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Molecular characterization of *Bifidobacterium longum* biovar *longum* NAL8 plasmids and construction of a novel replicon screening system

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Abstract In this study, we performed molecular characterization and sequence analysis of three plasmids from the human intestinal isolate *Bifidobacterium longum* biovar *longum* NAL8 and developed a novel vector screening system. Plasmids pNAL8H (10 kb) and pNAL8M (4.9 kb) show close sequence similarity to and the same gene organization as the already characterized *B. longum* plasmids. The *B. longum* plasmid pNAC1 was identified as being most closely related to pNAL8L (3.5 kb). However, DNA sequence analysis suggested that direct repeat-rich sites could have promoted several recombination events to diversify the two plasmid molecules. We verified the likely rolling circle replication of plasmid pNAL8L and studied the phylogenetic relationship in all the *Bifidobacterium* plasmids fully sequenced to date based on *in silico* comparative sequence analysis of their replication proteins and iteron regions. Our transformation experiments confirmed that the ColE1 replication origin from high-copy-number pUC vectors could interfere with the replication apparatus of *Bifidobacterium* plasmids and give rise to false positive clones. As a result, we developed a system suitable for avoiding possible interference by other functional replication modules on the vector and for screening functional replicons from wild-type plasmids.

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Introduction

Bifidobacteria are becoming commercially and economically significant ingredients in the fermented dairy products and food supplement industries because of their claimed probiotic properties (Stanton et al. 2001).

Studying bacterial plasmids can provide valuable information to help elucidate the ecological mechanisms of microbial adaptability and evolution, and a starting point for preparing instruments for biotechnological purposes.

Only a few *Bifidobacterium* species have been demonstrated to harbor extrachromosomal DNA molecules, and these species are *asteroides*, *B. breve*, *B. globosum*, *B. indicum*, *B. longum*, and *B. pseudocatenulatum* (Schürch 2002; Iwata and Morishita 1989; Sgorbati et al. 1982). Among the above species, plasmids have been most frequently identified in *B. longum*, a common human intestinal inhabitant that shows a plasmid-bearing frequency of about 68% of the strains (Sgorbati et al. 1982). Moreover, among the 17 *Bifidobacterium* plasmid sequences deposited in the GenBank, 12 belong to *B. longum*.

This study reports molecular characterization and computational sequence analysis of three plasmids in the human

intestinal isolate *B. longum* biovar *longum* NAL8 (Canzi et al. 2005) with an *in silico* comparison of all the *Bifidobacterium* plasmid sequences available to date.

As the genetic transformation of bifidobacteria is difficult (Corneau et al. 2004) and the expression of heterologous genes are often unsuccessful (Rossi et al. 1996), few molecular instruments are today available for study and genetic modification of bifidobacteria (Park et al. 1999; Rossi et al. 1996). When we developed various cloning vectors built in the *Escherichiacoli* strain XL1 based on the replication system ColE1 of pUC18 or pUC19 vectors, we obtained false positive clones not containing the expected recombinant molecule. Other authors (Schürch 2002; Corneau et al. 2004) have reported similar results. Therefore, for our study, we developed and used a novel system suitable to circumvent this problem.

Materials and methods

Plasmids, bacterial strains, and media

Table 1 lists the bacterial strains and plasmids used in this study. *Bifidobacterium* strains were grown in MRS (Difco, Detroit, MI, USA) supplemented with 0.05% L-cysteine hydrochloride (cMRS) anaerobically at 37°C. *E. coli* was cultivated in synthetic-oil-based medium at 37°C. Antibiotics (supplied by Sigma, St. Louis, MO) were employed in the following concentrations: *E. coli*, 200-μg erythromycin (Em) ml^{-1}, 10-μg kanamycin ml^{-1}, 6-μg chloramphenicol (Cm) ml^{-1}; *Bifidobacterium*, 5-μg Cm ml^{-1}, and 5-μg Em ml^{-1}.

Plasmid DNA isolation and analysis

Plasmid DNA isolation from 30 ml of *Bifidobacterium* broth culture was performed by extracting it with an equal volume of 5-M LiCl and by precipitating it with polyethylene glycol (Sambrook and Russell 2001). Plasmids were purified by centrifugation to equilibrium in a CsCl–EtBr density gradient. Subsequently, the plasmids from the strain NAL8 were separated by electrophoresis and excision from the agarose gel.

For the analysis of circular covalently closed (CCC) and open circle plasmid forms, second-dimension electrophoresis was performed according to Hintermann et al. (1981).

Characterization and sequencing of *B. longum* NAL8 plasmids

A complete DNA sequence was determined for plasmids pNAL8M and pNAL8L, whereas for plasmid pNAL8H, a restriction map was prepared by digesting it with restriction enzymes *Ava*I, *Eco*RI, *Eco*RV, *Kpn*I, *Hinc*II, *Hind*III, and *Pst*I (Sigma). Moreover, in pNAL8H, regions A and B were PCR-amplified with primers designed on the DNA sequence of plasmid pNAC3 (NC_004768). PCR reactions were performed with *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). Sequences were determined using an ABI Prism BigDye™ terminator technology in an ABI Prism™ 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

To predict the location of putative open-reading frames (ORFs), an ORF-finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) software was set at default for the bacterial code. The basic local alignment search tool (BLAST) programs (http://www.ncbi.nlm.nih.gov/blast/) were used to conduct similarity searches against the GenBank and EMBL sequence databases. The gene or protein sequence alignments and neighbor-joining phylogenetic analysis were run using ClustalW and Treecon v1.3b software, respectively.

Table 1 Bacterial strains and plasmids used in the study

	Pertinent characteristics	Source
Bacterial strain		
B. longum NAL8		Canzi et al. (2005)
B. animalis ScMa7	Isolated from commercial fermented milk	This study
B. longum NCC2705		Nestlé Culture Collection
E. coli VE7108	Gram host of pG^+host, pGHori and pGOSBif33	Maguin et al. (1996)
Plasmids		
PNAL8H	10 kb; natural plasmid from *B. longum* NAL8	This study
PNAL8M	4920 bp; natural plasmid from *B.longum* NAL8	This study
PNAL8L	3489 bp; natural plasmid from *B. longum* NAL8	This study
pG^+host	Em^r, thermosensitive replicon	Maguin et al. (1996)
PGHori	3202 bp; pG^+host without *repA* gene	This study
PGOSBif33	6691 bp; *E. coli–Bifidobacterium* shuttle vector	This study

Plasmid and vector maps were plotted using the Vector NTI 6 (InforMax, Oxford, UK) program.

Detection of single-stranded plasmid DNA

DNA was isolated from *B. longum* NAL8, treated with nuclease S1 (Roche Diagnostic), separated by agarose gel electrophoresis, and transferred onto a hybond-N nylon membrane (Amersham Biosciences, Milan, Italy; Sambrook and Russell 2001). Single-stranded DNA was identified by using as probe a DNA fragment of 394 bp obtained by PCR from the plasmid pNAL8L (primers employed were p3.5f, 5′-CGGACGGTACTTAGTACAA-3′ and SGD3.5r, 5′-CCGCAACGTCAACCAGAT-3′). After amplification, the DNA fragment obtained was DIG-dUTP-labeled by random priming with a labeling kit (Roche Diagnostic, Rotkreuz, Switzerland) and used as probe in the hybridization experiment. Filter hybridization was performed according to the supplier's instructions with pre-hybridization and hybridization steps in 50% (wt/vol) formamide at 42°C and stringency washes in 0.1× SSC, 0.1% SDS at 65°C.

Vector constructions

Unless otherwise stated, ligation reactions were performed in 10 µl with 2.5 U of T4 ligase (Fermentas) at 22°C for 60 min. *E. coli* transformations were performed by heat shock of inoue-competent cells (Sambrook and Russell 2001).

To construct the plasmid pGHori, we designed two divergent primers near the extremities of the replication gene of vector pG$^+$host9 (Maguin et al. 1996), pGhf (5′-TATCAGTGTG*GAGCTC*GAGCAAGTT-3′), and pGhr (5′-AGCCATAGAT*GAGCTC*AAACTCTCT-3′), which contained the restriction site *Sac*I (underlined; Fig. 1). The thermosensitive vector pG$^+$host9 that bears the ribosomal RNA adenine *N*-6-methyltransferase gene for erythromycin resistance was maintained in the *E. coli* strain VE7108, which has a non-thermosensitive copy of the plasmid replication gene *repA* on its chromosome. The PCR product we obtained via amplification with primers pGhf and pGhr, after restriction with *Sac*I, was self-ligated (5 U of Fermentas T4 ligase in a final volume of 50 µl) and cloned back into *E. coli* VE7108. To obtain vector pGOSBif33, we linearized plasmid pNAL8L with *Cla*I and cloned it in the same restriction site of vector pGHori (Fig. 1).

Electroporation of bifidobacteria

Electrocompetent *Bifidobacterium* cells were prepared as follows. Fresh cMRS broth (250 ml) supplemented with 0.2% glucose was inoculated with 10 ml of an overnight culture of the *Bifidobacterium* strain and incubated at 37°C for 1–5 h until an optical density (at 600 nm) of 0.2–0.4 was reached. The cells were chilled on ice and concentrated in further washing steps of 250, 50, and 10 ml (washing buffer: 1 mmol l^{-1} ammonium citrate, 0.5% sucrose, pH 6). After washing, the pellet was resuspended in 1.25 ml of the same buffer. Cell suspensions were stored at −4°C for 3 h and then maintained at −80°C until used for electroporation (12.5 kV/cm, 2-mm cuvettes, time constants obtained between 3.9 and 4.2 ms).

Accession numbers

The EMBL accession numbers of the DNA sequences determined in this study are as follows: region A of plasmid pNAL8H, AM183142; region B of plasmid pNAL8H, AM183143; complete sequence of plasmid pNAL8M, AM183144; complete sequence of plasmid pNAL8L, AM183145.

Results

Plasmids in *B. longum* NAL8

The two-dimensional gel electrophoresis revealed three different CCC DNA molecules in a nucleic acid extract of *B. longum* NAL8 after ultracentrifugation in a CsCl–EtBr density gradient (Supplementary Material S1). The super-coiled DNA ladder suggested the molecular weights of the three molecules labeled pNAL8H, pNAL8M, and pNAL8L to be 10, 4.9, and 3.5 kb, respectively.

Molecular characterization of pNAL8H

The restriction map of the plasmid pNAL8H showed a strong similarity with the already characterized *B. longum* plasmid pNAC3 (NC_004768; Corneau et al. 2004), with differences located only inside two small regions labeled A and B that were sequenced. The sequence analysis revealed that only two point mutations differentiate region A (675 bp) in pNAL8H from the corresponding region in pNAC3. Region B (1,381 bp) holds the nucleotides just upstream of the putative plasmid *rep* gene containing direct repeat sequences called iterons that constitute the putative plasmid replication origin. The putative replication origin of pNAC3 consists of eight direct repeats of 11 nucleotides with the sequence 5′-GGTGGCTAAAG-3′. In pNAL8H, this motif is present nine times because an insertion of 65 bp observed in region B includes an extra iteron sequence.

Nucleotide sequence analysis of plasmid pNAL8M

Plasmid pNAL8M was fully sequenced, consisting of 4,910 bp with a mean G+C content of 61.9mol%. A

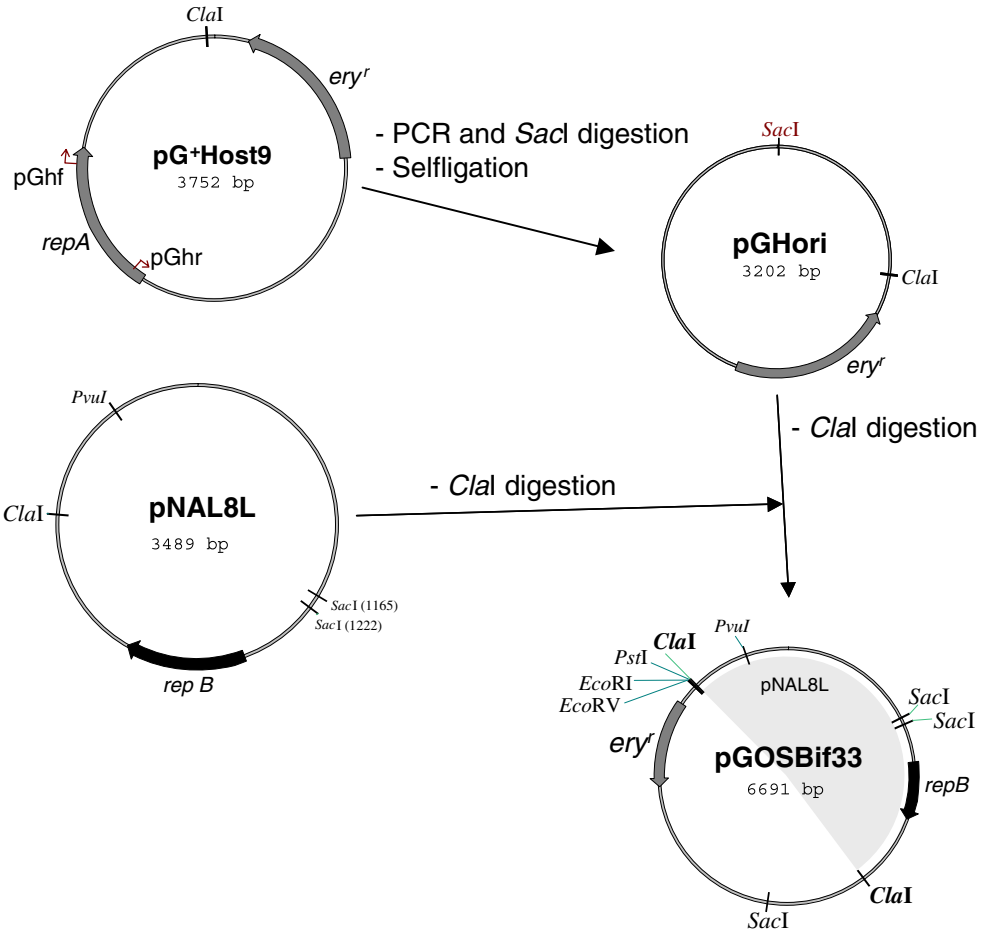


Fig. 1 Strategy adopted to construct *E. coli* VE7108–*Bifidobacterium* shuttle plasmid pGOSBif33

database search revealed that pNAL8M shares 97% nucleotide identity with pKJ50 (NC_004978), a *B. longum* plasmid already characterized (Park et al. 1999). Moreover, 4,405 bp of pNAL8M are present with 99% nucleotide identity in plasmid pDOJH10L. As stated by Lee and O'Sullivan (2006), plasmid pDOJH10L seems to result from the fusion of three different plasmid molecules (Supplementary Material S2). Interestingly, the junctions between the DNA regions of potentially different origin that constitute pDOJH10L are characterized by the 5′-CCGG-3′ motif that may represent the target sequence of recombination events (Supplementary Material S2).

Characterization and sequence analysis of plasmid pNAL8L

The hybridization experiments after S1 nuclease treatment of plasmid DNA revealed that pNAL8L probably replicates through a rolling circle mechanism since we detected the ssDNA of pNAL8L (Supplementary Material S3).

The DNA sequencing of both strands of the plasmid pNAL8L resulted in a single contiguous sequence of 3,489 bp (mean G+C content of 59.0mol%). Five ORFs identified on the molecule showed significant similarity with putative proteins already deposited in the databanks (Fig. 2).

Our DNA sequence analysis showed that pNAC1 is the plasmid molecule most closely related to pNAL8L. However, the new and composite organization of pNAL8L suggests that several recombination events occurred to diversify the two plasmid molecules (Fig. 2). Specifically, the pNAL8L region including *orfIII* occurs in an orientation opposite to that of the homologous region in pNAC1. This region is delimitated by two loci, DR^{II} and DR^{III}, constituted by direct repeats. DR^{III} comprises a 22-bp sequence 5′-ACTTAGTACAAAAGGGGAACGA-3′ (sequence Rp) repeated 7 times, whereas the corresponding region in pNAC1 contains the Rp sequence only once. On the other side of the region containing *orfIII*, DR^{II} comprises the sequence 5′-ATGGGCATCTCCATGTG GATGTCC-3′, repeated almost perfectly three times. The same 24-bp motif occurs in the corresponding region of pNAC1, repeated also three times. As suggested for other plasmids (Hofreuter and Haas 2002), such sequence repeats may act as hot spots for recombination and site-specific integration. This assumption is supported by the observation that the region of pNAL8L containing *orfV*, closely similar to a chromosomal region in *B. longum* NCC2705, is

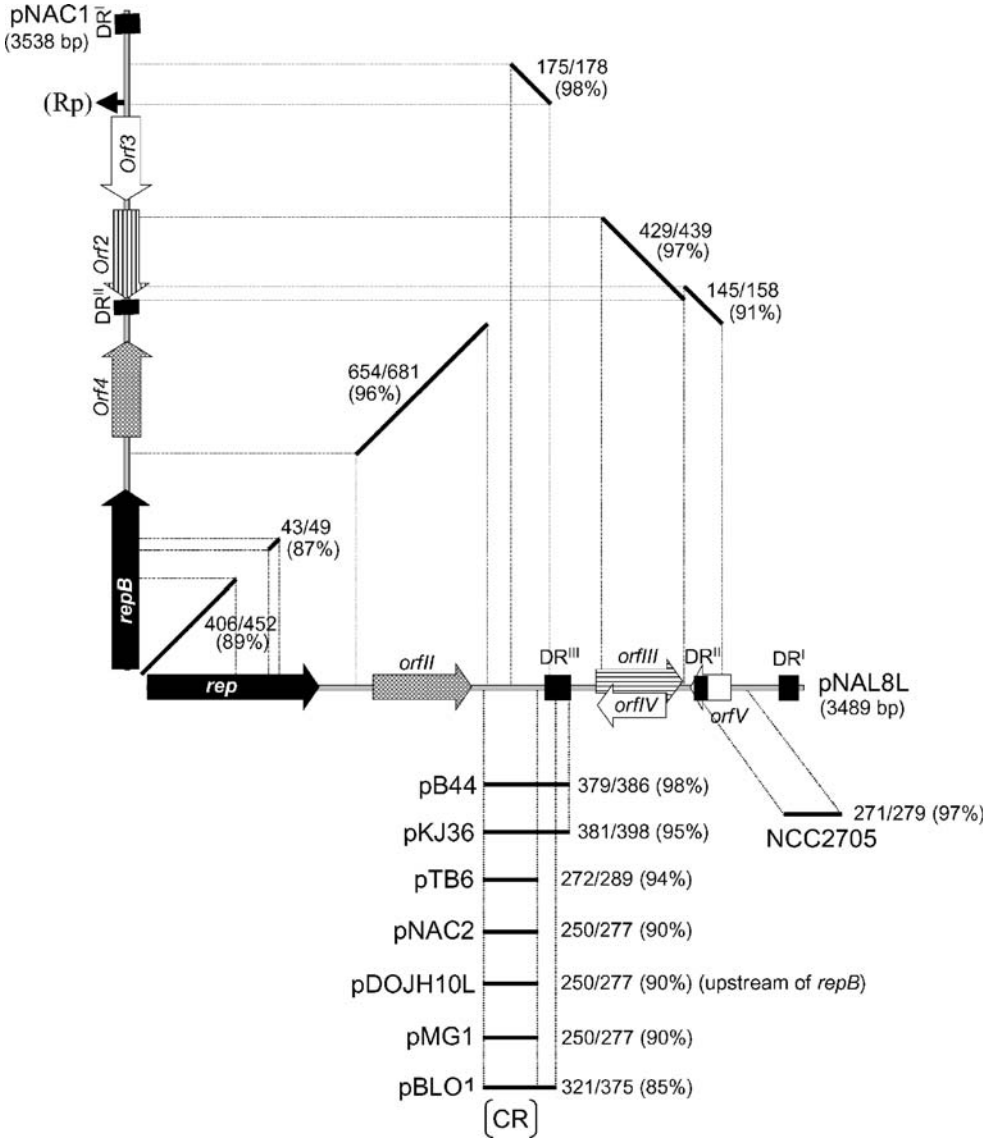


Fig. 2 Functional map of plasmid pNAL8L and bi-dimensional representation of pNAL8L regions with significant similarity with pNAC1; identity value reported for each pNAL8L fragment in nucleotide ratio and percentage; the region labeled CR and common to seven other *Bifidobacterium* plasmids is indicated. *orfV* codes for a presumptive peptide that is 62% identical with the hypothetical protein BL1470 found on the chromosome of *Bifidobacterium longum* NCC2705 (AAN25265). The putative product of *orfIV* showed similarity (83% identity) to the subunit K/omega (COG1758) of the DNA-directed RNA polymerase of *Bifidobacterium longum* DJO10A

located between two direct repeat-rich sites, DR^I and DR^{II} (Fig. 2).

Furthermore, a region of about 250 bp, upstream of the DRIII locus of pNAL8L, was strongly conserved among the 7 plasmids constituting the Rep cluster I (Supplementary Material S4; Fig. 3). This 250-bp DNA fragment contains a 36-bp AT-rich region at its 3′ terminus that may well be the site where DNA strands melt when the plasmids of Rep cluster I start replicating.

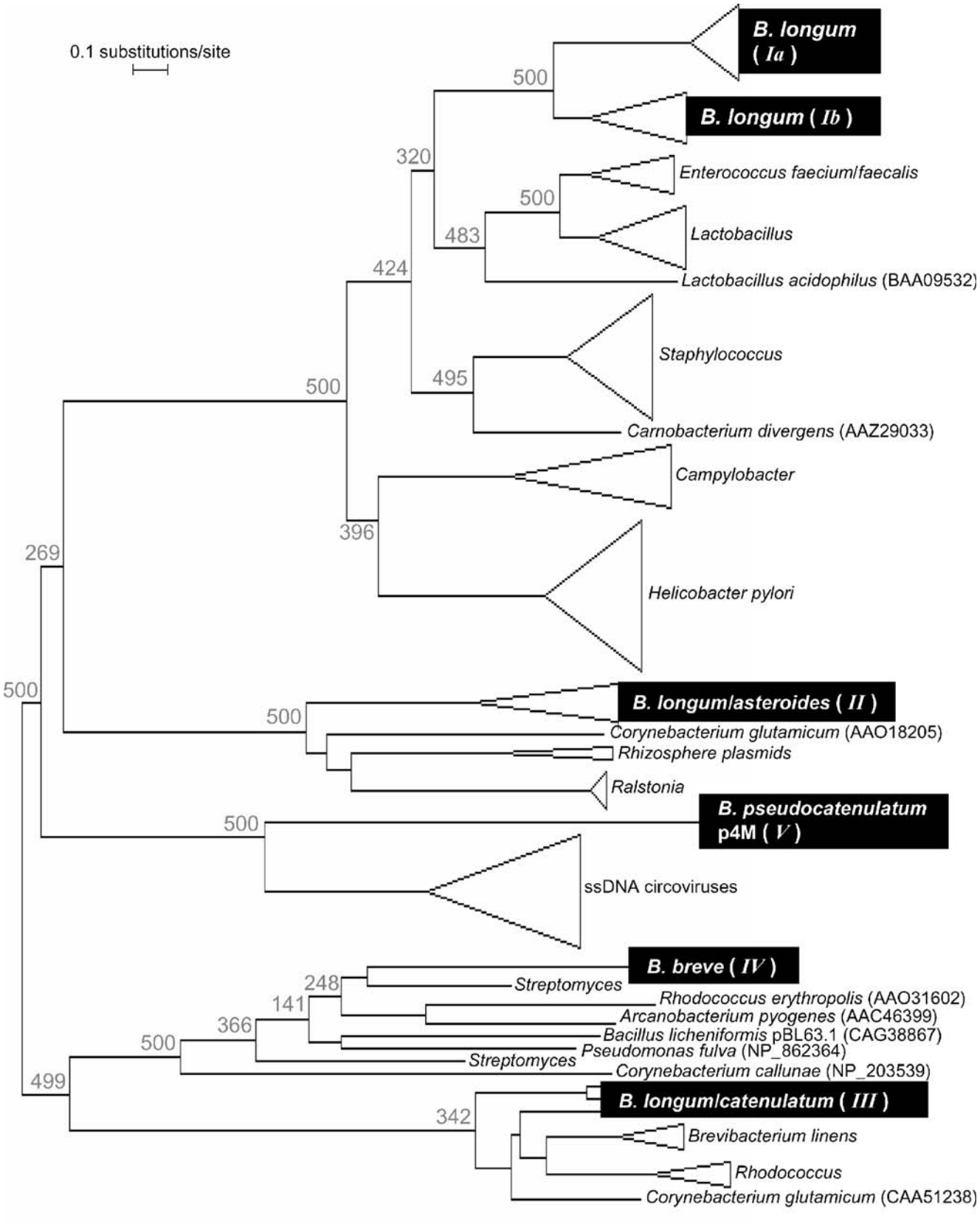
Phylogenetic relationship between *Bifidobacterium* plasmid Rep proteins

With a neighbor-joining dendrogram, Rep proteins from *Bifidobacterium* plasmids can be clustered into five main groups (Supplementary Material S4 and Fig. 3). *B. longum* Reps occur in three of these clusters (named I, II, and III), whereas only the replication proteins of two *B. breve* and one *B. pseudocatenulatum* plasmids constitute groups IV and V, respectively (Supplementary Material S4). This classification is supported by the homology between Reps belonging to a single group and specific Pfam families of initiator protein conserved domains (Supplementary Material S4).

The neighbor-joining dendrogram built, considering *Bifidobacterium* plasmid Reps and 54 other replication proteins most closely related in the BLAST-p analysis, revealed that each of the five typologies of *Bifidobacterium* Rep proteins has similarities with clusters of replication proteins from various other hosts (Fig. 3).

Iterons in pNAL8L and other *Bifidobacterium* plasmids

The iteron region DR^I of pNAL8L, located just upstream of the *rep* gene (Fig. 2), shows the typical organization of iteron-containing replication origins (Supplementary Mate-


0.1 substitutions/site
B. longum (Ia)
B. longum (Ib)
Enterococcus faecium/faecalis
Lactobacillus
Lactobacillus acidophilus (BAA09532)
Staphylococcus
Carnobacterium divergens (AAZ29033)
Campylobacter
Helicobacter pylori
B. longum/asteroides (II)
Corynebacterium glutamicum (AAO18205)
Rhizosphere plasmids
Ralstonia
B. pseudocatenulatum p4M (V)
ssDNA circoviruses
B. breve (IV)
Streptomyces
Rhodococcus erythropolis (AAO31602)
Arcanobacterium pyogenes (AAC46399)
Bacillus licheniformis pBL63.1 (CAG38867)
Pseudomonas fulva (NP_862364)
Streptomyces
Corynebacterium callunae (NP_203539)
B. longum/catenulatum (III)
Brevibacterium linens
Rhodococcus
Corynebacterium glutamicum (CAA51238)
500
320
500
483
424
500
495
500
396
269
500
500
500
248
141
366
500
499
342

◀ **Fig. 3** Neighbor-joining dendrogram of Rep proteins generated from ClustalW alignment of 54 Rep proteins most closely related to 22 *Bifidobacterium* plasmid Reps in Blast-p search; *Bifidobacterium* plasmid clusters shown in *white letters on black field*; bootstrap values of the main internodes (500 replicates) are shown; database accession numbers shown in *parentheses*. The accession numbers of the replication protein sequences not shown on the dendrogram are as follows: *Enterococcus faecium/faecalis*, AAL05545, AAL39167, AAM44830, NP_863270; *Lactobacillus*, AAL54831, AAZ13604, BAA08501, BAA87064, NP_862269, NP_862285; *Staphylococcus*, AAC63222, AAD02381, AAD02388, AAD02408A, AAN71829, AAK73551, BAE05993, NP_863200, NP_395563, NP_976281, YP_187551; *Campylobacter*, AAG40755, BAD95842, CAA57593, CAA57596, NP_862911, YP_247575; *Helicobacter pylori*, AAD11553, AAD11557, AAD11558, AAD11559, AAD11561, AAD11562, ABA26021, ABB51115, NP_044345, NP_858075, NP_862334, NP_862357, YP_025604; plasmids from rhizosphere, CAC79146, CAC82776; *Ralstonia*, AAZ64686, CAD17152, ZP_00166294, ZP_00596502; circoviruses, AAC69861, AAG30561, AAG30564, AAS93271, AAT00476, AAT00477, AAT00478, AAX10150, AAY81718, NP_573442; *Brevibacterium linens*, CAA72653, NP_065271, ZP_00377816; *Rhodococcus*, BAE06128, NP_858932

rial S5). It contains three fully identical 22 bp directly repeated sequences followed immediately by an almost perfect, inverted repeated sequence comprising the first 11 bp of the iteron. Just upstream of the direct repeated sequences is an AT-rich region, flanked by smaller GC-rich sequences, where DNA strands may melt at the onset of plasmid replication. Furthermore, plasmid pNAL8L contains two other regions with a typical organization of the iteron-containing origins, DR^{II} and DR^{III}, but located far from the replication gene (Fig. 2 and Supplementary Material S5).

When we aligned the iteron regions of the *Bifidobacterium* plasmids to construct a neighbor-joining dendrogram (Fig. 4), we found significant similarity between DR^{II} and iterons from the plasmids of Rep cluster III, whereas DR^{III} was significantly similar to iterons from the plasmids of Rep group Ia (Supplementary Material S4; Fig. 3). Globally, the dendrogram in Fig. 4 shows a clear correspondence between the iteron sequence typologies found in *Bifidobacterium* plasmids and the Rep clusters (Supplementary Material S4). As expected, each Rep protein appears to be associated with its specific iteron region, suggesting that a *rep* gene and its cognate iteron region should be considered a unique module in the mosaic structure of *Bifidobacterium* plasmids.

Transformation experiments

We used pGHori vector to prepare an *E. coli* VE7108–*Bifidobacterium* shuttle plasmid, labeled pGOSBif33, based on pNAL8L. We found no clones when we transformed *B. longum* NAL8L with pGOSBif33, a finding that suggested a possible incompatibility with plasmid pNAL8L, which is naturally present in NAL8. On the contrary, when we

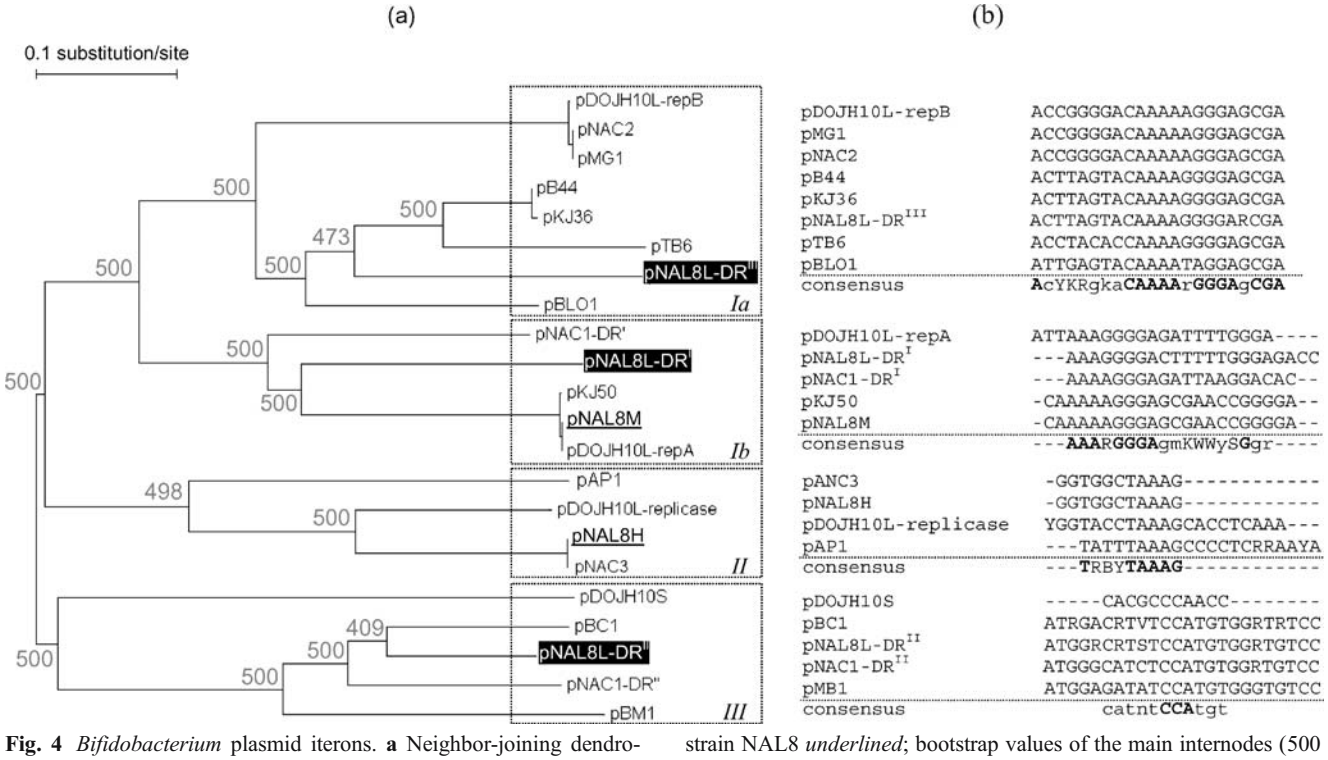


Fig. 4 *Bifidobacterium* plasmid iterons. **a** Neighbor-joining dendrogram generated from the ClustalW alignment of iteron-containing DNA regions from *Bifidobacterium* plasmids; pNAL8L iterons shown in *white letters on black field* with two other plasmids of *B. longum* strain NAL8 *underlined*; bootstrap values of the main internodes (500 replicates) are shown; cluster denominations refer to Rep protein groups of Supplementary Material S4. **b** ClustalW alignments of iteron sequences from *Bifidobacterium* plasmids

introduced pGOSBif33 via electroporation into *B. longum* NCC2705 and *B. animalis* ScMa7, we obtained transformation rates of 200 and 50 clones per microgram of recombinant DNA, respectively. The clones showed bifidobacterial morphology under the microscope, registered positive in the PCR for the erythromycin resistance gene, *ery*^{r}; and we could isolate the expected circular DNA molecule from their cultures (Supplementary Material S6). Moreover, we could accurately identify the plasmid molecules in recombinant cells in PCR experiments with several pairs of primers targeting different regions of pGOSBif33 (data not shown).

Discussion

Plasmid multiple colonization is quite common in *B. longum* so that Sgorbati et al. (1982) reported strains exhibiting as many as seven plasmid bands. Nevertheless, no more than two co-resident plasmids of a single strain have been fully characterized so far (Corneau et al. 2004; Lee and O'Sullivan 2006; Park et al 1997). That is why we decided to perform a molecular characterization of the three plasmids harbored by *B. longum* NAL8, a strain isolated as a member of the predominant bifidobacterial population in a single fecal sample from a healthy elderly woman (Canzi et al. 2005).

Plasmids pNAL8H and pNAL8M showed a close sequence similarity to and the same gene organization as the already characterized *B. longum* plasmids. Plasmids sharing close sequence similarity have already been isolated from *B. longum* strains of different origin (for example, plasmids pB44, pKJ36, pNAC2, pTB6, and pMG1), indicating that molecular recombination and rearrangements occur rarely. Yet, examples of evident rearrangements in *B. longum* plasmids have been detected by analyzing the pNAL8L DNA sequence or the recently characterized pDOJH10L plasmid (Lee and O'Sullivan 2006).

The phylogenetic analysis of replication proteins supports the hypothesis that ancestral replicons from different hosts may be acquired by and then developed in the *Bifidobacterium*, resulting in the five Rep types identified (Supplementary Material S4 and Fig. 3). It is also noteworthy that *Corynebacterium* replicons, known to replicate in bifidobacteria (Argnani et al. 1996; Schürch 2002), contain replication proteins similar to typologies II, III, and IV (Fig. 3), suggesting that these replicons may have begun replicating in members of *Bifidobacterium* and *Corynebacterium* before the two evolved into distinct genera. However, these speculations lack experimental proof because the direction of acquisition cannot be inferred from comparative analysis.

Shuttle vectors based on the ColE1 replication origin constitute the most commonly used system to isolate functional replicons of *Bifidobacterium* (Matteuzzi et al. 1990; Rossi et al. 1996; Matsumura et al. 1997; Park et al. 1997, 1999; Tanaka et al. 2005). However, when ColE1 was used successfully, it was obtained from pBR322, not from high-copy-number pUC vectors. In fact, the experimental data by Corneau et al. (2004) clearly indicate that ColE1 from the high-copy-number vector pUC18 can be rescued from *Bifidobacterium*. Further, the authors suggested that various replicon-screening systems should be developed to overcome such potential incompatibility problems. Despite several efforts in our laboratory to create *E. coli–Bifidobacterium* shuttle vectors based on the ColE1 replication origin from pUC vectors, we were equally unable to obtain stable *Bifidobacterium* transformants. The recombinants on selective agar plates showed bifidobacterial morphology under microscopic observation, were resistant to the antibiotic used for selection, and registered positive in PCR for the antibiotic resistance gene, yet, after plasmid DNA extraction, yielded no expected recombinant DNA molecules (data not shown).

We, thus, prepared a circular DNA molecule, pGHori, that did not code for any replication protein, yet could replicate in the *E. coli* strain VE7108. In fact, *E. coli* VE7108 has on the chromosome a constitutively expressed copy of the replication gene *repA*. The product of *repA* acting in *trans* recognizes the origin still present in pGHori and promotes its replication through a rolling circle mechanism. Based on the *E. coli* VE7108/pGHori system, we built the pGOSBif33 shuttle vector that was successfully employed for the transformation of *B. longum* NCC2705 and *B. animalis* ScMa7. In conclusion, pGHori and its host, *E. coli* VE7108, can serve as a system suitable for avoiding possible interference by other functional replication modules on the vector, such as the pUC origin, and consequently, for screening functional replicons from wild-type plasmids.

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