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Functional analysis of the pBC1 replicon from *Bifidobacterium catenulatum* L48

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Abstract To determine the minimal replicon of pBC1 (a 2.5-kb cryptic plasmid of *Bifidobacterium catenulatum* L48) and to check the functionality of its identified open reading frames (ORFs) and surrounding sequences, different segments of pBC1 were amplified by polymerase chain reaction (PCR) and cloned into pBif, a replication probe vector for bifidobacteria. The largest fragment tested in this manner encompassed most of the pBC1 sequence, while the shortest just included the *repB* gene and its immediate upstream sequences. Derivatives were all shown to allow replication in bifidobacteria. Surprisingly, both the transformation frequency and segregational stability in the absence of antibiotic selection decreased with reducing plasmid length. The relative copy number of the constructs (ranging from around 3 to 23 copies per chromosome equivalent, as compared to 30 copies for the original pBC1) was shown to be strain dependent and to decrease with reducing plasmid length. These results suggest that, although not essential, the *copG*-like and *orfX*-like genes of pBC1 play important roles in pBC1 replication. Interruption of *repB* produced a construct incapable of replicating in bifidobacteria. The analysis of pBC1 will allow its use in the construction of general and specific cloning vectors.

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Introduction

Starting soon after birth and during most of their live, bifidobacterial species form some of the most dominant bacterial populations of the human and animal gastrointestinal tract (GIT; Ventura et al. 2004). These bacteria are thought to contribute to health maintenance via beneficial metabolic (production of organic acids), protective (inhibition or exclusion of harmful bacteria, anti-toxin activity) and trophic (stimulation of the immune system) activities (Guarner and Malagelada 2003). Evidence of these beneficial effects is rapidly accumulating, and not surprisingly, bifidobacteria are major components of many commercial probiotic products (Tuohy et al. 2003; Leahay et al. 2005). However, fundamental knowledge is still scarce relating to the exact mechanisms by which bifidobacteria contribute to host health and well-being. Such knowledge is essential for any scientific support of their purported health benefits and consequent inclusion as probiotics in functional foods (Kullen and Klaenhammer 2000). Basic research on the interactions of bifidobacteria with the cells of the GIT and other bacteria is also needed (Vaughan et al. 1999). Recently, bifidobacteria have been investigated for novel biotechnological applications, such as the expression of genes encoding detoxifying activities (cholesterol oxidase, bile salt hydrolase; Rossi et al. 1996a) and tumor-suppressing factors (Li et al. 2003; Xu et al. 2007).

Molecular studies of *Bifidobacterium* strains and their modification by genetic engineering rely on the scant

availability of suitable cloning, expression, and/or integrative vectors that permit the efficient transformation, integration, and maintenance of DNA. The genome sequences of *Bifidobacterium longum* NCC 2705 (Schell et al. 2002), *B. longum* DJO10A (NZ_AABM00000000), *Bifidobacterium adolescentis* ATCC 15703 (NC_008618), and *B. adolescentis* L2–32 (NZ_AXD02000000) have recently been released, strengthening the need for general and purpose-specific cloning vectors for retrieving genes and operons for molecular analysis. However, the genetics of these microbes is poorly understood compared to others of industrial importance (Ventura et al. 2004)—bifidobacteria are fastidious, requiring rich media, and strict anaerobic conditions for growth (Scardovi 1986), and are therefore difficult to study in the laboratory. Further, genetic studies have been hampered by a lack of appropriate bacterial replicons (of either plasmid or phage origin). Indeed, the data available on the phages that infect this genus are scarce and fragmentary (Sgorbati et al. 1983; Ventura et al. 2005). Plasmids seem to be less abundant as compared to other intestinal species (Sgorbati et al. 1982; Iwata and Morishita 1989), although around 17–20 cryptic plasmid molecules have now been fully sequenced (reviewed in Álvarez-Martín et al. 2007; Guglielmetti et al. 2007; Sangrador-Vegas et al. 2007) and some *Bifidobacterium–Escherichia coli* shuttle vectors have been constructed. None of these, however, has been experimentally dissected, and in only a few has the mode of replication been analyzed (O'Riordan and Fitzgerald 1999; Park et al. 1999; Corneau et al. 2004; Tanaka et al. 2005; Lee and O'Sullivan, 2006). Therefore, basic knowledge of the biology of plasmids in bifidobacteria is still needed for the development of robust and efficient molecular tools. In particular, translated and untranslated plasmid sequences involved in structural and segregational stability need to be identified and characterized.

The aims of the present work were to identify the minimal replicon of plasmid pBC1 of *Bifidobacterium catenulatum* L48 (Álvarez-Martín et al. 2007) and to study the functionality of the different open reading frames (ORFs) and associated structures in its nucleotide sequence plus their effect on stability and copy number. Such knowledge will be very important for the design and construction of novel vectors for *Bifidobacterium* species derived from the pBC1 replicon.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Table 1 shows the bacterial strains and plasmids used. *B. catenulatum* and *Bifidobacterium pseudocatenulatum* strains were currently grown in MRS broth (Merck; VWR International, Darmstad, Germany) supplemented with 0.25% cysteine, while *Bifidobacterium breve* was usually grown in RCM broth (Merck). When required, bacteriological agar (Merck) was added to the media at 15 g l^{-1}. All incubations were performed at 37°C in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK; atmosphere: 10% H_2, 10% CO_2, and 80% N_2). *E. coli* One Shot® chemically competent cells (Invitrogen, Carlsbad, Ca., USA), used as a transformation host for cloning, were cultured at 37°C in Luria Bertani (LB) broth (Sambrook and Russell, 2001) with vigorous shaking. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) were incorporated into the LB agar at concentrations of 50 μg ml^{-1} and 40 μg ml^{-1}, respectively. Ampicillin, chloramphenicol, and tetracycline (all from Sigma-Aldrich, St. Louis, MO, USA), at 100, 10, and 5 μg ml^{-1}, respectively, were used to select for *E. coli* transformants; erythromycin (Sigma), chloramphenicol and tetracycline, at 5, 2, and 5 μg ml^{-1}, respectively, were used for bifidobacterial selection.

Plasmid isolation and analysis

Plasmid DNA from bifidobacteria was isolated according to the method of O'Sullivan and Klaenhammer (1993) with the following modification: pellets were suspended in TSE buffer (sucrose 25%, 50 mM Tris–HCl pH 8.0, and 10 mM EDTA pH 8.0) and incubated with lysozyme (30 mg ml^{-1}) at 37°C for 30 min. *E. coli* plasmid DNA was isolated using the Jet-Quick Plasmid Miniprep Kit (Genomed, Lohne, Germany) as recommended by the manufacturer. Plasmids were all analyzed by electrophoresis in TBE (89 mM Tris–HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.0) on 0.75–1.2% agarose gels (FMC Bioproducts, Philadelphia, PA, USA), followed by staining with ethidium bromide (0.5 μg ml^{-1}).

DNA manipulations and molecular cloning

The general procedures used for DNA manipulation were essentially those described by Sambrook and Russell (2001). Restriction endonucleases (Fermentas GMBH, St. Leon-Rot, Germany) and T4 DNA ligase (Roche, Mannheim, Germany) were used according to the manufacturers' instructions. The chemical transformation of plasmid DNA into *E. coli* was performed as described by Sambrook and Russell (2001), and electrotransformation (electroporation) of the plasmid DNA into *Bifidobacterium* performed as described by Rossi et al. (1996b) using a GenePulser apparatus (Bio-Rad Laboratories, Richmond, Ca., USA).

Table 1 Bacterial strains, plasmids and oligonucleotides utilized in this work

Item	Relevant genotype or phenotype	Source or reference
Strains		
Escherichia coli TOP10	F-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), F80*lacZΔM15*, *ΔlacX74*, *recA1, deoR*, *araD139*, *Δ(ara-leu)7697*, *galU*, *galK*, *rspL* (Str^r), *endA1*, *nupG*	Invitrogen (La Jolla, Ca., USA)
Bifidobacterium breve UCC2003	Human intestinal plasmid-free strain	APC-University College Cork
Bifidobacterium catenulatum L48	Human intestinal strain, containing pBC1	Álvarez-Martín et al. (2007)
Bifidobacterium pseudocatenulatum M115	Human intestinal plasmid-free strain	IPLA Laboratory Collection
Plasmid		
pBC1	Cryptic plasmid of 2.5 kbp	Álvarez-Martín et al. (2007)
pBif	Ap^r, Cm^r, 6.5 kbp	APC-University College Cork
pAM1	pBC1-pUC19E Em^r	Álvarez-Martín et al. (2007)
pAM5	pBC1-pUC19 Tet^r [tet(W)]	This work
pBC1.2	Segment of pBC1 in pBif lacking the putative promoter of *copG*-like	This work
pBC1.3	Segment of pBC1 in pBif lacking *copG*-like	This work
pBC1.4	Segment of pBC1 in pBif lacking *copG*-like and an IR after *orfX*-like	This work
pBC1.5	Segment of pBC1 in pBif lacking *copG*-like and *orfX*-like	This work
Oligonucleotides (5′-3′)		
pBC1-Ori	GTCACTGCATGCCCAGAGTAACACCACGGTCACAC	This work
pBC1-Orfx1	CGTCATGCATGCCGTCATTGCCCCATCATCTCCTTG	This work
pBC1-Orfx2	CTGATGGCATGCCACTTGCTACTCCGGCCTTGC	This work
pBC1-CopG1	CTGACTGCATGCTCGCCGGAGCCGTCTGAC	This work
pBC1-CopG2	GACTCAGCATGCGTGGAGATGGTCTACGAGCAGCG	This work
Fdxs	ACTCATTCCCCCGACTCAGG	This work
Rdxs	TCGCGGCATAGCTCATTCAG	This work
FrepB	GCCACGTTCGTCGCCATCCA	This work
RrepB	CCGACCAGCTCTGCCTTTTG	This work
Fxfp	GACGTCACCAACAAGCAGTG	This work
Rxfp	CTTCCATCTGGTGCTCGGAG	This work

Ap^r, Cm^r, St^r, and Tet^r, resistance to ampicillin, chloramphenicol, streptomycin, and tetracycline, respectively

Construction of pBC1 derivatives

Derivatives of pBC1 (pBC1.2, pBC1.3, pBC1.4, and pBC1.5) were constructed as outlined in Fig. 1. Purified DNA from pBC1 was used as a template in PCR reactions involving the Extensor Hi-Fidelity PCR Enzyme Mix (ABgene, Epsom, UK) and the synthetic oligonucleotide pairs shown in Table 1. All oligonucleotides were designed with a site for the *Sph*I restriction enzyme at their 5′ ends to allow direct cloning of amplicons after digestion with this enzyme into the unique *Sph*I site of the *E. coli* replication probe vector pBif (Table 1). pBif consists of a pBluescript II KS vector containing a chloramphenicol resistance cassette from plasmid pC194 (Sangrador-Vegas et al. 2007). Ligation mixtures were transferred into *E. coli* as described above; the selection of recombinant cells harboring pBC1-pBif derivatives was performed by blue-white screening on LB agar supplemented with X-gal, IPTG, and chloramphenicol. Derivatives selected in *E. coli* and verified by sequencing were then electrotransferred into *B. breve* UCC2003 and *B. pseudocatenulatum* M115.

Disruption of the *repB* gene

Two restriction enzymes were found to have unique recognition sites in pBC1 within the coding sequence of *repB*, namely *Mlu*I (at position 1,011) and *Aar*I (at position 1256), while no such recognition sites were found in the pUC19E or pBif vectors. The pBC1-pUC19E derivative pAM1 (Álvarez-Martín et al. 2007) was digested with both *Mlu*I and *Aar*I, the resulting fragment ends were filled in with the Klenow fragment of DNA polymerase I (Roche), and self ligated using T4 DNA ligase. Ligation mixtures were electrotransformed into *E. coli*, in which plasmids were analyzed for loss of the *Mlu*I restriction site. The expected construct was verified by sequencing and then used for transformation of *B. pseudocatenulatum* M115.

Segregational stability of the different constructs

The stability of the constructs was assayed by growing cells in non-selective media for approximately 100 generations. Cultures were plated on a daily basis onto non-selective agar plates, and plasmid maintenance of the resulting

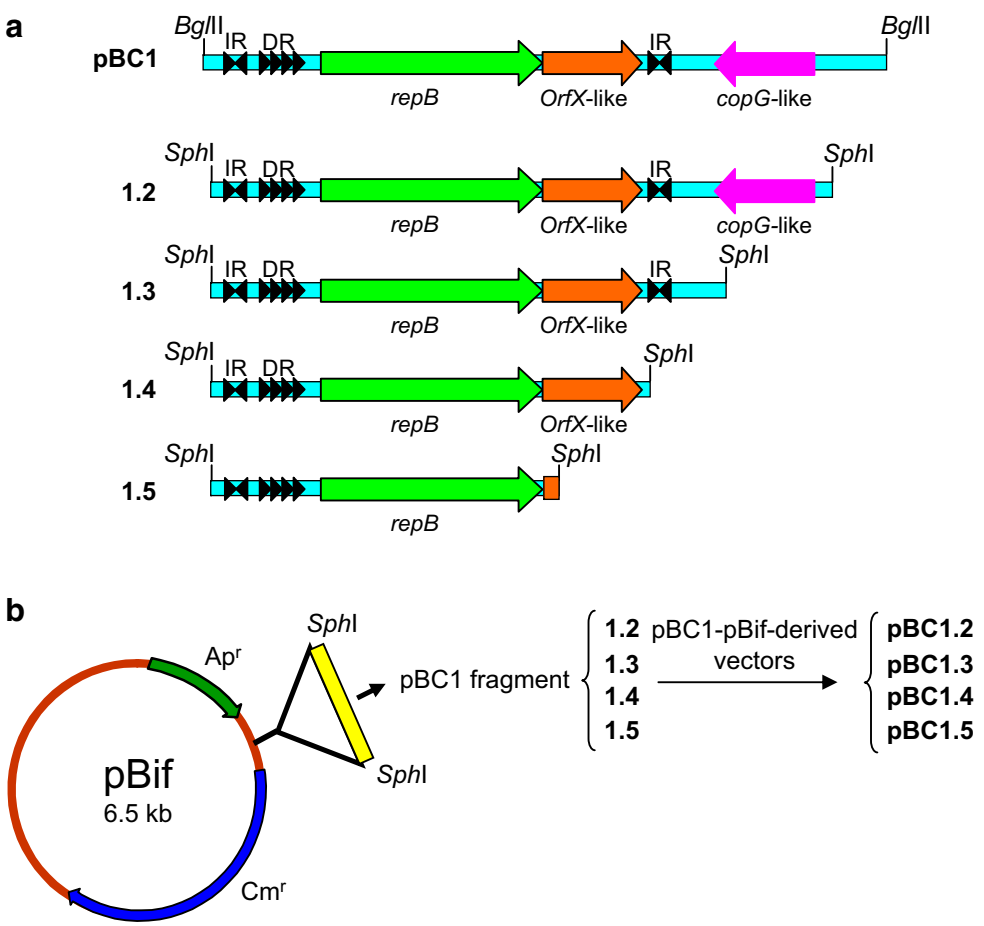

Fig. 1 **a** Physical and genetic map of pBC1 from *Bifidobacterium catenulatum* L48 and the pBC1 derivatives utilized in this work. Position of key features of pBC1 are indicated. The *Bgl*II site was arbitrarily taken as the numbering starting point of pBC1 sequence. The *Sph*I sites were introduced with the oligonucleotide primers used for amplification, as indicated on "Materials and methods." Arrows denote direction and approximate length of the different ORFs; thought figures are not drawn to scale. Facing black arrowheads indicate inverted repeats (IRs), and arrowheads with the same orientation indicate direct repeats (DRs). **b** Diagram showing the cloning strategy of the pBC1-derived amplicons into the replication-probe vector pBif

colonies was monitored by transfer colonies to antibiotic-containing agar plates. Plasmid content was then checked by electrophoresis of plasmid preparations.

Determination of the relative copy number

The copy number of the pAM5, a pBC1-pUC19E derivative in which the erythromycin resistance gene was substituted by a recently described bifidobacterial *tet*(W) gene (Flórez et al. 2006), and pBC1-pBif derivatives was assessed by quantitative real-time PCR (QPCR), using the culture and PCR conditions reported by Lee et al. (2006). Amplification and detection were performed in a Fast Real-Time PCR system (Applied Biosystems, Foster City, Ca., USA) 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 1-deoxy-d-xylulose 5-phosphate synthase (*dxs*) gene of *B. breve* UCC2003 was used as the comparator gene for copy number determination in this bacterium, assuming a copy number of one for this chromosomally encoded gene. A 119-bp segment of the *dxs* gene was amplified with primers Fdxs and Rdxs (Table 1). For *B. pseudocatenulatum* M115, a segment of 120 bp of the xylulose-5-phophate-fructose-6-phosphate-phosphoketolase gene (*xfp*) (GenBank Accession No. AY377401), amplified with primers Fxfp and Rxfp (Table 1), was used as the comparator gene. The relative copy number of the derivatives was calculated using the formula $N_{relative} = (1+E)^{-\Delta CT}$ (Lee et al. 2006), where E is the amplification efficiency of the target and reference genes, and ΔCT is the difference between the threshold cycle number (C_T) of the *dxs* reaction and that of *repB*. Experiments were performed in triplicate; mean results are provided.

Antibiotic resistance of the constructs

The antibiotic resistance of the constructs to chloramphenicol was measured using the Etest method, according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). This was performed in LSM medium (90% Isosensitest, 10% MRS; both from Oxoid, Oxoid Ltd., Basingstoke, Hampshire, UK; Klare et al. 2005) with cysteine (0.3 g l^{-1}).

Results

Functionality of the different ORFs of pBC1

To check the functionality of the identified ORFs and some of the surrounding sequences of pBC1, several segments of this plasmid were amplified by PCR and cloned into the pBif vector (Fig. 1). pBif is based on the *E. coli* pUC vector and does not replicate in bifidobacteria. However, it contains a chloramphenicol resistance gene allowing selection to be performed in both Gram-negative and Gram-positive organisms, including bifidobacteria (Sangrador-Vegas et al. 2007). Constructions were all performed and checked in *E. coli*, after which each of the construct was introduced into *B. breve* and *B. pseudocatenulatum* by electroporation.

The first construct, pBC1.2, harbors the complete sequence of the original plasmid, except for some 300 nucleotides (nt) around the single *Bgl*II site of pBC1, which is expected to contain the promoter of the *copG*-like gene. The second construct, pBC1.3, has a deletion of around 600 nt as compared to pBC1.2, including the complete *copG*-like gene. The third construct, pBC1.4, harbors the *repB* and the *orfX*-like genes but lacks the inverted repeat (IR) located downstream of the *orfX* stop codon. The position and sequence of this IR suggests that it may function as a Rho-independent transcription terminator. This structure seems to be well conserved in other bifidobacterial plasmids, such as in plasmid pMB1 from *B. longum* (Rossi et al. 1996a; although no function has been attributed to it). Finally, the smallest construct, pBC1.5, only harbors the *repB* gene and its upstream sequences, a region rich in secondary structures resembling the origin of replication of some theta-replicating plasmids (Álvarez-Martín et al. 2007). To exclude polar effects influencing copy number or antibiotic resistance, the selected constructs had the same relative orientation respect to the pBif molecule.

The recombinant plasmids pBC1.2, pBC1.3, pBC1.4, and pBC1.5 were then introduced into *B. breve* UCC2003 and *B. pseudocatenulatum* M115 by electroporation. In all cases, transformants were obtained, indicating that each of these constructs was capable of replication in these two different bifidobacterial strains. Surprisingly, the transformation frequency of the constructs increased with construct length: the lowest transformation efficiency (1.0×10^{1} cfu μg^{-1}) corresponded to the pBC1.5 plasmid, which only carries the *repB* gene, while the highest (1.2×10^{3} cfu μg^{-1}) to pBC1.2, a construct carrying an almost complete version of the pBC1 molecule (Table 2). The transformation frequency of this last construct was comparable to that of pAM5, 1.6×10^{3} μg^{-1}, which includes the whole pBC1 in a pUC-derived vector.

Disruption of the ORF encoding *repB* by digestion of the pAM1 with *Mlu*I and *Aar*I, filling in with Klenow and ligation resulted in a plasmid that did not allow transformation of the bifidobacteial strains used in this study (in contrast to pAM1). This indicates that this pMA1 derivative is unable to replicate in bifidobacteria, and that a functional RepB is essential for pBC1 replication.

Plasmid stability

Stability of each construct was analyzed twice and counts were done in duplicate. Average results are presented in Fig. 2. In the absence of selective pressure, nearly 100% of the cells retain pAM5, which contains the complete pBC1 sequence, after 100 generations in the two bifidobacterial strains. However, notable variability was observed in terms of the segregational stability of the different derivative

Table 2 Plasmid copy number and chloramphenicol resistance level of pBC1-derivatives in *Bifidobacterium breve* UCC2003 and *Bifidobacterium pseudocatenulatum* M115

pBC1-derived vectors	*B. breve* UCC2003			*B. pseudocatenulatum* M115	
	Relative copy number[a]	Transformation efficiency (ufc μg^{-1})[b]	MIC ($\mu g\ ml^{-1}$) to chloramphenicol	Relative copy number	MIC ($\mu g\ ml^{-1}$) to chloramphenicol
pAM5 (pBC1-pUC19E)	28±2.94	1.6×10^{3}	1[c]	31±4.28	0.75[c]
pBC1.2	3±0.18	1.2×10^{3}	48	23±3.52	24
pBC1.3	7±1.56	8.5×10^{2}	64	17±5.35	24
pBC1.4	8±0.98	2.5×10^{2}	48	11±2.17	16
pBC1.5	8±0.92	1.0×10^{1}	48	5±2.08	16

[a] The copy number of pBC1 per chromosome equivalent in its original host *Bifidobacterium catenulatum* L48 was determined to be 30.90±4.62 (Álvarez-Martín et al. 2007).

[b] Results are average of three independent transformations. The same tendency was observed for the *Bifidobacterium pseudocatenulatum* M115, although only one transformation experiment was done (data not shown).

[c] pAM5 harbors a *tet*(W) gene and, consequently, the minimum inhibitory concentration (MIC) to chloramphenicol of strains carrying this vector is identical to the plasmid-free strains.

constructs (Fig. 2). In most cases, a particular pBC1-derivative exhibited different segregational stability behavior in *B. pseudocatenulatum* as compared to *B. breve*, with a higher stability in the latter strain for all constructs during the first 60 generations; with the exception of pBC1.2 (showing identical stability to that of pAM5 in *B. pseudocatenulatum*). In general, plasmid length positively correlated with plasmid stability and constructs lacking the *copG*-like and/or *orfX*-like showed decreased stability as compared to the constructs that did contain these genes.

Relative copy number

The relative copy number of pAM5 and the constructs pBC1.2, pBC1.3, pBC1.4, and pBC1.5 was measured using exponentially growing cells by QPCR as outlined in the "Materials and methods." A tenfold serial dilution of total DNA from *B. breve* UCC2003 and *B. pseudocatenulatum* M115 was used to determine standard curves for the *repB*, *dxs*, and *xfp* genes. Theoretically, for a tenfold dilution in template DNA, a Ct value of 3.322 cycles should be expected (Lee et al. 2006). The standard curves obtained for *repB*, *dxs*, and *xfp* genes were linear (R^2>0.99) over the range tested; the slopes were 3.67 and 3.76, respectively, slightly higher than their theoretical values.

Table 2 shows the copy number results for *B. breve* and *B. pseudocatenulatum*. The copy number of the original plasmid pBC1 was determined to be around 30±4.62 copies per chromosome (Álvarez-Martín et al. 2007), and a similar value was obtained for pAM5 in each of these strains (Table 2). The copy number obtained for the pBC1 constructs was strongly reduced in *B. breve*, while the copy number in *B. pseudocatenulatum* was shown to decrease as the plasmid size decreased. Constructs lacking the *copG*-like and *orfX*-like genes showed the lowest copy numbers (except for the very low value obtained for pBC1.2 in *B. breve*).

Resistance to chloramphenicol appeared to be correlated to copy number in *B. pseudocatenulatum* M115 (Table 2) but not in *B. breve* UCC2003. In the latter, most pBC1-derivatives conferred a higher chloramphenicol resistance than in *B. pseudocatenulatum*; although the copy number was significantly lower. Influence of the culture medium of the inocula (MRS vs RCM) or differential growth kinetics of the strains may also account for the observed MIC differences. Nevertheless, the level of resistance allowed for the efficient selection of the vector in all cases.

Discussion

Genetic engineering projects involving *Bifidobacterium* species and strains require general and specialized cloning and expression vectors of small molecular size that are structurally stable, that allow efficient cloning, that permit the maintenance of homologous and heterologous DNA fragments, and that allow the expression of homologous and heterologous genes. A number of shuttle vectors have already been constructed, which can replicate in *Bifidobacterium* spp. and *E. coli* (Álvarez-Martín et al. 2007; Lee and O'Sullivan, 2006; Matsumura et al. 1997; Missich et al. 1994; Park et al. 1999; Rossi et al. 1996a, 1998). Many of these vectors are a result of cloning of complete bifidobacterial plasmids into an *E. coli* vector containing an antibiotic selection marker. However, the basic biology of bifidobacterial plasmids has remained largely unexplored. Furthermore, the functionality of the various ORFs and associated secondary structures on such plasmids and their minimal replicon have yet to be determined.

In this work, sequential deletions were used to study the functionality of two ORFs (*copG*-like and *orfX*-like) present in the pBC1 sequence and associated structures and to analyze their effect on stability and copy number.

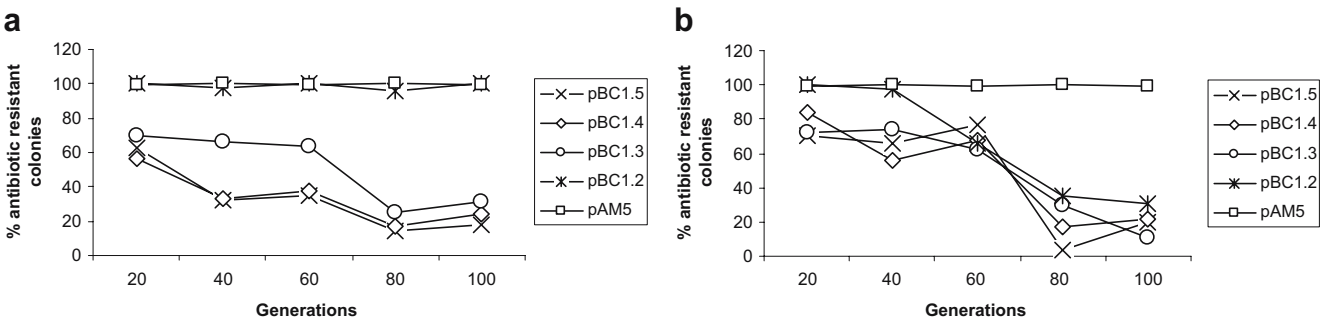

Fig. 2 Segregational stability of pBC1 derivatives in *B. pseudocatenulatum* M115 (**a**) and in *B. breve* UCC 2003 (**b**). Average results of two independent experiments for each construct are represented. Strains harboring the different constructs were cultured in the absence of selective pressure, plated under the same conditions, and assayed for plasmid maintenance by replica-plating onto antibiotic-containing media at, approximately, 20-, 40-, 60-, 80-, and 100-generation intervals. The presence of plasmids was finally checked by gel electrophoresis of plasmid preparations

Genes homologous to *orfX*-like genes (and with similar organization) have been identified in many lactococcal theta-replicating plasmids downstream of the essential *repB* gene (Frère et al. 1993; Gravesen et al. 1995; Hayes et al. 1991; Sánchez et al. 2000). Although their precise function remains unclear, some have been shown to participate in the regulation of plasmid copy number, plasmid stability, or both (Frère et al. 1993; Gravesen et al. 1995; Hayes et al. 1991; Sánchez et al. 2000). However, naturally occurring plasmids that carry interrupted *orfX* genes or even completely lack this ORF, have also been reported, e.g., pWVO2 (Kiewiet et al. 1993) and pNZ4000 (involving the genes *repB1* and *repB2*; van Kranenburg and de Vos 1998).

The deduced product of the *copG*-like gene of pBC1 contains a conserved domain found in proteins of the CopG family (Álvarez-Martín et al. 2007). This family of proteins is thought to be involved in the replication of plasmids that make use of the RC replicating mechanism (del Solar et al. 1998). For example, the minimal replicon of plasmid pMB02 from *Lactococcus lactis* subsp. *cremoris* was shown to include *copG* (Sánchez and Mayo 2003), whereas the minimal replicon of pMB1 from *B. longum* (Rossi et al. 1996a) and pBM300 from *Bacillus megaterium* (Kunnimalaiyaan and Vary 2005) was reported to include a *orfX*-like gene. In the latter two plasmids, constructs lacking either the *copG* or the *orfX*-like gene were shown to exhibit decreased stability (Kunnimalaiyaan and Vary, 2005). Still in some others, e.g., pGA1 and pXZ608, both from *Corynebacterium glutamicum* (Lei et al. 2002; Nesvera et al. 1997), the minimal replicon only includes the gene encoding their replication protein.

From our results, we conclude that neither the *orfX*-like nor *copG*-like genes are essential for the replication of pBC1, although the observed differences in transformation frequency, plasmid stability, and copy number indicates that they may play important roles in the replication process. Although unlikely, as pUC19E and pBif share an identical *E. coli* replicon, the different vectors used to clone the complete pBC1 sequence and its deleted derivatives might have affected copy number. The maintenance of small, functional, indigenous RC plasmids in Gram-positive bacteria is usually achieved by their having a large copy number, with no need for dedicated partitioning mechanisms (del Solar et al. 1998). Consequently, these two variables are usually strongly related. Indeed, segregational instability has often been observed in plasmids with replication defects, resulting in a reduction in plasmid copy number. In pBC1, a clear correlation was observed between a reduction in copy number and segregational instability, although it was not absolute. The differential behavior of pBC1.2 and pBC1.3 in stability might additionally be due to a read through of *copG* in pBC1.2. Nevertheless, it should also be noted that pBC1 may be a theta-replicating plasmid (Álvarez-Martín et al. 2007), and it may therefore behave differently from RC plasmids (del Solar et al. 1998). Also worth noting is the strong effect that the genetic background of the host cell has on stability, copy number, and the antibiotic resistance afforded by the constructs (Fig. 2; Table 2). Genetic background may include interference with plasmid-integrated remnants in particular strains (Schell et al. 2002). However, given the equal stability of pAM5 and pBC1.2 and their similar copy number in the two *Bifidobacterium* species, the observed differences in the stability of the smaller constructs must be due to varying host-specific replication interactions within the missing segments.

The functionality of *repB* was addressed by introducing a deletion into its ORF, which was shown to cause the loss of replication ability in bifidobacteria. This is not surprising, as plasmids that replicate by either the RC mechanism or the theta mode need specific replication proteins to recognize (bind) and cut the double-stranded origin of replication (*sdo*) for the process to begin (del Solar et al. 1998).

In conclusion, the present results show that the replication of pBC1 relies on a 1.5-kbp segment harboring the *repB* gene and its putative promoter sequence. Although not essential, the presence of both the *orfX*-like and *copG*-like genes and their surrounding sequences profoundly affects the transformation efficiency of the constructs, as well as their copy number and their segregational stability. Whether any of these two genes directly determines stability or whether this decreased stability is due to other, perhaps structural causes, remains to be determined. The construction of stable, multi-copy number vectors based on the pBC1 replicon should therefore include all three identified genes. However, the reduced copy number of several constructs could be exploited to develop low-copy number plasmids, best suited for studying and fine-tuning single-copy chromosomally encoded genes, as well as the construction of unstable vectors, which may be very useful if final curing of such a plasmid is required.

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