbrIII or *E. coli* BM1 were then used for *B. breve* UCC2003 transformation. pAM5 DNA isolated from *E. coli* harbouring pNZ-M.BbrII-M.BbrIII gave a 1000-fold higher transformation frequency as compared with pAM5 isolated from *B. breve* UCC2003.

Expression of M.BbrII and M.BbrIII in *E. coli* and methylation of plasmid DNA

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frequency as compared with pAM5 from *E. coli* pNZ8048 while plasmid DNA isolated from *E. coli* BM1 gave a 50-fold higher transformation frequency. pAM5 DNA isolated from EC101 pNZ-M.BbrII-M.BbrIII gave transformation efficiencies comparable to those obtained with plasmid DNA isolated from *B. breve* (Fig. S1B).

**Disruption of the *galA* and *apuB* genes of *B. breve UCC2003***

In order to establish if methylation of a non-replicating plasmid by the *B. breve* UCC2003 MTases would increase transformation efficiency to a sufficiently high level that would allow site-specific homologous recombination, two genes, *galA* and *apuB*, were selected as mutational targets. The *galA* and *apuB* genes encode an endogalactanase and an amylolpullulanase, respectively, which are involved in extracellular polysaccharide metabolism by *B. breve* UCC2003 (Hinz *et al.*, 2005; Ryan *et al.*, 2006; O’Connell Motherway *et al.*, 2008). To establish if gene disruption could be achieved using homologous recombination, DNA fragments of 476 and 744 bp, representing internal fragments of the *galA* gene, and a 939 bp internal fragment of the *apuB* gene were cloned in pORI19 and provided with a tetracycline resistance marker, generating plasmids pORI19-tet-G476, pORI19-tet-G476 and pORI19-tet-apuB respectively (see Experimental procedures). These plasmids, being derivatives of pORI19, cannot replicate in *B. breve* UCC2003 as they lack a functional replication protein (Law *et al.*, 1995). These pORI19 derivatives were introduced into *E. coli* EC101 harbouring pNZ-M.BbrII-M.BbrIII to facilitate methylation, and preparations of the resulting methylated pORI19-derived plasmids were then introduced into *B. breve* UCC2003 by electroporation. Tetracycline-resistant transformants were isolated at a frequency of 50 per µg of transformed DNA when greater than 700 bp of homologous DNA was used. The number of potential integrants was slightly reduced when the smaller region of homologous DNA was used. All transformants obtained were expected to carry *galA* or *apuB* gene disruptions, while no such transformants were obtained when unmethylated pORI19 constructs were introduced into *B. breve* UCC2003. The suspected chromosomal integration of the pORI constructs was verified by colony PCR on a selection of Tet® transformants using a forward primer upstream of the region of integration and a reverse primer based on pORI19 (galAp1 and pORI19A, or apuBp1 and pORI19B) (results not shown). Southern hybridizations confirmed the assumed integration of the individual pORI-derived plasmids by homologous recombination. For the presumed *galA* disruptions of *B. breve* UCC2003, Southern hybridizations were performed using SphI-digested genomic DNA and employing a 2.6 kb PCR fragment encompassing *galA* as a probe. SphI was selected for the genomic digests since there are no corresponding restriction sites within the *galA* sequence. The *galA* fragment hybridized to a 6.1 kb fragment of UCC2003 genomic DNA, while in the UCC2003 derivatives with a presumed pORI-tet-G476 or pORI-tet-G744 integration this band was absent, and expected hybridization signals of 10.5 kb and 557 bp, or 10.8 kb and 848 bp, respectively, were observed (Fig. 5). For two of each of the UCC2003 mutant strains examined the *galA* probe also hybridized to a 5.3 kb or 5.5 kb SphI fragment for the pORI19-tet-G476 and pORI19-tet-G744 integrants respectively [Fig. 5B(i), lanes 4 and 5; Fig. 5B(ii), lanes 5 and 6]. These hybridization signals indicate that duplication of pORI19-tet-galA plasmids had occurred after integration of the plasmid into the bacterial chromosome in these mutant strains. For the suspected *apuB* integrants of strain UCC2003, Southern hybridizations were performed using BamHI-digested genomic DNA and a 1 kb probe encompassing an internal fragment of *apuB*. The *apuB* fragment hybridized to a 3.6 kb fragment of UCC2003 genomic DNA. For the *apuB* mutant strains the anticipated hybridization signals of 2.1 and 7.2 kb were obtained (Fig. S2).

Collectively these results demonstrate that methylation of plasmid DNA by the *B. breve* UCC2003 MTases M.BbrII and M.BbrIII in *E. coli* circumvents the BbrII and BbrIII REase activities in *B. breve* UCC2003 and allows a sufficiently high transformation efficiency so as to allow reliable homologous recombination in *B. breve* UCC2003. In addition, these data illustrate that chromosomal integration in *B. breve* UCC2003 can be achieved with less than 500 bp of homologous DNA.

**Phenotypic analysis of the *B. breve* UCC2003 plasmid integrants***

In order to verify the expected phenotypic consequences of the created gene disruptions in *galA* and *apuB*, strains *B. breve* UCC2003, and individual representatives of *B. breve* UCC2003 mutants generated by insertion of pORI19-tet-G744 or pORI19-tet-G476, designated here as UCC2003-*galA*-476 and UCC2003-*galA*-744, respectively, were analysed for their ability to grow on galactan as the sole carbohydrate source (Fig. 6A). Similarly, *B. breve* UCC2003 and a derivative with an integrated pORI19-tet-apuB (designated UCC2003-apuB-939) were analysed for the ability to grow on starch, amyllopectin, glycogen or pullulan as the sole carbohydrate source (Fig. 6B). In contrast to the wild-type *B. breve* UCC2003, the *B. breve* UCC2003-*galA*-476 or UCC2003-*galA*-744 mutant strains failed to grow on potato galactan, while comparable growth of the parent and *galA* mutant strains was observed when glucose was the sole carbohydrate source. In a similar manner it was shown that *B. breve*
UCC2003-apuB-939 failed to grow on starch, amylopectin, glycogen or pullulan, which contrasted with observed good growth on these substrates by the parent strain. Comparable growth for parent and mutant strains was observed when glucose was used as the sole carbohydrate source. These results confirm that the chromosomal plasmid integrations in UCC2003 cause a demonstrable phenotype and clearly illustrate the importance of the extracellular enzymes specified by \textit{galA} and \textit{apu} in the metabolism of specific high-molecular-weight polysaccharides by \textit{B. breve} UCC2003.

**Discussion**

Bifidobacterial strains demonstrate substantial variability in the efficiency of transformation by plasmids from \textit{E. coli}, while many strains exhibit complete resistance to transformation (Lee and O'Sullivan, 2006). Progress in the evaluation of probiotic factors in bifidobacteria has been slow due to the lack of efficient and versatile systems for genetic manipulation (Ventura \textit{et al.}, 2004). While quite a number of \textit{E. coli}-bifidobacterial shuttle vectors have been developed, it has been noted that widespread application of these plasmids among bifidobacterial species is limited (Lee and O'Sullivan, 2006).

As shown here, R–M systems are one of the major obstacles hindering progress in the genetic accessibility and analysis of \textit{B. breve} UCC2003, and are likely to do this in other (bifido)bacteria as well. Convincing evidence to support this notion can be obtained from the available bifidobacterial genome sequences. Genes specifying R–M systems can be identified in all sequenced bifidobacterial genomes. The genomes of \textit{B. longum} subsp. \textit{longum} NCC2705 (Schell \textit{et al.}, 2002) and \textit{B. longum} subsp.
longum DJ010A (Lee et al., 2008) both harbour a single type I R–M system, two type II R–M systems and one type IV R–M system. The type II REases specified by blo_1473 and bld_0356 are predicted to be isoschizomers of EcoRII, which restricts within the sequence ↓CCWGG, while the REases specified by blo_564 and bln_1359 are predicted to be isoschizomers of Sau3A1, which recognizes the sequence ↓GATC. The recognition sequence of the type I and type IV R/M systems in the sequenced B. longum genomes are unknown. The genome of B. adolescentis ATCC15703 (Suzuki et al., 2006) specifies two MTase subunits and six REase subunits. The restriction subunits specified by bad_1283 and bad_1232 are predicted to be isoschizomers of KpnII and Sau3AI, respectively, while the remaining four are as yet unknown. The sequenced genomes of B. dentium ATCC27678 (Sudarsanam et al., 2008) and B. animalis HN019 (Collett et al., 2008) both harbour a single type II R–M system, where the REase is predicted to be an isoschizomer of Avall, which recognizes the sequence G↓GWCC (Sutcliffe and Church, 1978). Based on the results obtained for B. breve UCC2003, it is tempting to speculate that exploiting the MTases encoded by the aforementioned sequenced bifidobacterial strains would allow the transformation efficiencies of these strains to be improved. For bifidobacterial strains that are particularly recalcitrant to transformation or where the complete genome sequence is not known it may be possible to methylate plasmid DNA isolated from E. coli by incubating the DNA with crude cell extracts of the Bifidobacteria in the presence of S-adenosylmethionine thereby possibly improving the transformation efficiency.

An alternative method that would circumvent bifidobacterial R–M systems would be to introduce plasmid DNA by conjugation. To date conjugation has not been conclusively demonstrated for the genus Bifidobacterium. Until recently the only evidence supporting the possibility of conjugation in bifidobacteria was the identification of genes encoding proteins potentially involved in the conjugation process on various bifidobacterial plasmids. Putative relaxase-encoding genes have been identified on plasmids pJK36 and pJK50 from B. longum subsp. longum (Park et al., 1999; 2000), while homologues of septal DNA translocator (Tra) proteins have been identified on the B. breve plasmid pCIBb1 (O’Riordan and Fitzgerald, 1999) and the B. pseudocatenulatum plasmid p4M (Gibbs et al., 2006). Recently, Shkoporov and colleagues (2008) sequenced three plasmids of bifidobacterial origin: pB44 from B. longum, pB90 from B. bidifudum and pB21a from B. breve. Both pB44 and pB90 harbour genes encoding potential mobilization functions while pB21A encodes a putative Tra protein. These proteins were exploited in efforts to achieve conjugation in bifidobacter-
ria, and although antibiotic-resistant, PCR-positive and thus putative transconjugants were obtained, plasmid transfer has as yet not been demonstrated.

The difficulties associated with obtaining sufficiently high transformation efficiencies so as to allow insertional mutagenesis in B. breve UCC2003 through homologous recombination led us to believe that R–M systems were the barrier that needed to be overcome in order to achieve this. In the present study we describe three different R–M systems specified by the genome of UCC2003: Bbrl, an isoschizomer of BbeI; Bbrll, a neoschizomer of Sall; and Bbrlll, an isoschizomer of Psll. Restriction analysis of chromosomal DNA from UCC2003 showed that the DNA is protected from restriction with BbeI and Psll, but not Sall. The observed restriction of DNA by Sall can be explained by M.Sall being a N6-adenosine-methylase, while M.Bbrll is predicted to be cytosine-specific MTase, which may therefore not confer (full) protection against Sall restriction. However, the finding that M.Bbrll does provide full protection against Sall restriction when it is expressed from a multicopy plasmid in B. breve JCM 7017 would indicate that M.Bbrll in such circumstances is more abundant, thereby eliciting complete methylation and concomitant protection of the DNA. The three R–M systems identified in B. breve UCC2003 do not appear to be highly conserved among B. breve strains, just one strain examined in this study, B. breve NCIMB 8815, was shown to exhibit protection of Bbrl and Bbrlll recognition sites indicating that this species and indeed the genus Bifidobacterium is likely to harbour a very diverse range of R–M activities.

The contribution of each R–M system in impeding plasmid transformation of B. breve UCC2003 was determined and established that all three systems impact on transformation efficiency, with Bbrlll, at least under the circumstances used here, providing the biggest hurdle to incoming DNA. To facilitate methylation of plasmid DNA by M.Bbrll and M.Bbrlll, thereby enhancing the transformation frequency of B. breve UCC2003, two E. coli strains were constructed, where bbrlllM and bbrlllM were expressed in different ways, either from their own promoter on plasmid pNZ8048 or from an IPTG-inducible promoter on the E. coli chromosome. The observed higher transformation efficiency for pAM5 DNA isolated from E. coli pNZ-M.Bbrlll-M.Bbrlll may be attributed to the high copy number of pNZ8048 plasmids in E. coli and resulting higher expression levels of the MTases as compared with expression from single copy on the E. coli chromosome in E. coli BM1.

Having established that the use of M.Bbrlll- and M.Bbrlll-methylated plasmid DNA results in a significantly increased transformation efficiency of B. breve UCC2003, we conclusively showed that gene disruptions in B. breve UCC2003 can be created using a non-replicating and M.Bbrlll- and M.Bbrlll-methylated plasmid. We have previously produced a gene disruption in the apuB gene of B. breve UCC2003 by adaptation of the lactococcal two plasmid homologous recombination system (O’Connell Motherway et al., 2008). However, in our hands this system was very tedious, time-consuming and not reliable (O’Connell Motherway et al., 2008; our unpublished results). Therefore, insertional mutagenesis of the apuB gene was deemed an appropriate control to evaluate the validity and reliability of the plasmid methylation strategy.

By M.Bbrlll-M.Bbrlll-mediated methylation of plasmid DNA in E. coli prior to transformation into B. breve UCC2003, gene disruptions not only in apuB, but also in galA were successfully and reliably created, as verified by genetic and phenotypic analyses.

This, to the best of our knowledge, therefore represents the first reliable system for creating insertional mutation in a member of the genus Bifidobacterium. The ability to achieve chromosomal integration of a non-replicative plasmid with less than 500 bp of homologous DNA also opens the opportunity for the creation of a bank of B. breve UCC2003-derived mutants carrying random chromosomal integrations, which in turn will provide a range of possibilities to further advance fundamental knowledge on the physiology, biochemistry and genetics of this strain. Such information will obviously be relevant to other bifidobacteria and will be crucial to understand the health-promoting properties that have been attributed to various members of this genus.

Experimental procedures

The description of the experimental procedures resides in Appendix S1 in Supporting information.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A. Restriction analysis of E. coli JM101 and two representative JM101 bbrIM and bbrIIIM methylase integration strains. Lane 1, molecular weight marker X (Roche). Lanes 2–4, PstI digest of total DNA isolated from JM101 following induction with 0, 1 or 10 mM IPTG. Lanes 5–7 and lane 8–10, PstI digests of total DNA isolated from two representative JM101 bbrIM and bbrIIIM methylase integration strains after induction with 0, 1 or 10 mM IPTG. B. Transformation efficiency of B. breve UCC2003 with pAM5 plasmid DNA isolated from B. breve UCC2003, E. coli pNZ-M.Bbr-III, E. coli BM1 or E. coli pNZ8048.

Fig. S2. A. Schematic representation of the relevant regions of the B. breve UCC2003 and UCC2003-apuB-939 chromosome. Chromosomal DNA is represented by a thin line, the apuB gene is represented by a black arrow, the internal apuB fragment is indicated by a solid grey line and pORI19 is indicated by a boxed line. BamHI sites relevant to the Southern hybridization analysis are indicated. B. Southern hybridization analysis of BamHI-digested chromosomal DNAs of B. breve UCC2003 (lane 1) and four representative B. breve UCC2003-apuB-939 mutants (lanes 2–5). The molecular weight of the relevant hybridization signals are indicated to the left of the panel. The internal 1 kb PCR amplicon of apuB was used as a probe for the hybridization.

Table S1. Oligonucleotide primers used in this study. Table S2. Restriction analysis of genomic DNA from B. breve strains with BbeI and PstI.

Appendix S1. Experimental procedures.

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