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Mobilome and genetic modification of bifidobacteria

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Abstract

Until recently, proper development of molecular studies in *Bifidobacterium* species has been hampered by growth difficulties, because of their exigent nutritive requirements, oxygen sensitivity and lack of efficient genetic tools. These studies, however, are critical to uncover the cross-talk between bifidobacteria and their hosts' cells and to prove unequivocally the supposed beneficial effects provided through the endogenous bifidobacterial populations or after ingestion as probiotics. The genome sequencing projects of different bifidobacterial strains have provided a wealth of genetic data that will be of much help in deciphering the molecular basis of the physiological properties of bifidobacteria. To this end, the purposeful development of stable cloning and expression vectors based on robust replicons – either from temperate phages or resident plasmids – is still needed. This review addresses the current knowledge on the mobile genetic elements of bifidobacteria (prophages, plasmids and transposons) and summarises the different types of vectors already available, together with the transformation procedures for introducing DNA into the cells. It also covers recent molecular studies performed with such vectors and incipient results on the genetic modification of these organisms, establishing the basis that would allow the use of bifidobacteria for future biotechnological applications.

Keywords: *Bifidobacterium*, bifidobacteria, plasmid, vector, genetic engineering

1. Introduction

The members of the genus *Bifidobacterium* are anaerobic, fermentative, high G+C Gram-positive bacteria belonging to the phylum *Actinobacteria* and the order *Bifidobacteriales* (Scardovi, 1986). Bifidobacterial species form a coherent phylogenetic group showing over 93% similarity of the 16S rRNA sequences among them. At present, more than 40 species are included in the genus *Bifidobacterium* (http://old.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Bifidobacterium), of which *B. adolescentis, B. angulatum, B. breve, B. bifidum, B. catenulatum, B. dentium, B. longum* and *B. pseudocatenolatum* are the dominant species in the human gastrointestinal tract (GIT) (Fanaro *et al.*, 2003; Mueller *et al.*, 2006). These and *B. animalis* subsp. *lactis*, the typical species isolated from functional foods (Masco *et al.*, 2005), are therefore the first target for health-related studies.

Bifidobacteria are considered to exert a vast array of beneficial health effects, including establishment of a healthy microbiota in infants, competitive exclusion against intestinal pathogens and modulation of the immune functions (Leahy *et al.*, 2005; Turroni *et al.*, 2009). In humans, bifidobacteria represent up to 90% of the total gut microbiota in breast-fed babies (Fanaro *et al.*, 2003; Turroni *et al.*, 2012) and up to 5% in healthy adults (Claesson *et al.*, 2011; Mueller *et al.*, 2006). Colonisation of the human intestinal tract by bifidobacteria starts soon after birth and lasts lifelong. Thus, unsurprisingly, bifidobacteria have

become a common component of probiotic products designed for human or animal consumption (Leahy *et al.*, 2005; Parvez *et al.*, 2006; Tuohy *et al.*, 2003). Probiotic products represent a strong growth area within the functional foods market and currently have a significant economic impact on the dairy sector. However, as pointed out by Stanton *et al.* (2001)

> long-term exploitation of probiotics as health promoters is dependent on several factors, including sound, scientifically-proven clinical evidence of health-promoting activity, accurate consumer information, effective marketing strategies and, above all, a quality product that fills consumer expectations.

While clinical evidence for the purported beneficial effects is rapidly accumulating (Guglielmetti *et al.*, 2011; Ishikawa *et al.*, 2011; Leahy *et al.*, 2005; Tuohy *et al.,* 2003), there still is a lack of fundamental knowledge on the molecular mechanisms by which bifidobacteria interact with other bacteria and with their hosts, while contributing to health and well-being (Kullen and Klaenhammer, 2000; Ventura *et al.*, 2009a). Combined clinical and molecular data are essential to proof the beneficial physiological effects needed for allowing probiotics to be marketed with health claims (Katan, 2012).

2. Genetics of bifidobacteria

Compared with other microbes of industrial importance, the genetics of bifidobacteria is poorly understood (Ventura *et al.*, 2004). Bifidobacteria are difficult to handle in the laboratory because they are exigent microorganisms demanding rich media and requiring strict anaerobic conditions for growth (Scardovi, 1986). In addition, genetic studies have further been hampered by a lack of appropriate bacterial replicons of either plasmid or phage origin with which to construct suitable genetic tools. Moreover, until recently, bifidobacteria were considered recalcitrant to transformation and genetic engineering techniques were simply unknown.

In the last decade, whole genome sequencing has revolutionised the genetic, biochemical and molecular biological research in bacteria and in many higher organisms, constituting an essential step for generating primary genetic information for downstream functional applications, such as comparative genomics, transcriptomics and/or proteomics, which in turn can address fundamental and applied questions. Specifically, operons and genes encoding several cell envelope-associated structures such as exopolysaccharides (Barrangou *et al.*, 2009; Fanning *et al.*, 2012a; Schell *et al.*, 2002; Ventura *et al.*, 2009b), fimbriae-like glycoproteins (Fanning *et al.*, 2012b; Schell *et al.*, 2002; Ventura *et al.*, 2009b), serpin-like protease inhibitors (Álvarez-Martín *et al.*, 2012a; Ivanov *et al.*, 2006; Turroni *et al.*, 2010), adhesins (Guglielmetti *et al.*, 2008b) and pilus-like structures (Foroni *et al.*, 2011; O'Connell Motherway *et al.*, 2011a) have been identified. In addition, genes dealing with diverse stresses that bifidobacteria face in their environment (acid, bile), genes encoding adaptive functions to the intestinal niche or others contributing to ecological fitness have also been identified (Barrangou *et al.*, 2009; Kim *et al.*, 2009; Ruiz *et al.*, 2012a; Schell *et al.*, 2002; Sela *et al.*, 2008). Concerning the adaptation to intestinal environment, largely the best studied bifidobacterial strain is *B. breve* UCC2003, thanks to the molecular and functional characterisation of many genetic loci that have been identified in its genome (Álvarez-Martín *et al.*, 2012a,b; Fanning *et al.*, 2012; O'Connell Motherway *et al.*, 2008, 2011a,b; Pokusaeva *et al.*, 2011; Ruiz *et al.*, 2012b).

Until now, the genome sequences publically available of 17 *Bifidobacterium* strains belonging to five species, i.e. *B. adolescentis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. dentium* and *B. longum* of both *infantis* and *longum* subspecies, have all been concluded and analysed (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). In addition, many other *Bifidobacterium* genome projects are in progress worldwide and, due to the progressive cost reduction of the sequencing technologies, this list is exponentially growing. To manage this enormous wealth of genetic data and prove unequivocally the biological role of each particular operon or gene, cloning, expression, knock-out and transfer of the determinants are needed. To this aim, suitable vectors and other genetic tools based on the replication units and insertional machinery of phages, plasmids and/or transposons are essential.

3. The mobilome of bifidobacteria

Genomes are the result of the adaptive evolution of microorganisms to their ecological niche (Ventura *et al.*, 2007). In this context, various genetic events, such as gene duplication, horizontal gene transfer (HGT), gene decay and chromosomal rearrangements, have all determined the shape of bacterial genomes. As suggested by Philippe and Douady (2003), plasmids, bacteriophages (phages), and transposons are considered major agents for shaping the bifidobacterial genomes through HGT processes. The above mentioned mobile genetic elements as well as others, such as group II introns and jumping genes, constitute what has recently been defined as the mobilome (Frost *et al.*, 2005; Siefert, 2009).

Plasmids of bifidobacteria

Plasmids are extra-chromosomal, autonomously replicating genetic elements found in bacteria, archaea and eukaryotic cells. Despite their independent replication, plasmids make use of cellular enzymes to ensure both replication and maintenance (Hayes, 2003). Plasmids display an enormous

diversity of features, such as size, host range and the repertoire of genes they carry. By definition, plasmids do not encode essential genes for growth, nonetheless they can provide a wide variety of phenotypes to the cells that harbour them, including antibiotic resistance, bacteriocin production, virulence and pathogenesis or degradation of complex and recalcitrant (toxic) compounds found in some ecosystems. They can also encode for the ability to use carbohydrates and/or protein substances as a source of carbon and energy (Thomas *et al.*, 2004). All these properties have endowed plasmids with the title of primarily adaptative entities (Siezen *et al.*, 2005). Apart from the traits with an impact on host physiology and ecology, important characteristics of plasmids include copy number (low, medium, high), host range (narrow, broad) and capability to spread (conjugation, mobilisation) (Hayes, 2003). Small plasmids (i.e. smaller than 15-20 kb in size) often do not encode any selectable trait and are therefore denominated 'cryptic'. It seems, however, plausible to presume that they simply benefit its host by promoting recombination and, consequently, enhance the ecological adaptability of the bacterial population (Guglielmetti *et al.*, 2007a; Thomas *et al.*, 2004). Plasmids can be, consequently, depicted as accessory and/or adaptive gene pools shared by bacteria.

Analyses of bifidobacteria have indicated that extra-chromosomal elements are scarcer than in other intestinal bacterial species (Iwata and Morishita, 1989; Park *et al.*, 1997; Sgorbati *et al.*, 1982) and, where found, they have a size generally smaller than 15 kb. Strains from the species *B. longum* subsp. *longum* (hereafter *B. longum*, if not differently specified), *B. globosum*, *B. asteroides* and *B. indicum* seem to harbour more plasmids than those from other bifidobacteria species (Sgorbati *et al.*, 1982). At present, the nucleotidic sequence of more than 30 bifidobacterial plasmid molecules is available in GenBank (Table 1; http://www.ncbi.nlm.nih.gov/sites/entrez/). The majority of these molecules (up to 19) were isolated from *B. longum* strains, which includes pMB1 (Rossi *et al.*, 1996), the first bifidobacterial plasmid to be analysed at a molecular level. Three other plasmids have been characterised from strains of *B. breve*, two from *B. asteroides* and *B. bifidum*, and single plasmids have been analysed from strains of *B. catenulatum*, *B. pseudocatenulatum*, and *B. pseudolongum* subsp. *globosum*. The obtained sizes of the studied plasmids show a range between 1.8 kb for pMB1 to 10.2 kb for pNAC3. In most cases, strains harbour a single plasmid, except for three strains of *B. longum* in which two different plasmid molecules were identified (Table 1). In addition, *B. longum* NAL8 and *B. longum* FI10564 have been reported to contain three different plasmids each. The significance of these autonomously replicating DNA elements in bifidobacteria remains unclear, since no obvious phenotypic traits have been associated to plasmids, except for the production of the bacteriocin bifidocin B by *B. bifidum* NCFB 1454, which was associated to a 8 kb plasmid (Yildirim *et al.*, 1999). Nevertheless, this plasmid has never been sequenced or characterised further. More recently, another plasmid from *B. bifidum* (pBIF10) has been found to contain *tetQ*, a gene encoding a ribosome protection protein providing tetracycline resistance (DQ093580).

The basic biology of bifidobacterial plasmids remains poorly understood and most of the information derives from *in silico* investigation. Indeed, the mode of replication has been experimentally analysed for only a few of them (Corneau *et al.*, 2004; Guglielmetti *et al.*, 2007b; Lee and O'Sullivan, 2006; Moon *et al.*, 2009; O'Riordan and Fitzgerald, 1999; Park *et al.*, 1999, 2008). Furthermore, dissection of open reading frames (ORFs) and analysis of untranslated sequences and structures have been undertaken for only two plasmids, pBC1 (Álvarez-Martín *et al.*, 2007a) and pCIBA089 (Cronin *et al.*, 2007). Sequence comparison suggests that most bifidobacterial plasmids probably replicate by means of a rolling-circle mechanism, with the exception represented by eight plasmids that appear to use the theta-replicating mode (Table 1; Álvarez-Martín *et al.*, 2008; Cronin *et al.*, 2007; Klijn *et al.*, 2006; Lee and O'Sullivan, 2006; Moon *et al.*, 2009; Rossi *et al.*, 1996). Phylogenetic analysis of their replication (Rep) initiator proteins revealed that *Bifidobacterium* plasmids could be clustered into six different groups (Table 1 and Figure 1). Homology of bifidobacterial Rep proteins has shown that, in some cases, their closest relatives are found in plasmids from phylogenetically distant bacterial groups (Álvarez-Martín *et al.*, 2007b; Guglielmetti *et al.*, 2007b). For instance, the Rep protein of plasmid pBIF10 from *B. bifidum* M203049 (type IV) is strictly related with the replication proteins of plasmids commonly harboured by the *Cytophaga – Flavobacterium – Bacteroides* group of Gram-negative bacteria. The same plasmid also contains two other DNA regions, respectively of 1,966 and 2,569 bp displaying strong similarity with DNA from *Bacteroides* (DQ093580). The former region includes mobilisation genes *mobA* and *mobB*, while the latter comprises a *tetQ* gene (Figure 2). These similarities leave to suppose that plasmid pBIF10 could have been relatively recently acquired by strain *B. bifidum* M203049 from *Bacteroides* by an HGT event. More surprisingly, the Rep protein encoded by plasmid p4M from *B. pseudocatenulatum* VMKB4M (type VI) displays its highest level of similarity to the replication initiator protein of the eukaryotic circoviruses/cicloviruses (Gibbs *et al.*, 2006), a feature that is unprecedented in any known bacterial plasmid.

Upstream of the Rep protein, *Bifidobacterium* plasmids contains non-coding regions characterised by tandem direct and inverted repeats sequences, in an organisation that resembles the so-called DNA iteron structures observed in the origin of replication of some theta and rolling circle replicating plasmids (Del Solar *et al.*, 1998). The tandem repeat organisation is similar in all plasmids

Table 1. Plasmids of *Bifidobacterium* species whose whole nucleotide sequence is known.

Bifidobacterium species[1]	Strain	Plasmid	Size (bp)	Putative replication mechanism (Rep type[2])	Reference/GenBank accession number
B. asteroides	DSM20089	pCIBAO89	2,111	Theta (II)	Cronin *et al.*, 2007 / EU030683
B. asteroides	DSM20089	pAP1	2,140	Theta (II)	Y11549
B. bifidum	B80	pB80	4,898	RC (Ia)	Shkoporov *et al.*, 2008a / DQ305402
B. bifidum	CCTCC M203049	pBIF10	9,275	RC (IV)	DQ093580
B. breve	NCFB2258	pCIBb1	5,750	RC (III)	O'Riordan and Fitzgerald, 1999 / AF085719
B. breve	B21a	pB21a	5,206	RC (III)	Shkoporov *et al.*, 2008a / DQ497626
B. breve	-	pNBb1	2,297	RC (III)	E17316
B. catenulatum	L48	pBC1	2,540	Theta (V)	Alvarez-Martin *et al.*, 2007 / DQ011664
B. longum	KJ	pKJ36	3,625	RC (Ib)	Park *et al.*, 1997 / AF139129
B. longum	KJ	pKJ50	4,960	RC (Ia)	Park *et al.*, 1999 / BLU76614
B. longum	NCC2705	pBLO1	3,626	RC (Ib)	Schell *et al.*, 2002 / AF540971
B. longum	MG1	pMG1	3,682	RC (Ib)	Park *et al.*, 2003 / AY210701
B. longum	RW048	pNAC1	3,538	RC (Ia)	Corneau *et al.*, 2004 / AY112724
B. longum	RW041	pNAC2	3,684	RC (Ib)	Corneau *et al.*, 2004 / AY112723
B. longum	RW041	pNAC3	10,224	Theta (II)	Corneau *et al.*, 2004 / AY112722
B. longum	BK51	pTB6	3,624	RC (Ib)	Tanaka *et al.*, 2005 / AB187597
B. longum	B2577	pMB1	1,847	Theta (V)	Rossi *et al.*, 2006 / X84655
B. longum	DJO10A	pDOJH10L[3]	10,073	Theta (II)	Lee and O'Sullivan, 2006 / AF538868
B. longum	DJO10A	pDOJH10S	3,661	Theta (V)	Lee and O'Sullivan, 2006 / AF538869
B. longum	NAL8	pNAL8L	3,489	RC (Ia)	Guglielmetti *et al.*, 2007 / AM183145
B. longum	NAL8	pNAL8M	4,910	RC (Ia)	Guglielmetti *et al.*, 2007 / AM183144
B. longum	VMKB44	pB44	3,624	RC (Ib)	Shkoporov *et al.*, 2008a / AY066026
B. longum	FI10564	pFI2576	2,197	Theta (V)	Moon *et al.*, 2009 / DQ452864
B. longum	BK28	pBK283	4,537	RC (Ia)	Fukiya *et al.*, 2010 / AB495342
B. longum	DPC6043	p6043A	4,896	RC (Ia)	DQ458911
B. longum	DPC6043	p6043B	3,680	RC (Ib)	DQ458910
B. longum	M62	pSP02	4,896	RC (Ia)	GU256055
B. pseudocatenulatum	VMKB4M	p4M	4,488	RC (VI)	Gibbs *et al.*, 2006 / AF359574
B. pseudolongum subsp. *globosum*	DPC479	pASV479	4,815	RC (III)	Sangrador Vegas *et al.*, 2007 / DQ103758
Bifidobacterium sp.	A24	pBIFA24	4,892	RC (Ia)	Park *et al.*, 2008 / DQ286581

[1] *B. longum* stands for *Bifidobacterium longum* subsp. *longum*.

[2] Replication protein typology of RC- (rolling-circle) and theta-type plasmids are in accordance with dendrograms in Figure 1.

[3] DNA sequence assessment suggests that pDOJH10L is a cointegrate involving plasmids very similar to pKJ50 (96% identity) and pNAC2 (98% identity).

but sequences of the repeats are variable, thus, conferring specific interaction between Rep proteins and their cognate DNA sequences.

Differences in nucleotide sequences and gene organisation have been encountered among the 30 known bifidobacterial plasmids, leading to the identification of 13 different modular structures, represented by the genetic maps of characteristic plasmids shown in Figure 2. Apart from Rep, many plasmids contains accessory ORFs encoding hypothetical proteins, some of which, such as the mobilisation-like proteins (Figure 2), may be involved, together with their accompanying *oriT* sequences, in plasmid spread. In a few bifidobacterial plasmids are also present putative genes encoding non-essential proteins, such as OrfX and CopG, which are involved in the control of replication, copy number and/or plasmid stability (Álvarez-Martín *et al.*, 2007a; Del Solar *et al.*, 1998). Finally, it should be mentioned that whole genome analyses of *Bifidobacterium* strains reveal the presence of integrated plasmid remnants in the chromosome, such as those discovered in *B. longum* NCC2705 (Schell *et al.*, 2002) and F8 (GenBank Acc. No. FP929034).

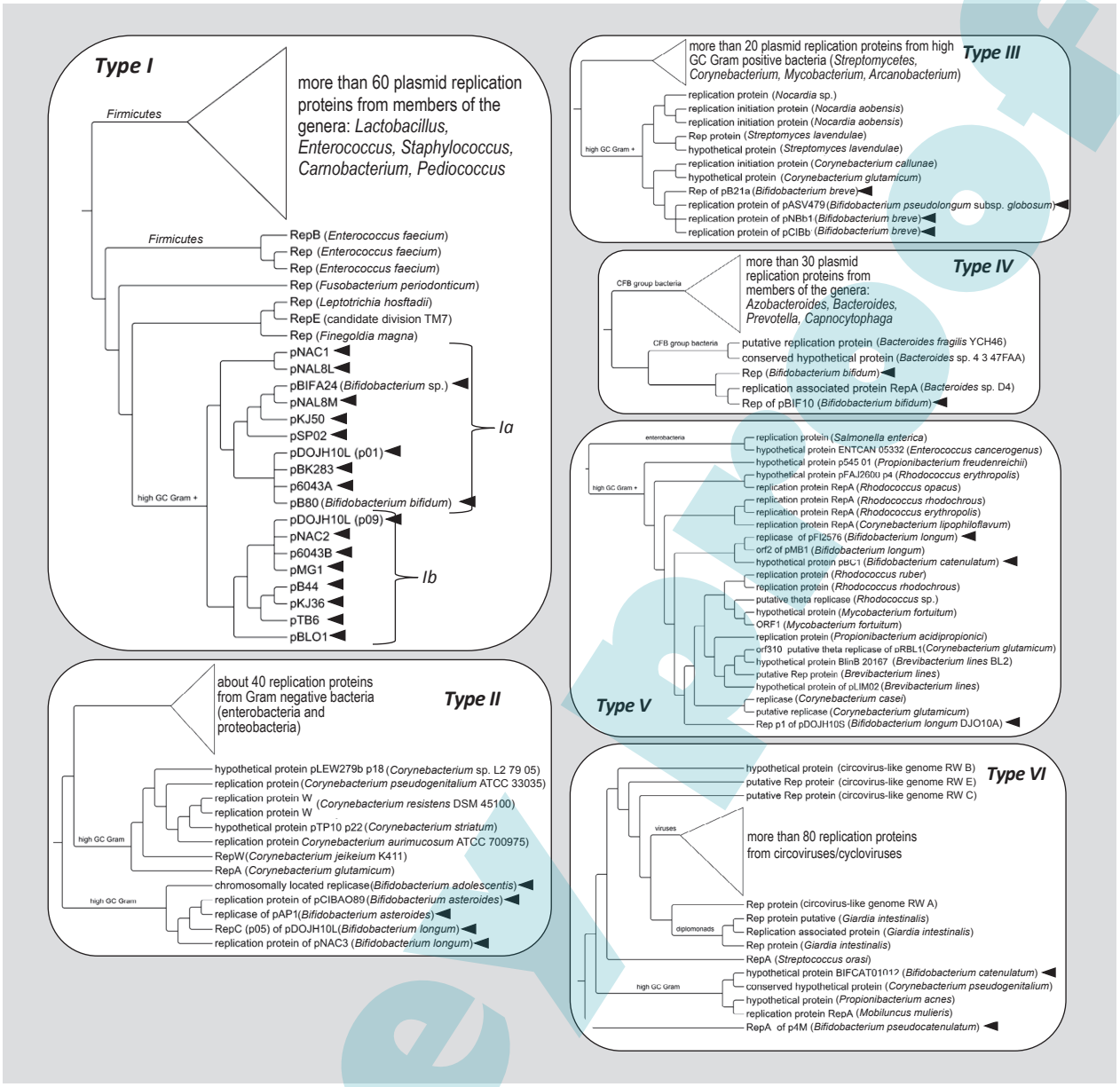

Figure 1. Trees produced by BLAST using a pairwise alignment between a query (an arbitrarily selected Rep protein from a *Bifidobacterium* plasmid) and the database sequences searched. Type I: this group includes 18 Rep proteins from *Bifidobacterium* plasmids. Plasmids without species indication belong to *Bifidobacterium longum* susp. *longum*. Types II-VI: Rep stands for replication initiation protein. Types II and IV: *Bifidobacterium longum* stands for *Bifidobacterium longum* susp. *longum*. CFB stands for the bacterial group *Cytophaga – Flavobacterium – Bacteroides*. Red triangles evidence Rep proteins from *Bifidobacterium* plasmids.

Phages of bifidobacteria

Phages are widely distributed among eubacteria, where they are thought to influence the genomic evolution and adaptive capabilities of their hosts (Canchaya *et al.*, 2003). The first report of *Bifidobacterium* phages dates back to 1966, when they were detected in rumen (Youseff *et al.*, 1966). However, in this work the morphology and other characteristics of the phage particles were unreported. The work of Youseff *et al.* was followed by an electron microscopy observation of a lytic phage from the so-called *Bifidobacterium ruminale* (today reclassified as *Bifidobacterium thermophilum*) strain RU271 by Matteuzzi and Sozzi (1970). Further pioneering reports include that of Sgorbati *et al.* (1983), where inducible prophages from strains of *B. longum* were released by mitomycin C and further characterised by electron microscopy. However, no further studies on bifidobacterial phages appeared until the analysis of whole genomes. Three highly related prophage-like elements have been reported to be present in the genome of *B. breve* UCC2003, *B. longum* NCC2705 and *B. longum* DJO10A (Ventura *et al.*, 2005). These elements, designated Bbr-1, Bl-1 and Blj-1,

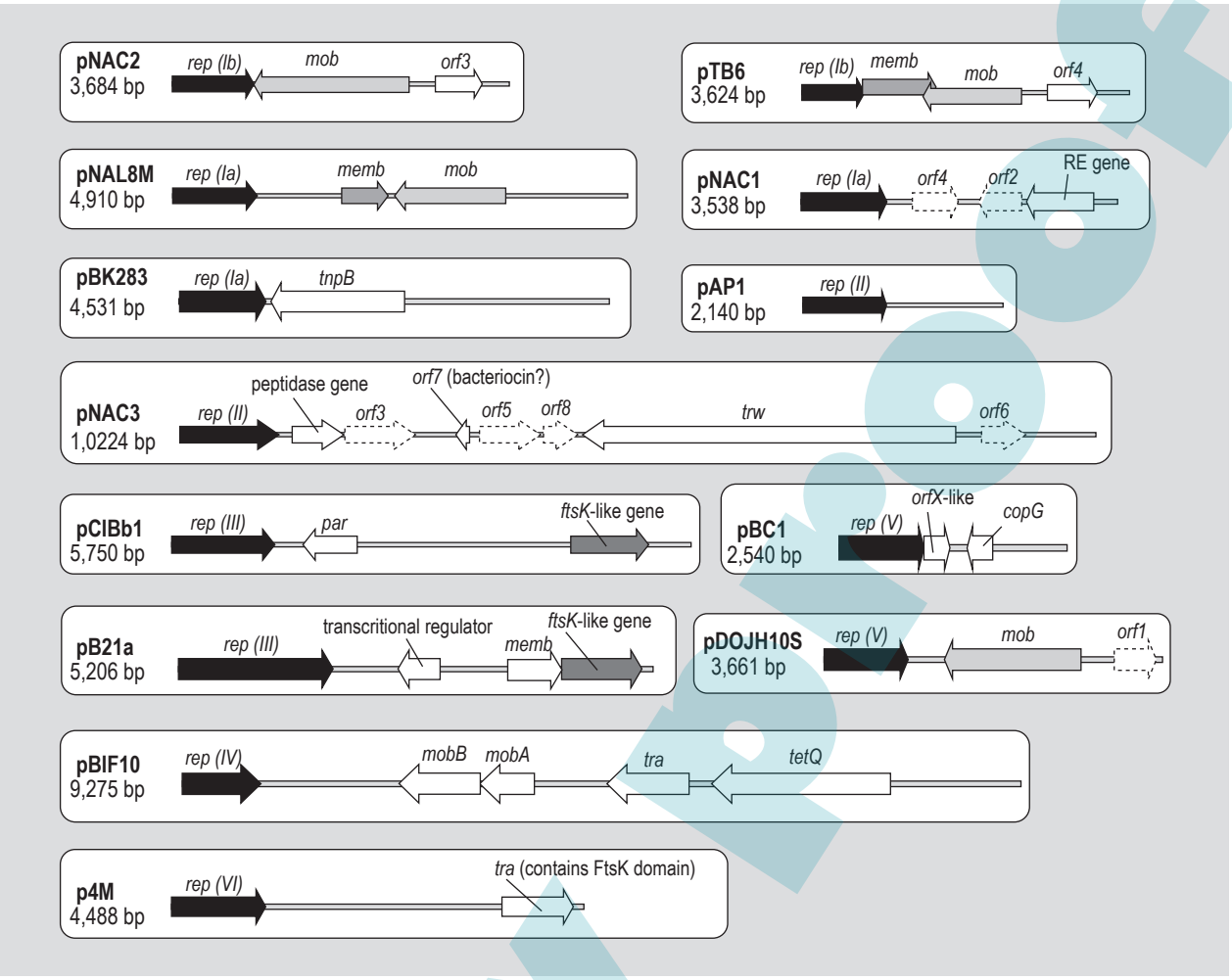

Figure 2. Linear functional maps of a representative plasmid from all the *Bifidobacterium* plasmid structures up to now recognised (in accordance with Table 1). In these maps, all the open reading frames from *Bifidobacterium* plasmids with a putatively assigned biological function are included. Dotted arrows refer to hypothetical conserved proteins with unknown function. Genes represented with the same colours/motif share a significant sequence similarity. Genes represented with white arrows are harboured by only one of the shown plasmids. Rep typology in accordance to Figure 1 is indicated between brackets.

Legend of the gene symbols indicated in the maps:

***rep* = gene encoding a replication initiation protein.**

***mob* = gene encoding a putative plasmid mobilisation protein.**

***memb* = gene encoding an integral transmembrane protein.**

***tnpB* = gene encoding for the transposase of an insertion sequence-like element of the IS200/IS605 family, named ISBlo15.**

***tra* = transposase gene.**

***trw* = gene encoding for a putative protein containing the conjugative relaxase domain TrwC/TraI (Conserved Domain Database, CDD, accession code TIGR02686).**

***ftsK*-like gene = gene putatively encoding for a domain of the FtsK/SpoIIIE family. This domain contains a putative ATP binding P-loop motif. A mutation in FtsK causes a temperature sensitive block in cell division and it is involved in peptidoglycan synthesis or modification. The SpoIIIE protein is implicated in intercellular chromosomal DNA transfer (CDD accession code pfam01580).**

***tet*(Q) = tetracycline resistance gene encoding a ribosomal protection protein.**

***copG* = ORF encoding a putative protein that shares similarity with the plasmid pMV158-encoded transcriptional repressor CopG (CDD accession code pfam01402).**

***par* = gene encoding for a putative protein belonging to the ParA conserved family of bacterial proteins (CDD accession code cd02042), implicated in chromosome segregation (involved in the plasmid replication and partition).**

RE gene = gene encoding a putative protein with a type II restriction endonuclease domain (*Eco*RII, CDD accession code pfam09019).

peptidase gene = gene encoding for a putative member of peptidase family C39 (cd02549). Peptidase family C39 mostly contains bacteriocin-processing endopeptidases from bacteria.

***mobA* and *mobB* = mobilisation protein genes harboured by plasmids from the *Cytophaga – Flavobacterium – Bacteroides* group of bacteria.**

respectively, share nucleotide and organisation homology with double-stranded DNA bacteriophages infecting low G+C Gram-positive bacteria, arguing for a common evolution of phages within the GIT ecosystem (Ventura *et al.*, 2005). The Blj-1 prophage is 36.9 kb long and is excised from the chromosome when *B. longum* DJO10A is exposed to mitomycin C or hydrogen peroxide (Ventura *et al.*, 2005). Thus, Blj-1 appears to constitute the first inducible prophage whose sequence is entirely known. In contrast, Bbr-1 and Bl-1 elements are not inducible, suggesting they may represent non-functional prophages. Though defective, they may still constitute functional satellite phages, whose mobility depends on helper phages in a similar manner to that described for the cryptic mycophages Rv1 and Rv2 (Hendrix *et al.*, 1999). All bifidobacterial prophages are integrated in a tRNAMet gene, which had not previously been shown to act as an *attB* site in Gram-positive bacteria (Campbell, 1992). Analysis of the distribution of this integration site in bifidobacterial species has revealed that the *attB* site is well conserved. In addition, in the genome of *B. longum* subsp. *infantis* ATCC 15697 and those of *B. animalis* subsp. *lactis* DSM10140 and Bl-04, prophage genes have also been encountered (Barrangou *et al.*, 2009; Sela *et al.*, 2008), although these remnant elements are not adjacent to tRNAMet sequences. Ventura *et al.* (2009c) have recently extended the study of the genetic organisation, phylogenetic relationships, and, for some prophages, the transcription profiles and inducibility of 19 phage particles integrated in the chromosomes of nine bifidobacterial strains. More prophages were shown to be non-inducible, but they are thought to contribute to shaping the bacterial genome and to increase the fitness of the lysogenic hosts, thus guaranteeing a more efficient colonisation of human intestines.

The use of conserved *attB* sequences and their associated *int* genes from these prophages might allow the construction of efficient recombination modules analogous to the *Streptomyces* integrative plasmid pSE211 (Brown *et al.*, 1990). This module may represent an ideal source for integration systems, enabling future development of food-grade, single copy integration of foreign DNA at specific sites within the bifidobacterial chromosome without disturbing host functions. Similar systems have been developed for lactic acid bacteria, such as lactobacilli (Martín *et al.*, 2000), and for high G+C bacteria, such as *Streptomyces* and *Mycobacterium* (Combes *et al.*, 2002).

Transposons and insertion sequence elements in bifidobacteria

Transposons and insertion-sequence (IS) elements are mobile genetic units that move from one to another position in the genome by a process referred to as transposition. Transposition occurs via one of two mechanisms: cut-and-paste transposition or replicative transposition (Roberts *et al.*, 2008), leaving one copy on the target DNA or two copies on both donor and target DNA. Both transposons and ISs are potent, broad spectrum mutators contributing to the shape of the function, structure and dynamics of genes and genomes (Philippe and Douady, 2003). Transposons and ISs can be converted into powerful genetic tools, with which to explore the functionality of genes. In addition, transposons integrating at preferred, neutral sites can be used for genetic modification of bacteria. Sixteen IS elements of five classes have been reported to be spread in the *B. longum* NCC2705 genome (Schell *et al.*, 2002). Although present at similar numbers in the genome of all other strains analysed, ISs have not been considered in other studies (Barrangou *et al.*, 2009; Garrigues *et al.*, 2010; O'Connell Motherway *et al.*, 2011a; Sela *et al.*, 2008; Ventura *et al.*, 2009b). An IS of 1,047 bp long was identified in the upstream region of the tetracycline resistant gene *tet*(W) found on the chromosome of *B. longum* F8 (Kazimierczak *et al.*, 2006). A similar IS of 1,163 bp was also found to interrupt the structural *tet*(W) gene in the susceptible *B. longum* M21 strain (Ammor *et al.*, 2008). The spreading process of the ISs involves excision and integration into a new place, at which position a short nucleotide duplication is usually found. Interestingly, five out of the six bp sequences duplicated in F8 (CAATGC) seem to mirror the 5 bp duplication in M21 (GTTAC) (B. Mayo, unpublished results), suggesting the presence of active insertion sites in bifidobacterial genomes.

Recently, Fukiya *et al.* (2010) characterised an insertion sequence-like element of the IS*200*/IS*605* family, which was inserted into a 5.0 kb pKJ50-like plasmid resulting in the size-increased cryptic plasmid pBK283 from *B. longum* strain BK28. The element, named IS*Blo15*, was 1,593 bp in length and contained a single ORF encoding a putative transposase, *tnpB* (Figure 2). The same authors also reported that sequences similar to IS*Blo15* are widely distributed among the nine *Bifidobacterium* species they tested. Finally, a copy of the transposon Tn*5432*, which encodes resistance to erythromycin and clindamycin, has been identified in several *B. thermophilum* strains isolated from pig faeces (Van Hoek *et al.*, 2008). Tn*5432* was first isolated from *Corynebacterium xerosis* and rescued copies on plasmids were shown to be able to transpose in *Corynebacterium glutamicum* causing several mutations (Tauch *et al.*, 1995). The transposition ability of Tn*5432* from *B. thermophilum* remains however to be determined.

Knowledge on *Bifidobacterium* transposons and ISs is strongly needed, since they can bring to the development of high-efficiency transposon mutagenesis systems that could greatly facilitate the molecular study of bifidobacteria. However, tools for bifidobacteria based on these elements are yet not available. Finally, a new kind of mobile genetic element has been described in *B. longum* (Lee *et al.*, 2008; Schell *et al.*, 2002), named mobile integrase cassette (MIC). MICs are constituted by a conserved 20 bp palindrome

sequence and two insertion sequences separated by three contiguous but different *xerC* integrase genes (Lee *et al.*, 2008). Interestingly, one MIC of the strain *B. longum* DJO10A was shown to be active during the adaptation of *B. longum* DJO10A to *in vitro* fermentation conditions (continous growth up to about 1000 generations) (Lee *et al.*, 2008).

4. General and specialised vectors for bifidobacteria

Some natural bifidobacterial plasmids have provided the basis for the construction of *Escherichia coli* – *Bifidobacterium* shuttle vectors, mostly resulting from the direct cloning of whole plasmids into an *E. coli* vector containing selectable antibiotic resistance genes such as spectinomycin, erythromycin and chloramphenicol (Álvarez-Martín *et al.*, 2008; Guglielmetti *et al.*, 2007b; Klijn *et al.*, 2006; Lee and O'Sullivan, 2006; Matsumura *et al.*, 1997; Missich *et al.*, 1994; Park *et al.*, 1999; Rossi *et al.*, 1996, 1998). In this way, the plasmid pBC1 from *B. catenulatum* L48 has been used for the construction of a series of *E. coli* – *Bifidobacterium* shuttle vectors with innovative characteristics such as the presence of a tetracycline resistance gene of bifidobacterial origin [*tet*(W)] and the cloning of the β-galactosidase complementing peptide gene for a convenient blue/white screening of recombinant clones in *E. coli* (Álvarez-Martín *et al.*, 2008). The functionality of the vectors was further checked by cloning and overexpression of an α-l-arabinofuranosidase gene from *B. longum* B667 in *E. coli* and *Bifidobacterium* strains.

As plasmid maintenance constitutes a major problem for vector utilisation, González Vara *et al.* (2003) studied the segregational and structural stability of pMB1-derived constructs in *B. animalis* by continuous culture. These authors reported a high correlation between instability and plasmid size, while no major deletions and rearrangements were observed. However, some constructs did not behave as expected (González Vara *et al.*, 2003), a result that agrees with observations by other authors (Álvarez-Martín *et al.*, 2007a), suggesting that beyond plasmid size, secondary structure of the constructs may further influence stability.

It is worth noting that in spite of a limited knowledge of plasmid biology, a number of vectors for heterologous expression of desirable foreign genes have already been developed. As an example, the reporter vector pMDY23 expresses the *gusA* gene of *E. coli* (Klijn *et al.*, 2006); vector pBES2 has been used to express the α-amylase gene of *B. adolescentis* in *B. longum* (Rhim *et al.*, 2006); pBLES100 (Matsumura *et al.*, 1997) has been employed for the expression of the *Salmonella* flagellin gene (Takata *et al.*, 2006); and pBV22210 has been used to express the anticancer protein endostatin (Xu *et al.*, 2007).

5. Genetic modification of bifidobacteria

Since the mid-eighties, research efforts have focused on establishing effective protocols for the genetic modification of bifidobacteria. Currently, electrotransformation (electroporation) of bifidobacteria by plasmid DNA is commonly being reported, whereas little or nothing is known about other recombinant DNA technologies such as conjugation. In fact, the members of the genus *Bifidobacterium* have traditionally been considered refractory to efficient and reproducible genetic manipulation. Potentially, several factors can contribute, to various extents in different strains, to bifidobacterial recalcitrance for acquiring exogenous DNA: (1) the presence of a thick (multi-layered) and complex cell wall (Fischer, 1987); (2) intracellular restriction/modification barriers (Hartke *et al.*, 1996; O'Connell Motherway *et al.*, 2009; Schell *et al.*, 2002; Yasui *et al.*, 2009); and (3) sensitivity to environmental stresses (principally oxygen) during preparation of competent cells and transformation.

Full exploitation of genomic data requires the use of general and specialised vectors for gene overexpression, integration, knock-out and gene expression studies. Such molecular studies can substantiate the wide use of bifidobacteria as probiotic by explaining the molecular mechanisms governing the interaction with the host. In addition, bifidobacteria have recently been appointed as biotechnological agents for *in situ* production and delivery of therapeutic compounds, such as antigens (for live vaccine development) and tumour-suppressing substances (Cronin *et al.*, 2010; Fujimori, 2006; Xu *et al.*, 2007) and as a means of increasing beneficial detoxifying activities into the GIT (Park *et al.*, 2007). Traditional and new applications, therefore, require utilisation of robust genetic tools and improved genetic transformation techniques.

Genetic transformation by electroporation

The first scientific proof of genuine genetic transformation of *Bifidobacterium* dates back to 1994, when Missich *et al.* (1994) introduced by electroporation pRM2, a derivative of the small *B. longum* cryptic plasmid pMB1 (Sgorbati *et al.* 1982), into a cured *B. longum* strain that originally harboured the plasmid pMB1. The small theta replicating plasmid pMB1 represents, so far, the replicon most commonly used to construct *Bifidobacterium* vectors (Missich *et al.*, 1994). The protocols available for preparing electrocompetent cells and subsequent electroporation are based mainly on the comprehensive studies by Argnani *et al.* (1996) and Rossi *et al.* (1997), who considered and optimised several conditions such as growth medium, washing solutions, incubation temperatures and voltage.

Preparation of electrocompetent cells

The preparation of electrocompetent bacterial cells consists in weakening the cell wall and making the bacteria permeable to DNA during an electrical discharge while preserving their viability. The general strategies for achieving this goal comprise use of bacterial cells in the exponential growth phase, growth in presence of high sugar concentration, osmotic stabilisers in washing and electroporation buffers or maintaining cells at low temperature.

Growth phase. Since the composition of the cell wall plays a key role in DNA uptake, numerous studies have reported the importance of harvesting bacterial cells at a specific stage of their growth. Some studies showed that bifidobacteria could be effectively transformed only when they were in the early exponential phase ($OD_{600\ nm}$ 0.2-0.4) (Argnani *et al.*, 1996; Missich *et al.*, 1994; Rossi *et al.*, 1997), whereas efficiencies dropped for older cells, reaching zero for cells from the stationary growth phase (Rossi *et al.*, 1997). In contrast, other researchers have observed maximal transformation efficiency with cells in the middle to late log phase. For instance, Matsumura *et al.* (1997) found that with the vector pBLESS100 the transformation efficiency of *B. longum* 105-A was about one order of magnitude higher when cells were approaching the stationary phase as compared to early-log. Similarly, in more recent studies, midlogarithmic-phase cells were used (optical density at 600 nm 0.5 to 0.7) to effectively transform different *Bifidobacterium* species (Álvarez Martín *et al.*, 2008; Cronin *et al.*, 2007; MacConaill *et al.*, 2003; Sangrador-Vegas *et al.*, 2007).

Growth media. The addition to the growth medium of sugars in high concentrations is traditionally recognised as an effective strategy to improve the transformation yield by affecting the composition of the cell wall. Argnani *et al.* (1997) cultivated the cells in MRS broth supplemented with 0.5 M sucrose and washed them in a buffered sucrose solution at the same concentration. Rossi *et al.* (1996) showed a 100-fold increase in transformation efficiency when *Bifidobacterium* cells were grown in Iwata medium (IM) supplemented with raffinose 0.3 M or, especially, 16% Actilight®P (Marckolsheim, France) as compared with cells grown in IM broth with or without glucose. Actilight®P comprises of a mix of short-chain fructooligosaccharides (1-kestose, nystose, and fructosylnystose; FOS), which are metabolised by bifidobacteria and may protect cells from stress (Guglielmetti *et al.*, 2008a). Using this sugar product in a growth broth and washing buffer can thus improve transformation efficacy by preserving the cells' physiological condition during the preparation of competent bifidobacteria. This statement has recently been confirmed in a study, in which the use of 16% FOS or 10% galactooligosaccharides allowed the transformation of *B. bifidum* and *B. asteroides*, two bifidobacterial species known for their recalcitrance for acquiring exogenous DNA (Serafini *et al.*, 2012).

Electroporation buffers. Argnani *et al.* (1997) showed that a few-hour storage of bacterial cells before electroporation at 4 °C in an electroporation buffer composed of 0.5 M sucrose, 1 mM ammonium citrate, pH 6, significantly improved the transformation efficiency of bifidobacteria. They suggested that in the conditions they had established, the presence of low-molarity ammonium citrate (more than HEPES and phosphate buffers) as the osmotic stabiliser may support the right degree of cell autolysis without limiting cell viability, resulting in improved cell wall permeability for exogenous DNA. In addition, Rossi *et al.* (1997) showed the importance of the incubation step in the electroporation buffer at 0 °C overnight. Their electroporation buffer, named KMR, was composed of KH_2PO_4 5 mM, $MgCl_2$ 1 mM and raffinose 0.3 M, pH 4.8. However, the higher salt concentration of the KMR buffer may favour arcing events during the electrical discharge (S. Guglielmetti, unpublished data).

Efficiency of electrotransformation in bifidobacteria

In general, the rate of electroporation-mediated transformation in bifidobacteria is extremely low and constitutes the main limitation on successfully applying traditional genetic manipulation strategies to members of this genus. Wide variation in transformation efficiencies have been reported in the literature, ranging from about 10^0 to more than 10^6 transformants per µg of recombinant DNA (Table 2). Besides the protocols adopted for preparing competent cells and subsequent electroporation, considerable differences can be obtained depending on the strain under study (Table 2). Nonetheless, valuable progress has recently been made in improving transformation rates, thanks to studies on the restriction/modification systems of bifidobacteria, which have been shown to be the main obstacle in the acquisition of foreign DNA by these bacteria.

Optimisation strategies for the electro-transformation of bifidobacteria

To improve efficiency it is crucial to preserve cell viability during electroporation. One main reason for cell mortality during these experiments is oxidative stress. To overcome this problem, Park *et al.* (2003) added Oxyrase® (Mansfield, OH, USA), an enzyme system removing oxygen from its environment, to the incubation buffer after the electric pulse was given to the competent cells. This strategy allowed *B. longum* MG1 to transform at 100-fold improved electroporation efficiency.

In general, DNA introduced into bacteria by electroporation is more vulnerable to restriction nucleases than that transferred by conjugation or natural transformation.

Table 2. Summary of protocols for electrotransformation (electroporation) of bifidobacteria.

Vector (size, kb) (marker)[1]	Replicon(s)	Growth medium[2]	Washing buffer	Electroporation buffer	Preincubation[3]	Voltage and resistance	Recovering medium	Transformation rate (transformants/µg DNA)[4]	Reference
pDG7 (7.3 kb) (Cm^R)	pMB1-pBR322	MRS, 0.5 M sucrose, 0.05% cysteine-HCl ($OD_{600\ nm}$ 0.2)	0.5 M sucrose	1 mM ammonium citrate buffer pH 6, 0.5 M sucrose	3.5 h at 4 °C in electroporation buffer	12 kV/cm, 200 Ω	MRS, 0.5 M sucrose, 0.05% cysteine	*B. animalis* ATCC 27536 (5.0×10^3) *B. breve* 4 (1.3×10^4) *B. breve* AS (2.0×10^2) *B. bifidum* U3 (3.0×10^2) *B. bifidum* ATCC 15696 (7.4×10^3) *B. infantis* U1 (2.5×10^2) *B. infantis* ATCC 27920 (4.0×10^4) *B. longum* U2 (2.6×10^3) *B. longum* Wiesby 2 (7.0×10^4)	Argnani *et al.*, 1996
pNC7 (4.9 kb) (Cm^R)	pMB1 (non replicative in *E. coli*)	Iwata Medium (IM; Iwata and Morishita, 1989), 16% Actilight®P ($OD_{600\ nm}$ 0.2-0.3)	5 mM K-phosphate buffer pH 7	KMR buffer (5 mM KH_2PO_4, 1 mM $MgCl_2$, 0.3 M raffinose, pH 4.8)	Overnight at 0 °C in electroporation buffer	12.5 kV/cm, 200 Ω	IM, 16% Actilight®P	*B. animalis* ATCC 27536 (3.0×10^4) *B. breve* MB226 (6.6×10^4) *B. breve* MB252 (2.3×10^4) *B. bifidum* MB254 (7.2×10^4) *B. infantis* MB208 (1.2×10^5) *B. infantis* MB263 (9.3×10^3) *B. longum* MB231 (2.8×10^2) *B. pseudocatenulatum* MB264 (5.0×10^1) *B. ruminale* MB266 (7.2×10^2) *B. dentium* MB269 (3.6×10^1) *B. magnum* MB267 (1.8×10^3)	Rossi *et al.*, 1997 [5]
pRM2 (7.5 kb) (Sp^R)	pMB1-pBR322	TPY + glucose ($OD_{660\ nm}$ 0.6)	10% glycerol	10% glycerol	Freezing at -135 °C and storage at -70 °C	10 kV/cm, 200 Ω	TPY + glucose	*B. longum* B2577 (3.8×10^2)	Missich *et al.*, 1994
pBLES100 (9.1 kb) (Sp^R)	pTB6[6]-pBR322	Briggs Medium (Briggs, 1953) supplemented with 2% lactose instead of glucose (cells in middle to late log phase)	10% glycerol	10% glycerol	Freezing at -135 °C and storage at -70 °C	10 kV/cm, 200 Ω	Briggs Medium	*B. longum* 105-A (2.2×10^4)	Matsumura *et al.*, 1997
pBKJ50F (8.1 kb) (Cm^R)	pKJ50-pBR322	According to Argnani *et al.* (1996), with the only modification of pulse at 10 kV/cm.						*B. animalis* ATCC 27536 (2.0×10^2)	Park *et al.*, 1999
pBES2 (7.6 kb) (Cm^R)	pMG1[6]-pUC (ColE1)	According to Argnani *et al.* (1996), with the addition of Oxyrase® in the recovering medium.						*B. longum* MG1 (7.3×10^3)	Park *et al.*, 2003

Table 2. Continued.

Vector (size, kb) (marker)[1]	Replicon(s)	Growth medium[2]	Washing buffer	Electroporation buffer	Preincubation[3]	Voltage and resistance	Recovering medium	Transformation rate (transformants/µg DNA)[4]	Reference
pBRASTA101 (5.0 kb) (Sp^R)	pTB6[6]-pUC (ColE1)	According to Missich *et al.* (1994) and Matsumura *et al.* (1997).						*B. longum* 105-A (2.5×10^6)	Tanaka *et al.*, 2005
pFUN (8.1 kb) (Ery^R)	pAMβ1-(*E. faecalis*) pBluescript (ColE1)	IM, (Mid-log phase cells, $OD_{600\ nm}$ 0.5 to 0.7)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	-	10 kV/cm, 200 Ω	Iwata Medium	*B. breve* UCC2003 (10^2-10^3)	MacConaill *et al.*, 2003
pPKCm1 (6.2 kb) (Cm^R)	pCIBA089-pBluescript (ColE1)	IM, (Mid-log phase cells, $OD_{600\ nm}$ 0.5 to 0.7)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	-	10 kV/cm, 200 Ω	Iwata Medium	*B. breve* UCC2003 (3.8×10^6) *B. animalis* subsp. *lactis* (10^1) *B. longum* NCIMB8809 (10^2) *B. pseudolongum* NCIMB2244 (10^2) *B. globosum* JCM5820 (10^3) *B. pseudocatenulatum* LMG10505 (10^3) *B. dentium* NCFB2843 (10^4)	Cronin *et al.*, 2007
pASV480 (9.0 kb) (Cm^R)	pASV479-pBluescript (ColE1)	IM, (Mid-log phase cells, $OD_{600\ nm}$ 0.5 to 0.7)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	-	10 kV/cm, 200 Ω	Reinforced Clostridial Medium (RCM)	*B. breve* NCIMB 8807 ($\sim10^5$) *B. breve* NCFB 2258 ($\sim10^5$)	Sangrador Vegas *et al.*, 2007
pAM4 (7.6 kb) (Tet^R)	pBC1-pUC	MRS, 0.05% cysteine (Mid-log phase cells, $OD_{600\ nm}$ 0.5 to 0.7)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	0.5 M sucrose, 1 mM citrate buffer (pH 5.8)	20 min in ice	10 kV/cm, 200 Ω	Reinforced Clostridial Medium (RCM)	*B. adolescentis* LMG10502 (9.2×10^2) *B. animalis* LMG10508 (4.0×10^1) *B. animalis* subsp. *lactis* Bb12 (1.6×10^2) *B. breve* LMG13208 (1.0×10^2) *B. breve* UCC2003 (1.4×10^2) *B. dentium* F101 (9.5×10^1) *B. longum* L25 (6.6×10^1) *B. pseudolongum* LMG11571 (6.3×10^1) *B. pseudocatenulatum* M115 (1.0×10^5) *B. thermophilus* LMG11571 (4.6×10^1) *C. glutamicum* LMG19741 (3.0×10^0)	Álvarez-Martín *et al.*, 2008

[1] Cm^R: chloramphenicol acetyl transferase (*cat*); Tet^R: tetracycline resistance [*tet*(W)]; Sp^R: spectinomycin resistance; Ery^R: erythromycin resistance.

[2] Phase of growth at which bifidobacterial cells are collected before washing steps.

[3] Incubation step of competent cells before electroporation or storing at -80 °C.

[4] *B. animalis* ATCC 27536 is also known as *B. animalis* MB209; *B. infantis* stands for *Bifidobacterium longum* subsp. *infantis*.

[5] Transformation experiments were performed with vector DNA isolated from *B. animalis* MB209.

[6] pTB46 and pMG1 plasmids are isogenic to pB44, pNAC2, pBLO1, pDOJ10L, and pKJ36.

This is particularly important for bifidobacteria, whose perhaps most immediate obstacle to acquiring exogenous DNA are their restriction/modification (R-M) systems. DNA R-M gene clusters coding for methyltransferases and restriction enzymes can be recognised in all sequenced bifidobacterial genomes and to date several proven or potential R-M systems belonging to either Type I, II, and IV have already been identified (Hartke *et al.*, 1996; Lee *et al.*, 2008; O'Connell-Motherway *et al.*, 2009; Roberts, 1980; Schell *et al.*, 2002; Yasui *et al.*, 2009; http://rebase.neb.com/rebase). Based on the above, several studies have reported a significant increase in transformation efficiency when transformed DNA has been isolated from *Bifidobacterium* instead of *E. coli* (O'Connell-Motherway *et al.*, 2009; Rossi *et al.*, 1997, 1998; Yasui *et al.*, 2009). For instance, Rossi *et al.* (1998) found that only vector DNA prepared from bifidobacteria could successfully transform some strains of *B. longum*, *B. animalis* and *B. bifidum*. Therefore, proper modification of plasmid DNA can help to bypass the restriction barriers and favour the acquisition of foreign recombinant DNA by bifidobacteria. This assumption has been verified by a recent study, in which site-directed mutagenesis and *in vitro* methylation were applied to remove or modify restriction sites from a vector pYBamy59 before electrotransformation into *B. longum* MG1 (Kim *et al.*, 2010). In this study, sequence analysis of pYBamy59 fragments originated by incubation of recombinant DNA with cell extracts of MG1 revealed the presence of a *Sac*II-like endonuclease activity, recognising the palindromic sequence 5'-CCGCGG-3'. When pYBamy59 from *E. coli* was methylated *in vitro* by CpG or GpC methyltransferases or when *Sac*II sites were removed from pYBamy59 through site-directed mutagenesis, the transformation efficiency showed 8- to 15-fold increment as compared to the original plasmid (Kim *et al.*, 2010).

Another strategy to modify recombinant DNA before introduction into bifidobacterial cells was recently adopted in two independent studies (O'Connell-Motherway *et al.*, 2009; Yasui *et al.*, 2009) with the aim to boost the transformation efficiency of *B. adolescentis* ATCC 15703 and *B. breve* UCC2003. In this strategy, a shuttle vector was pre-methylated in *E. coli* cells carrying the genes encoding the DNA modification enzymes of the target *Bifidobacterium* before electroporation (Figure 3). In fact, Yasui *et al.* (2009) developed a system called 'Plasmid Artificial Modification' (PAM) and demonstrated its efficacy for the target host *B. adolescentis* ATCC 15703, a strain that could be transformed only at an extremely low level. In the ATCC 15703 genome, they identified two Type II DNA methyltransferase genes, BAD_1233 and BAD_1383, which they cloned in *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) by means of a low copy number vector, obtaining the so-called 'PAM host.' The *E. coli* TOP10 laboratory strain was shown to be the most suitable because it lacks the Type IV restriction enzymes *mrr* and *mcrBC*, which degrade DNA methylated by the R-M system of other bacteria, and the methylases *dam*, *dcm*, and *hsdMS*, which can make the DNA sensitive to possible Type IV restriction systems. Subsequently, an *E. coli-Bifidobacterium* shuttle vector based on the pTB6 *B. longum* replicon (Tanaka *et al.*, 2005) was introduced into the PAM host. Transformation efficiency improved considerably when *B. adolescentis* was electroporated with the shuttle vector DNA isolated from the PAM host, jumping from $1\text{-}3\times10^0$ to 10^5 transformants/μg. This confirms that the shuttle vector was methylated by the modification enzyme encoded by the PAM plasmid in the *E. coli* host and consequently protected against restriction by *B. adolescentis* (Figure 3).

The same approach was adopted by O'Connell-Motherway *et al.* (2009) with *B. breve* UCC2003 as the target host. In the annotated genome of this strain, they found three different R-M gene clusters, including the methylase genes *bbrIM*, *bbrIIM* and *bbrIIIM*. The role of these modification genes in the acquisition of exogenous DNA by *B. breve* was studied in transformation experiments with pAM5-derived vectors (based on the pBC1 replicon from *B. catenulatum*; Álvarez-Martín *et al.*, 2007b). The authors observed a 1000-, 10-, and 5-fold higher transformation frequency for pAM5 DNA isolated from *E. coli* expressing M.BbrIII, M.BbrII, and M.BbrI, respectively, which indicates that, although differently, all three DNA methylation systems affected the transformation efficiency.

The above studies demonstrated the usefulness of artificially modified DNA by means of the host methylases to increase the electroporation efficiency. Genome analyses and experimental data, however, have shown that *Bifidobacterium* strains harbour a very diverse range of R-M activities, even within the same species (O'Connell-Motherway *et al.*, 2009). Therefore, this strategy is at least partly limited to the strains whose whole-genome sequence is known. To overcome this problem, O'Connell-Motherway *et al.* (2009) proposed the possibility of methylating exogenous DNA isolated from *E. coli in vitro,* through incubation of the DNA with cell extracts of the target host in the presence of S-adenosylmethionine. However, experimental data of the practicability and effectiveness of this strategy are not yet available.

Conjugation in bifidobacteria

The R-M barriers of a bacterial strain can also be bypassed by introducing foreign DNA through conjugation. Conjugation may occur when *cis* (*oriT*) and *trans* (transfer proteins, Mob and Tra) elements found in mobilising plasmids are recognised by cellular components, which can be supplied by the host cell. Putative Mob and Tra protein coding genes and characteristic *cis* elements have

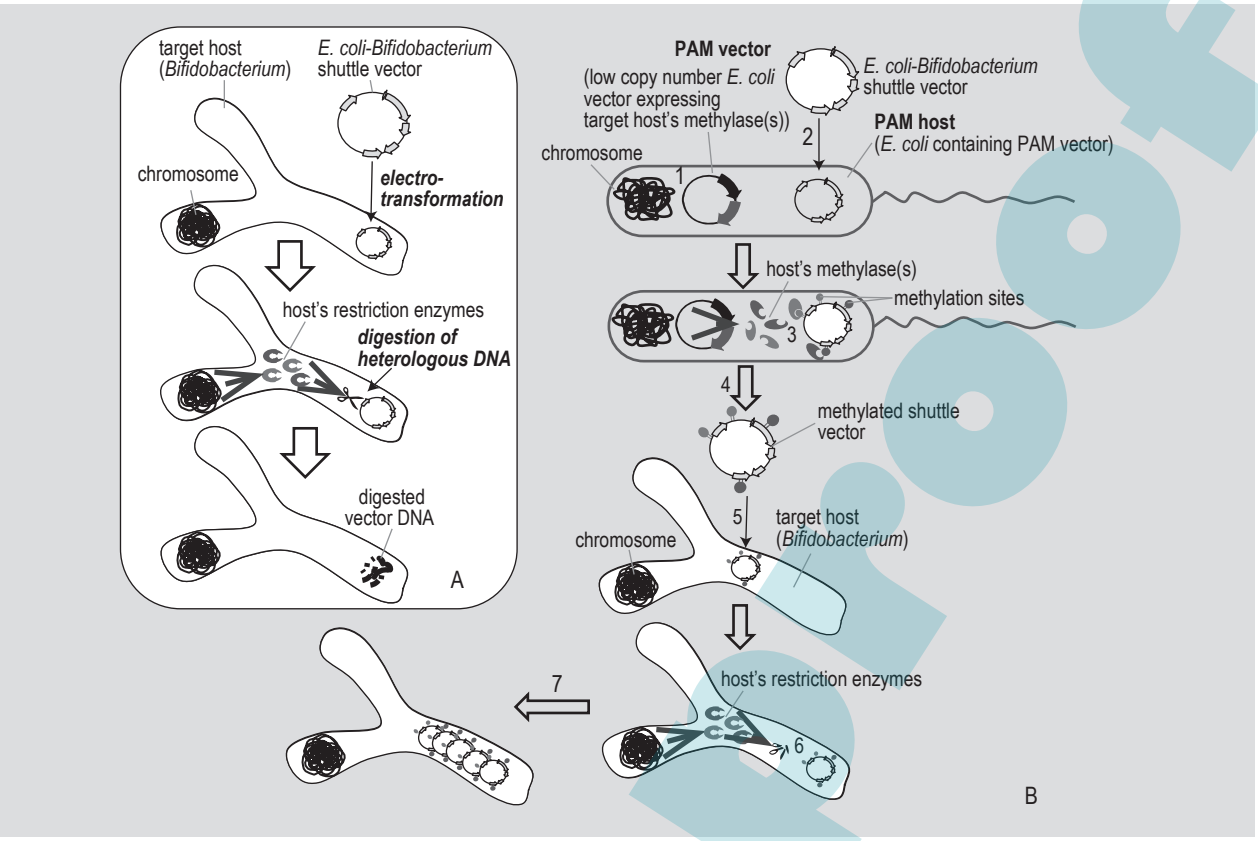

Figure 3. Strategies for the preparation of vector DNA to introduce by electroporation in bifidobacteria. (A) Conventional strategy involving the extraction of shuttle vector DNA from *Escherichia coli* and direct introduction in *Bifidobacterium* cells. (B) Plasmid Artificial Modification (PAM) strategy: (1) preparation of the PAM host consisting of an appropriate *E. coli* strain (e.g. *E. coli* TOP10) harbouring PAM vector (a low copy number vector coding for the methylase(s) of the target host); (2) introduction of an *E. coli – Bifidobacterium* shuttle vector into the PAM host; (3) modification of shuttle vector DNA by methylase(s) coded by PAM vector; (4) extraction of the now methylated vector and (5) introduction into the target host (*Bifidobacterium*) by electroporation; (6) target host restriction system(s) cannot digest the methylated shuttle vector; (7) shuttle vector replicates inside target host.

been found in several bifidobacterial plasmids (Corneau *et al.*, 2004; Gibbs *et al.*, 2006; O'Riordan and Fitzgeralds, 1999; Shkoporov *et al.*, 2008a; Table 1), suggesting their mobilisation potential. Nevertheless, up to now, no DNA transfer system based on conjugation is available for members of the genus *Bifidobacterium* and no conjugation events have been irrefutably demonstrated. The only documented systematic attempt to achieve conjugation in bifidobacteria was made by Shkoporov *et al.* (2008a), who exploited the mobilisation functions of three different *Bifidobacterium* plasmids to develop genetic tools based on the well-characterised intergeneric conjugative element RP4 (IncPα) (Simon *et al.*, 1983) to transfer DNA into *B. pseudocatenulatum*. They produced antibiotic-resistant clones that, though PCR-positive, did not contain the expected plasmid DNA. Consequently, development of effective conjugation systems for bifidobacteria, albeit potentially useful, remains in its infancy.

Expression of heterologous genes among bifidobacteria

The development of heterologous expression and secretion systems is strategically important for studying the properties of bifidobacteria, because of strain improvement and delivery into the human digestive tract of useful gene products such as vaccines or anticarcinogenic polypeptides. Information on regulated promoters, inducers and repressors is extremely limited in bifidobacteria (Ruiz *et al.*, 2012a). Additional efforts are, therefore, needed to identify strong, weak and regulated promoters for controlled gene expression in bifidobacteria under different environmental conditions.

Several antibiotic resistance genes, used in preparing cloning and expression vectors, have been shown to be functional in bifidobacteria even under the control of their own regulatory elements. These include, for instance, the chloramphenicol acetyl transferase (*cat*) of the plasmid pC194, the erythromycin resistance gene of pE194 from *Staphylococcus aureus* and the spectinomycin

resistence gene from *Enterococcus faecalis*. In contrast, the thiostrepton resistance gene from *Streptomyces* is not functional in bifidobacteria (Guglielmetti *et al.*, 2007b; Rossi *et al.*, 1998). Similarly, the genes coding for *Pseudomonas fluorescens* lipase, *Bacillus licheniformis* α-amylase and *Streptomyces* sp. cholesterol oxidase are stably maintained in the bifidobacterial host, but under the control of their own promoters they are not expressed (Rossi *et al.*, 1998).

Heterologous genes from a diverse group of organisms have already been expressed in bifidobacteria under the control of either homologous or heterologous or promoters (Table 3). The current list includes genes and promoters mainly from Gram-negative and Gram-positive bacteria, but with exceptions, as the luciferase gene from *Pyrophorus plagiophthalamus* (Guglielmetti *et al.*, 2008a) and several human genes (Xu *et al.*, 2007). In addition to gene expression, excretion and secretion processes should be studied to develop efficient export systems for heterologous proteins or enzymes. In this context, MacConaill *et al.* (2003) investigated protein export in *B. breve* UCC2003 by means of the export-specific vector pFUN, based on the use of the staphylococcal nuclease (Nuc) as a reporter enzyme (Poquet *et al.*, 1998). Due to the removal of its native signal peptide, the Nuc reporter protein is translocation-competent but unable to direct its own secretion. In this study, translational fusions were constructed with a *nuc* gene and the export signal provided by inserted *B. breve* chromosomal DNA fragments. By this strategy, seven signal peptides have been identified for *B. breve* UCC2003 (MacConaill *et al.*, 2003).

Recently, a secretion system has also been developed based on the α-amylase expression and secretion signals isolated from *B. adolescentis* INT57 (Park *et al.*, 2005b; Rhim *et al.*, 2006). Park and collaborators constructed a secretion vector, pBESAF2, containing the promoter and the signal peptide of the α-amylase gene *amyB*. The gene encoding an intracellular phytase from *E. coli* was introduced in this vector and transcriptionally fused to the signal sequence and finally introduced by electroporation in *B. longum* MG1. The authors demonstrated that, by using this system, phytase enzyme was successfully expressed and secreted by *B. longum* into the culture broth. Furthermore, this system was employed for expression and secretion by *B. longum* MB1 of the bacteriocin pediocin PA-1 from *Pediococcus acidilactici* K10 (Moon *et al.*, 2005).

Analogously, Deng *et al.* (2009) selected through computational analysis the signal peptide sequence from the endo-1,5-α-L-arabinosidase gene of *B. longum* NCC2705, by which they obtain expression and secretion of the human interferon-α2b protein (IFN-α2b) in *B. longum* ATCC 15707. This study showed that 65% of the total IFN-α2b was secreted from *B. longum* in the presence of the arabinosidase signal peptide, while only 15% of the protein was secreted without the signal peptide. Surprisingly, this experimentat was carried out without a conventional *E. coli-Bifidobacterium* shuttle plasmid, but by means of the commercial pBR322-based vector pBAD-gIIIA (Invitrogen), in which expression of the recombinant protein is arabinose-inducible by the presence of the promoter of the arabinose operon from *E. coli*. The maximal level of induction was obtained after addition of 0.2% arabinose (Deng *et al.*, 2009). Finally, Long *et al.* (2010) demonstrated that the exo-xylanase signal peptide sequence from *B. longum* was suitable to guide secretion of the mature peptide of the human gut hormone oxyntomodulin.

Reporter gene systems

Some reporter gene systems have already been developed and shown useful for several applications. To study promoter strength and regulation analysis or to identify genomic fragments containing active promoters, Klijn *et al.* (2006) developed the reporter vector pMDY23 based on the *E. coli* β-glucuronidase gene *gusA* and the small cryptic *B. longum* plasmid pNCC293. After introducing the vector in *B. longum* NCC2705, they demonstrated the suitability of pMDY23 as a reporter plasmid for promoter study by analysing the promoter activity of three DNA fragments (Klijn *et al.*, 2006; Table 3). More recently, two studies showed the application of bioluminescence reporter genes in *Bifidobacterium* spp. (Cronin *et al.*, 2008; Guglielmetti *et al.*, 2008a). Guglielmetti *et al.* (2008a) transformed by electroporation the human intestinal strain *B. longum* subsp. *longum* NCC2705 with a vector (pGBL8b) containing the insect luciferase gene *lucGR* from a click beetle (*P. plagiophthalamus*). The same vector, however, was incapable of transforming *B. animalis* subsp. *lactis* BB12 and *B. bifidum* MIMBb75 (Guglielmetti *et al.*, 2008a; S. Guglielmetti, unpublished results). The resulting bioluminescent *B. longum* was used to analyse variations in intracellular ATP concentration at acidic pH in the presence of different sugars, a technique proving to be a valuable tool for the rapid and sensitive study of the physiological state of bacterial cells under different environmental conditions. Nonetheless, the need to add exogenous D-luciferin as a substrate in this reporter system limits considerably its *in vivo* application.

The bacterial luciferase system (coded by the *luxABCDE* operon) is generally less sensitive than insect luciferases, yet bacterial luciferase requires as substrate a long-chain fatty aldehyde, which is intracellularly synthesised by a fatty acid reductase complex encoded by *luxCDE*. Therefore, the intracellular expression of the *lux* operon circumvents the disadvantage of exogenous addition of luciferin and is thus more suitable for *in vivo* applications. Cronin *et al.* (2008) adopted the *lux* operon to develop the non-invasive luciferase reporter vector pLuxMC1, which was introduced in *B. breve* UCC2003. Once mice were orally inoculated

Table 3. Expression of heterologous genes in bifidobacteria.

Protein (gene)	Origin	Expression host	Promoter	Reference(s)
Cytosine deaminase	*E. coli*	*B. longum* 105-A	Promoter of *hup* gene, coding for the histone like protein of *B. longum*	Nakamura *et al.*, 2002
Secreted nuclease (*nuc*)	*Staphylococcus aureus*	*B. breve* UCC2003	Seven different promoters from *B. breve* UCC2003	MacConaill *et al.*, 2003
Endostatin (Liver cDNA); TNF-related apoptosis-inducing ligand (TRAIL)	Human	*B. adolescentis*; *B. longum*	Coliphage lambda P_RP_L promoter regions	Fu *et al.*, 2005; Hu *et al.*, 2009; Li *et al.*, 2003; Xu *et al.*, 2007
Phytase (*appA*) fused with the signal sequence of the *amyB* gene from *B. adolescentis* Int-57	*E. coli* MC4100	*B. longum* MG1	Promoter of the *amyB* gene from *B. adolescentis* Int-57	Park *et al.*, 2005b
Green fluorescent protein (*gfp*)	Vector pEGFP (Clontech, USA)	*B. longum* MG1	Expressed with two promoters from *Bifidobacterium* spp. GE65 (sequence analysis revealed similarity with *Lactobacillus johnsonii*)	Ji *et al.*, 2005
Glutamate decarboxylase	Rice	*B. longum* MG1	Not known	Park *et al.*, 2005a
Flagellin (*fliC*)	*Salmonella* Typhimurium ATCC14028	*B. animalis* ATCC27536	Promoter of *hup* gene from *B. longum*	Takata *et al.*, 2006; Yamamoto *et al.*, 2010
β-glucuronidase (*gusA*)	*E. coli*	*B. longum* NCC2705	Putative promoters of genes BL1363, BL1613 and BL1518 from *B. longum* NCC2705	Klijn *et al.*, 2006
PTEN tumor suppressor	Human	*B. longum* L17	Promoter region of *hup* gene from *B. longum*	Hou *et al.*, 2006
Interleukin-10 (rhIL-10)	Human	*B. longum* ATCC15707	Promoter and terminator sequences from *hup* gene from *B. longum* NCC2705	Reyes Escogido *et al.*, 2007
Interleukin-10 (hIL-10)	Human	*B. breve* UCC2003	Promoters of *hup* and *gap* genes from *B. longum*	Khokhlova *et al.*, 2010
β-glucuronidase (*gusA*)	*E. coli* (from pNZ272)	*B. breve* NCIMB 8807	rRNA gene promoter from *B. breve* 8807	Sangrador-Vegas *et al.*, 2007
Luciferase (*lucGR*)	*Pyrophorus plagiophthalamus*	*B. longum* NCC2705	Promoter from phage T5	Guglielmetti *et al.*, 2008
Cholesterol oxidase (*choPA* operon)	*Streptomyces* spp.	*B. longum* MG1	16S rRNA gene promoter from *B. longum* MG1	Park *et al.*, 2008
α-l-arabinofuranosidase (*abfB*)	*B. longum* B667	*B. pseudocatenulatum* M115	Native	Álvarez-Martín *et al.*, 2008
Bacterial luciferase (*luxABCDE* operon)	*Photorhabdus luminescens*	*B. breve* UCC2003	*repC* promoter from *B. catenulatum* plasmid pBC1 and promoter P*help* from *Listeria monocytogenes*	Cronin *et al.*, 2008
Bile resistance mechanism BilE (*bilE* operon)	*L. monocytogenes* EGD-e	*B. breve* UCC2003	Native	Watson *et al.*, 2008
Synthetic human fibroblast growth factor (FGF-2) fused with signal peptide of Sec2 from *B. breve* UCC2003	pkFGFB	*B. breve* UCC2003	Promoter and terminator regions of *hup* gene from *B. longum* VMKB44 Promoter/TIR of *B. longum* VMKB44 gene *gap*	Shkoporov *et al.*, 2008b
Interferon-α2b	Human	*B. longum* ATCC 15707	*E. coli araBAD* promoter from commercial vector pBAD-gIIIA	Deng *et al.*, 2009; Yu *et al.*, 2010, 2011
Thymidine kinase	*Herpes simplex*	*B. infantis*	*tac* promoter of commercial vector pGEX-5X-1	Tang *et al.*, 2009
Granulocyte colony-stimulating factor (GCSF)	Human	*B. longum*	Coliphage lambda P_RP_L promoter regions	Zhu *et al.*, 2009
Oxyntomodulin (OXM)	Human	*B. longum* NCC2705	*E. coli araBAD* promoter	Long *et al.*, 2010
Interleukin-12 (mIL-12)	Mouse	*B. longum* NCC2705	*E. coli araBAD* promoter from commercial vector pBAD-gIIIA	Yu *et al.*, 2012

with bioluminescent *B. breve*, the reporter system allowed a real-time tracking of the colonisation and persistence of this probiotic strain *in vivo*.

Drug delivery systems

Due to their safety and ability to colonise specific areas of the human GIT, bifidobacteria may turn out to be optimal vectors for *in situ* delivery of biologically active substances. Of particular interest is the fact that certain anaerobic bacteria, including species of *Clostridium* and *Bifidobacterium*, can selectively germinate and grow in the hypoxic regions of solid tumours (Malmgren and Flanigan, 1955; Kimura *et al.*, 1980; Yazawa *et al.*, 2000, 2001), such as those of most primary breast and uterine cervix cancers. This fact was exploited by the Japanese research team of Prof. Fujimori, who developed a strategy called '*Bifidobacterial Selective Targeting*' (BEST) (Fujimori, 2006). The Fujimori team's BEST therapy involved the strain *B. longum* 105-A that was genetically engineered via electro-transformation with pBLES100-S-eCD, a plasmid based on the shuttle vector pBLES100 (Matsumura *et al.*, 1997) and comprising the cytosine deaminase gene (CD) under the *hup* gene promoter of *B. longum* (Nakamura *et al.*, 2002; Table 3) coding for a histone-like DNA-compacting protein. The CD enzyme converts the non-toxic prodrug 5-fluorocytosine (5-FC) to chemotherapeutic 5-fluorouracil (5-FU), which is systemically administrated to treat solid tumours. In conventional therapy, its clinical effectiveness is very limited by its high systemic toxicity, particularly toward the bone marrow. Fujimori's studies demonstrated that recombinant *B. longum* selectively produced CD in mammary tumour tissues in rats and that CD could convert 5-FC into 5-FU *in vivo* both after intratumoural injection and also by systemic administration (Sasaki *et al.*, 2006). Furthermore, no adverse effects were observed in animal models during the use of *B. longum* as a gene delivery vector (Sasaki *et al.*, 2006), a finding supporting the potential effectiveness of this novel approach for cancer gene therapy in humans. More recently, an improved version of pBLES100-S-eCD able to display 10-fold increased CD activity in *B. longum* has been developed (Hamaji *et al.*, 2007; Hidaka *et al.*, 2007). These authors demonstrated that the BEST approach works well even with a different bifidobacterial species such as *B. breve* I-53-8w.

Bifidobacteria have been used as a gene delivery vehicle of CD also by Chinese researchers, who expressed the CD gene in *B. longum* subsp. *infantis* by means of the vector pGEX-1λT. The recombinant bacterium was then used to inhibit melanoma in mice (Yi *et al.*, 2005). As for the Invitrogen vector pBAD-gIIIA mentioned above, the functionality of the vector pGEX-1λT is surprising, because it carries no bifidobacterial replicon but only the pBR322 *ori* region. Moreover, Yi *et al.* (2005) claimed that the recombinant bifidobacteria were selected with ampicillin through the β-lactamase gene encoded by pGEX-1λT, an antibiotic marker generally considered not to be active in Gram-positive bacteria. The same research team used this approach to clone the *Herpes simplex* virus-thymidine kinase in *B. longum* subsp. *infantis*. In this system, the thymidine kinase expressed specifically in tumour tissues by bacterial cells can convert the non-toxic precursor ganciclovir into the ganciclovir-3-phosphate, a toxic substance that kills the tumoural cells. The efficacy of this gene therapy system was demonstrated *in vivo* in a rat model of bladder cancer. After tail vein injection of 4.4×10^9 recombinant *Bifidobacterium* cells with a concomitant daily intraperitoneal injection of Ganciclovir, rat bladder tumour growth was inhibited on the 15th day after the beginning of the treatment (Tang *et al.*, 2009).

The BEST strategy was adopted also by Prof. Xu's research team, who employed *B. adolescentis* and *B. longum* as gene delivery vectors to transport the anti-angiogenic factor endostatin into a hypoxic solid liver tumour in mice (Fu *et al.*, 2005; Li *et al.*, 2003; Xu *et al.*, 2007). Originally, they claimed that expression of the human liver endostatin gene was achieved in *Bifidobacterium* spp. by means of the expression plasmid pBV220. The vector pBV220 (Zhang *et al.*, 1990) is a derivative of pBR322 and contains P_RP_L promoters of the λ bacteriophage, the *cI857ts* gene encoding the temperature-sensitive λ repressor and two strong transcriptional terminators (rrnBT1T2) of *E. coli*. This vector only contains the origin of replication of pBR322 and a unique antibiotic selection for ampicillin (β-lactamase gene). As for the vectors pBAD-gIIIA and pGEX-1λT, it is therefore once again unexpected that pBV220 was found functional in bifidobacteria. The same authors reported, however, that this construct was highly unstable in *B. longum* (Xu *et al.*, 2007). Therefore, it was modified by introducing the pMB1 *Bifidobacterium* replicon (Rossi *et al.*, 1996) and a chloramphenicol resistance gene. The resulting vector, called pBV22210, was much more stably maintained in *B. longum* than pBV220 (Xu *et al.*, 2007). Very recently, the plasmid pBV22210 has also been used in *B. longum* as an expression vehicle of the extracellular domain of TNF-related apoptosis-inducing ligand (TRAIL). The resulting recombinant strain was shown to have a specific anti-tumour effect on mouse osteosarcoma (Hu *et al.*, 2009). In a following study, vector pBV22210 was also used to express in *B. longum* the granulocyte colony-stimulating factor (G-CSF), a molecule frequently used as a co-adjuvant agent in tumour chemotherapy. When *B. longum*-pBV22210-GCSF was applied to treat H22 and S180 sarcoma-bearing mice, it was observed to have an effective antagonistic effect on bone marrow inhibited by cyclophosphamide and an over 65% inhibition of tumour growth (Zhu *et al.*, 2009). Finally, the BEST strategy was adopted by Hou and colleagues (2006), who observed a significant inhibition of the growth of solid tumours in knock-out mice lacking the phosphatase and tensin (PTEN)

homologue with a genetically engineered *B. longum*, transformed with a pMB1-derived vector expressing the PTEN tumour suppressor gene under the *hup* gene promoter from *B. longum*.

Other gene delivery systems have recently been developed in bifidobacteria. For instance, Takata *et al.* (2006) have developed a vaccine delivery system based on *B. animalis* ATCC 27536 genetically modified through transformation with the vector pBLES100, in which they cloned the flagellin gene *fliC* of *Salmonella* Typhimurium ATCC 14028 under the *B. longum hup* gene promoter. Significantly higher levels of flagellin-specific IgA in the serum and stools of mice treated by oral administration of this recombinant *B. animalis* were detected than in those treated with parental *B. animalis*. In a recent study, these authors investigated the potential effectiveness of genetically modified bifidobacteria as oral vaccines by protecting mice from a lethal challenge with *Salmonella* in a typhoid fever model (Yamamoto *et al.*, 2010). Another pioneering application of bifidobacteria as a drug delivery system was that of Long *et al.* (2010), who developed engineered bifidobacteria as oral carriers of oxyntomodulin, a gut hormone that is used to reduce food intake and body weight through intravenous administration. Interestingly, the results of this study showed that oxyntomodulin-transformed *B. longum* reduced food intake, body weight and decreased blood lipid in overweight mice. The benefits were identical to those obtained by oral administration of Orlistat, a gastrointestinal lipase inhibitor drug employed in obesity therapy (Long *et al.*, 2010).

As suggested by recent studies, bifidobacteria may also be used for the *in situ* delivery of human cytokines. The anti-inflammatory interleukin (IL)-10 has been expressed in its mature form by *B. longum* ATCC 15707 (Reyes Escogido *et al.*, 2007; Table 3) and *B. breve* UCC2003 (Khokhlova *et al.*, 2010). In the latter study, the gene coding for the mature form of human IL-10 was translationally fused to previously described *Bifidobacterium* signal peptides and placed under the control of bifidobacterial constitutive promoters. Specifically, a pB80 replicon-based shuttle vector carrying active promoter and terminator regions of *B. longum* gene *hup* was used to clone gene IL-10, which was fused with the signal peptide regions of genes *sec2*, *apuB* or *amyB* coding for *B. breve* secreted protein and extracellular amylopullulanase and *B. adolescentis* secreted alfa-amylase. Sec2 signal peptide was also placed under the control of constitutive promoter/terminator regions from *B. longum* gene *gap*, coding for enzyme glyceraldehyde-3-phosphate dehydrogenase. Interestingly, RT-qPCR experiments demonstrated that the expression level of IL-10 driven by *gap* promoter was higher than that under the control of *hup* promoter. Moreover, substitution of the Sec2 signal peptide-coding region with the signal sequence from *amyB* gene resulted in an intensely elevated level of IL-10 mRNA (Khokhlova *et al.*, 2010).

Knock-out of bifidobacterial genes

The prime method for studying the function of a gene and its impact on the overall cellular physiology and morphology consists in removing or disrupting the gene from its host (gene knock-out). This is generally accomplished by means of modification systems based on homologous recombination. A low occurrence of homologous recombination has been reported in bifidobacteria, which agrees well with the absence of the major prokaryotic DNA recombination pathway encoded by *recBCD* in the genome of some strains, such as *B. longum* NCC2705 (Schell *et al.*, 2002). As discussed previously, bifidobacteria display relatively low transformation efficiency. Evidently, the recombination frequency in *Bifidobacterium* is thus generally lower than that of transformation, limiting effective chromosomal integration of DNA. For these reasons, successful homologous recombination in bifidobacteria has only recently been reported.

In 2008, successful knockouts of *B. breve* UCC2003 genes by single-crossover recombination were published, employing two different strategies (O'Connel-Motherway *et al.*, 2008). First, O'Connel-Motherway and co-workers adopted a double-vector integration strategy to disrupt the amylopullulanase gene *apuB*. The first vector contained the origin of replication but lacked the *rep* gene coding for its replication protein (Ori+/Rep- vector). The second vector, bearing a different antibiotic resistance, harbours the *rep* gene of the former plasmid (Rep+ vector). An internal 1 kb DNA region of the *apuB* gene was cloned in the Ori+/Rep- plasmid and both vectors (Ori+/Rep- and Rep+) were then introduced in *B. breve* UCC2003 under double antibiotic selection. In such recombinant cells, the Ori+/Rep- plasmid could replicate only in the presence of the other vector, which supplied the Rep protein *in trans*. Once the recombinant *B. breve* was cultivated at a high temperature (42 °C) and with selection only for the Ori+/Rep- vector, the Rep+ plasmid was lost from *B. breve* cells due to its temperature sensitivity and segregational instability. Accordingly, under selective conditions, the Ori+/Rep- plasmid has to integrate into the chromosome. Thanks to this approach, the authors found by replica plaiting some *B. breve apuB* disruption isolates, which exhibited the expected phenotype and in which they verified plasmid integration by PCR and Southern hybridisation. However, O'Connell-Motherway *et al.* (2008) emphasised that this system is very tedious, time-consuming and unreliable.

In a later publication, the same researchers described insertional mutagenesis of the *apuB* gene and the endogalactanase gene (*galA*) of *B. breve* UCC2003 by means of plasmid methylation (O'Connell-Motherway *et al.*, 2009).

This strategy produced gene disruptions by single-crossover chromosomal integration of non-replicative plasmids containing internal fragments of 476 and 744 bp of the *gal*A gene, and a 939 bp internal fragment of the *apuB* gene. These three plasmids were first introduced into an *E. coli* host harbouring two *B. breve* UCC2003 methylase genes (*bbrIIM* and *bbrIIIM*). The resulting methylated plasmids were then introduced into *B. breve* UCC2003 by electroporation. Antibiotic-resistant transformants were isolated for all methylated vectors at a frequency of up to 50 per µg of transformed DNA. No transformants were obtained when unmethylated constructs were introduced into strain UCC2003. The expected integration in the chromosome was finally verified by genetic and phenotypic analyses. These results by O'Connell-Motherway *et al.* (2009) showed that methylation of the non-replicating plasmid by *B. breve* UCC2003 methylases increased transformation efficiency to a level sufficiently high to allow site-specific homologous recombination to occur. This strategy has allowed the dissection of many genes from the UCC2003 strain, including gene components of clusters involved in utilisation of ribose (Pokusaeva *et al.*, 2010), insoluble cellulose (cellodextrin) (Pokusaeva *et al.*, 2011) and galactans (O'Connell Motherway *et al.*, 2011b). More recently, the expression of the serpin protease inhibitor in *B. breve* UCC2003 has been shown to be autoregulated under the control of a two-component regulatory system (2-CRS), as reported by Dr. Van Sinderen's group (Álvarez-Martín *et al.*, 2012a). Furthermore, another 2-CRS controlling response to inorganic phosphate (P_i) starvation in this strain has been identified and characterised at a molecular level (Álvarez-Martín *et al.*, 2012b).

Also recently, Arigoni and Delley (2008) patented a gene deletion method in *Bifidobacterium* by two consecutive events of homologous recombination. The authors reported the deletion of the tetracycline resistance gene *tet*(W) from *B. animalis* subsp. *lactis* NCC2818 (commercially known as strain BB12). The *tet*(W) gene of BB12 is on the chromosome, adjacent to a transposase gene (genes BIF_01560 and BIF_02030; Garrigues *et al.*, 2010). Analysis of its flanking DNA sequences, however, strongly suggests that the *tet*(W) gene is not contained in a functional mobile element. In spite of this, their method comprised the following steps. DNA fragments of approximately 3 kb flanking the *tet*(W) gene were amplified and joined via the start and stop codon of *tet*(W). The resultant 6 kb DNA fragment was cloned in pJL74 (LeDeaux and Grossman, 1995), a spectinomycin resistance vector unable to replicate in bifidobacteria; the resulting plasmid was introduced in *B. animalis* subsp. *lactis* NCC2818. Spectinomycin-resistant ($spec^R$) colonies were shown to harbour the plasmid integrated into the *B. animalis* chromosome via a single cross-over event. $Spec^R$ transformants were then cultivated for about 100 generations without antibiotic selection to promote loss of the plasmid. Spectinomycin-sensitive ($spec^S$) colonies were then selected by replica plating on MRS agar with or without added spectinomycin. Twenty-one percent of the tested colonies were $spec^S$, indicating that a second cross-over event had occurred, which resulted in excision of the vector from the chromosome. Finally, out of the 135 $Spec^S$ colonies tested by PCR, two had received the deletion of the *tet*(W) gene. The same strategy was used to delete a protease inhibitor (serpin-like) gene (BL0108) from *B. longum* NCC2705 with two positive recombinant colonies out of 12 colonies tested in the final PCR screening. This study showed for the first time an *in frame* deletion of a specific entire gene in *Bifidobacterium*, achieved by targeted double cross-over recombination. It is anticipated that the application of PAM system to this knock-out strategy is likely to be a powerful tool for future gene deletion/replacement in *Bifidobacterium*.

6. Concluding remarks and future perspectives

A bunch of bifidobacterial plasmids have already been sequenced and analysed from several *Bifidobacterium* species, which has allowed the development of some cloning and expression vectors. However, there is still a lack of knowledge about the basic biology of plasmids for them to be used with confidence. Dissection of translated and untranslated sequences will aid to define the functionality of the different plasmid elements found. This will help the designing of high-copy and low-copy number vectors for the fine-tuning expression of homologous and heterologous genes, while increasing stability of the constructs. Polishing and refining currently-in-use vectors and broaden their positive selection will also be useful for many molecular studies, as well as for the development of compatible systems allowing introduction of two vectors in a single cell. Characterisation of inducible promoters would allow controlled gene expression in bifidobacteria (Ruiz *et al.*, 2012a). The construction of food-grade vectors, i.e. having no foreign DNA and free of antibiotic resistance makers, would further facilitate the future industrial use of genetically modified bifidobacteria.

Nonetheless, at present, the lack of efficient gene knock-out protocols that can be efficiently applied to virtually all bifidobacterial species is the main limitation. Gene knock-out is, in fact, the golden procedure to unambiguously understand the role of a specific coding sequence. A few studies (Álvarez-Martín *et al.*, 2012; Arigoni and Delley, 2008; O'Connell-Motherway *et al.*, 2008) demonstrated that standard knock-out procedures based on homologous recombination can be practicable in bifidobacteria, provided the frequency of transformation is sufficiently high. A suitable transformation rate could be potentially reached by combining steps from different protocols or introducing new ones, as can be deduced from the investigations presented in this review article. Among these, the use of strict anaerobic conditions during preparation of competent

cells and electroporation (to increase viability) and the employment of any possible strategy useful to overcome bifidobacterial restriction barriers seems to be paramount. To this aim, the set-up of procedures for the *in vitro* methylation of recombinant DNA before transformation appears promising, in spite of the improved transformation efficiency obtained by the laborious method of the PAM strategy.

Nowadays, the European Regulation 1924/2006 concerning health claims on product labels (EC, 2006) ratifies the need for an approval from the European Food Safety Authority (EFSA) for the efficacy of any specific probiotic product. As a consequence, industrial producers are demanding new efficient research instruments that can permit effective verification and demonstration of the health-promoting properties associated to probiotic microorganisms, such as bifidobacteria, otherwise claims will be rejected (Katan, 2012). Due to both technological advances (e.g. the rapidly growing number of whole genome sequence data) and the increasing interest of food and pharmaceutical industries on probiotics, research on bifidobacteria is boosting. It is, therefore, expected that in the next few years further achievements will be obtained in the genetic modification of these organisms.

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