APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2008, p. 4656–4665 Vol. 74, No. 15
0099-2240/08/$08.00+0 doi:10.1128/AEM.00074-08

Improved Cloning Vectors for Bifidobacteria, Based on the *Bifidobacterium catenulatum* pBC1 Replicon▽

Pablo Álvarez-Martín,[1] Ana Belén Flórez,[1] Abelardo Margolles,[1] Gloria del Solar,[2] and Baltasar Mayo[1]*

Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (CSIC), 33300 Villaviciosa, Asturias, Spain,[1] and Departamento de Ciencia de Proteínas, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maetzu 9, 28040 Madrid, Spain[2]

Received 10 January 2008/Accepted 7 April 2008

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 4.0×10^1 to 1.0×10^5 transformants μg^{-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 to 100 generations in the absence of selection. Vectors contain multiple cloning sites of different lengths, and the *lacZ*α peptide gene was introduced into one of the molecules, thus allowing the 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 α-L-arabinofuranosidase gene belonging to *Bifidobacterium longum*. *E. coli* and *Bifidobacterium pseudocatenulatum* recombinant clones were stable and showed an increase in α-arabinofuranosidase activity of over 100-fold compared to that of the untransformed hosts.

Bifidobacterium species are among the dominant microbial populations of the gastrointestinal tract of humans and other mammals (8, 33), where they are considered to exert many beneficial health effects (for a review, see reference 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 to be effective in alleviating constipation, reducing the symptoms of lactose intolerance, enhancing immune functions, reducing cholesterol levels, and suppressing tumorigenesis (19, 29).

Unfortunately, our basic knowledge of the mechanisms by which bifidobacteria interact and communicate with other bacteria and host cells remains poor. 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, has only rarely been addressed (3, 36, 37). Bifidobacteria belong to the phylum *Actinobacteria*, gram-positive microorganisms with high G+C content that have complex nutritional requirements and that 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 (GenBank accession number NZ_AABM00000000), *Bifidobacterium adolescentis* ATCC 15703 (accession number NC_008618), and *B. adolescentis* L2-32 (accession number NZ_AXD02000000) have been released, providing a vast array of genetic data that 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 identified only 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. Extra-

* Corresponding author. Mailing address: Departamento de Microbiología y Bioquímica de Productos Lácteos, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain. Phone: 34 985 89 21 31. Fax: 34 985 89 22 33. E-mail: baltasar.mayo@ipla.csic.es.

▽ Published ahead of print on 6 June 2008.

chromosomal elements seem to not 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, the mode of replication has been analyzed for only a few of them (4, 21, 27, 30, 32). Furthermore, the dissection of the open reading frames and the analysis of untranslated sequences and structures has been undertaken for only a couple of plasmids (2, 5). In spite of this, many 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 α-amylase gene of *B. adolescentis* in *B. longum* (34); pBLES100 (25) has been used in tumor suppressor studies (55) 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 anticancer protein endostatin in cancer gene therapy (53).

The present study reports the further characterization of plasmid pBC1 from *Bifidobacterium catenulatum* L48 (1, 2) and its use in the development of improved cloning vectors. The plasmid was cloned entirely in a pUC derivative reported 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 to be true cloning vectors because they have several unique restriction enzyme sites at nonessential 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 α-galactosidase complementing peptide gene for a convenient blue-white screening of recombinant clones in *E. coli*. The study of the copy number, stability, and host range of some vectors was also addressed. To check the functionality of these vectors, an α-L-arabinofuranosidase gene from *B. longum* B667 was cloned and overexpressed in both *E. coli* and *Bifidobacterium* strains.

MATERIALS 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 (VWR International, Darmstadt, Germany) or in RCM broth (VWR International, Darmstadt, Germany) supplemented with 0.25% (wt/vol) L-cysteine (MRSC). *Lactococcus* and *Enterococcus* strains in Table 2 were grown in M17 medium (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 Chemical Co., St. Louis, MO) were added to the appropriate media at the following concentrations: 100 μg ml^{-1} ampicillin, 250 μg ml^{-1} erythromycin, and 5 μg ml^{-1} tetracycline for *E. coli* and 5 μg ml^{-1} erythromycin, 5 μg ml^{-1} tetracycline, and 2 μg ml^{-1} chloramphenicol for bifidobacteria.

DNA isolation and analysis. Plasmid DNA from bifidobacteria was isolated according to a method described previously by O'Sullivan and Klaenhammer (28), with the following modification: pellets were suspended in TSE buffer (25% sucrose, 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) according to 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% to 1.2% agarose gels (FMC Bioproducts, Philadelphia, PA) and then stained with ethidium bromide (0.5 μg ml^{-1}) and photographed.

Total DNA from *B. longum* B667 was prepared according to a procedure described previously by Tanaka et al. (47) and analyzed as described above.

DNA manipulation and molecular techniques. The general procedures used for DNA manipulation were essentially those described previously by Sambrook and Russell (38). Restriction endonucleases and *Taq* DNA polymerase came from Takara (Otsu, Shiga, Japan), T4 DNA ligase was obtained from Invitrogen (Carlsbad, CA), and the Klenow fragment of *E. coli* polymerase I was obtained from Roche (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, United Kingdom). When required, purified plasmids and amplicons were sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA).

Plasmid transfer. Plasmids were introduced into *E. coli* DH5α electrocompetent cells by electrotransformation (electroporation) (38) using a Gene-Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Electroporation of strains of lactic acid bacteria (*Lactococcus lactis*, *Lactobacillus casei*, and *Enterococcus durans*) was done essentially as reported previously by Leenhouts et al. (22), using plasmid p210 from *L. lactis* as a positive control (39). *Corynebacterium glutamicum* and *P. 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 a culture (8% inoculum) of the bifidobacterial strain grown overnight and incubated at 37°C for 3 to 4 h until the culture reached an optical density at 600 nm of 0.5 to 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 at 25 μF, 200 Ω, and 10 kV. The cells were immediately diluted in 950 μl of RCM broth and incubated for 2.5 h before plating onto the same agarified medium with the appropriate antibiotic. Plates were incubated for 2 to 3 days at 37°C 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 rifampin was isolated essentially as described previously by te Riele et al. (48). The DNA was electrophoresed in a 0.7% agarose gel and transferred onto Hybond-N nylon membranes (Amersham Biosciences, Uppsala, Sweden) under denaturing and nondenaturing conditions. Single-stranded DNA (ssDNA) intermediates were detected by hybridization using pBM02-derived and pBC1-derived DNA probes internally labeled with [α-^{32}P]dCTP (GE Healthcare).

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

Antibiotic resistance of vectors. The MICs of erythromycin and tetracycline supported by the constructs in different hosts were measured by the Etest method according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). MIC assays were performed using LSM medium (90% Isosensitest, 10% MRS [both from Oxoid Ltd., Basingstoke, Hampshire, United Kingdom]) with cysteine (0.3 g liter^{-1}) as previously reported (17).

Determination of relative plasmid copy number. The relative copy number of the pBC1-derived vectors was assessed by quantitative real-time PCR using the culture and PCR conditions reported previously by Lee et al. (20). Amplification and detection were performed using a Fast real-time PCR system (Applied Biosystems, Foster City, CA) with Power Sybr green PCR master mix (Applied Biosystems). Primers FrepB and RrepB (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 *Bifidobacterium 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 $N_{\text{relative}} = (1 + E)^{-\Delta C^T}$ (19), where E is the amplification efficiency of the target and reference genes and ΔCT is the difference between the threshold cycle number of the *xfp* reaction and that of *repB*. Experiments were performed in triplicate; mean results are provided.

TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers utilized in this work[a]

Strain, plasmid, or oligonucleotide	Genotype, phenotype, or sequence (5′–3′)	Source or reference
Strains		
Escherichia coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
Escherichia coli DH11S	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ(*lac-proAB*) Δ(*rec1398*) *deoR rpsL srl-thi-F′ proAB*$^+$ *lacI*^{q}*Z*ΔM15	Invitrogen
Escherichia coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) F 80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rspL* (Str^r) *endA1 nupG*	Invitrogen
Bifidobacterium longum B667	Human intestinal plasmid-free strain containing an α-L-arabinofuranosidase gene	24
Bifidobacterium pseudocatenulatum M115	Human intestinal isolate; plasmid free	IPLA Laboratory Collection
Bifidobacterium breve UCC2003	Human intestinal isolate; plasmid free	APC, University College Cork, Cork, Ireland
Bifidobacterium dentium F101	Human intestinal isolate; plasmid free	IPLA Laboratory Collection
Bifidobacterium longum L25	Human intestinal isolate; plasmid free	IPLA Laboratory Collection
Bifidobacterium adolescentis LMG 10502	Human intestinal isolate; plasmid free	LMG, Universiteit Gent, Gent, Belgium
Bifidobacterium animalis subsp. *animalis* LMG 10508	Human intestinal isolate; plasmid free	LMG
Bifidobacterium animalis subsp. *lactis* Bb12	Commercial fermented milk; plasmid free	Chr. Hansen A/S, Hørsholm, Denmark
Bifidobacterium breve LMG 13208	Human intestinal isolate; plasmid free	LMG
Bifidobacterium thermophilus LMG 11571	Human intestinal isolate; plasmid free	LMG
Bifidobacterium pseudolongum subsp. *pseudolongum* LMG 11571	Human intestinal isolate; plasmid free	LMG
Corynebacterium glutamicum LMG 19741	Sewage	LMG
Pediococcus acidilactici LMG 11384	Barley	LMG
Plasmids		
pUC19	Ap^r *lacZ*α; general cloning vector, MCS with 11 restriction enzyme sites	54
pUK21	Ap^r *lacZ*α; general cloning vector, MCS with 28 restriction enzyme sites	52
pCR4-TOPO	Ap^r *lacZ*α; TA cloning vector	Invitrogen, Carlsbad, CA
pAM1	*E. coli-Bifidobacterium* shuttle cloning vector; Ap^r Em^r	1
pAM2	*Bifidobacterium* cloning vector; Em^r	1
pAM3	*E. coli-Bifidobacterium* shuttle cloning vector; Ap^r Em^r Tet^r [*tet*(W)]	This work
pAM4	*E. coli-Bifidobacterium* shuttle cloning vector; Ap^r Tet^r [*tet*(W)]	This work
pAM-abfB	α-L-Arabinofuranosidase gene in pAM1; Ap^r Em^r	This work
Oligonucleotides		
Fxfp	GACGTCACCAACAAGCAGTG	1
Rxfp	CTTCCATCTGGTGCTCGGAG	1
FrepB	GCCACGTTCGTCGCCATCCA	1
RrepB	CCGACCAGCTCTGCCTTTTG	1
LacZF	CGTATGTTGTGTGGAATTGTGAG	This work
LacZR	GAAATACCGCACAGATGCGTAAG	This work
tet(W)-SacIF	CCCTGGAGCTCATGCTCATCATGTAC	This work
tet(W)-SacIR	CCATCGGAGCTCCATAACTTCTG	This work
abfBF-SphI (α-L-arabinofuranosidase gene)	CGAATCCCGCATGCGTACGAGGCAGTGTGGA ATCC	This work
abfBR-PstI (α-L-arabinofuranosidase gene)	TGTTCGCGCTGCAGGCTTCGATGACGTGGAGG AATC	This work

[a] Ap^r, Em^r, and Tet^r, resistance to ampicillin, erythromycin, and tetracycline, respectively. Underlined oligonucleotide sequences show artificial restriction enzyme sites introduced for cloning.

Cloning and expression of an α-L-arabinofuranosidase gene from *B. longum*. The α-L-arabinofuranosidase gene (*abfB*) in *B. longum* strain B667 was characterized previously 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 12°C. The ligation mixture was electrotransfomed into *E. coli* DH11S cells in which the construct (pAM-abfB) was obtained (it proved to not be viable in *E. coli* TOP10) and verified by the use of restriction enzymes and sequencing. pAM-abfB was then transferred into *B. pseudocatenulatum* M115 cells by electroporation.

Determination of α-L-arabinofuranosidase activity. α-L-Arabinofuranosidase activity in the cloning hosts and recombinant cells was determined according to methods described previously by Gueimonde et al. (11). Briefly, pellets were

TABLE 2. Host ranges and transformation frequencies of pBC1-derived vectors

Species and strain	No. of transformants per μg of DNA[b]		
	pAM1	pAM2[a]	pAM4
B. adolescentis LMG 10502	—	—	9.2×10^2
B. animalis subsp. *animalis* LMG 10508	—	—	4.0×10^1
B. animalis subsp. *lactis* Bb12	—	—	1.6×10^2
B. breve LMG 13208	—	—	1.0×10^2
B. breve UCC 2003	2.3×10^2	6.4×10^4	1.4×10^2
B. dentium F101	—	—	9.5×10^1
B. longum L25	—	—	6.6×10^1
B. pseudolongum subsp. *pseudolongum* LMG 11571	—	—	6.3×10^1
B. pseudocatenulatum M115	2.5×10^5	8.3×10^5	1.0×10^5
B. thermophilus LMG 11571	—	—	4.6×10^1
Corynebacterium glutamicum LMG 19741	—	—	3.0×10^0
Enterococcus durans L72	—	—	0
Lactobacillus casei ATCC 393	—	—	0
Lactococcus lactis subsp. *cremoris* MG 1363	0	0	0
Lactococcus lactis subsp. *lactis* IL-1403	—	0	0
Pediococcus acidilactici LMG 11384	—	—	0
Escherichia coli DH5α	—	0	—

[a] Whenever possible, pAM2 DNA was isolated from the same strain to which it was transformed.
[b] —, not done or not applicable.

suspended in 2 ml of potassium phosphate buffer (pH 6.8), and the cells were disrupted with a cell disruptor (Constant Systems Ltd., Daventry, Northants, United Kingdom). Activity was measured in triplicate using independent cell extracts.

RESULTS

Construction of vectors based on the pBC1 replicon. The pAM1 shuttle vector resulting from the cloning of pBC1 into 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 *Staphylococcus aureus* plasmid pE194 (14), was replaced by a recently characterized *tet*(W) gene identified in an intestinal isolate of *B. longum* (9). A 2,467-bp segment of DNA including the *tet*(W) gene and its upstream promoter sequences was amplified by PCR with specific primers into which the SacI sites were incorporated. The *tet*(W) gene was inserted into the unique SacI site of pAM1. The new construct, pAM3 (Fig. 1), was recovered in *E. coli* and then transformed into *B. pseudocatenulatum* cells. The erythromycin resistance gene of pAM3 was finally removed (to give pAM4) (Fig. 1) by partial digestion with SalI, 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 into *B. pseudocatenulatum* cells and other bifidobacteria. New single restriction enzyme sites were introduced into 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 the digestion of pUK21 with SpeI and then ligated into pAM4 digested with XbaI. As usual, the construct was first 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 *lacZ*α peptide gene, disrupted in pUC19E (23), in a pAM1 derivative. To this end, a 327-bp segment from pUC19 (54), carrying the *lacZ*α gene and the MCS, was amplified with primers LacZF and LacZR (Table 1), purified, and cloned into the pCR4-TOPO vector. The construct was then digested with the restriction enzyme NotI and treated with the Klenow fragment of *E. coli* polymerase I to make blunt ends. After the inactivation of the Klenow fragment, the plasmid was digested once again with SpeI. The resulting fragment was purified from a gel and ligated into a pAM1 vector subjected to a similar process of digestion with HindIII, treatment with Klenow fragment, and subsequent digestion with XbaI (Fig. 1). The construct, pAM6, was first identified in *E. coli*, verified by sequencing, and introduced into *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 1 μg of a unique DNA sample from pAM4. Transformants were recovered for all eight species with the two vectors, although the frequencies ranged from 4.0×10^1 μg^{-1} in *B. animalis* LMG 10508 to 1.0×10^5 μg^{-1} in *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 2 $\log_{10}$ units (data not shown). At a low frequency, pBM4 was also shown to transform *Corynebacterium glutamicum* strain LMG 19741. However, using the same amount of DNA, transformant colonies of several lactic acid bacterial strains of our laboratory collection, belonging to *Lactococcus lactis*, *Lactobacillus casei*, and *Enterococcus durans*, were never recovered (Table 2). Transformants were not obtained for *Propionibacterium acidilactici* strain LMG 11384. Finally, pAM2 (a pBC1-derived construct lacking the pUC part) (1) was used to transform electrocompetent cells of *E. coli*, but transformants were not recovered; therefore, the pBC1 replicon was assumed to be incapable of replicating in this species.

The MIC resistance values (obtained by the Etest method) for erythromycin and tetracycline conferred upon *B. pseudocatenulatum* M115 by pAM1 and pAM4 vectors were 8 to 12 μg ml^{-1} and 48 μg ml^{-1}, respectively. These values contrast with the high level of susceptibility shown by the original plasmid-free strain M115 (0.064 and 0.125 μg ml^{-1}, respectively).

The copy number for pBC1 and pAM1 was previously established to be around 30 copies per chromosome equivalent in *B. pseudocatenulatum* M115 (1). In a similar way, the copy numbers of pAM5 and pAM6 in this strain were shown to be 31.5 ± 0.37 and 28.4 ± 0.64 copies per chromosome equivalent, respectively. The copy numbers for these two vectors in *B. breve* UCC2003 were estimated to be 29.1 ± 0.96 and 27.6 ±

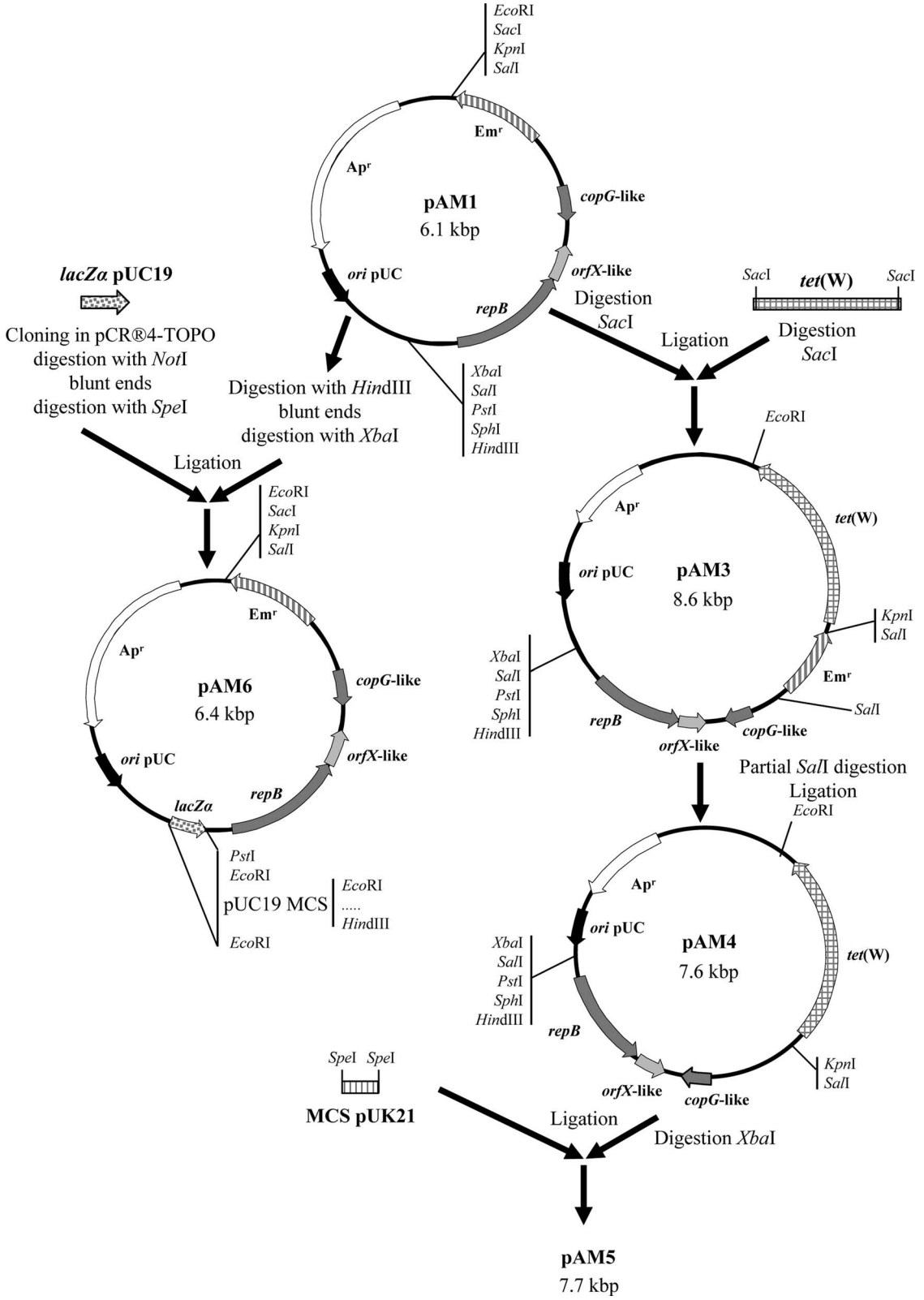

FIG. 1. Physical maps of the plasmids utilized and the constructs obtained from the pBC1 replicon in this work. The key tracks the origins, lengths, and direction of the open reading frames and other structures, as indicated. Only relevant restriction enzyme sites are indicated. Molecules are proportional but not drawn to scale.

0.54 copies per chromosome equivalent, respectively, per cell. These results agree well with those reported previously (1, 2).

Constructs pAM2 and pAM4 were both checked for stability under nonselective conditions in *B. pseudocatenulatum* M115. Twenty-four colonies of both antibiotic-resistant and -susceptible phenotypes were examined for plasmid maintenance after five overnight transfers (approximately 80 to 100 generations). All antibiotic-resistant colonies retained the constructs, while antibiotic-susceptible ones 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 frequencies for these two constructs were observed for *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 suggest that pBC1 might replicate by a theta-type mechanism, although elements of both the theta and rolling-circle (RC)-type mechanisms have been reported for pBC1 (1). To gain further insight into its mode of replication and the involvement of the RNA polymerase in this process, the whole plasmid pBC1 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 *L. lactis*, p210 (39), was run under the same conditions. Comparison of the hybridization results of gels transferred under denaturing and nondenaturing conditions can be found in Fig. 2. As expected, ssDNA accumulated in the samples corresponding to p210 treated with both chloramphenicol and rifampin (Fig. 2B and D, lines 8), but no such DNA was seen in samples involving pBC1 or its derivatives.

Cloning and expression of an 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 into both *B. pseudocatenulatum* and *E. coli*, and its level of expression was 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 SphI and PstI 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 genes equivalent to *abfB*, but they show negligible levels of expression in the absence of plasmid (<0.12 specific activity units [SAUs] [min^{-1} μg $protein^{-1}$]). 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 SAUs), an increase of more than 100-fold. Moreover, no segregant lost this activity after a week of daily transferring of the *B. pseudocatenulatum* strain under nonselective conditions. Indeed, all 24 colonies examined by plasmid analysis retained the pAM-abfB construct; thus it was therefore considered to be 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. Maintenance of the whole plasmid was based on the observation that although *repB* is the only gene considered to be 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 that exploit cryptic plasmids in a procedure similar to that followed in this study have been constructed. 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 been investigated for only a few plasmids (4, 21, 27, 32). Indeed, apart from pBC1 (2), only the recently reported plasmid pCIBA089 from *Bifidobacterium 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) (7). The ability of the pBC1 replicon to replicate in many 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 a rather high level of segregation stability. Differences in the transformation efficiencies 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 in 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

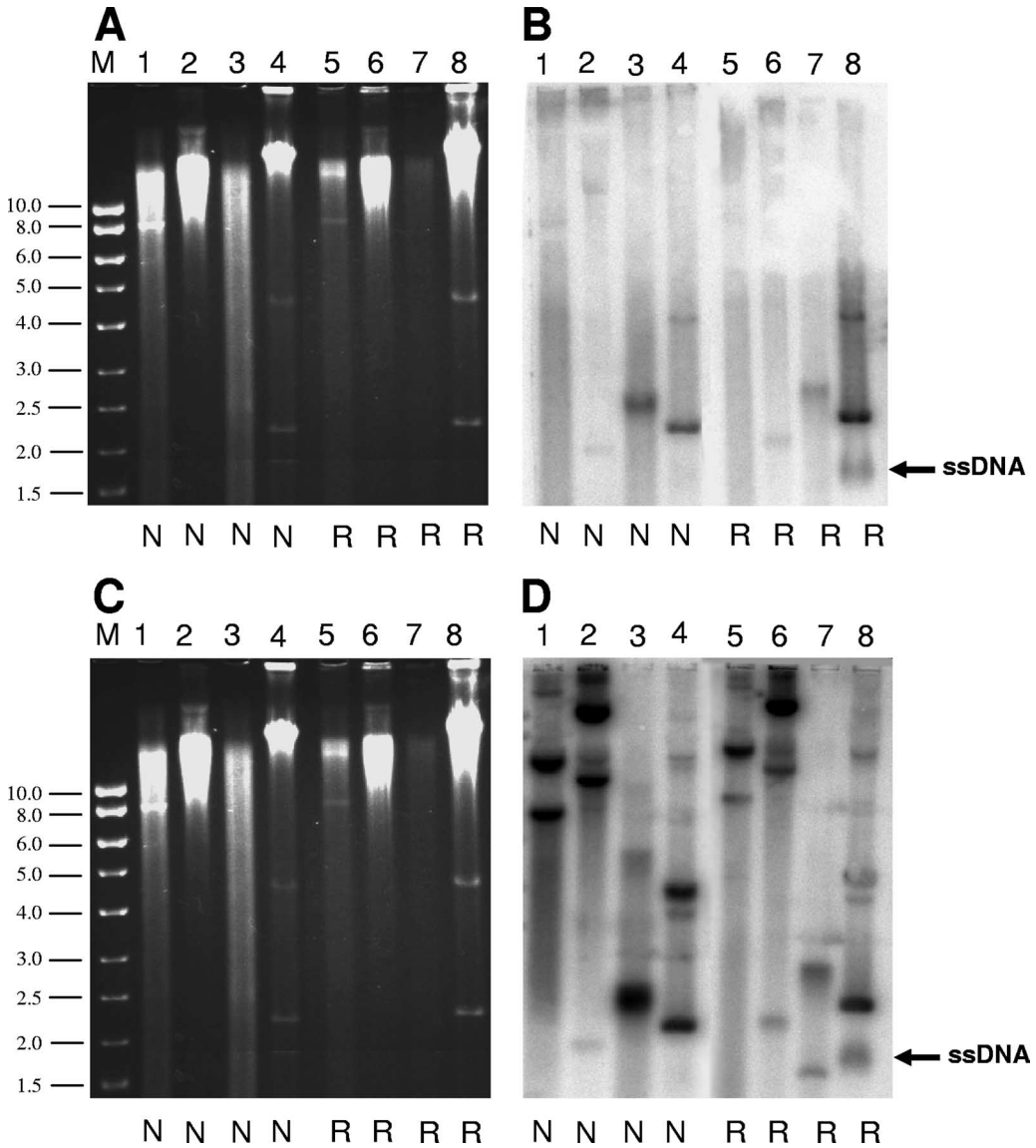

FIG. 2. Hybridization experiments aimed to analyze pBC1 replication intermediates using internal segments of *repB* genes from pBC1 from *B. catenulatum* and pBM02 from *Lactococcus lactis* (positive control for the detection of ssDNA) as probes (36). (A and C) Ethidium bromide-stained gels showing total 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), and *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 1 h with both rifampin and chloramphenicol or erythromycin (R). (B) Autoradiogram after hybridization of a gel transferred under nondenaturing conditions (which favors the transfer of ssDNA). (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 rifampin and chloramphenicol (B and D, lanes 8) is indicated.

through gene dosage. Furthermore, the pBC1 replicon has proven to be nonfunctional in some bacterial species, including nonrelated gram-positive (*L. lactis*, *L. casei*, *E. durans*, and *P. acidilactici*) and gram-negative (*E. coli*) bacteria, 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 to not to spread the (beneficial) properties, which might provide selective advantages to competitors (7). The fact that pAM4 could replicate in *C. glutamicum* is not surprising, as this bacterium belongs, as do the bifidobacteria, to the phylum *Actinobacteria* and are thus 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 Laboratories, Inc., Palo

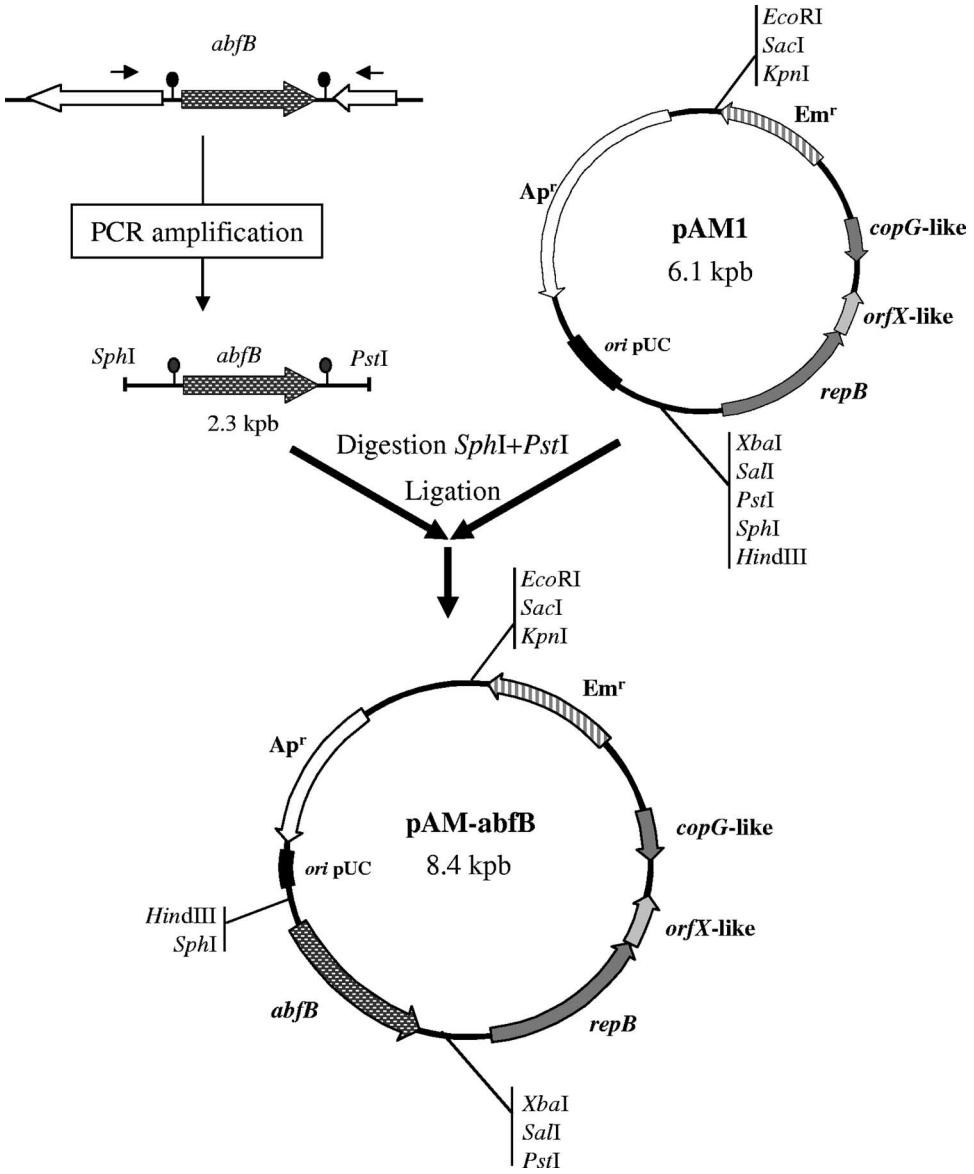

FIG. 3. Amplification and cloning of the α-L-arabinofuranosidase gene *abfB* from *Bifidobacterium longum* B667 and cloning in pAM1. Molecules are proportional but not drawn to scale.

Alto, CA) (13), would further allow the cloning of DNA fragments without the need of a large 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 *lacZ*α 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 (6, 16). As mentioned above, 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 been suggested only for the bifidobacterial plasmids pMB1 (35) and pDOJH10S (21) from *B. longum* and, more recently, for pCIBAO89 and pCIBA43 from *B. asteroides* (5). Although the theta mode of replication has yet to be proven, 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 nondigestible (i.e., by humans) dietary carbohydrates by bifidobacteria (24). Therefore, this enzymatic activity is related to the utilization of nondigestible 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 gastrointestinal tract 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 with respect to the activity of the original *B. longum* B667 grown in glucose, as its enzymatic activity seemed 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.

ACKNOWLEDGMENTS

This work was partially supported by a project from the Spanish Ministry of Education and Science (reference number AGL2007-61869-ALI).

B. breve UCC 2003 was kindly provided by Douwe van Sinderen, Department of Microbiology, University College Cork, Cork, Ireland. LMG strains were obtained from the Belgium Co-ordinated Collections of Microorganisms, Universiteit Gent, Gent, Belgium.

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