Improved cloning vectors for bifidobacteria based on the *Bifidobacterium catenulatum* pBC1 replicon

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This study reports the development of several cloning vectors for bifidobacteria based on the replicon of pBC1, a cryptic plasmid from *Bifidobacterium catenulatum* L48 thought to replicate via the theta mode. These vectors, in which antibiotic resistance genes encoding either erythromycin or tetracycline resistance acted as selection markers, were able to replicate in a series of eight *Bifidobacterium* species at frequencies ranging from $4.0 \times 10^1$ to $1.0 \times 10^5$ transformants $\mu g^{-1}$, but not in *Lactococcus lactis* or *Lactobacillus casei*. They showed a relative copy number of around 30 molecules per chromosome equivalent, and a good segregational stability, with more than 95% of the cells retaining the vectors after 80-100 generations in the absence of selection. Vectors contain multiple cloning sites of different length, and the *lacZa* peptide gene was introduced in one of the molecules, thus allowing easy selection of colonies harboring recombinant plasmids in *Escherichia coli*. The functionality of the vectors for engineering *Bifidobacterium* strains was assessed by cloning and examining the expression of an $\alpha$-L-arabinofuranosidase gene belonging to *Bifidobacterium longum*. *E. coli* and *Bifidobacterium pseudocatenulatum* recombinant clones were stable and showed an increase in $\alpha$-arabinofuranosidase activity of over 100-fold compared to the untransformed hosts.

**INTRODUCTION**

*Bifidobacterium* species are among the dominant microbial populations of the gastrointestinal tract (GIT) of humans and other mammals (8, 33), where they are considered to exert many beneficial health effects (for a review see 19) including the establishment of a healthy microbiota in infants, the development of a competent...
immune system, the production of short chain fatty acids, and the inhibition of pathogens (19, 45). Not surprisingly, bifidobacteria are major components of many commercial probiotic products that have been shown effective in alleviating constipation, reducing the symptoms of lactose intolerance, enhancing immune functions, reducing cholesterol levels, and the suppression of tumorigenesis (19, 29).

Unfortunately, our basic knowledge of the mechanisms by which bifidobacteria interact and communicate with other bacteria and host cells remains poorly understood. Such knowledge is essential for the scientific support of their purported health benefits and their rational inclusion as probiotics in functional foods (19), but the study of these organisms’ probiotic properties and their contribution to host health and well-being has been hampered by a lack of molecular tools (50). In addition, the study of the variables affecting the transformation of plasmid DNA in *Bifidobacterium* species, and the optimization of the transformation process, have only rarely been addressed (3, 36, 37).

Bifidobacteria belong to the phylum *Actinomycetaceae* -Gram-positive microorganisms with high G+C content that have complex nutritional requirements and which are very sensitive to oxygen (41); these characteristics (strict anaerobes, nutritionally fastidious, and instable DNA cloning in *Escherichia coli*) may have limited the study of their genetics.

Recently, the genome sequences of *Bifidobacterium longum* NCC 2705 (42), *B. longum* DJO10A (NZ_AABM00000000), *B. adolescentis* ATCC 15703 (NC_008618) and *B. adolescentis* L2–32 (NZ_AXD02000000) have been released, providing a vast array of genetic data which may help us better understand the mode of action behind their probiotic properties (15). However, the genomic data available cannot be fully exploited due to the limitations of our current molecular tools for the analysis of gene
function and regulation. Therefore, new, improved vectors for cloning, integration, knockout and gene expression studies are required. Molecular studies are also required for the future improvement of Bifidobacterium strains by genetic engineering, i.e., the construction of strains with enhanced probiotic characteristics and/or that better retain their viability during storage. Furthermore, bifidobacteria are thought to be promising systems for the delivery of therapeutic agents, such as antigens (for live vaccine development) and tumor-suppressing substances (10, 53), and as a means of increasing beneficial detoxifying activities (31).

Until recently, only fragmentary information on the bacteriophages infecting Bifidobacterium species was available (44). Moreover, phages infecting bifidobacteria have never been isolated and characterized. Indeed, genome sequencing has only identified a single related prophage-like element in each of the genomes of the sequenced strains B. breve UCC 2003 (not yet released), B. longum NCC 2705 and B. longum DJO10A (51). Thus, bifidobacterial plasmids are the only available source of replicons for bifidobacterial vectors. Extrachromosomal elements seem not to be very common among Bifidobacterium strains (43). Nonetheless, 14 fully-sequenced plasmids from eight bifidobacterial species are reported in the GenBank database (http://www.ncbi.nlm.nih.gov/sites/entrez). However, the basic biology of plasmids in this genus remains poorly understood; indeed, in only a few has the mode of replication been analyzed (4, 21, 27, 30, 32). Further, the dissection of the open reading frames (ORFs) and the analysis of untranslated sequences and structures has been undertaken in only a couple of plasmids (2, 5). In spite of this, a bunch of all-purpose and specific vectors have already been constructed and used in different studies. As an example, pMDY23, a reporter vector, carries the gusA gene of E. coli (18); vector pBES2 has
been used to express the \( \alpha \)-amylase gene of *B. adolescentis* in *B. longum* (34); pBLES100 (25) has been used in tumor suppressor studies (54) and for the expression of the flagellum protein gene(s) of *Salmonella* (for mucosal immunization purposes) (46); and pBV22210 has been used to express and deliver the anti-cancer protein endostatin in cancer gene therapy (53).

The present study reports on the further characterization of plasmid pBC1 from *B. catenulatum* L48 (1, 2) and its use in the development of improved cloning vectors. The plasmid has already been cloned entirely in a pUC derivative in a previous work (1). Furthermore, the pUC part of the resulting shuttle vector was removed, demonstrating that necessary replicating elements were all within the pBC1 DNA (1). These two constructs can be considered true cloning vectors because they have several unique restriction enzyme sites at non-essential positions in their sequences and antibiotic resistance genes allowing selection. In this work, the construction of a series of new *E. coli*-Bifidobacterium shuttle vectors is reported. These include the replacement of the erythromycin resistance gene by a tetracycline resistance gene of bifidobacterial origin, the insertion of a large polylinker, and the cloning of the \( \alpha \)-galactosidase complementing-peptide gene for a convenient blue-white screening of recombinant clones in *E. coli*. The study of copy number, stability and host range of some vectors was also addressed. To check the functionality of these vectors, an \( \alpha \)-L-arabinofuranosidase gene from *B. longum* B667 was cloned and overexpressed in both *E. coli* and Bifidobacterium strains.
MATERIAL AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bifidobacteria, lactobacilli and *Pediococcus acidilactici* strains were routinely cultivated under anaerobic conditions at 37°C in MRS broth (Merck; VWR International, Darmstad, Germany) or in RCM broth (Merck; VWR International, Darmstad, Germany), supplemented with 0.25% (w/v) L-cysteine (MRSC). *Lactococcus* and *Enterococcus* strains in Table 2 were grown in M17 (Scharlau, Scharlau Chemie SA, Barcelona, Spain). *E. coli* and *Corynebacterium glutamicum* strains were grown at 37°C in Luria Bertani (LB) broth (38) with vigorous shaking.

Antibiotics (supplied by Sigma, Sigma Chemical Co., St. Louis, MO) were added to the appropriate media at the following concentrations: ampicillin 100 µg ml\(^{-1}\), erythromycin 250 µg ml\(^{-1}\) and tetracycline 5 µg ml\(^{-1}\) for *E. coli*; erythromycin 5 µg ml\(^{-1}\), tetracycline 5 µg ml\(^{-1}\) and chloramphenicol 2 µg ml\(^{-1}\) for bifidobacteria.

DNA isolation and analysis. Plasmid DNA from bifidobacteria was isolated by the method of O’Sullivan and Klaenhammer (28) with the following modification: pellets were suspended in TSE buffer (sucrose 25%, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and incubated with lysozyme (30 mg/ml) at 37°C for 30 min. Plasmid DNA from *E. coli* was isolated using the commercial Plasmid Miniprep Kit (Eppendorf AG, Hamburg, Germany), following the manufacturer’s recommendations. Plasmids were analyzed by electrophoresis in TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) on 0.75-1.2% agarose gels (FMC Bioproducts, Philadelphia, PA), and then stained with ethidium bromide (0.5 µg ml\(^{-1}\)) and photographed.
Total DNA from the *B. longum* B667 was prepared according to the procedure of Tanaka et al. (47), and analyzed as above.

**DNA manipulation and molecular techniques.** The general procedures used for DNA manipulation were essentially those described by Sambrook and Russell (38). Restriction endonucleases and *Taq* DNA polymerase came from Takara (Takara, Otsu, Shiga, Japan), T4 DNA ligase from Invitrogen (Invitrogen, Carlsbad, CA), and the Klenow fragment of *E. coli* polymerase I from Roche (Roche Applied Sciences, Basel, Switzerland). All were used according to the manufacturers’ instructions. Amplicons were purified by using the GFX PCR DNA Gel Band Purification kit (GE Healthcare Biosciences, Buckinghamshire, UK). When required, purified plasmids and amplicons were sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

**Plasmid transfer.** Plasmids were introduced into *E. coli* DH5α electrocompetent cells by electroporation (electrotransformation) (38) using a Gene-Pulser Apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer’s instructions. Electrotransformation of lactic acid bacteria strains (*Lactococcus lactis, Lactobacillus casei* and *Enterococcus durans*) was essentially done as reported by Leenhouts et al. (22), using as a positive control plasmid p210 from *L. lactis* (39). *Corynebacterium glutamicum* and *Pediococcus acidilactici* strains were electrotransformed as previously reported (49). Electrocompetent *Bifidobacterium* cells were prepared by optimizing previously reported methods (1, 2). In short, fresh MRSC broth (50 ml) was inoculated with an overnight culture (8% inoculum) of the bifidobacterial strain and incubated at 37°C for 3-4 h until the culture reached an OD$_{600 \text{ nm}}$ of 0.5-0.7. The cells were then chilled for 20 min, washed twice in ice-cold sucrose-citrate buffer (0.5 M sucrose, 1
mM ammonium citrate, pH 5.8) and suspended in 100 µl of the same buffer. The cell suspension was stored on ice for 20 min. Electroporation was performed under the following conditions: 25 µF, 200 Ω and 10 kV. The cells were immediately diluted in 950 µl of RCM and incubated for 2.5 h before plating onto the same agarified medium with the appropriate antibiotic. Plates were incubated for 2-3 days at 37ºC under anaerobic conditions.

**Detection of ssDNA intermediates by hybridization.** Total DNA from *Bifidobacterium* and *E. coli* cells grown in the presence or absence of both chloramphenicol and rifampicin was isolated essentially as described by te Riele et al. (48). The DNA was electrophoresed in a 0.7% agarose gel, and transferred under denaturing and non-denaturing conditions to Hybond-N nylon membranes (Amersham Biosciences, Uppsala, Sweden). ssDNA intermediates were detected by hybridization using pBM02-derived and pBC1-derived DNA probes internally labeled with [α-32P]dCTP (GE Healthcare).

**Segregation stability of vectors in bifidobacteria.** The stability of the constructs was assayed by growing the cells in non-selective media for approximately 100 generations and plating daily onto non-selective agar plates. Antibiotic resistance maintenance was monitored by transference of the resulting colonies to antibiotic-containing agar plates. Finally, plasmids was monitored by plasmid extraction from antibiotic resistant and susceptible colonies as above.

**Antibiotic resistance of the vectors.** The minimum inhibitory concentration (MIC) of erythromycin and tetracycline supported by the constructs in different hosts was measured by the Etest method, according to the manufacturer’s instructions (AB Biodisk, Solna, Sweden). MIC assays were performed in LSM medium (90%
Isosensitest, 10% MRS; both from Oxoid, Oxoid Ltd., Basingstoke, Hampshire, UK) with cysteine (0.3 g l\(^{-1}\)) as previously reported (17).

**Determination of the relative plasmid copy number.** The relative copy number of the pBC1-derived vectors was assessed by quantitative real-time PCR (QPCR), using the culture and PCR conditions reported by Lee et al. (20). Amplification and detection were performed in a Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) using Power SYBER® Green PCR Master Mix (Applied Biosystems). The FrepB and RrepB primers (Table 1) were designed based on the pBC1 repB sequence (in which their oligonucleotide sequences were 113 bp apart). The xylulose-5-phosphate-fructose-6-phosphate-phosphoketolase gene (\(xfp\)) (GenBank Accession No. AY377401) of \(B.\) pseudocatenulatum M115 was used as the single-copy reference gene. A 120 bp segment of the \(xfp\) gene was amplified with primers Fxfp and Rxfp (Table 1). The relative copy number of the derivatives was calculated using the formula \(N_{\text{relativa}}=(1+E)^{-\Delta C T}\) (19), where E is the amplification efficiency of the target and reference genes, and \(\Delta C T\) is the difference between the threshold cycle number (CT) of the \(xfp\) reaction and that of \(repB\). Experiments were performed in triplicate; mean results are provided.

**Cloning and expression of an \(\alpha\)-L-arabinofuranosidase gene from \(B.\) longum.** The \(\alpha\)-L-arabinofuranosidase gene (\(abfB\)) in the \(B.\) longum B667 strain has been characterized by Margolles and de los Reyes-Gavilán (24). Amplification of the \(abfB\) gene was accomplished with primers abfBF and abfBR, in which \(SphI\) and \(PstI\) sites were inserted, using genomic DNA from \(B.\) longum B667 as a template. The PCR product was purified, digested with \(SphI\) and \(PstI\), cloned into pAM1 digested with these two enzymes, and ligated overnight at 12ºC. The ligation mixture was electrotransfomed into \(E.\) coli DH11S, in which the construct (pAM-abfB) was obtained.
(it proved to be not viable in *E. coli* TOP10) and verified by the use of restriction
enzymes and sequencing. pAM-abfB was then transferred by electroporation into *B.
*pseudocatenulatum* M115 cells.

**Determination of α-L-arabinofuranosidase activity.** α-L-arabinofuranosidase
activity in the cloning hosts and recombinant cells was determined according to
Gueimonde et al. (11). Briefly, pellets were suspended in 2 ml of potassium phosphate
buffer (pH 6.8) and the cells disrupted with a Cell Disruptor (Constant Systems Ltd.,
Daventry, Northants, UK). Activity was measured in triplicate using independent cell-
free extracts.

**RESULTS**

**Construction of vectors based on the pBC1 replicon.** The pAM1 shuttle vector
resulting for the cloning of pBC1 in pUC19E (1) was taken at the starting material for
the construction of more versatile pBC1-derived vectors. Firstly, the heterologous
erythromycin resistance gene in pAM1, originally isolated from the *Staphylococcus
aureus* plasmid pE194 (14), was replaced by a recently characterized *tet*(*W*) gene
identified in an intestinal isolate of *B. longum* (9). A 2467 bp segment of DNA
including the *tet*(*W*) gene and its upstream promoter sequences was amplified by PCR
with specific primers, into which the *Sac*I sites were incorporated. The *tet*(*W*) gene was
inserted into the unique *Sac*I site of pAM1. The new construct, pAM3 (Fig. 1), was
recovered in *E. coli* and then transformed into *B. pseudocatenulatum*. The erythromycin
resistance gene of pAM3 was finally removed (to give pAM4, Fig. 1) by partial
digestion with *Sal*I, isolation of the right fragment from an agarose gel, intramolecular
ligation and electroporation of the ligation mixture into *E. coli*. The new construct was
finally electrotransformed in *B. pseudocatenulatum* and other bifidobacteria. New single restriction enzyme sites were introduced in pAM4 by cloning the 28 unique recognition sequences from the multiple cloning site (MCS) of pUK21 (52). The MCS was recovered from a gel after digestion of pUK21 with *SpeI* and then ligated to pAM4 digested with *XbaI*. As usual, the construct was firstly obtained in *E. coli*, checked by restriction enzyme analysis and sequenced. Following electroporation, the new construct, pAM5, was then recovered from bifidobacterial strains.

For convenient blue/white screening of recombinant molecules in *E. coli*, we restored the original *lacZα*-peptide gene, disrupted in pUC19E (23), in a pAM1 derivative. To this end, a 327-bp segment from pUC19 (55), carrying the *lacZα* gene and the MCS, was amplified with primers LacZF and LacZR (Table 1), purified, and cloned into the pCR®4-TOPO vector. The construct was then digested with the restriction enzyme *NotI* and treated with the Klenow fragment of the *E. coli* polymerase I to make blunt ends. After inactivation of Klenow, the plasmid was digested once again with *SpeI*. The resulting fragment was purified from a gel and ligated to a pAM1 vector subjected to a similar process of digestion with *HindIII*, treatment with Klenow, and subsequent digestion with *XbaI* (Fig. 1). The construct, pAM6, was first identified in *E. coli*, verified by sequencing and introduced in *B. pseudocatenulatum* M115, to check for replication in bifidobacteria.

**Construct host range, antibiotic resistance, copy number and stability.** To study the host range of the pBC1 derivatives, competent cells belonging to strains of eight different *Bifidobacterium* species (*B. adolescentis, B. animalis, B. breve, B. dentium, B. longum, B. pseudolongum, B. pseudocatenulatum, and B. thermophilum*) were electrotransformed with one µg of a unique DNA sample from pAM4. Transformants
were recovered for all eight species with the two vectors, although the frequencies ranged from $4.0 \times 10^1 \mu g^{-1}$ in \textit{B. animalis} LMG 10508 to $1.0 \times 10^5$ in \textit{B. pseudocatenulatum} M115 (Table 2). Transformation was found to be strain-dependent rather than species-dependent, as different strains of the same species showed dissimilar transformation frequencies of more than two log$_{10}$ units (data not shown). At low frequency, \textit{pBM4} was also shown to transform the strain \textit{Corynebacterium glutamicum} LMG 19741. However, using the same amount of DNA, transformant colonies of several lactic acid bacteria strains of our laboratory collection, belonging to \textit{Lactococcus lactis}, \textit{Lactobacillus casei} and \textit{Enterococcus durans} species, were never recovered (Table 2). Transformants were neither obtained for the strain \textit{Propionibacterium acidilactici} LMG 11384. Finally, \textit{pAM2} (a \textit{pBC1}-derived construct lacking the pUC part) (1) was used to transform electrocompetent cells of \textit{E. coli}, but transformants were not recovered, therefore the \textit{pBC1} replicon was assumed incapable of replicating in this species.

The MIC resistance values (obtained by the Etest method) for erythromycin and tetracycline conferred upon \textit{B. pseudocatenulatum} M115 by \textit{pAM1} and \textit{pAM4} vectors were $8-12 \ \mu g \ ml^{-1}$ and $48 \ \mu g \ ml^{-1}$ respectively. These values contrast with the high susceptibility shown by the original plasmid-free M115 strain (0.064 and 0.125 $\mu g \ ml^{-1}$ respectively). \textit{pBC1} and \textit{pAM1} copy number has already been established to be around 30 copies per chromosome equivalent in \textit{B. pseudocatenulatum} M115 (1). In a similar way, the copy number of \textit{pAM5} and \textit{pAM6} in this strain was shown to be of $31.5 \pm 0.37$ and $28.4 \pm 0.64$, respectively. Copy number for these two vectors in \textit{B. breve} UCC2003 was...
estimated in 29.1±0.96 and 27.6±0.54, respectively, per cell. These results agree well with those reported previously (1, 2).

Constructs pAM2 and pAM4 were both checked for stability under non-selective conditions in *B. pseudocatenulatum* M115. Twenty four colonies of both antibiotic resistant and susceptible phenotype were examined for plasmid maintenance after five overnight transfers (approximately 80-100 generations). All antibiotic resistant colonies retained the constructs, while antibiotic susceptible were shown to be plasmid free.

Based on these data, more than 96% and 98% of the colonies retained the pAM2 and pAM4 constructs respectively. Similar stability frequency for these two constructs was observed in *B. longum* L25 and *B. animalis* LMG 10508 (data not shown).

**Analysis of the intracellular presence of pBC1 ssDNA.** Comparisons of pBC1 translated and untranslated sequences with those in databases suggests that pBC1 might replicate by a theta type mechanism, although elements of both the theta and rolling circle (RC) type mechanisms have been reported in pBC1 (1). To gain further insight into its mode of replication, and on the involvement of the RNA polymerase in this process, the whole pBC1 plasmid and its derivatives pBC1.5 (lacking the putative promoter region of a *copG*-like gene) and pBC1.2 (lacking both *copG*-like and *orfX*-like genes; 1) were analyzed by hybridization using an internal segment of *repB* from pBC1 (obtained by PCR) as a probe. As a positive control of RC replication (in which ssDNA appears as a replication intermediate), a derivative of plasmid pBM02 from *Lactococcus lactis* -p210- (39) was run under the same conditions. Comparison of the hybridization results of gels transferred under denaturing and non-denaturing conditions can be found in Fig. 2. As expected, ssDNA accumulated in the samples corresponding to p210
treated with both chloramphenicol and rifampicin (Fig. 2, line 8 in panels B and D), but no such DNA was seen in samples involving pBC1 or its derivatives.

Cloning and expression of a α-L-arabinofuranosidase gene from *B. longum*. To demonstrate the functionality of pBC1-derived vectors, the *abfB* gene encoding an α-L-arabinofuranosidase from *B. longum* B667 (24) was cloned in both *B. pseudocatenulatum* and *E. coli* and its expression assessed. The *abfB* gene from this strain and its regulatory sequences were amplified from purified total DNA of *B. longum* B667 using two primers with added *Sph*I and *Pst*I sites (Table 1). This allowed directional cloning in pAM1 digested with these two enzymes (Fig. 3). The construct, pAM-abfB, was obtained in *E. coli* DH11S and was subjected to restriction enzyme analysis and sequencing before its electrotransformation into *B. pseudocatenulatum* M115. These two strains might have equivalent genes to *abfB*, but they show negligible expression in the absence of plasmid (< 0.12 specific activity units [SAU; min⁻¹µg protein⁻¹]). In contrast, in the *E. coli* and *B. pseudocatenulatum* clones harboring the *abfB* gene of *B. longum* B667, the α-arabinofuranosidase activity ranged from 9.45 to 16.15 SAUs (average 12.95), an increase of more than 100-fold. Moreover, no segregant lost this activity after a week of daily transferring the *B. pseudocatenulatum* strain in non-selective conditions. Indeed, all 24 colonies examined by plasmid analysis retained the pAM-abfB construct, thus considering it was therefore stable in this host.

**DISCUSSION**

The lack of suitable tools for use in bifidobacteria for cloning, integration, gene disruption and gene expression analysis is delaying the analysis of their gene-related functions and the molecular mechanisms underlying their probiotic properties - and such
tools will be necessary if we are to exploit the potential of the vast array of data provided by genome sequencing projects. In this work, pAM1, a previously developed *E. coli*-Bifidobacterium shuttle vector harboring the pBC1 replicon (1), was modified to produce a new series of pBC1-derived vectors, giving versatility and adding new possibilities for cloning and expression in bifidobacteria. Maintaining of the whole plasmid was based on the observation that, although repB is the only gene considered essential for pBC1 replication, orfX-like and copG-like genes influence the stability and copy number of the constructs in at least some strains (1, 2).

At present, several Bifidobacterium-*E. coli* shuttle vectors have been constructed that exploit cryptic plasmids in a procedure similar to that followed in this study. These included, among others, general *E. coli*-Bifidobacterium shuttle vectors (1, 5, 18, 21, 25, 26, 30, 35, 37), replicon screening vectors (12), and expression vectors (40). However, the majority of these are based on poorly characterized replicons since the mode of replication has only been investigated in a small number of plasmids (4, 21, 27, 32). Indeed, apart from pBC1 (2), only the recently reported plasmid pCIBA089 from *B. asteroides* has been characterized at the molecular level (5).

One of the key factors in vector development is the plasmid host range. A broad host range is necessary if genes are to be transferred among different species and genera, but a narrow host range is preferred to ensure the confinement of plasmid-engineered traits (i.e., to prevent the dissemination of genes among competitors and harmful microorganisms inhabiting the same environment [6]). The ability of the pBC1 replicon to replicate in a large number of bifidobacterial species, including the well-known commercial probiotic strain *B. animalis* subsp. *lactis* Bb12 (Chr. Hansen A/S, Hørsholm, Denmark), renders pBC1-derivatives easily transferable among species of
this genus, in which they were found to show rather high segregation stability.

Differences in the transformation efficiency among strains may be a result of different genetic backgrounds, and may be related to interference with integrated plasmid remnants (e.g., in *B. longum* NCC 2075) (42) or to the presence of restriction/modification systems (as in *B. breve* UCC 2003) (D. van Sinderen, personal communication). In fact, the transformation efficiency is better when the DNA of the constructs is isolated from bifidobacteria (Table 2). Alternatively, the transformation frequency might also be affected by a differential sensitivity of the strains to oxygen, as the preparation of electrocompetent cells demands excessive handling at open air. Although transformation frequencies are rather high for at least some strains, higher transformation frequencies are needed for most genetic purposes, and the improvement of current gene transfer systems or the development of new transformation strategies remains a necessity.

The high relative copy number of pBC1 and its derived vectors (around 30 copies per chromosome equivalent) could complement pCIBA089-derivatives (approximately 4 copies per cell) (5), allowing the fine tuning of gene expression through gene dosage. Further, the pBC1 replicon has proven to be non-functional in some bacterial species, including non-related Gram-positive (*L. lactis, L. casei, E. durans,* and *P. acidilactici*) and Gram-negative bacteria (*E. coli*), a prerequisite for the future development of food grade vectors. These, apart from the absence of antibiotic resistance genes, should preferably not replicate in bacteria from the same ecosystem in order of not to spread the (beneficial) properties, which might provide selective advantages to competitors (6). The fact that pAM4 could replicate in *C. glutamicum* is not surprising, as this bacterium belongs as the bifidobacteria to the phylum *Actinobacteria*, thus being phylogenetically
related. The replication of pBC1-derivatives in other Actinobacteria is currently being tested.

The number of useful restriction enzymes in some of the vectors developed in this study are certainly limited (XbaI, SalI, PstI, SphI and HindIII in most of them). However, the availability of several complete genome sequences allows the easy cloning of PCR-amplified DNA fragments to which desired restriction enzyme sites can be added. The use of ligase-independent cloning methods, such as the recently developed PCR In-fusion™ technique (Clontech Lab. Inc., Palo Alto, CA) (13), would further allow the cloning of DNA fragments without the need of a large multi-cloning site (MCS). Nevertheless, one of the vectors, pAM5, has been endowed with a long MCS with more than 20 single restriction enzyme sites. The incorporation of the lacZα gene into pAM6 would further allow a convenient color screening (blue-white) for plasmids carrying inserts in E. coli.

The mode of replication is another key factor in a vector, as it affects the structural stability, the host range, and the size of the DNA fragments that can successfully be cloned. In general, vectors that follow a theta type replication mechanism are preferred over those replicating by the RC mode; they are usually more stable, accept larger DNA fragments and have a narrower host range (7, 16). As mentioned, sequence comparisons and phylogenetic analyses have indicated that pBC1 might replicate via a theta-type mechanism. Based on sequence similarities, this mode of replication has only been suggested for the bifidobacterial plasmids pMB1 (35) and pDOJH10S (21) from B. longum, and more recently for pCIBAO89 and pCIBA43 from B. asteroides (5).

Though the theta mode of replication has yet to be proved, the present study provides
further indications that pBC1 may use the theta type of replication since no ssDNA intermediates were detected during plasmid replication.

Arabinofuranosidases are involved in the breakdown of many non-digestible (i.e., by humans) dietary carbohydrates by bifidobacteria (24). Therefore, this enzymatic activity is related to the utilization of non-digestible carbohydrates as fermentative prebiotic substrates for bifidobacteria. Engineering of probiotic strains with greater arabinofuranosidase activity might lead to a better competition of probiotic strains in the human GIT ecosystem and/or allow the future use of strain-specific prebiotics. In this example, by the use of a pBC1-derived vector, the specific activity of this enzyme was increased more than 100-fold from the low basal level of the untransformed *B. pseudocatenulatum* M115. The same α-arabinofuranosidase increase was observed respect to the activity of the original *B. longum* B667 grown in glucose, as its enzymatic activity seems to be subjected to induction by arabinose-containing substrates (11). The success of this experience suggests a great potential of these vectors for cloning and expressing desirable genes in bifidobacteria, and the feasibility of modifying strains of this commercially and medically important bacterial group.

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<td>F, φ80dλZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(k, m’k’), pheA, supE44, λ, thi-1, gyrA96, relA1 mcrA, Δ(mrr-hsdRMS-mcrBC), Δ(lac-proAB), Δ(rec1398), deoR, rpsL, rrl-thi-F/ProAB+ lacIqZΔM15</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH11S</td>
<td>F′mcrA, Δ(mrr-hsdRMS-mcrBC) F′lacZΔM15, recA1, deoR, araD139, Δ(ara-leu)7697, galU, galK, rpsL (Su8), endA1, nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>Escherichia coli</em> TOP10</td>
<td>Human intestinal plasmid-free strain; containing an α-L-arabinofuranosidase gene</td>
<td>Margolles and de los Reyes Gavilán (24)</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> B667</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium pseudocatenulatum</em> M115</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>IPLA Laboratory Collection</td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em> UCC2003</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>APC, University College Cork; Ireland</td>
</tr>
<tr>
<td><em>Bifidobacterium dentium</em> F101</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>IPLA Laboratory Collection</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> L25</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>IPLA Laboratory Collection</td>
</tr>
<tr>
<td><em>Bifidobacterium adolescentis</em> LMG 10502</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>LMG, Universiteit Gent, Belgium</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em> subsp. <em>animalis</em> LMG 10508</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>LMG</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em> subsp. <em>lactis</em> Bb12</td>
<td><em>Commercial fermented milk</em></td>
<td>IPLA Laboratory Collection</td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em> LMG 13208</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>LMG</td>
</tr>
<tr>
<td><em>Bifidobacterium thermophilus</em> LMG 11571</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>LMG</td>
</tr>
<tr>
<td><em>Bifidobacterium pseudolongum</em> subsp. <em>pseudolongum</em> LMG 11571</td>
<td>Human intestinal isolate, plasmid-free</td>
<td>LMG</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em> LMG 19741</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pediococcus acidilactici</em> LMG 11384</td>
<td>Sewage</td>
<td>Barley</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pUC19</td>
<td>Ap’, lacZα, general cloning vector, MCS with 11 restriction enzyme sites</td>
<td>Yanisch-Perron et al. (55)</td>
</tr>
<tr>
<td>pUK21</td>
<td>Ap’, lacZα, general cloning vector, MCS with 28 restriction enzyme sites</td>
<td>Vieira and Messing (52)</td>
</tr>
<tr>
<td>pCR®4-TOPO</td>
<td>Ap’, lacZα, TA cloning vector</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>pAM1</td>
<td><em>E. coli-Bifidobacterium</em> shuttle cloning vector; Ap’, Em’</td>
<td>Álvarez-Martín et al. (1)</td>
</tr>
<tr>
<td>pAM2</td>
<td><em>Bifidobacterium</em> cloning vector; Em’</td>
<td>Álvarez-Martín et al. (1)</td>
</tr>
<tr>
<td>pAM3</td>
<td><em>E. coli-Bifidobacterium</em> shuttle cloning vector; Ap’, Em’, Tet’ [tet(W)]</td>
<td>This work</td>
</tr>
<tr>
<td>pAM4</td>
<td><em>E. coli-Bifidobacterium</em> shuttle cloning vector; Ap’, Tet’ [tet(W)]</td>
<td>This work</td>
</tr>
<tr>
<td>pAM-abfB</td>
<td>α-L-arabinofuranosidase gene in pAM1; Ap’, Em’</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Oligonucleotides (5’-3’)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fxfp</td>
<td>GACGTCACAAACAAGCAGTG</td>
<td>Álvarez-Martín et al. (1)</td>
</tr>
<tr>
<td>Rxfp</td>
<td>CTTCCATCTGGTCTGCGGAG</td>
<td>Álvarez-Martín et al. (1)</td>
</tr>
<tr>
<td>FrepB</td>
<td>GCCACGTCTCGCATCCA</td>
<td>Álvarez-Martín et al. (1)</td>
</tr>
<tr>
<td>RrepB</td>
<td>CGGACCGCTCTGCTTGTGT</td>
<td>Álvarez-Martín et al. (1)</td>
</tr>
<tr>
<td>LacZF</td>
<td>CGTATGTTGTTGGAATTGTGAG</td>
<td>This work</td>
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<tr>
<td>LacZR</td>
<td>GAAATACCGCACAGATGCATGGA</td>
<td>This work</td>
</tr>
<tr>
<td>tet(W)-SacIF</td>
<td>CCCTGGAGCTCAGCTATCATCATGTCG</td>
<td>This work</td>
</tr>
</tbody>
</table>
Ap<sup>r</sup>, Em<sup>r</sup>, and Tet<sup>r</sup>, resistance to ampicillin, erythromycin, and tetracycline, respectively.

Underlined oligonucleotide sequences show artificial restriction enzyme sites introduced for cloning.

MCS, multiple cloning site
Table 2.- Host range and transformation frequencies of pBC1-derived vectors.

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Transformants per µg of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pAM1</td>
</tr>
<tr>
<td><strong>B. adolescentis</strong> LMG 10502</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. animalis</strong> subsp. <strong>animalis</strong> LMG 10508</td>
<td>-</td>
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<tr>
<td><strong>B. animalis</strong> subsp. <strong>lactis</strong> Bb12</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. breve</strong> LMG 13208</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. breve</strong> UCC 2003</td>
<td>2.3x10(^2)</td>
</tr>
<tr>
<td><strong>B. dentium</strong> F101</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. longum</strong> L25</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. pseudolongum</strong> subsp. <strong>pseudolongum</strong> LMG 11571</td>
<td>-</td>
</tr>
<tr>
<td><strong>B. pseudocatenulatum</strong> M115</td>
<td>2.5x10(^5)</td>
</tr>
<tr>
<td><strong>B. thermophilus</strong> LMG 11571</td>
<td>-</td>
</tr>
<tr>
<td><strong>Corynebacterium glutamicum</strong> LMG 19741</td>
<td>-</td>
</tr>
<tr>
<td><strong>Enterococcus durans</strong> L72</td>
<td>-</td>
</tr>
<tr>
<td><strong>Lactobacillus casei</strong> ATCC 393</td>
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<tr>
<td><strong>Lactococcus lactis</strong> subsp. <strong>cremoris</strong> MG 1363</td>
<td>0</td>
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<tr>
<td><strong>Lactococcus lactis</strong> subsp. <strong>lactis</strong> IL1403</td>
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<td><strong>Pediococcus acidilactici</strong> LMG 11384</td>
<td>-</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong> DH5(α)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Whenever possible, pAM2 DNA was isolated from the same strain to which it was transformed.

-, not done or not applicable.
Cloning in pCR®4-TOPO digestion with NotI blunt ends digestion with SpeI

Digestion with HindIII blunt ends digestion with XbaI

pAM1 6.1 kbp

EcoRI SacI KpnI SalI

pAM2 6.1 kbp

EcoRI SacI KpnI SalI

pAM3 8.6 kbp

EcoRI KpnI SalI

pAM4 7.6 kbp

KpnI SalI

pAM5 7.7 kbp

KpnI SalI
PCR amplification

Digestion SphI+PstI

Ligation

EcoRI
SacI
KpnI

SphI
PstI

2.3 kbp

ApR
EmR

copG-like
orfX-like
ori pUC
repB

EcoRI
SacI
KpnI

XbaI
SalI
PstI
SphI
HindIII

abfB

pAM1
6.1 kbp

abfB

SphI

2.3 kbp

HindIII
SphI

pAM-abfB
8.4 kpb

XbaI
SalI
PstI

ApR
EmR

copG-like
orfX-like
ori pUC
repB

EcoRI
SacI
KpnI

XbaI
SalI
PstI
SphI
HindIII

abfB

SphI

2.3 kbp

HindIII
SphI
FIGURE LEGENDS

Figure 1. Physical maps of the plasmids utilized and the constructs obtained in this work from the pBC1 replicon. The color key tracks the origin, length and direction of the open reading frames (ORFs) and other structures, as indicated. Only relevant restriction enzyme sites are indicated. Molecules are proportional but not drawn to scale. MCS, multiple cloning site.

Figure 2.- Hybridization experiments aimed to analyze pBC1 replication intermediates using as probes internal segments of repB genes from pBC1 from B. catenulatum and pBM02 from Lactococcus lactis (positive control for the detection of ssDNA) (36). Panels A and C. Ethidium bromide stained gels showing total DNA preparations from B. pseudocatenulatum M115 harboring construct pBC1.2 (8.0 kbp) (lanes 1 and 5); B. pseudocatenulatum M115 carrying construct pBC1.5 (8.7 kbp) (lanes 2 and 6); B. catenulatum L48 containing the original pBC1 plasmid (2.5 kbp) (lanes 3 and 7); E. coli DH5α carrying construct p210 from L. lactis (3.8 kbp) (lanes 4 and 8). M, molecular weight marker. Plasmids were isolated before (N) and after incubation of the cells for one h with both rifampicin and chloramphenicol or erythromycin (R). Panel B. Autoradiogram after hybridization of a gel transferred under non-denaturing conditions (which favors the transfer of ssDNA). Panel D. Autoradiogram after hybridization of a gel transferred under denaturing conditions. The position of ssDNA of plasmid p210 from L. lactis in the sample treated with rifampicin and chloramphenicol (Panels B and D, lane 8) is indicated.
Figure 3.- Amplification and cloning of the α-L-arabinofuranosidase gene \textit{ahfB} from \textit{Bifidobacterium longum} B667 and cloning in pAM1. Molecules are proportional but not drawn to scale.