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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 SalI, 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; Turroni *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 (Sudarsanam *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

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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, MG^{2+} 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 MG^{2+} 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). BbeI, the first bifidobacterial REase to be described, was isolated from *Bifidobacterium breve* YIT4006, recognizing and cleaving the sequence 5′-GGCGC$^{\downarrow}$C-3′. However, two copies of the BbeI 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′$^{\downarrow}$CCWGG-3′), while BinSII exhibits the same restriction specificity as BbeI (5′-GGCGC$^{\downarrow}$C-3′). BinI was isolated from *B. longum* subsp. *infantis* 659, and recognizes the asymmetric pentanucleotide sequence 5′-GGATCNNNN$^{\downarrow}$N-3′ (Khosaka and Kiwaki, 1984). Skrypina and colleagues (1988) showed that four out of 12 bifidobacterial strains exhibited REase activity, of which two, BadI from *B. adolescentis* LVA1 and BbfI from *B. bifidum* LVA3, are isoschizomers of XhoI (5′-C$^{\downarrow}$TCGAG-3′), while the REases Bbf7411I from *B. bifidum* 7411 and Bla7920I from *B. lactentis* 7920 are neoschizomers of BspMII (5′-T$^{\downarrow}$CCGGA-3′). Hartke and colleagues (1996) identified two REases from *B. longum* subsp. *longum* BL2: BlоI is an isoschizomer of XhoII (5′-R$^{\downarrow}$GATCY-3′), while BloII is an isoschizomer of PstI (5′-CTGCA$^{\downarrow}$G-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, BbrII and BbrIII 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, BbrII and BbrIII (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, *bbrIM*, codes for a protein (M.BbrI; 43.2 kDa) with 60% and 53% identity to cytosine-specific MTases from Clavibacter *michiganesis* 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. michiganesis* and *P. luminescens* are known to methylate of the sequence 5′-GGC$^{(m5)}$GCC-3′, which is also the recognition sequence of the BbeI REase identified by Khosaka and colleagues (1982) from *B. breve* YIT4006. The protein product of the second ORF, *bbr0215*, exhibits 94% identity to a hypothetical protein encoded by *B. longum* subsp. *longum* NCC2705 (Schell

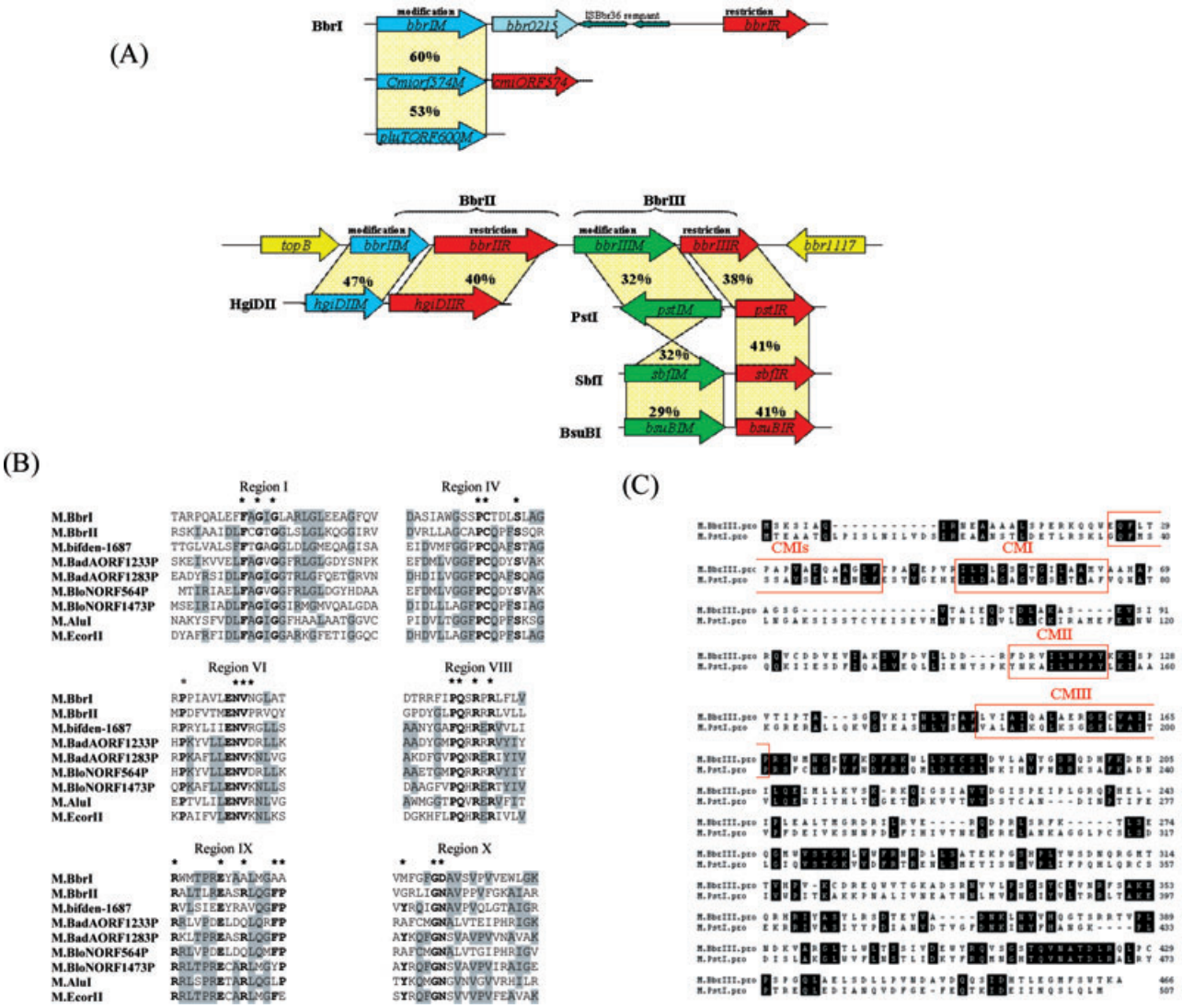

Fig. 1. A. Schematic representation of R–M systems encoded by *B. breve* UCC2003. Each arrow indicates an ORF. Predicted protein function is indicated by M (modification) or R (restriction) in the gene name. The percentage amino acid (aa) identity is indicated.
B. Alignment of the six highly conserved motifs of MTases M.BbrI and M.BbrII from *B. breve* UCC2003 with annotated cytosine MTases from *B. dentium* ATCC27678, *B. adolescentis* ATCC15703 and *B. longum* NCC2705 and M.AluI and M.EcoRII. Highly conserved aa are marked with an asterisk above the sequence and indicated in bold, while aa are shaded in grey if at least four of the depicted protein sequences contain an identical residue at a particular position.
C. Sequence alignment of M.BbrIII and M.PstI. Amino acids are shaded if both of the depicted proteins contain identical residues at corresponding positions. The boxed sequences CMIs, CMI, CMII and CMIII represent the four highly conserved regions of N6-adenine MTases.

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$^{\downarrow}$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.

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

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli strains		
EC101	Cloning host, repA$^+$ km^r	Law *et al.* (1995)
JM109	*F' traD36 proAB lacI*^{q}Z M15 *recA1 relA1 endA1 thi hsdR17*	
JM101	*supE*, *thi* (*lacproAB*) *(F' traD36 proAB lacI*^{q}Z M15	Yanisch-Perron *et al.* (1985)
BM101	*E. coli* JM101with *bbrIIM* and *bbrIIIM* 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 nursling 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
UCC2004	Isolate from nursling stool	UCC
UCC2003	Isolate from nursling stool	Mazé *et al.* (2007)
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^r, 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 *bbrIIM* (*bbr1121)*	This study
pNZ-M.BbrIII	pNZ8048 derivative containing *bbrIIIM* (*bbr1119)*	This study
pNZ-M.BbrII + M.BbrIII	pNZ8048 derivative containing *bbrIIM* and *bbrIIIM*	This study
pAM5	pBC1-puC19-Tc^r	Alvarez-Martín *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.BbrII + M.BbrIII	pQE60 derivative containing *bbrIIM* and *bbrIIIM* transcriptionally fused to IPTG-inducible promoter	This study
pKVB2	Tc^r, Km^r containing internally deleted *E. coli glg*B gene 11.7 kb	Kiel *et al.* (1987)
pKVB2-M.BbrII-M.BbrIII	pKVB2 derivative containing *bbrIIM* and *bbrIIIM* transcriptionally fused to IPTG-inducible promoter	This study
pORI19	Em^r, repA$^-$, ori$^+$, cloning vector	Law *et al.* (1995)
pORI19-tet-G744	Internal 744 bp fragment of *gal*A and *tet*W cloned in pORI19	This study
pORI19-tet-G476	Internal 476 bp fragment of *gal*A and *tet*W cloned in pORI19	This study
pORI19-tet-apuB	Internal 939 bp fragment of *apu*B and *tet*W cloned in pORI19	This study

JCM, Japan Collection of Microorganisms; NCFB, National Collection of Food Bacteria, Reading, UK; UCC, University College Cork, Cork, Ireland.

The third identified R–M system on the genome of *B. breve* UCC2003, BbrIII, is predicted to encode an isoschizomer of PstI and BloII, the latter representing a REase identified from *B. longum* subsp. *longum* BL2 (Hartke *et al.*, 1996). The first gene, *bbrIIIM*, encodes a 315-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, CMI, CMII and CMIII (Timinskas *et al.*, 1995), can be identified in M.BbrIII (Fig. 1C). The second gene *bbrIIIR* encodes a 355-amino-acid protein (36.6 kDa), exhibiting 38% identity to the REase Pst1 (5′-CTGCA$^{\downarrow}$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

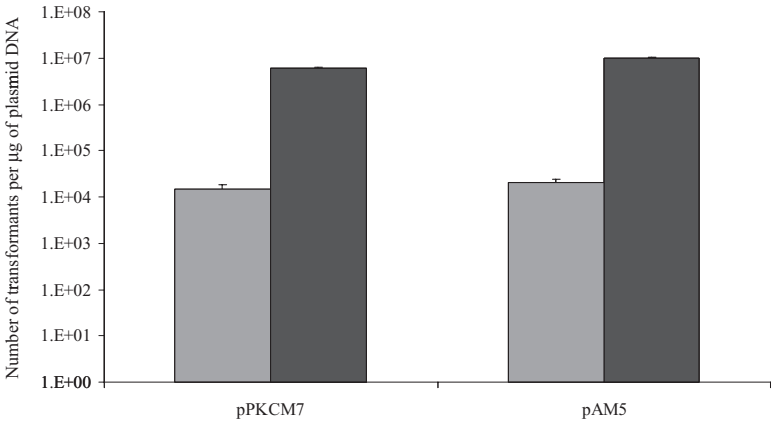

Fig. 2. Transformation efficiency of pPKCM7 or pAM5 DNA isolated from *E. coli* (grey bars) or *B. breve* UCC2003 (black bars).

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.BrrIII represent distinct MTases that protect, based on their similarities to characterized R–M systems, DNA sequences cut by BbeI, 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 BbeI 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*, *bbrIIM*, *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 BbeI, PstI and SalI 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 BbeI, 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 BbeI or PstI (Table S2). Only for three strains, *B. breve* UCC2004, NCFB 2258 and NCFB 8815, the DNA was protected from restriction with BbeI. In

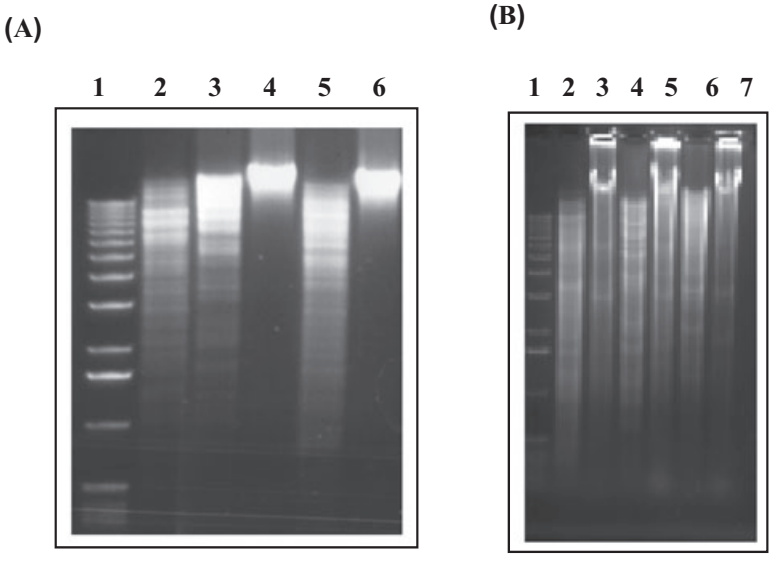

Fig. 3. A. Restriction analysis of total DNA from *B. breve* UCC2003. Lane 1, molecular weight marker X (Roche). Lanes 2–6: total *B. breve* UCC2003 DNA restricted with lane 2, BamHI; lane 3, HindIII; lane 4, BbeI; lane 5, SalI; lane 6, PstI.
B. Restriction analysis of total DNA from *B. breve* JCM7017 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 BbeI; lane 3, JCM7017 harbouring pNZ-M.BbrI restricted with BbeI; lane 4, JCM7017 harbouring pNZ8048 restricted with SalI; lane 5, JCM7017 harbouring pNZ-M.BbrII restricted with SalI; lane 6, JCM7017 harbouring pNZ8048 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.

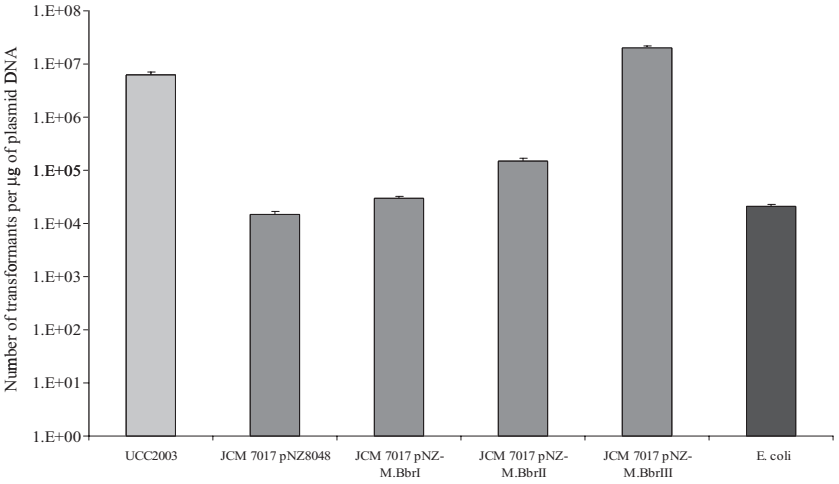

Fig. 4. Transformation efficiency of *B. breve* UCC2003 using pAM5 plasmid DNA isolated from UCC2003, *B. breve* JCM7017 harbouring pNZ8048, pNZ-M.BbrI, pNZ-M.BbrII, pNZ-M.BbrIII or *E. coli*.

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 SalI and three BbeI sites, into *B. breve* JCM7017 strains harbouring either pNZ8048, 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 48h of anaerobic incubation at 37°C on RCA with tetracycline selection (Fig. 4). pAM5 DNA isolated from JCM7017 expressing M.BbrIII allowed an almost 1000-fold higher transformation frequency as compared with pAM5 isolated from *E. coli* or JCM 7017 harbouring pNZ8048. A 10- and 5-fold higher transformation efficiency was observed for pAM5 isolated from JCM7017 expressing M.BbrII and M.BbrI respectively. The transformation frequency obtained with pAM5 DNA isolated from JCM 7017 expressing M.BbrIII was comparable to the transformation frequency obtained with pAM5 plasmid DNA isolated from *B. breve* UCC2003. However, in the latter case DNA preparations contain just the pAM5 plasmid, while in the former case the DNA preparation would have contained a mixture of pAM5 and pNZ-M.BbrIII. These results demonstrate that the BbrIII restriction endonuclease (isoschizomer of PstI) is highly active in *B. breve* UCC2003 and that the activity of this restriction endonuclease appears to represent the main limitation to the genetic accessibility of *B. breve* UCC2003, at least for plasmid pAM5.

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

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 pNZ8048 (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 *bbrIIM* and *bbrIIIM* under the control of an IPTG-inducible promoter integrated into the *glg*B 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 SalI restriction was not observed (results not shown) and this may be due to the lower level expression of *bbrIIM* from the *E. coli* chromosome as compared with expression from plasmid pNZ-M.BbrII-M.BbrIII. In addition, SalI can restrict hemi-methylated DNA, therefore the observed restriction by SalI 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 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 gal*A and* apu*B 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, *gal*A and *apu*B, were selected as mutational targets. The *gal*A and *apu*B genes encode an endogalactanase and an amylopullulanase, 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 *gal*A gene, and a 939 bp internal fragment of the *apu*B gene were cloned in pORI19 and provided with a tetracycline resistance marker, generating plasmids pORI19-tet-G744, 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 *gal*A or *apu*B 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^r 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 *gal*A disruptions of *B. breve* UCC2003, Southern hybridizations were performed using SphI-digested genomic DNA and employing a 2.6 kb PCR fragment encompassing *gal*A as a probe. SphI was selected for the genomic digests since there are no corresponding restriction sites within the *gal*A sequence. The *gal*A 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 *gal*A probe also hybridized to a 5.3 kb or 5.5 kb Sph1 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 *apu*B integrants of strain UCC2003, Southern hybridizations were performed using BamHI-digested genomic DNA and a 1 kb probe encompassing an internal fragment of *apu*B. The *apu*B fragment hybridized to a 3.6 kb fragment of UCC2003 genomic DNA. For the *apu*B 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 *gal*A and *apu*B, 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, amylopectin, 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 *gal*A mutant strains was observed when glucose was the sole carbohydrate source. In a similar manner it was shown that *B. breve*

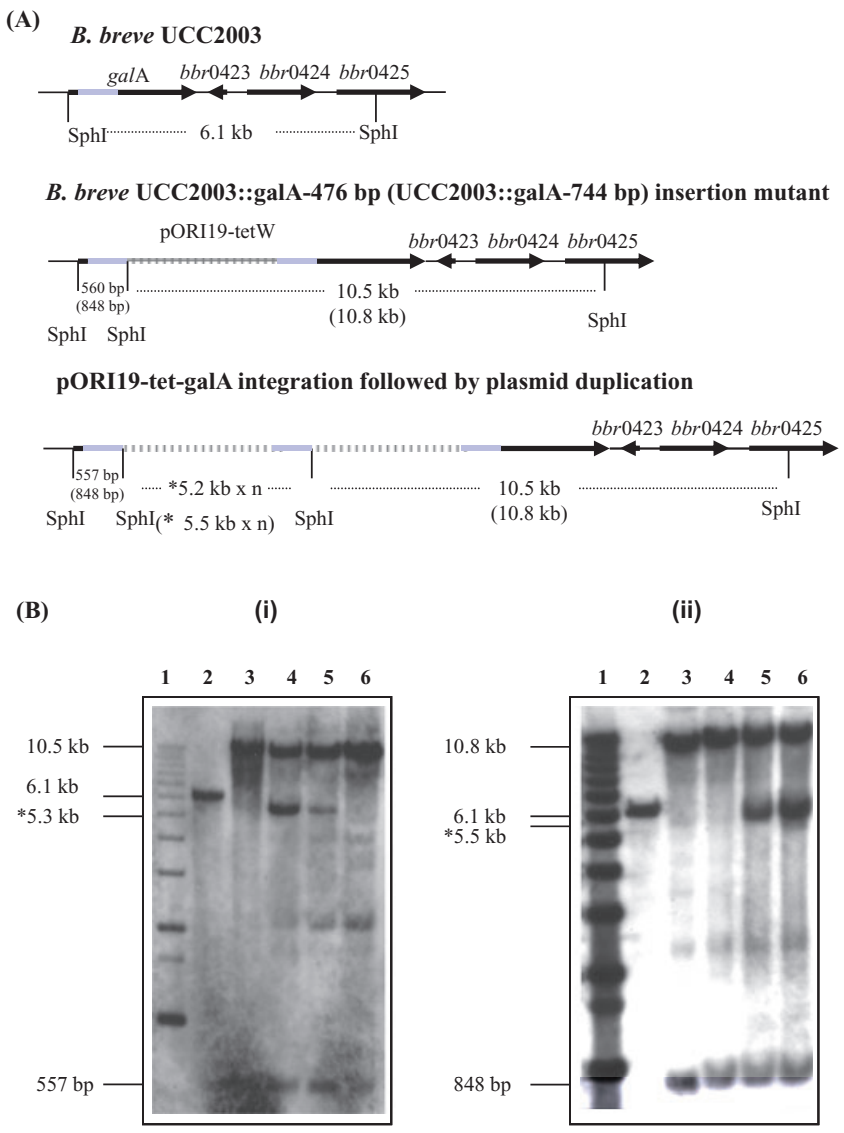

Fig. 5. A. Schematic representation of the relevant regions of the *B. breve* UCC2003 and UCC2003-galA-476 and UCC2003-galA-744 (in brackets) chromosomes. Chromosomal DNA is represented by a thin line, the *gal*A gene is represented by a black arrow, the internal *gal*A fragment is indicated by a solid grey line and pORI19 is indicated by a boxed line. SphI sites relevant to the Southern hybridization analysis are indicated.
B. Southern hybridization analysis of SphI chromosomal DNAs of (i) *B. breve* UCC2003 (lane 2) and four representative *B. breve* UCC2003-galA-476 mutants (lanes 3–6); (ii) *B. breve* UCC2003 (lane 2) and four representative *B. breve* UCC2003-galA-744 mutants (lanes 3–6). The hybridization signals for molecular weight marker X (Roche) are in lane 1 and the molecular weight of the relevant hybridization signals are indicated to the left of the panel. A PCR product of 2.6 kb encompassing *gal*A was used as a probe for the hybridization.

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 *gal*A and *apu*B in the metabolism of specific high-molecular-weight polysaccharides by *B. breve* UCC2003.

Discussion

Bifidobacterial strains demonstrate substantial variability in the efficiency of transformation by plasmids from *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 *et al.*, 2004). While quite a number of *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 *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 *B. longum* subsp. *longum* NCC2705 (Schell *et al.*, 2002) and *B. longum* subsp.

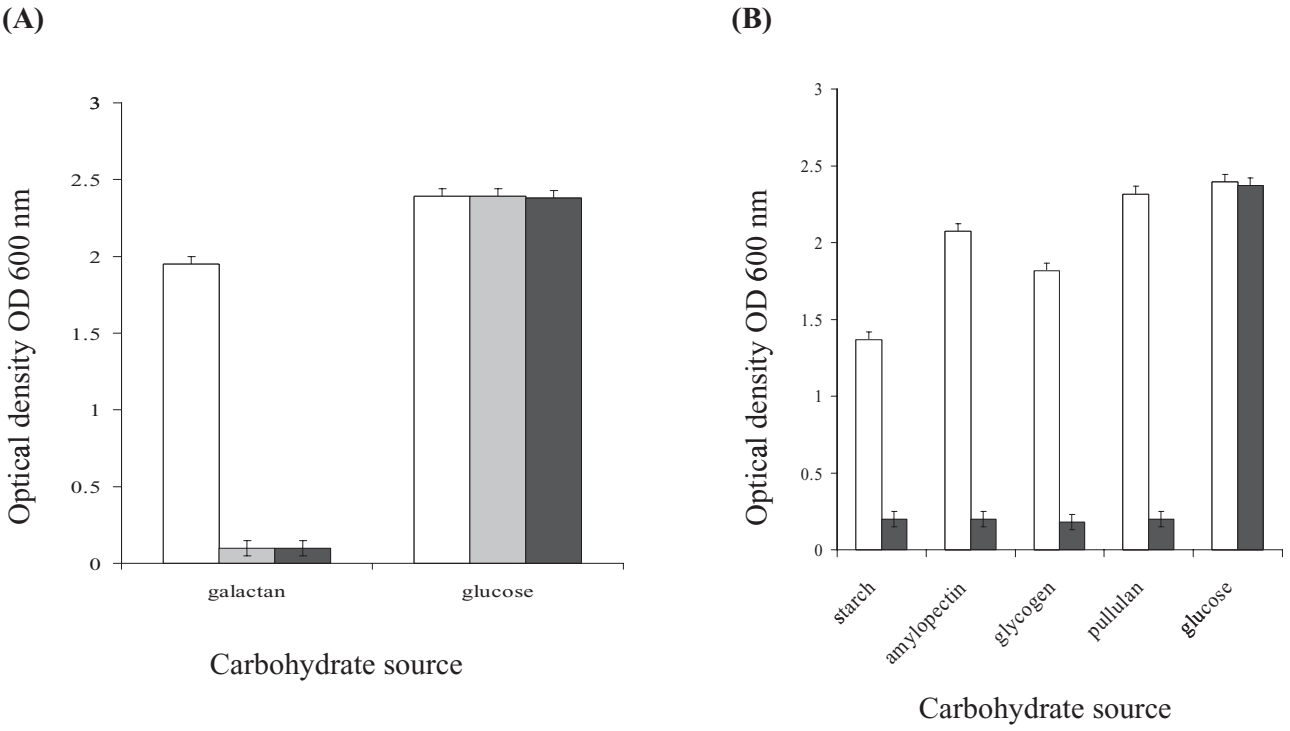

Fig. 6. A. Growth profile analysis of *B. breve* UCC2003 (white), *B. breve* UCC2003-galA-476 (grey) and *B. breve* UCC2003-galA-744 (black) in modified Rogosa broth supplemented with potato galactan or glucose.
B. Growth profile analysis of *B. breve* UCC2003 (white) and *B. breve* UCC2003-apuB (black) in modified Rogosa broth supplemented with starch, amylopectin, glycogen, pullulan or glucose as indicated.

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 AvaII, 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. bifidum* 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 bifidobacte-

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: BbrI, an isoschizomer of BbeI; BbrII, a neoschizomer of SalI; and BbrIII, an isoschizomer of PstI. Restriction analysis of chromosomal DNA from UCC2003 showed that the DNA is protected from restriction with BbeI and PstI, but not SalI. The observed restriction of DNA by SalI can be explained by M.SalI being a N6-adenosine-methylase, while M.BbrII is predicted to be cytosine-specific MTase, which may therefore not confer (full) protection against SalI restriction. However, the finding that M.BbrII does provide full protection against SalI restriction when it is expressed from a multicopy plasmid in *B. breve* JCM 7017 would indicate that M.BbrII 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 BbrI and BbrIII 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 BbrIII, at least under the circumstances used here, providing the biggest hurdle to incoming DNA. To facilitate methylation of plasmid DNA by M.BbrII and M.BbrIII, thereby enhancing the transformation frequency of *B. breve* UCC2003, two *E. coli* strains were constructed, where *bbrIIM* and *bbrIIIM* 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.BbrII-M.BbrIII 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.BbrII- and M.BbrIII-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.BbrII- and M.BbrIII-methylated plasmid. We have previously produced a gene disruption in the *apu*B 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 *apu*B gene was deemed an appropriate control to evaluate the validity and reliability of the plasmid methylation strategy. By M.BbrII-M.BbrIII-mediated methylation of plasmid DNA in *E. coli* prior to transformation into *B. breve* UCC2003, gene disruptions not only in *apu*B, but also in *gal*A 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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References

Alvarez-Martín, P., O'Connell-Motherway, M., van Sinderen, D., and Mayo, B. (2007) Functional analysis of the pBC1 replicon from *Bifidobacterium catenulatum* L48. *Appl Microbiol Biotechnol* **76:** 1395–1402.

Collett, M.A., Depree, K.M., Rand, C.J., Mason, C., and Stanton, J.-A.L. (2008) *Bifidobacterium animalis* subsp. *lactis* HN019, whole genome shotgun sequence. *NCBI Database*.

Cronin, M., Knobel, M., O'Connell-Motherway, M., Fitzgerald, G.F., and van Sinderen, D. (2007) Molecular dissection of a bifidobacterial replicon. *Appl Environ Microbiol* **73:** 7858–7866.

Düsterhöft, A., and Kröger, M. (1991) Cloning, sequence and characterization of m5C-methyltransferase-encoding

gene, hgiDIIM (GTCGAC), from *Herpetosiphon giganteus* strain Hpa2. *Gene* **106:** 87–92.

Fulton, L., Clifton, S., Fulton, B., Xu, J., Minx, P., Pepin, K.H., *et al.* (2007) *Bifidobacterium adolescentis* (L2-32) genome sequence. NCBI Database.

Gibbs, M.J., Smeianov, V.V., Steele, J.L., Upcroft, P., and Efimov, B.A. (2006) Two families of rep-like genes that probably originated by interspecies recombination are represented in viral, plasmid, bacterial, and parasitic protozoan genomes. *Mol Biol Evol* **6:** 1097–1100.

Hartke, A., Benachour, A., Bouibonnes, P., and Auffray, Y. (1996) Characterisation of a complex restriction/modification system detected in a *Bifidobacterium longum* strain. *Appl Microbiol Biotechnol* **45:** 132–136.

Hinz, S.W., Pastink, M.I., van den Broek, L.A., Vincken, J.P., and Voragen, A.G. (2005) *Bifidobacterium longum* endogalactanase liberates galactotriose from type I galactans. *Appl Environ Microbiol* **71:** 5501–5510.

Janscak, P., Sandmeier, U., Szczelkun, M.D., and Bickle, T.A. (2001) Subunit assembly and mode of DNA cleavage of the type III restriction endonucleases EcoP1I and EcoP15I. *J Mol Biol* **306:** 417–431.

Khosaka, T., and Kiwaki, M. (1984) BinI: a new site-specific endonuclease from *Bifidobacterium infantis*. *Gene* **31:** 251–255.

Khosaka, T., Sakurai, T., Takahashi, H., and Saito, H. (1982) A new site-specific endonuclease BbeI from *Bifidobacterium breve*. *Gene* **17:** 117–122.

Khosaka, T., Kiwaki, M., and Rak, B. (1983) Two site-specific endonucleases BinSI and BinSII from *Bifidobacterium infantis*. *FEBS Lett* **163:** 170–174.

Kiel, J.A., Vossen, J.P., and Venema, G. (1987) A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation. *Mol Gen Genet* **207:** 294–301.

Kobayashi, I. (2001) Behaviour of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Res* **29:** 3742–3756.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R.J., and Wilson, G.G. (1984) The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res* **22:** 1–10.

Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G., and Leenhouts, K. (1995) A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol* **177:** 7011–7018.

Leahy, S.C., Higgins, D.G., Fitzgerald, G.F., and van Sinderen, D. (2005) Getting better with bifidobacteria. *J Appl Microbiol* **98:** 1303–1315.

Lee, J.H., and O'Sullivan, D.J. (2006) Sequence analysis of two cryptic plasmids from *Bifidobacterium longum* DJO10A and construction of a shuttle cloning vector. *Appl Environ Microbiol* **72:** 527–535.

Lee, J.H., Karamychev, V.N., Kozyavkin, S.A., Mills, D., Pavlov, A.R., Pavlova, N.V., *et al.* (2008) Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. *BMC Genomics* **9:** 247.

Lenoir-Wijnkoop, I., Sanders, M.E., Cabana, M.D., Caglar, E., Corthier, G., Rayes, N., *et al.* (2007) Probiotic and prebiotic influence beyond the intestinal tract. *Nutr Rev* **65:** 469–489.

Mazé, A., O'Connell-Motherway, M., Fitzgerald, G.F., Deutscher, J., and van Sinderen, D. (2007) Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* **73:** 545–553.

Murray, N.E. (2002) Immigration control of DNA in bacteria: self versus non-self. *Microbiology* **148:** 3–20.

O'Connell Motherway, M., Fitzgerald, G.F., Neirynck, S., Ryan, S., Steidler, L., and van Sinderen, D. (2008) Characterisation of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* **74:** 6271–6279.

O'Hara, A.M., and Shanahan, F. (2006) The gut flora as a forgotten organ. *EMBO Rep* **7:** 688–693.

Oishi, K., Sato, T., Yokoi, W., Yoshida, Y., Ito, M., and Sawada, H. (2008) Effect of probiotics, *Bifidobacterium breve* and *Lactobacillus casei*, on bisphenol A exposure in rats. *Biosci Biotechnol Biochem* **72:** 1409–1415.

O'Riordan, K., and Fitzgerald, G.F. (1999) Molecular characterization of a 5.75 kb cryptic plasmid from *Bifidobacterium breve* NCFB 2258 and determination of the mode of replication. *FEMS Microbiol Lett* **174:** 285–294.

Park, M.S., Shin, D.W., Lee, K.H., and Ji, G.E. (1999) Sequence analysis of plasmid pKJ50 from *Bifidobacterium longum*. *Microbiology* **145:** 585–592.

Park, M.S., Shin, D.W., Lee, K.H., and Ji, G.E. (2000) Characterisation of plasmid pKJ36 from *Bifidobacterium* longum and construction of an *E. coli–Bifidobacterium* shuttle vector. *J Microbiol Biotechnol* **10:** 310–320.

Pingoud, A., Fuxreiter, M., Pingoud, V., and Wende, W. (2005) Type II restriction endonucleases: structure and mechanism. *Cell Mol Life Sci* **62:** 685–707.

Roberts, R.J., Belfort, M., Bestor, T., Bhagwat, A.S., Bickle, T.A., Bitinaite, J., *et al.* (2003) A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res* **31:** 1805–1812.

de Ruyter, P.G., Kuipers, O.P., and de Vos, W.M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* **62:** 3662–3667.

Ryan, S.M., Fitzgerald, G.F., and van Sinderen, D. (2006) Screening for and identification of starch-, amylopectin-, and pullulan-degrading activities in bifidobacterial strains. *Appl Environ Microbiol* **72:** 5289–5296.

Sangrador-Vegas, A., Stanton, C., Van Sinderen, D., Fitzgerald, G.F., and Ross, R.P. (2007) Characterization of plasmid pAS479 from *Bifidobacterium pseudolongum* subsp. *globosum* and its use for expression vector construction. *Plasmid* **58:** 140–147.

Saxelin, M., Tynkkynen, S., Sandholm, T.M., and de Vos, W.M. (2005) Probiotic and other functional microbes: from markets to mechanisms. *Curr Opin Biotechnol* **16:** 204–211.

Schell, M.A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., *et al.* (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA* **99:** 14422–14427.

Sgorbati, B., Scardovi, V., and Leblanc, D.J. (1982) Plasmids in the genus *Bifidobacterium*. *J Gen Microbiol* **128:** 2121–2131.

Shkoporov, A.N., Efimov, B.A., Khokhlova, E.V., Steele, J.L., Kafarskaia, L.I., and Smeianov, V.V. (2008) Characterization of plasmids from human infant *Bifidobacterium* strains: sequence analysis and construction of *E. coli–Bifidobacterium* shuttle vectors. *Plasmid* **60:** 136–148.

Skrypina, N.A., Kramarov, V.M., Liannaia, A.M., and Smolianinov, V.V. (1988) Restriction endonucleases from *Bifidobacteria*. *Mol Gen Mikrobiol Virusol* **5:** 15–16.

Sudarsanam, P., Ley, R., Guruge, J., Turnbaugh, P.J., Mahowald, M., Liep, D., and Gordon, J. (2008) Draft genome sequence of *Bifidobacterium dentium* (ATCC 27678). NCBI Database.

Sutcliffe, J.G., and Church, G.M. (1978) The cleavage site of the restriction endonuclease Ava II. *Nucleic Acids Res* **5:** 2313–2319.

Suzuki, T., Tsuda, Y., Kanou, N., Inoue, T., Kumazaki, K., Nagano, S., *et al.* (2006) *Bifidobacterium adolescentis* ATCC 15703 complete genome sequence. NCBI Database.

Timinskas, A., Butkus, V., and Janulaitis, A. (1995) Sequence motifs characteristic for DNA (cytosine-N4) and DNA (adenine0N6) methyltransferases. Classification of all DNA methyltransferases. *Gene* **157:** 3–11.

Tock, M.R., and Dryden, D.T.F. (2005) The biology of restriction and anti-restriction. *Curr Opin Microbiol* **8:** 466–472.

Turroni, F., Ribbera, A., Foroni, E., van Sinderen, D., and Ventura, M. (2008) Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek* **94:** 35–50.

Ventura, M., van Sinderen, D., Fitzgerald, G.F., and Zink, R. (2004) Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek* **86:** 205–223.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F., and van Sinderen, D. (2007) Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* **71:** 495–548.

Walder, R.Y., Walder, J.A., and Donelson, J.E. (1984) The organization and complete nucleotide sequence of the PstI Restriction-Modification System. *J Biol Chem* **259:** 8015–8026.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–109.

Zoetendal, E.G., and Vaughan, E. E., and de Vos, W.M. (2006) A microbial world within us. *Mol Microbiol* **59:** 1639–1650.

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 *bbrIIM* and *bbrIIIM* methylase integration strains. Lane 1, molecular weight marker X (Roche). Lanes 2–4, Pst1 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 *bbrIIM* 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.BbrII-M.BbrIII, *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 *apu*B 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 *apu*B 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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