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 the growth difficulties, because of their exigent nutritive requirements and oxygen sensitivity, and a lack of efficient genetic tools. These studies, however, are critical to uncover the cross-talk between bifidobacteria and their hosts' cells, and also to prove unequivocally the supposed beneficial effects they provide through endogenous bifidobacterial populations or after their ingestion as probiotics. The genome sequencing projects of different bifidobacterial strains have provided a wealth of genetic data, which will be of much help to decipher 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 summarizes 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.

1. General introduction

The members of the genus *Bifidobacterium* are anaerobic, fermentative bacteria, high G+C, Gram-positive bacteria belonging to order Bifidobacteriales inside the phylum Actinobacteria (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 dominant *Bifidobacterium* 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 the establishment of 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 (Mueller et al., 2006; Claesson et al., 2011). The colonization of human intestinal tract by bifidobacteria starts soon after birth and lasts all lifelong. Thus, unsurprisingly bifidobacteria have become a common component of probiotic products designed for human or animal consumption (Tuohy et al., 2003; Leahy et al., 2005; Parvez et al., 2006). Probiotic products represent a strong growth area within the functional foods market and are currently having a significant economic impact on the dairy sector. However, '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' (Stanton et al., 2001). While clinical evidence for the purported beneficial effects is rapidly accumulating (Tuohy et al., 2003; Leahy et al., 2005; Guglielmetti et al., 2011; Guglielmetti et al., 2011; Ishikawa et al., 2011), there still is a lack of fundamental

knowledge on the molecular mechanisms by which bifidobacteria interact with other bacteria and their hosts, while contributing to their health and well-being (Kullen and Klaenhammer, 2000; Ventura et al., 2009).

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 for growth rich media and requiring strict anaerobic conditions (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 revolutionized 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 (EPS) (Schell et al., 2002; Barrangou et al., 2009; Ventura et al., 2009b), fimbriae-like glycoproteins (Schell et al., 2002; Ventura et al., 2009b), serpin-like protease inhibitors (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 (Schell et al., 2002; Sela et al., 2008; Barrangou et al., 2009; Kim et al., 2009). Concerning the adaptation to intestinal environmental, largely the best studied bifidobacterial strain is *B. breve* UCC2003, thanks to the molecular and functional characterization of many genetic loci, which have been identified in its genome (O'Connell et al., 2008; Zomer and van Sinderen, 2010; O'Connell Motherway et al., 2011a; O'Connell Motherway et al., 2011b; Pokusaeva et al., 2011; Fanning et al., 2012; Ruiz et al., 2012).

Up till now, the genome sequences publically available of 17 *Bifidobacterium* strains belonging to five species, namely *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 these aims, 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 determined the shape of bacterial genomes. As suggested by

126 Philippe and Douady (2003), plasmids, bacteriophages (phages), and transposons are
127 considered major agents for shaping the bifidobacterial genomes through horizontal
128 gene transfer (HGT) processes. The above mentioned mobile genetic elements, as well
129 as others, such as group II introns and jumping genes, constitute what has recently been
130 defined as the mobilome (Frost et al., 2005; Siefert, 2009).
131
132 **3.1. Plasmids of bifidobacteria**
133 Plasmids are extra-chromosomal, autonomously replicating genetic elements
134 found in bacteria, archaea, and eukaryotic cells. Despite their independent replication,
135 plasmids make use of cellular enzymes to ensure both replication and maintenance
136 (Hayes, 2003). Plasmids display an enormous diversity of features, such as size, host
137 range, and the repertoire of genes that they carry. By definition, plasmids do not
138 encode essential genes for growth, nonetheless they can provide a wide variety of
139 phenotypes to the cells that harbour them, including antibiotic resistance, bacteriocin
140 production, virulence and pathogenesis or degradation of complex and recalcitrant
141 (toxic) compounds found in some ecosystems. They can also encode for the ability to
142 use carbohydrates and/or protein substances as a source of carbon and energy (Thomas
143 et al., 2004). All these properties have endowed plasmids with the title of primarily
144 adaptative entities (Siezen et al., 2005). Apart from the traits with an impact on host
145 physiology and ecology, important characteristics of plasmids include copy number
146 (low, medium, high), host range (narrow, broad), and capability to spread (conjugation,
147 mobilization) (Hayes, 2003). Small plasmids (i.e. smaller than 15-20 kb in size) often
148 do not encode any selectable trait and are therefore denominated “cryptic”. It seems,
149 however, plausible to presume that they simply benefit its host by promoting
150 recombination and, consequently, enhance the ecological adaptability of the bacterial

151 population (Guglielmetti et al., 2007a; Thomas et al., 2004). Plasmids can be,
152 consequently, depicted as accessory and/or adaptive gene pools shared by bacteria.
153 Analyses of bifidobacteria have indicated that extrachromosomal elements are
154 scarcer than in other intestinal bacterial species (Sgorbati et al., 1982; Iwata and
155 Morishita, 1989; Park et al., 1997) and, where found, they have a size generally smaller
156 than 15 kb. Strains from the species *B. longum* subsp. *longum* (hereafter *B. longum*, if
157 not differently specified), *B. globosum*, *B. asteroides*, and *B. indicum* seem to harbour
158 more plasmids than those from other species (Sgorbati et al., 1982). At present, the
159 nucleotidic sequence of more than 30 bifidobacterial plasmid molecules is available in
160 GenBank (Table 1; http://www.ncbi.nlm.nih.gov/sites/entrez/). The majority of these
161 molecules (up to 19) were isolated from *B. longum* strains, which includes pMB1
162 (Rossi et al., 1996), the first plasmid to be analysed at a molecular level from a
163 member of this genus. Three other plasmids have been characterized from strains of *B.*
164 *breve*, two from *B. asteroides* and *B. bifidum*, and single plasmids have been analysed
165 from strains of *B. catenulatum*, *B. pseudocatenulatum*, and *B. pseudolongum* subsp.
166 *globosum*. The obtained sizes of the studied plasmids show a range between 1.8 kb for
167 pMB1 to 10.2 kb for pNAC3. In most cases, strains harbour a single plasmid, except
168 for three strains of *B. longum* in which two different plasmid molecules were identified
169 (Table 1). In addition, *B. longum* NAL8 and *B. longum* FI10564 have been reported to
170 contain three different plasmids each. The significance of these autonomously-
171 replicating DNA elements in bifidobacteria remains unclear, since no obvious
172 phenotypic traits have been associated to plasmids, except for the production of the
173 bacteriocin bifidocin B by *B. bifidum* NCFB 1454, which was associated to a 8 kb
174 plasmid (Yildirim et al., 1999). Nevertheless, this plasmid has never been sequenced or
175 characterized further. More recently, a further plasmid of this species (pBIF10) has

176 been found to contain *tetQ*, a gene encoding a ribosome protection protein providing
177 tetracycline resistance (DQ093580).
178 The basic biology of bifidobacterial plasmids remains poorly understood and most
179 of the information derive from *in silico* investigation. Indeed, the mode of replication
180 has been experimentally analysed for only a few of them (Moon et al., 2009; Park et
181 al., 2008; Guglielmetti et al., 2007b; Lee and O'Sullivan, 2006; Corneau et al., 2004;
182 O'Riordan and Fitzgerald, 1999; Park et al., 1999). Furthermore, dissection of open
183 reading frames (ORFs) and analysis of untranslated sequences and structures have been
184 undertaken for only two plasmids: pBC1 (Álvarez-Martín et al., 2007a) and pCIBA089
185 (Cronin et al., 2007). Sequence comparison suggests that most bifidobacterial plasmids
186 probably replicate by means of a rolling-circle mechanism, with the exception
187 represented by eight plasmids that appear to use the theta-replicating mode (Table 1;
188 Moon et al., 2009; Álvarez-Martín et al., 2008; Cronin et al., 2007; Klijn et al., 2006;
189 Lee and O'Sullivan, 2006; Rossi et al., 1996). Phylogenetic analysis of their replication
190 (Rep) initiator proteins revealed that *Bifidobacterium* plasmids could be clustered into
191 six different groups (Table 1 and Fig. 1). Homology of bifidobacterial Rep proteins has
192 shown that, in some cases, their closest relatives are found in plasmids from
193 phylogenetically distant bacterial groups (Álvarez-Martín et al., 2007b; Guglielmetti et
194 al., 2007b). For instance, Rep protein of plasmid pBIF10 from *B. bifidum* M203049
195 (type *IV*) is strictly related with the replication proteins of plasmids commonly
196 harbored by the *Cytophaga–Flavobacterium–Bacteroides* group of Gram-negative
197 bacteria. The same plasmid also contains two other DNA regions, respectively of 1966
198 and 2569 bp displaying strong similarity with genetic regions of *Bacteroides* intestinal
199 strains (DQ093580). The former region includes mobilization genes *mobA* and *mobB*,
200 while the latter comprises *tetQ* gene (Fig. 2). These similarities leave to suppose that

plasmid pBIF10 could have been relatively recently acquired by strain *B. bifidum* M203049 from *Bacteroides* thorough horizontal DNA transfer.

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, a feature that is unprecedented in any known bacterial plasmid (Gibbs et al., 2006).

Upstream of the Rep protein, *Bifidobacterium* plasmids contains non-coding regions characterized by tandem direct and inverted repeats sequences, in an organization 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 organization is similar in all plasmids but sequences of the repeats are variable, thus, conferring specific interaction between Rep proteins and DNA sequences.

Differences in nucleotide sequences and gene organization 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 Fig. 2. Apart from Rep, many plasmids contains accessory ORFs encoding hypothetical proteins, some of which, such as the mobilization-like proteins (Fig. 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 are revealing 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).

3.2. 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 Youse et al. was followed by an electron microscopy observation of a lytic phage from the so-called *B. ruminale* (today reclassified as *B. 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 characterized 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 respectively, share nucleotide and organization 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 three bifidobacterial prophages are integrated in a tRNAMet gene, which had not previously been shown to act as an *attB* site in Gram-positives (Campbell, 1992). Analysis of the distribution of this integration site in bifidobacterial species has revealed that *attB* sites are 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 (Sela et al., 2008; Barrangou et al., 2009), although these remnant elements are not adjacent to tRNAMet sequences. The use of conserved *attB* sequences and their associated *int* genes 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).

3.3. Transposons and insertion sequence (IS) elements in bifidobacteria

Transposons and 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, respectively, 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 analyzed, ISs have not been considered in other works. An IS of 1047 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 1163 bp was also found to interrupt the structural *tet*(W) gene in the susceptible *B. longum* M21 strain (Ammor et al., 2008). The *tet(W)* gene is also located in the chromosome of the largely diffused commercial probiotic strain *B. animalis* subsp. *lactis* BB12 and was found to be adjacent to a transposase gene (genes BIF_01560 and BIF_02030 of the annotated genome of strain BB12; Garrigues et al., 2010). However, analysis of the flanking DNA sequences shows that the *tet(W)* gene should not be contained in a functional mobile element.

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), suggesting the presence of active insertion sites in bifidobacterial genomes.

Recently, Fukiya et al. (2010) characterized 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 1593 bp in length and contained a single ORF encoding

301 a putative transposase, *tnpB* (Fig. 2). The same authors also reported that sequences
302 similar to IS*Blo15* are widely distributed among the nine *Bifidobacterium* species they
303 tested.
304 Finally, a copy of the transposon Tn5432, which encodes resistance to
305 erythromycin and clindamycin, has been identified in several *B. thermophilum* strains
306 isolated from pig faeces (van Hoek et al., 2008). Tn5432 was first isolated from
307 *Corynebacterium xerosis* and rescued copies on plasmids were shown to be able to
308 transpose in *Corynebacterium glutamicum* causing several mutations (Tauch et al.,
309 1995). The transposition ability of Tn5432 from *B. thermophilum* remains however to
310 be determined.
311 Knowledge on *Bifidobacterium* transposons and ISs is strongly needed, since they
312 can bring to the development of high-efficiency transposon mutagenesis systems that
313 could greatly facilitate the molecular study of bifidobacteria. However, tools for
314 bifidobacteria based on these elements are yet not available.
315 Finally, a new mobile genetic element has been described in *B. longum* (Schell et
316 al., 2002; Lee et al., 2008), named mobile integrase cassette (MIC). MIC elements are
317 constituted by a conserved 20 bp palindrome sequence and two insertion sequences
318 separated by three contiguous but different *xerC* integrase genes (Lee et al., 2008).
319 Interestingly, one MIC of the strain *B. longum* DJO10A was shown to be active during
320 the adaptation of *B. longum* DJO10A to *in vitro* fermentation conditions (continous
321 growth up to about 1000 generations) (Lee et al., 2008).
322
323 **4. General and specialized vectors for bifidobacteria**
324 Some natural bifidobacterial plasmids have provided the basis for the construction
325 of *Escherichia coli–Bifidobacterium* shuttle vectors, mostly resulting from the direct

326 cloning of whole plasmids into an *E. coli* vector containing selectable antibiotic
327 resistance genes such as spectinomycin, erythromycin and chloramphenicol (Álvarez-
328 Martín et al., 2008; Guglielmetti et al., 2007b; Klijn et al., 2006; Lee and O'Sullivan,
329 2006; Park et al., 1999; Rossi et al., 1996, 1998; Matsumura et al., 1997; Missich et al.,
330 1994; Table 13.2). In this way, the plasmid pBC1 from *B. catenulatum* L48 has been
331 used for the construction of a series of *E. coli–Bifidobacterium* shuttle vectors with
332 innovative characteristics such as the presence of a tetracycline resistance gene of
333 bifidobacterial origin [*tet*(W)] and the cloning of the β-galactosidase complementing
334 peptide gene for a convenient blue/white screening of recombinant clones in *E. coli*
335 (Álvarez-Martín et al., 2008). The functionality of the vectors was further checked by
336 cloning and overexpression of an α-l-arabinofuranosidase gene from *B. longum* B667
337 in *E. coli* and *Bifidobacterium* strains.
338 As plasmid maintenance constitutes a major problem for vector utilization,
339 González Vara and co-workers studied the segregational and structural stability of
340 pMB1–derived constructs in *B. animalis* by continuous culture (González Vara et al.,
341 2003). These authors reported a high correlation between instability and plasmid size,
342 while no major deletions and rearrangements were observed. However, some
343 constructs did not behave as expected (González Vara et al., 2003), a result that agrees
344 with observations by other authors (Álvarez-Martín et al., 2007a), suggesting that
345 beyond plasmid size, secondary structure of the constructs may further influence
346 stability.
347 It is worth noting that in spite of a limited knowledge of plasmid biology, a
348 number of vectors for heterologous expression of desirable foreign genes have already
349 been developed. As an example, the reporter vector pMDY23 expresses the *gusA* gene
350 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: (i) the presence of a thick (multi-layered) and complex cell wall (Fischer, 1987), (ii) intracellular restriction/modification barriers (Hartke et al., 1996; Schell et al., 2002; Yasui et al., 2009), and (iii) sensitivity to environmental stresses (principally oxygen) during preparation of competent cells and transformation.

Full exploitation of genomic data requires the use of general and specialized 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 (Fujimori, 2006; Xu et al.,

2007), and as a means of increasing beneficial detoxifying activities into the gastrointestinal tract (Park et al., 2007). Traditional and new applications, therefore, require utilization of robust genetic tools and improved genetic transformation techniques.

5.1. Genetic transformation by electroporation

The first scientific proof of genuine genetic transformation of *Bifidobacterium* dates back to 1994, when Missich and collaborators introduced by electroporation pRM2 (Missich et al., 1994), 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 electrocompent cells and subsequent electroporation are based mainly on the comprehensive studies by Argnani et al. (1996) and Rossi et al. (1997), who considered and optimized several conditions such as growth medium, washing solutions, incubation temperatures, and voltage.

5.1.1. Preparation of electro-competent cells

The preparation of electro-competent 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 stabilizers in washing and electroporation buffers, or maintaining cells at low temperature.

400 Growth phase. Since the composition of the cell wall plays a key role in DNA
401 uptake, numerous studies have reported the importance of harvesting bacterial cells at a
402 specific stage of their growth. Some studies showed that bifidobacteria could be
403 effectively transformed only when they were in the early exponential phase ($OD_{600\ nm}$
404 0.2-0.4) (Missich et al., 1994; Argnani et al., 1996; Rossi et al., 1997), whereas
405 efficiencies dropped for older cells, reaching zero for cells from the stationary growth
406 phase (Rossi et al., 1997). In contrast, other researchers have observed maximal
407 transformation efficiency with cells in the middle to late log phase. For instance,
408 Matsumura et al. found that with the vector pBLESS100 the transformation efficiency
409 of *B. longum* 105-A was about one order of magnitude higher when cells were
410 approaching the stationary phase as compared to early-log phase (Matsumura et al.,
411 1997). Similarly, in more recent studies, midlogarithmic-phase cells were used (optical
412 density at 600 nm 0.5 to 0.7) to effectively transform different *Bifidobacterium* species
413 (MacConaill et al. 2003; Cronin et al., 2007; Sangrador-Vegas et al., 2007; Álvarez
414 Martín et al., 2008).
415 Growth media. The addition to the growth medium of sugars in high
416 concentrations is traditionally recognized as an effective strategy to improve the
417 transformation yield by affecting the composition of the cell wall. Argnani and
418 collaborators (1997) cultivated the cells in MRS broth supplemented with 0.5 M
419 sucrose and washed them in a buffered sucrose solution at the same concentration.
420 Rossi et al. (1996) showed a 100-fold increase in transformation efficiency when
421 *Bifidobacterium* cells were grown in Iwata medium (IM) supplemented with raffinose
422 0.3 M or, especially, 16% Actilight®P, as compared with cells grown in IM broth with
423 or without glucose. Actilight®P is a commercial product comprising a mix of short-
424 chain fructooligosaccharides (1-kestose, nystose, and fructosylnystose; FOS), which

425 are metabolised by bifidobacteria and may protect cells from stress (Guglielmetti et al.,
426 2008a). Using this sugar product in a growth broth and washing buffer can thus
427 improve transformation efficacy by preserving the cells' physiological condition
428 during the preparation of competent bifidobacteria. This statement has been recent
429 confirmed in a study, in which the use of 16% FOS or 10% GOS allowed the
430 transformation of *B. bifidum* and *B. asteroides*, two bifidobcaterial species known for
431 their recalcitrance for acquiring exogenous DNA (Serafini et al., 2012).
432 Electroporation buffers. Argnani and collaborators showed that a few-hour storage
433 of bacterial cells before electroporation at 4 °C in an electroporation buffer composed
434 of 0.5 M sucrose, 1 mM ammonium citrate, pH 6, significantly improved the
435 transformation efficiency of bifidobacteria. Argnani et al. (1997) suggested that in the
436 conditions they had established, the presence of low-molarity ammonium citrate (more
437 than HEPES and phosphate buffers) as the osmotic stabilizer may support the right
438 degree of cell autolysis without limiting cell viability, resulting in improved cell wall
439 permeability for exogenous DNA. In addition, Rossi and collaborators showed the
440 importance of the incubation step in the electroporation buffer at 0°C overnight (Rossi
441 et al., 1997). Their electroporation buffer, named KMR, was composed of KH_2PO_4 5
442 mM, $MgCl_2$ 1 mM and raffinose 0.3 M, pH 4·8. However, the higher salt concentration
443 of the KMR buffer may favour *arcing* events during the electrical discharge (S.
444 Guglielmetti, unpublished).
445
446 *5.1.2. Efficiency of electro-transformation in bifidobacteria*
447 In general, the rate of electroporation-mediated transformation in bifidobacteria is
448 extremely low and constitutes the main limitation on successfully applying traditional
449 genetic manipulation strategies to members of this genus. Wide variation in

450 transformation efficiencies have been reported in the literature, ranging from about 10^0
451 (e.g., Rossi et al., 1997) to more than 10^6 (e.g., Tanaka et al., 2005) transformants per
452 µg of recombinant DNA (Table 2). Besides the protocols adopted for preparing
453 competent cells and subsequent electroporation, considerable differences can be
454 obtained depending on the strain under study (see Table 2). Nonetheless, valuable
455 progress has recently been made in improving transformation rates, thanks to studies
456 on the restriction/modification systems of bifidobacteria, which have been shown to be
457 the main obstacle in the acquisition of foreign DNA by these bacteria.
458
459 *5.1.3. Optimization strategies for the electro-transformation of bifidobacteria*
460 To improve efficiency it is crucial to preserve cell viability during electroporation.
461 One main reason for cell mortality during these experiments is oxidative stress. To
462 overcome this problem, Park and colleagues added Oxyrase®, and enzyme system
463 removing oxygen from its environment, to the incubation buffer after the electric pulse
464 was given to the competent cells. This strategy allowed *B. longum* MG1 to transform at
465 100-fold improved electroporation efficiency (Park et al., 2003).
466 In general, DNA introduced into bacteria by electroporation is more vulnerable to
467 restriction nucleases than that transferred by conjugation or natural transformation.
468 This is particularly important for bifidobacteria, whose perhaps most immediate
469 obstacle to acquiring exogenous DNA are their restriction/modification (R-M) systems.
470 DNA R-M gene clusters coding for methyltransferases and restriction enzymes can be
471 recognized in all sequenced bifidobacterial genomes, and to date several proven or
472 potential R-M systems belonging to either Type I, II, and IV have already been
473 identified (Roberts, 1980; Hartke et al., 1996; Schell et al., 2002; Lee et al., 2008;
474 O'Connell-Motherway et al., 2009; Yasui et al., 2009; http://rebase.neb.com/rebase).

475 Based on the above, several studies have reported a significant increase in
476 transformation efficiency when transformed DNA has been isolated from
477 *Bifidobacterium* instead of *E. coli* (Rossi et al., 1997; Rossi et al., 1998; O'Connell-
478 Motherway et al., 2009; Yasui et al., 2009). For instance, Rossi et al. (1998), found that
479 only vector DNA prepared from bifidobacteria could successfully transform some
480 strains of *B. longum*, *B. animalis*, or *B. bifidum*. Therefore, proper modification of
481 plasmid DNA can help to bypass the restriction barriers and favour the acquisition of
482 foreign recombinant DNA by bifidobacteria. This assumption has been verified by a
483 recent study, in which site-directed mutagenesis and *in vitro* methylation were applied
484 to remove or modify restriction sites from a vector pYBamy59 before
485 electrotransformation into *B. longum* MG1 (Kim et al., 2010). In this study, sequence
486 analysis of pYBamy59 fragments originated by incubation of recombinant DNA with
487 cell extracts of MG1, revealed the presence of a *Sac*II-like endonuclease activity,
488 recognizing the palindromic sequence 5'-CCGCGG-3'. When pYBamy59 from *E. coli*
489 was methylated *in vitro* by CpG or GpC methyltransferases, or when *Sac*II sites were
490 removed from pYBamy59 through site-directed mutagenesis, the transformation
491 efficiency showed 8- to 15-fold increment as compared to the original plasmid (Kim et
492 al., 2010).

493 Another strategy to modify recombinant DNA before introduction into
494 bifidobacterial cells was recently adopted in two independent studies (Yasui et al.,
495 2009; O'Connell-Motherway et al., 2009), with the aim to boost the transformation
496 efficiency of *B. adolescentis* ATCC 15703 and *B. breve* UCC2003, respectively. In this
497 strategy, a shuttle vector was pre-methylated in *Escherichia coli* cells carrying the
498 genes encoding the DNA modification enzymes of the target *Bifidobacterium* before
499 electroporation (Fig. 3). In fact, Yasui and coworkers (2009) developed a system called

500 ”Plasmid Artificial Modification” (PAM) and demonstrated its efficacy for the target
501 host *B. adolescentis* ATCC 15703, a strain that could be transformed only at an
502 extremely low level. In the ATCC 15703 genome, they identified two Type II DNA
503 methyltransferase genes, BAD_1233 and BAD_1383, which they cloned in *E. coli*
504 TOP10 (Invitrogen) by means of a low copy number vector, obtaining the so-called
505 “PAM host.” The *E. coli* TOP10 laboratory strain was shown to be the most suitable
506 because it lacks the Type IV restriction enzymes *mrr* and *mcrBC* (which degrade DNA
507 methylated by the R-M system of other bacteria) and the methylases *dam*, *dcm*, and
508 *hsdMS* (which can make the DNA sensitive to possible Type IV restriction systems).
509 Subsequently, an *E. coli-Bifidobacterium* shuttle vector, based on the pTB6 *B. longum*
510 replicon (Tanaka et al., 2005), was introduced into the PAM host. Transformation
511 efficiency improved considerably when *B. adolescentis* was electroporated with the
512 shuttle vector DNA isolated from the PAM host, jumping from $1–3x10^0$ to 10^5
513 CFU/μg. This confirms that the shuttle vector was methylated by the modification
514 enzyme encoded by the PAM plasmid in the *E. coli* host and consequently protected
515 against restriction by *B. adolescentis* (Fig. 3).
516 The same approach was adopted by O’Connell-Motherway et al. (2009) with *B.*
517 *breve* UCC2003 as the target host. In the annotated genome of this strain, they found
518 three different R-M gene clusters, including the methylase genes *bbrIM*, *bbrIIM* and
519 *bbrIIIM*. The role of these modification genes in the acquisition of exogenous DNA by
520 *B. breve* was studied in transformation experiments with pAM5-derived vectors (based
521 on the pBC1 replicon from *B. catenulatum*; Álvarez-Martín et al., 2007b). The authors
522 observed a 1000-, 10-, and 5-fold higher transformation frequency for pAM5 DNA
523 isolated from *E. coli* expressing M.BbrIII, M.BbrII, and M.BbrI, respectively, which

524 indicates that, although differently, all three DNA methylation systems affected the
525 transformation efficiency.
526 The above studies demonstrated the usefulness of artificially modified DNA by
527 means of the host methylases to increase the electroporation efficiency. Genome
528 analyses and experimental data, however, have shown that *Bifidobacterium* strains
529 harbour a very diverse range of R-M activities, even within the same species
530 (O'Connell-Motherway et al., 2009). Therefore, this strategy is at least partly limited to
531 the strains whose whole-genome sequence is known. To overcome this problem,
532 O'Connell-Motherway et al. (2009) proposed the possibility of methylating exogenous
533 DNA isolated from *E. coli in vitro,* through incubation of the DNA with cell extracts of
534 the target host in the presence of S-adenosylmethionine. However, experimental data
535 of the practicability and effectiveness of this strategy are not yet available.
536
537 *5.2. Conjugation in bifidobacteria*
538 The R-M barriers of a bacterial strain can also be bypassed by introducing foreign
539 DNA through conjugation. Conjugation may occur when *cis* (*oriT*) and *trans* (transfer
540 proteins, Mob and Tra) elements found in mobilizing plasmids are recognized by
541 cellular components, which can be supplied by the host cell. Putative Mob and Tra
542 protein coding genes and characteristic *cis* elements have been found in several
543 bifidobacterial plasmids (O'Riordan and Fitzgeralds, 1999; Corneau et al., 2004; Gibbs
544 et al., 2006; Shkoporov et al., 2008a; Table 1), suggesting their mobilization potential.
545 Nevertheless, up to now, no DNA transfer system based on conjugation is available for
546 members of the genus *Bifidobacterium*, and no conjugation events have been
547 irrefutably demonstrated. The only documented systematic attempt to achieve
548 conjugation in bifidobacteria was made by Shkoporov and collaborators (2008a), who

549 exploited the mobilization functions of three different *Bifidobacterium* plasmids to
550 develop genetic tools based on the well characterized intergeneric conjugative element
551 RP4 (IncPα) (Simon et al., 1983) to transfer DNA into *B. pseudocatenulatum*. They
552 produced antibiotic-resistant clones that, though PCR-positive, did not contain the
553 expected plasmid DNA. Consequently, development of effective conjugation systems
554 for bifidobacteria, albeit potentially useful, remains in its infancy.
555
556 *5.3. Expression of heterologous genes among bifidobacteria*
557 The development of heterologous expression and secretion systems is strategically
558 important for studying the properties of *Bifidobacterium* because of strain
559 improvement and delivery into the human digestive tract of useful gene products such
560 as vaccines or anticarcinogenic polypeptides.
561 Heterologous genes from a diverse group of organisms have been expressed in
562 bifidobacteria under the control of either heterologous or homologous promoters
563 (Table 3). The current list includes genes and promoters mainly from Gram-negative
564 and Gram-positive bacteria, but with exceptions, as the luciferase gene from
565 *Pyrophorus plagiophthalamus* (Guglielmetti et al., 2008a) and several human genes
566 (Xu et al., 2007). In addition to the genes reported in Table 3, several antibiotic
567 resistance genes, used in preparing cloning and expression vectors, have been shown to
568 be functional in bifidobacteria even under the control of their own regulatory elements.
569 They include, for instance, the chloramphenicol acetyl transferase (*cat*) of the plasmid
570 pC194, the erythromycin resistance gene of pE194 from *Staphylococcus aureus*, and
571 the spectinomycin resistence gene from *Enterococcus faecalis*. In contrast, the
572 thiostrepton resistance gene from *Streptomyces* is not functional in bifidobacteria
573 (Rossi et al., 1998, Guglielmetti et al., 2007b). Similarly, the genes coding for

574 *Pseudomonas fluorescens* lipase, *Bacillus licheniformis* α-amylase and *Streptomyces*
575 sp. cholesterol oxidase, are stably maintained in the bifidobacterial host, but under the
576 control of their own promoters they are not expressed (Rossi et al., 1998).
577 With respect to protein expression in bifidobacteria, excretion and secretion
578 processes should be studied to develop export systems for expressed heterologous
579 proteins or enzymes. In this context, MacConaill and collaborators (2003) investigated
580 protein export in *B. breve* UCC2003 by means of the export-specific vector pFUN,
581 based on the use of the staphylococcal nuclease (Nuc) as a reporter enzyme (Poquet et
582 al., 1998). Due to the removal of its native signal peptide, the Nuc reporter protein is
583 translocation-competent but unable to direct its own secretion. In this study,
584 translational fusions were constructed with a *nuc* gene and the export signal provided
585 by inserted *B. breve* chromosomal DNA fragments. By this strategy, seven signal
586 peptides have been identified for *B. breve* UCC2003 (MacConaill et al., 2003).
587 Recently, a secretion system has also been developed based on the α-amylase
588 expression and secretion signals isolated from *B. adolescentis* INT57 (Park et al.,
589 2005b; Rhim et al., 2006). Park and collaborators constructed a secretion vector,
590 pBESAF2, containing the promoter and the signal peptide of the α-amylase gene
591 *amyB*. The gene encoding an intracellular phytase from *E. coli* was introduced in this
592 vector and transcriptionally fused to the signal sequence and finally introduced by
593 electroporation in *B. longum* MG1. The authors demonstrated that, by using this
594 system, phytase enzyme was successfully expressed and secreted by *B. longum* into the
595 culture broth. Furthermore, this system was employed for expression and secretion by
596 *B. longum* MB1 of the bacteriocin pediocin PA-1 from *Pediococcus acidilactici* K10
597 (Moon et al., 2005).

598 Analogously, Deng and collaborators (2009) selected through computational
599 analysis the signal peptide sequence from the endo-1,5-α-L-arabinosidase gene of *B.*
600 *longum* NCC2705, by which they obtain expression and secretion of the human
601 interferon-α2b protein (IFN-α2b) in *B. longum* ATCC 15707. This study showed that
602 65% of the total IFN-α2b was secreted from *B. longum* in the presence of the
603 arabinosidase signal peptide, while only 15% of the protein was secreted without the
604 signal peptide. Surprisingly, this experimentation was carried out without a
605 conventional *E. coli-Bifidobacterium* shuttle plasmid, but by means of the commercial
606 pBR322-based vector pBAD-gIIIA (Invitrogen), in which expression of the
607 recombinant protein is arabinose-inducible by the presence of the promoter of the
608 arabinose operon from *E. coli*. The maximal level of induction was obtained after
609 addition of 0.2 % arabinose (Deng et al., 2009). Finally, Long et al. (2010)
610 demonstrated that the exo-xylanase (XynF) signal peptide sequence from *B. longum*
611 was suitable to guide secretion of the mature peptide of the human gut hormone
612 oxyntomodulin.
613 Information on regulated promoters, inducers, and repressors is extremely limited
614 in bifidobacteria. Additional efforts are, therefore, needed to identify strong, weak, and
615 regulated promoters for controlled gene expression in bifidobacteria under different
616 environmental conditions. However, current knowledge on the expression of
617 heterologous genes in *Bifidobacterium* has enabled development of reporter gene and
618 drug delivery systems as potentially promising tools for future research and
619 pharmaceutical applications.
620
621 5.3.1. Reporter gene systems

622 Some reporter gene systems have already been developed and shown useful for
623 several applications. To study promoter strength and regulation analysis or to identify
624 genomic fragments containing active promoters, Klijn and co-workers (2006)
625 developed the reporter vector pMDY23 based on the *Escherichia coli* β-glucuronidase
626 gene *gusA* and the small cryptic *B. longum* plasmid pNCC293. After introducing the
627 vector in *B. longum* NCC2705, they demonstrated the suitability of pMDY23 as a
628 reporter plasmid for promoter study by analyzing the promoter activity of three DNA
629 fragments (Klijn et al., 2006; Table 3).
630 More recently, two studies showed the application of bioluminescence reporter
631 genes in *Bifidobacterium* spp. (Guglielmetti et al., 2008a; Cronin et al., 2008).
632 Guglielmetti and collaborators transformed by electroporation the human intestinal
633 strain *B. longum* subsp. *longum* NCC2705 with a vector (pGBL8b) containing the
634 insect luciferase gene *lucGR* from a click beetle (*Pyrophorus plagiophthalamus*). The
635 same vector, however, was incapable of transforming *B. animalis* subsp. *lactis* BB12
636 and *B. bifidum* MIMBb75 (Guglielmetti et al., 2008a; S. Guglielmetti, unpublished).
637 The resulting bioluminescent *B. longum* was used to analyze variations in intracellular
638 ATP concentration at acidic pH in the presence of different sugars, a technique proving
639 to be a valuable tool for the rapid and sensitive study of the physiological state of
640 bacterial cells under different environmental conditions. Nonetheless, the need to add
641 exogenous D-luciferin as a substrate in this reporter system limits considerably its *in*
642 *vivo* application.
643 The bacterial luciferase system (coded by the *luxABCDE* operon) is generally less
644 sensitive than insect luciferases, yet bacterial luciferase requires as substrate a long-
645 chain fatty aldehyde, which is intracellularly synthesized by a fatty acid reductase
646 complex encoded by *luxCDE*. Therefore, the intracellular expression of the *lux* operon

647 circumvents the disadvantage of exogenous addition of luciferin and is thus more
648 suitable for *in vivo* applications. Cronin et al. (2008) adopted the *lux* operon to develop
649 the non-invasive luciferase reporter vector pLuxMC1, which was introduced in *B.*
650 *breve* UCC2003. Once mice were orally inoculated with bioluminescent *B. breve*, the
651 reporter system allowed a real-time tracking of the colonisation and persistence of this
652 probiotic strain *in vivo*.
653
654 5.3.2. Drug delivery systems
655 Due to their safety and ability to colonize specific areas of the human
656 gastrointestinal tract, bifidobacteria may turn out to be optimal vectors for *in situ*
657 delivery of biologically active substances. Of particular interest is the fact that certain
658 anaerobic bacteria, including species of *Clostridium* and *Bifidobacterium*, can
659 selectively germinate and grow in the hypoxic regions of solid tumors (Malmgren and
660 Flanigan, 1955; Kimura et al., 1980; Yazawa et al., 2000; Yazawa et al., 2001), such as
661 those of most primary breast and uterine cervix cancers. This fact was exploited by the
662 Japanese research team of Prof. Fujimori, who developed a strategy called
663 "*Bifidobacterial Selective Targeting*" (BEST) (Fujimori, 2006). The Fujimori team's
664 BEST therapy involved the strain *B. longum* 105-A, which was genetically engineered
665 via electro-transformation with pBLES100-S-eCD, a plasmid based on the shuttle
666 vector pBLES100 (Matsumura et al., 1997) and comprising the cytosine deaminase
667 gene (CD) under the *hup* gene promoter of *B. longum* (Nakamura et al., 2002; Table 3),
668 which codes for a histone-like DNA-compacting protein. The CD enzyme converts the
669 non-toxic prodrug 5-fluorocytosine (5-FC) to chemotherapeutic 5-fluorouracil (5-FU),
670 which is systemically administrated to treat solid tumors. In conventional therapy, its
671 clinical effectiveness is very limited by its high systemic toxicity, particularly toward

672 the bone marrow. Fujimori's studies demonstrated that recombinant *B. longum*
673 selectively produced CD in mammary tumor tissues in rats, and that CD could convert
674 5-FC into 5-FU *in vivo* both after intratumoral injection and also by systemic
675 administration (Sasaki et al., 2006). Furthermore, no adverse effects were observed in
676 animal models during the use of *B. longum* as a gene delivery vector (Sasaki et al.,
677 2006), a finding supporting the potential effectiveness of this novel approach for
678 cancer gene therapy in humans. More recently, it was developed an improved version
679 of pBLES100-S-eCD, able to display 10-fold increased CD activity in *B. longum*
680 (Hamaji et al., 2007). They demonstrated that the BEST approach works well even
681 with a different bifidobacterial species, such as *B. breve* I-53-8w (Hidaka et al., 2007).
682 Bifidobacteria have been used as a gene delivery vehicle of CD also by Chinese
683 researchers, who expressed the CD gene in *B. longum* subsp. *infantis* by means of the
684 vector pGEX-1λT. The recombinant bacterium was then used to inhibit melanoma in
685 mice (Yi et al., 2005). As for the Invitrogen vector pBAD-gIIIA mentioned above, the
686 functionality of the vector pGEX-1λT is surprising, because it carries no bifidobacterial
687 replicon but only the pBR322 *ori* region. Moreover, Yi and collaborators claimed that
688 the recombinant bifidobacteria were selected with ampicillin through the β-lactamase
689 gene encoded by pGEX-1λT (Yi et al., 2005), an antibiotic marker generally
690 considered not to be active in Gram-positives. The same research team used this
691 approach to clone the *Herpes simplex* virus-thymidine kinase (HSV-TK) in *B. longum*
692 subsp. *infantis*. In this system, the thymidine kinase expressed specifically in tumor
693 tissues by bacterial cells, can convert the non-toxic precursor ganciclovir into the
694 ganciclovir-3-phosphate, a toxic substance that kills the tumoral cells. The efficacy of
695 this gene therapy system was demonstrated *in vivo* in a rat model of bladder cancer.
696 After tail vein injection of 4.4×10^9 recombinant *Bifidobacterium* cells with a

concomitant daily intraperitoneal injection of Ganciclovir, on the 15th day after the beginning of the treatment, rat bladder tumor growth was inhibited (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 tumor in mice (Li et al., 2003, Fu et al., 2005, 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 promotors 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 antitumor 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 coadjuvant agent in tumor chemotherapy. When *B. longum*-pBV22210-GCSF was applied to treat H22 and S180 sarcoma-bearing mice, it was

722 observed an effective antagonistic effect on bone marrow inhibited by
723 cyclophosphamide and an over 65% inhibition of tumor growth (Zhu et al., 2009).
724 Finally, the BEST strategy was adopted by Hou and colleagues (2006), who observed a
725 significant inhibition of the growth of solid tumors in a knock-out mice lacking the
726 phosphatase and tensin (PTEN) homolog with a genetically engineered *B. longum*,
727 transformed with a pMB1-derived vector expressing the PTEN tumor suppressor gene
728 under the *hup* gene promoter from *B. longum.*

729 Other gene delivery systems have recently been developed in bifidobacteria. For
730 instance, it was developed a vaccine delivery system based on *B. animalis* ATCC
731 27536, genetically modified through transformation with the vector pBLES100, in
732 which they cloned the flagellin gene *fliC* of *Salmonella* Typhimurium ATCC 14028
733 under the *B. longum hup* gene promoter (Takata et al., 2006). Significantly higher
734 levels of flagellin-specific IgA in the serum and stools of mice treated by oral
735 administration of this recombinant *B. animalis* than in those treated with parental *B.*
736 *animalis*, were detected (Takata et al., 2006). In a recent work, these authors studied
737 the potential effectiveness of genetically modified bifidobacteria as oral vaccines by
738 protecting mice from a lethal challenge with *Salmonella* in a typhoid fever model
739 (Yamamoto et al., 2010).

740 Another pioneering application of bifidobacteria as drug delivery system was that
741 of Long and collaborators (2010), who developed engineered bifidobacteria as oral
742 carriers of oxyntomodulin, a gut hormone that is used to reduce food intake and body
743 weight through intravenous administration. Interestingly, the results of this study
744 showed that oxyntomodulin-transformed *B. longum* reduced food intake, body weight
745 and decreases blood lipid in overweight mice. The benefits were identical to those

746 obtained by oral administration of Orlistat, a gastrointestinal lipase inhibitor drug
747 employed in obesity therapy (Long et al., 2010).
748 As suggested by recent studies, bifidobacteria may also be used for the *in situ*
749 delivery of human cytokines. The ani-inflammatory interleukin (IL)-10 has been
750 expressed in its mature form by *B. longum* ATCC 15707 (Reyes Escogido et al., 2007;
751 Table 3) and *B. breve* UCC2003 (Khokhlova et al., 2010). In the latter study, the gene
752 coding for the mature form of human IL-10 was translationally fused to previously
753 described *Bifidobacterium* signal peptides and placed under the control of
754 bifidobacterial constitutive promoters. Specifically, a pB80 replicon-based shuttle
755 vector carrying active promoter and terminator regions of *B. longum* gene *hup*, was
756 used to clone gene IL-10, which was fused with the signal peptide regions of genes
757 *sec2*, *apuB* or *amyB*, coding for *B. breve* secreted protein and extracellular
758 amylopullulanase and *B. adolescentis* secreted alfa-amylase, respectively. Sec2 signal
759 peptide was also placed under the control of constitutive promoter/terminator regions
760 from *B. longum* gene *gap*, coding for enzyme glyceraldehyde-3-phosphate
761 dehydrogenase. Interestingly, RT-qPCR experiments demonstrated that the expression
762 level of IL-10 driven by *gap* promoter was higher than that under the control of *hup*
763 promoter. Moreover, substitution of the Sec2 signal peptide-coding region with the
764 signal sequence from *amyB* gene resulted in an intensely elevated level of IL-10
765 mRNA (Khokhlova et al., 2010).
766
767 *5.4. Knock-out of bifidobacterial genes*
768 The prime method for studying the function of a gene and its impact on the overall
769 cellular physiology and morphology consists in removing or disrupting the gene from
770 its host (gene knock-out). This is generally accomplished by means of modification

771 systems based on homologous recombination. A low occurrence of homologous
772 recombination has been reported in bifidobacteria, which agrees well with the absence
773 of the major prokaryotic DNA recombination pathway encoded by *recBCD* in the
774 genome of some strains, such as *B. longum* NCC2705 (Schell et al., 2002). As
775 discussed previously, bifidobacteria display relatively low transformation efficiency.
776 Evidently, the recombination frequency in *Bifidobacterium* is thus generally lower than
777 that of transformation, limiting effective chromosomal integration of DNA. For these
778 reasons, successful homologous recombination in bifidobacteria has only recently been
779 reported.
780 In 2008, it was published successful knockouts of *B. breve* UCC2003 genes by
781 single-crossover recombination, employing two different strategies (O'Connel-
782 Motherway et al., 2008). First, O'Connel-Motherway and co-workers adopted a
783 double-vector integration strategy to disrupt the amylopullulanase gene *apuB*. The first
784 vector contained the origin of replication but lacked the *rep* gene coding for its
785 replication protein (Ori+/Rep- vector). The second vector, bearing a different antibiotic
786 resistance, was a derivative of the lactococcal temperature-sensitive plasmid pVE6007
787 and harboured the *rep* gene of the former plasmid (Rep+ vector). An internal 1 kb
788 DNA region of the *apuB* gene was cloned in the Ori+/Rep- plasmid, and both vectors
789 (Ori+/Rep- and Rep+) were then introduced in *B. breve* UCC2003 under double
790 antibiotic selection. In such recombinant cells, the Ori+/Rep- plasmid could replicate
791 only in the presence of the other vector, which supplied the Rep protein *in trans*. Once
792 the recombinant *B. breve* was cultivated at a high temperature (42 °C) and with
793 selection only for the Ori+/Rep- vector, the Rep+ plasmid was lost from *B. breve* cells
794 due to its temperature sensitivity and segregational instability. Accordingly, under
795 selective conditions, the Ori+/Rep- plasmid has to integrate into the chromosome.

796 Thanks to this approach, the authors found by replica plaiting some *B. breve apuB*
797 disruption isolates, which exhibited the expected phenotype and in which they verified
798 plasmid integration by PCR and Southern hybridization. However, O'Connell-
799 Motherway and collaborators emphasized that this system is very tedious, time-
800 consuming, and unreliable (O'Connell-Motherway et al., 2008).
801 In a later publication, the same researchers described insertional mutagenesis of
802 the *apuB* gene and the endogalactanase gene (*galA*) of *B. breve* UCC2003 by means of
803 plasmid methylation (O'Connell-Motherway et al., 2009). This strategy produced gene
804 disruptions by single-crossover chromosomal integration of non-replicative plasmids
805 containing internal fragments of 476 and 744 bp of the *gal*A gene, and a 939 bp
806 internal fragment of the *apuB* gene. These three plasmids were first introduced into an
807 *E. coli* host harbouring two *B. breve* UCC2003 methylase genes (*bbrIIM* and *bbrIIIM*).
808 The resulting methylated plasmids were then introduced into *B. breve* UCC2003 by
809 electroporation. Antibiotic-resistant transformants were isolated for all methylated
810 vectors at a frequency of up to 50 per μg of transformed DNA. No transformants were
811 obtained when unmethylated constructs were introduced into strain UCC2003. The
812 expected integration in the chromosome was finally verified by genetic and phenotypic
813 analyses. These results by O'Connell-Motherway et al. (2009) showed that methylation
814 of the non-replicating plasmid by *B. breve* UCC2003 methylases increased
815 transformation efficiency to a level sufficiently high to allow site-specific homologous
816 recombination to occur. This strategy has allowed the dissection of many genes from
817 the UCC2003 strain, including gene components of clusters involved in utilization of
818 ribose (Pokusaeva et al., 2010), insoluble cellulose (cellodextrin) (Pokusaeva et al.,
819 2011), and galactans (O'Connell Motherway et al., 2011b).

Also recently, Arigoni and Delley (2008) patented a gene deletion method in *Bifidobacterium* by two consecutive events of homologous recombination. The authors reported deletion of the tetracycline resistance gene *tet*(W) from *B. animalis* subsp. *lactis* NCC2818 (commercially known as strain BB12). 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. The application of plasmid artificial modification (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 rudimentary 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. 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 to the study of physiological and probiotic properties of bifidobacteria. Gene knock-out is, in fact, the golden procedure to unambiguously understand the role of a specific coding sequence. A few studies (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 different expedients, which can be deduced from the investigations presented in this review article, such as 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. To this aim, it appears promising the set-up of protocols for the *in vitro* methylation of recombinant DNA before transformation, in spite of the improved transformation efficiency obtained by the laborious method of the PAM strategy.

Nowadays, the European regulation concerning health claims on product labels (EC regulation 1924/2006) 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. In addition to technological reasons, such rapidly growing whole genome sequence data, the increasing interest of food and pharmaceutical industries on probiotics is boosting the research on bifidobacteria. It is, therefore, expected that in the next few years further steps will be done in the genetic modification of these organisms.

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Disclosure

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Figure Legends

Fig. 1. Trees produced by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) 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.

Fig. 2. Linear functional maps of a representative plasmid from all the *Bifidobacterium* plasmid structures up to now recognized (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 colors/motif share a significant sequence similarity. Genes represented with white arrows are harbored by only one of the shown plasmids. Rep typology in accordance to Fig. 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 mobilization 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

1333 domain of the FtsK/SpoIIIE family. This domain contains a putative ATP binding P-
1334 loop motif. A mutation in FtsK causes a temperature sensitive block in cell division
1335 and it is involved in peptidoglycan synthesis or modification. The SpoIIIE protein is
1336 implicated in intercellular chromosomal DNA transfer (CDD accession code
1337 pfam01580). *tet*(Q), tetracycline resistance gene encoding a ribosomal protection
1338 protein. *copG*, orf ecoding a putative protein that shares similarity with the plasmid
1339 pMV158-encoded transcriptional repressor CopG (CDD accession code pfam01402).
1340 *par*, gene encoding for a putative protein belonging to the ParA conserved family of
1341 bacterial proteins (CDD accession code cd02042), implicated in chromosome
1342 segregation (involved in the plasmid replication and partition). RE gene, gene encoding
1343 a putative protein with a type II restriction endonuclease domain (*Eco*RII, CDD
1344 accession code pfam09019). peptidase gene, encoding for a putative member of
1345 peptidase family C39 (cd02549). Peptidase family C39 mostly contains bacteriocin-
1346 processing endopeptidases from bacteria. *mobA* and *mobB*, mobilization protein genes
1347 harbored by plasmids from the *Cytophaga–Flavobacterium–Bacteroides* group of
1348 bacteria.
1349 **Fig. 3.** Strategies for the preparation of vector DNA to introduce by electroporation in
1350 bifidobacteria. (A) Conventional strategy, involving the extraction of shuttle vector
1351 DNA from *E. coli* and direct introduction in *Bifidobacterium* cells. (B) Plasmid
1352 Artificial Modification (PAM) strategy: 1 – preparation of the PAM host, consisting of
1353 an appropriate *E. coli* strain (e.g. *E. coli* TOP10) harbouring PAM vector (a low copy
1354 number vector coding for the methylase(s) of the target host); 2 – introduction of an *E.*
1355 *coli-Bifidobacterium* shuttle vector into the PAM host; 3 – modification of shuttle
1356 vector DNA by methylase(s) coded by PAM vector; 4 – extraction of the now
1357 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.

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	2111	Theta (*II*)	Cronin et al. 2007 / EU030683
B. asteroides	DSM20089	pAP1	2140	Theta (*II*)	Y11549
B. bifidum	B80	pB80	4898	RC (*Ia*)	Shkoporov et al. 2008a / DQ305402
B. bifidum	CCTCC M203049	pBIF10	9275	RC (*IV*)	DQ093580
B. breve	NCFB2258	pCIBb1	5750	RC (*III*)	O'Riordan and Fitzgerald 1999 / AF085719
B. breve	B21a	pB21a	5206	RC (*III*)	Shkoporov et al. 2008a / DQ497626
B. breve	-	pNBb1	2297	RC (*III*)	E17316
B. catenulatum	L48	pBC1	2540	Theta (*V*)	Alvarez-Martin et al. 2007 / DQ011664
B. longum	KJ	pKJ36	3625	RC (*Ib*)	Park et al. 1997 / AF139129
B. longum	KJ	pKJ50	4960	RC (*Ia*)	Park et al. 1999 / BLU76614
B. longum	NCC2705	pBLO1	3626	RC (*Ib*)	Schell et al. 2002 / AF540971
B. longum	MG1	pMG1	3682	RC (*Ib*)	Park et al. 2003 / AY210701
B. longum	RW048	pNAC1	3538	RC (*Ia*)	Corneau et al. 2004 / AY112724
B. longum	RW041	pNAC2	3684	RC (*Ib*)	Corneau et al. 2004 / AY112723
B. longum	RW041	pNAC3	10224	Theta (*II*)	Corneau et al. 2004 / AY112722
B. longum	BK51	pTB6	3624	RC (*Ib*)	Tanaka et al. 2005 / AB187597
B. longum	B2577	pMB1	1847	Theta (*V*)	Rossi et al. 2006 / X84655
B. longum	DJO10A	pDOJH10L[3]	10073	Theta (*II*)	Lee and O'Sullivan 2006 / AF538868
B. longum	DJO10A	pDOJH10S	3661	Theta (*V*)	Lee and O'Sullivan 2006 / AF538869
B. longum	NAL8	pNAL8L	3489	RC (*Ia*)	Guglielmetti et al. 2007 / AM183145
B. longum	NAL8	pNAL8M	4910	RC (*Ia*)	Guglielmetti et al. 2007 / AM183144
B. longum	VMKB44	pB44	3624	RC (*Ib*)	Shkoporov et al. 2008a / AY066026
B. longum	FI10564	pFI2576	2197	Theta (*V*)	Moon et al. 2009 / DQ452864
B. longum	BK28	pBK283	4537	RC (*Ia*)	Fukiya et al. 2010 / AB495342
B. longum	DPC6043	p6043A	4896	RC (*Ia*)	DQ458911
B. longum	DPC6043	p6043B	3680	RC (*Ib*)	DQ458910
B. longum	M62	pSP02	4896	RC (*Ia*)	GU256055
B. pseudocatenulatum	VMKB4M	p4M	4488	RC (*VI*)	Gibbs et al. 2006 / AF359574
B. pseudolongum subsp. *globosum*	DPC479	pASV479	4815	RC (*III*)	Sangrador-Vegas et al. 2007 / DQ103758
Bifidobacterium sp.	A24	pBIFA24	4892	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 is in accordance with dendrograms in Fig. 1. [3]DNA sequence assessment suggests that pDOJH10L is a cointegrate involving plasmids very similar to pKJ50 (96% identity) and pNAC2 (98% identity).

1369 **Table 2.-** Summary of protocols for electrotransformation (electroporation) of bifidobacteria.

Vector (size, kb) (marker)	Replicon(s)	Growth medium[1]	Washing buffer	Electroporation buffer	Preincubation[2]	Voltage and resistance	Recovering medium	Transformation rate (transformants/µg DNA)	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 pH6, 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 $5.0x10^3$ *B. breve* 4 $1.3x10^4$ *B. breve* AS $2.0x10^2$ *B. bifidum* U3 $3.0x10^2$ *B. bifidum* ATCC 15696 $7.4x10^3$ *B. infantis* U1 $2.5x10^2$ *B. infantis* ATCC 27920 $4.0x10^4$ *B. longum* U2 $2.6x10^3$ *B. longum* Wiesby 2 $7.0x10^4$	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 pH7	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 $3.0x10^4$ *B. breve* MB226 $6.6x10^4$ *B. breve* MB252 $2.3x10^4$ *B. bifidum* MB254 $7.2x10^4$ *B. infantis* MB208 $1.2x10^5$ *B. infantis* MB263 $9.3x10^3$ *B. longum* MB231 $2.8x10^2$ *B. pseudocatenulatum* MB264 $5.0x10^1$ *B. ruminale* MB266 $7.2x10^2$ *B. dentium* MB269 $3.6x10^1$ *B. magnum* MB267 $1.8x10^3$	Rossi et al. 1997[3]
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 $3.8x10^2$	Missich et al. 1994
pBLES100 (9.1 kb) (Sp^R)	pTB6[4]-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 $2.2x10^4$	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 $2.0x10^2$	Park et al. 1999
pBES2 (7.6 kb) (Cm^R)	pMG1[4]-pUC (ColE1)	According to Argnani et al. (1996), with the addition of Oxyrase® (Oxyrase inc. Ohio) in the recovering medium						*B. longum* MG1 $7.3x10^3$	Park et al. 2003

pBRASTA101 (5.0 kb) (Sp^R)	pTB6[4]-pUC (ColE1)	According to Missich et al. (1994) and Matsumura et al. (1997)						*B. longum* 105-A 2.5x10^6	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 3.8x10^6 *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 ~10^5 *B. breve* NCFB 2258 ~10^5	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 9.2x10^2 *B. animalis* LMG10508 4.0x10^1 *B. animalis* subsp. *lactis* Bb12 1.6x10^2 *B. breve* LMG13208 1.0x10^2 *B. breve* UCC2003 1.4x10^2 *B. dentium* F101 9.5x10^1 *B. longum* L25 6.6x10^1 *B. pseudolongum* LMG11571 6.3x10^1 *B. pseudocatenulatum* M115 1.0x10^5 *B. thermophilus* LMG11571 4.6x10^1 *C. glutamicum* LMG19741 3.0x10^0	Álvarez-Martín et al. 2008

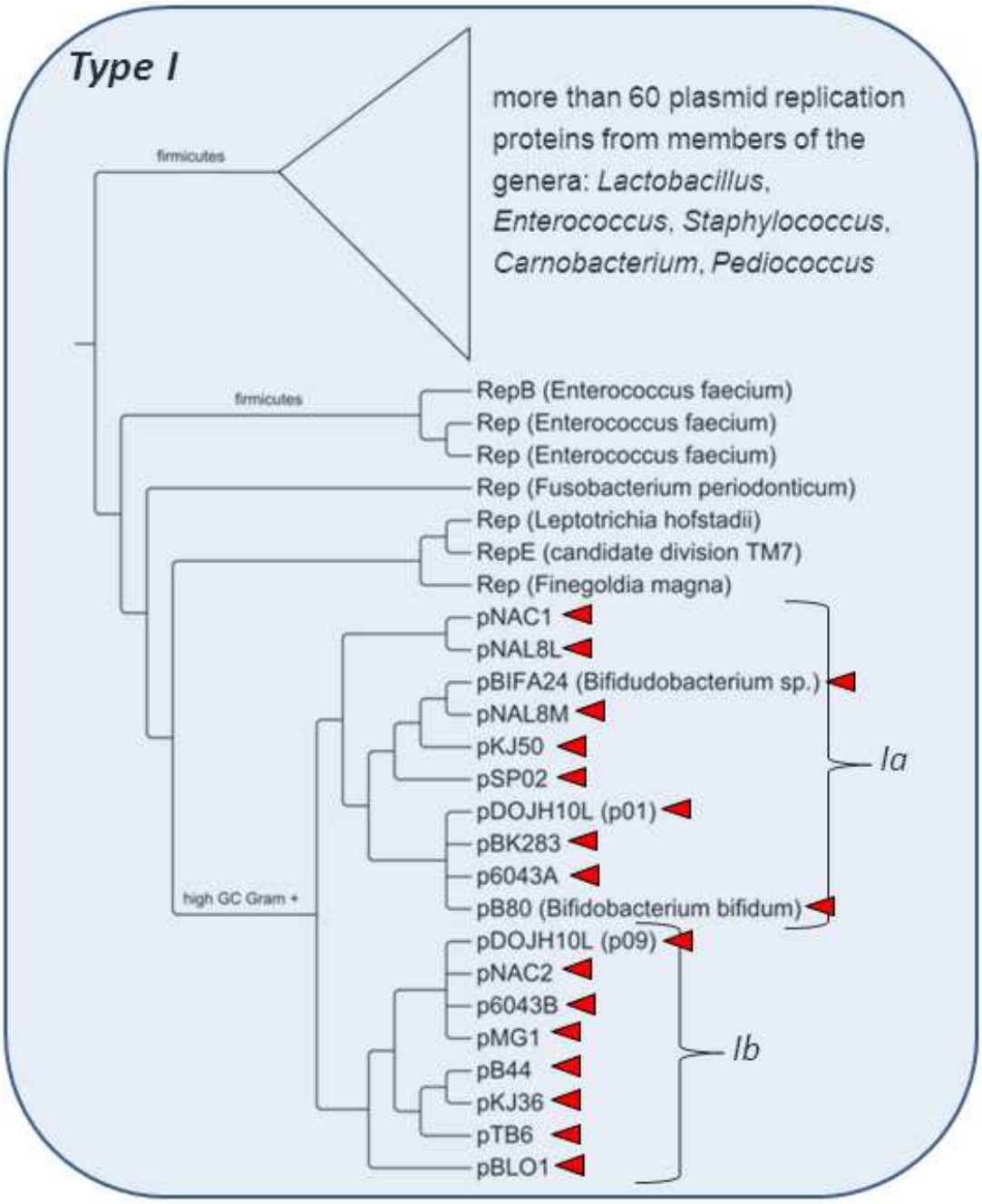
[1]Phase of growth at which bifidobacterial cells are collected before washing steps.
[2]Incubation step of competent cells before electroporation or storing at -80°C.
[3]Trasformation experiments were performed with vector DNA isolated from *B. animalis* MB209.
[4]pTB46 and pMG1 plasmids are isogenic to pB44, pNAC2, pBLO1, pDOJ10L, and pKJ36.
B. animalis ATCC 27536 is also known as *B. animalis* MB209; *B. infantis* stands for *Bifidobacterium longum* subsp. *infantis*.
Key of antibiotic markers: Cm^R, chloramphenicol acetyl transferase (*cat*); Tet^R, tetracycline resistance [*tet*(W)]; Sp^R, spectinomycin resistance; Ery^R, erythromycin resistance.

1379 **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	Li et al. 2003; Fu et al. 2005; Xu et al. 2007; Hu et al., 2009
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

1380 **Fig.1**



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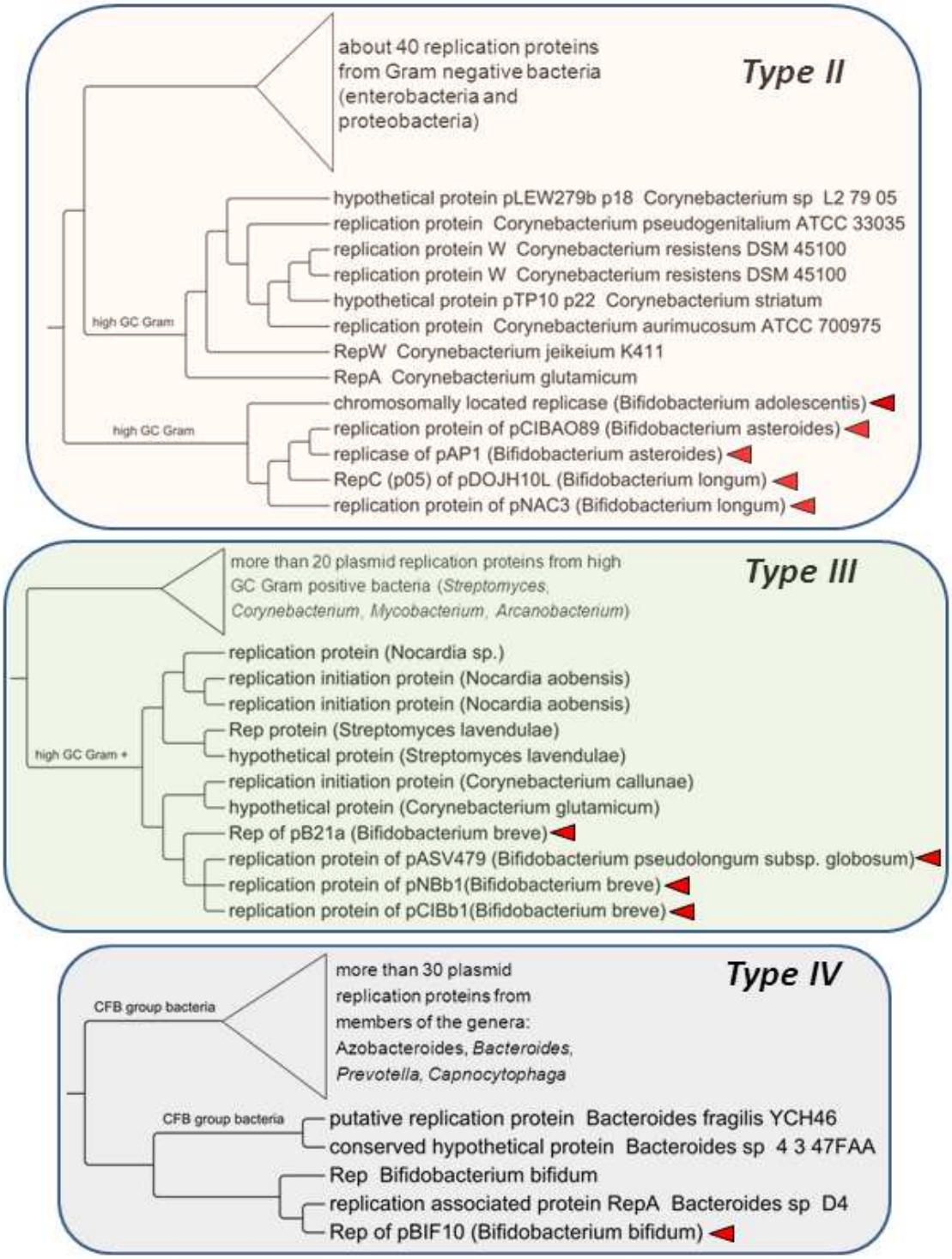
Type II
about 40 replication proteins from Gram negative bacteria (enterobacteria and proteobacteria)
hypothetical protein pLEW279b p18 Corynebacterium sp L2 79 05
replication protein Corynebacterium pseudogenitalium ATCC 33035
replication protein W Corynebacterium resistens DSM 45100
replication protein W Corynebacterium resistens DSM 45100
hypothetical protein pTP10 p22 Corynebacterium striatum
replication protein Corynebacterium aurimucosum ATCC 700975
RepW Corynebacterium jeikeium K411
RepA Corynebacterium glutamicum
chromosomally located replicase (Bifidobacterium adolescentis)
replication protein of pCIBAO89 (Bifidobacterium asteroides)
replicase of pAP1 (Bifidobacterium asteroides)
RepC (p05) of pDOJH10L (Bifidobacterium longum)
replication protein of pNAC3 (Bifidobacterium longum)
high GC Gram
high GC Gram
Type III
more than 20 plasmid replication proteins from high GC Gram positive bacteria (Streptomyces, Corynebacterium, Mycobacterium, Arcanobacterium)
replication protein (Nocardia sp.)
replication initiation protein (Nocardia aobensis)
replication initiation protein (Nocardia aobensis)
Rep protein (Streptomyces lavendulae)
hypothetical protein (Streptomyces lavendulae)
replication initiation protein (Corynebacterium callunae)
hypothetical protein (Corynebacterium glutamicum)
Rep of pB21a (Bifidobacterium breve)
replication protein of pASV479 (Bifidobacterium pseudolongum subsp. globosum)
replication protein of pNBb1(Bifidobacterium breve)
replication protein of pCIBb1(Bifidobacterium breve)
high GC Gram +
Type IV
more than 30 plasmid replication proteins from members of the genera: Azobacteroides, Bacteroides, Prevotella, Capnocytophaga
CFB group bacteria
CFB group bacteria
putative replication protein Bacteroides fragilis YCH46
conserved hypothetical protein Bacteroides sp 4 3 47FAA
Rep Bifidobacterium bifidum
replication associated protein RepA Bacteroides sp D4
Rep of pBIF10 (Bifidobacterium bifidum)

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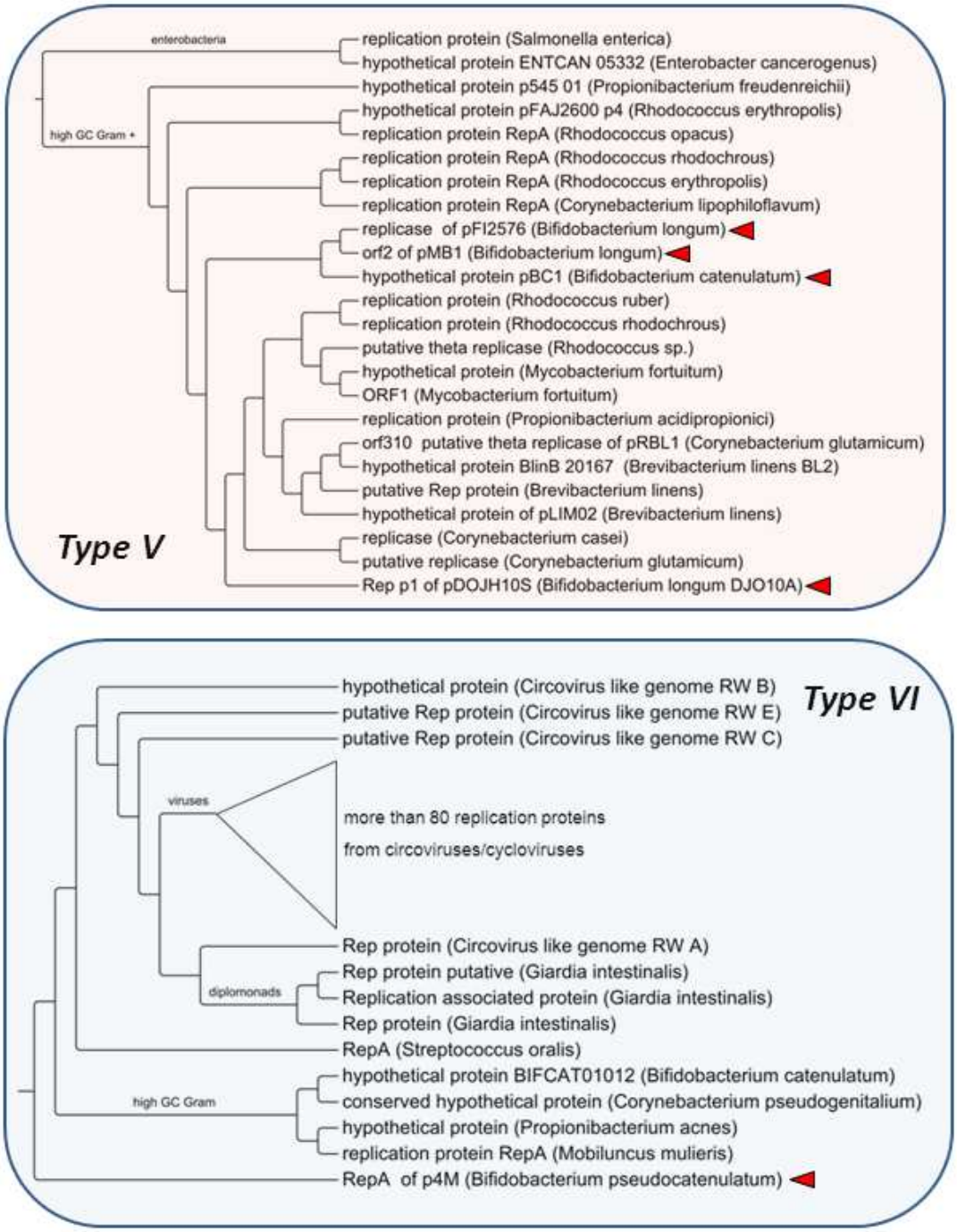
enterobacteria
high GC Gram +
replication protein (Salmonella enterica)
hypothetical protein ENTCAN 05332 (Enterobacter cancerogenus)
hypothetical protein p545 01 (Propionibacterium freudenreichii)
hypothetical protein pFAJ2600 p4 (Rhodococcus erythropolis)
replication protein RepA (Rhodococcus opacus)
replication protein RepA (Rhodococcus rhodochrous)
replication protein RepA (Rhodococcus erythropolis)
replication protein RepA (Corynebacterium lipophiloflavum)
replicase of pFI2576 (Bifidobacterium longum)
orf2 of pMB1 (Bifidobacterium longum)
hypothetical protein pBC1 (Bifidobacterium catenulatum)
replication protein (Rhodococcus ruber)
replication protein (Rhodococcus rhodochrous)
putative theta replicase (Rhodococcus sp.)
hypothetical protein (Mycobacterium fortuitum)
ORF1 (Mycobacterium fortuitum)
replication protein (Propionibacterium acidipropionici)
orf310 putative theta replicase of pRBL1 (Corynebacterium glutamicum)
hypothetical protein BlinB 20167 (Brevibacterium linens BL2)
putative Rep protein (Brevibacterium linens)
hypothetical protein of pLIM02 (Brevibacterium linens)
replicase (Corynebacterium casei)
putative replicase (Corynebacterium glutamicum)
Rep p1 of pDOJH10S (Bifidobacterium longum DJO10A)
Type V
hypothetical protein (Circovirus like genome RW B)
putative Rep protein (Circovirus like genome RW E)
putative Rep protein (Circovirus like genome RW C)
Type VI
viruses
more than 80 replication proteins
from circoviruses/cycloviruses
Rep protein (Circovirus like genome RW A)
diplomonads
Rep protein putative (Giardia intestinalis)
Replication associated protein (Giardia intestinalis)
Rep protein (Giardia intestinalis)
RepA (Streptococcus oralis)
high GC Gram
hypothetical protein BIFCAT01012 (Bifidobacterium catenulatum)
conserved hypothetical protein (Corynebacterium pseudogenitalium)
hypothetical protein (Propionibacterium acnes)
replication protein RepA (Mobiluncus mulieris)
RepA of p4M (Bifidobacterium pseudocatenulatum)

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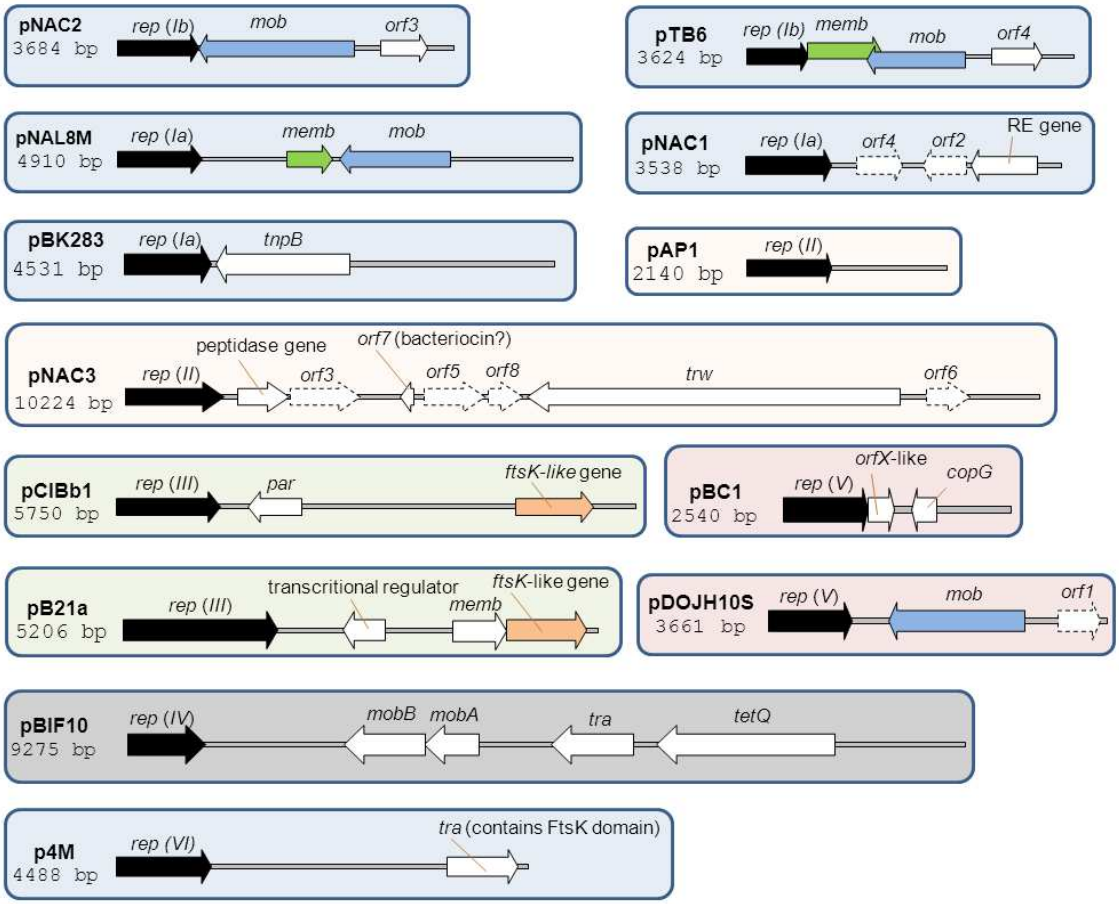
1385 **Fig. 2**



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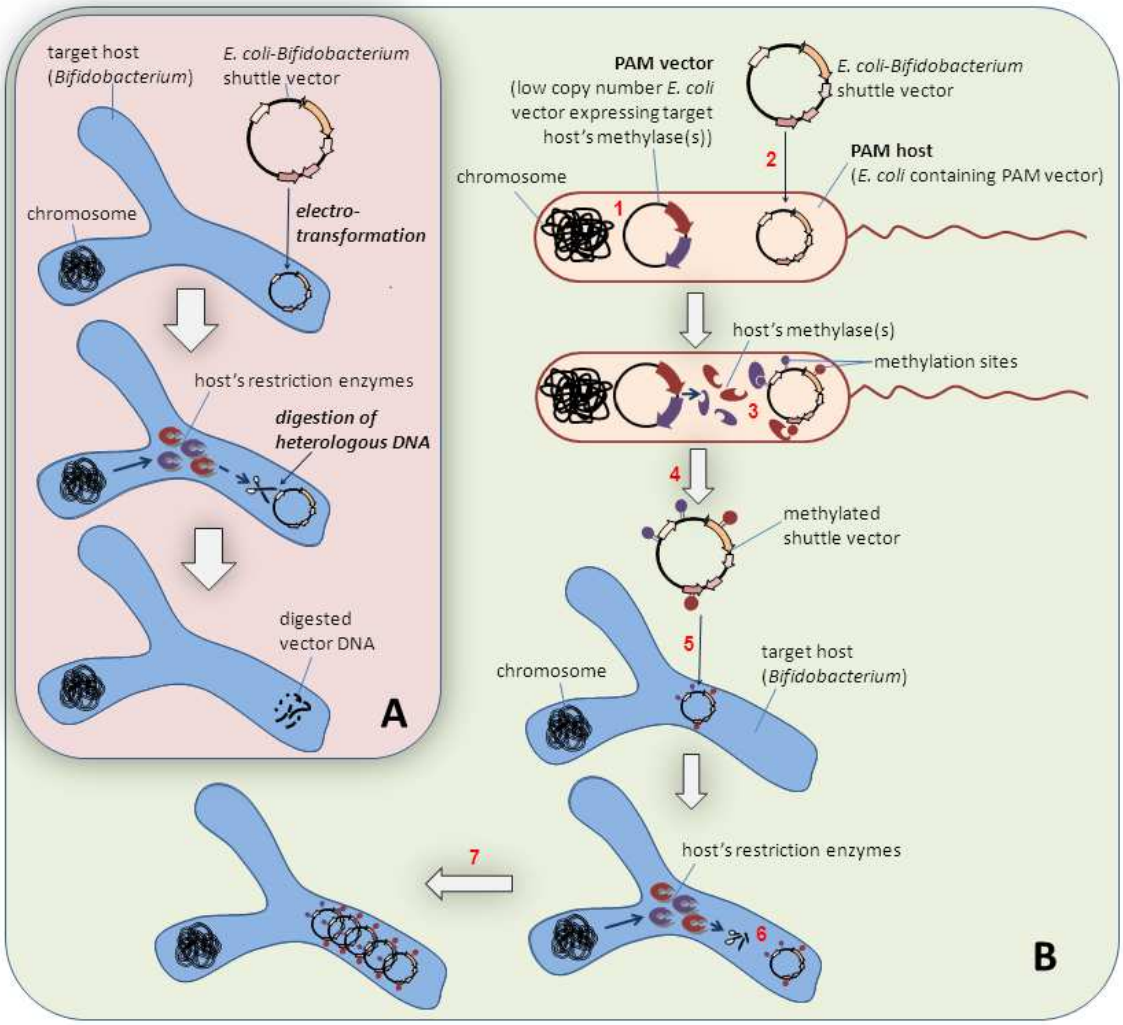
1388 **Fig. 3**



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