

Discovery of a Conjugative Megaplasmid in *Bifidobacterium breve*

Francesca Bottacini,^a Mary O'Connell Motherway,^{a,b} Eoghan Casey,^b Brian McDonnell,^b Jennifer Mahony,^b Marco Ventura,^c Douwe van Sinderen^{a,b}

Alimentary Pharmabiotic Centre, National University of Ireland, Cork, Ireland^a; School of Microbiology, National University of Ireland, Cork, Ireland^b; Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy^c

Bifidobacterium breve is a common and sometimes very abundant inhabitant of the human gut. Genome sequencing of *B. breve* JCM 7017 revealed the presence of an extrachromosomal element, designated pMP7017 consisting of > 190 kb, thus representing the first reported bifidobacterial megaplasmid. *In silico* characterization of this element revealed several genomic features supporting a stable establishment of the megaplasmid in its host, illustrated by predicted CRISPR-Cas functions that are known to protect the host against intrusion of foreign DNA. Interestingly, pMP7017 is also predicted to encode a conjugative DNA transfer apparatus and, consistent with this notion, we demonstrate here the conjugal transfer of pMP7017 to representative strains of *B. breve* and *B. longum* subsp. *longum*. We also demonstrate the presence of a megaplasmid with homology to pMP7017 in three *B. longum* subsp. *longum* strains.

Bifidobacterium breve is a common inhabitant of the infant gut, in addition to *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium bifidum* (1), and the less frequently encountered *Bifidobacterium kashiwanohense* (2). Furthermore, representatives of this species have been isolated in other environments, including human milk, the adult gut, and human vagina (3). The presence of bifidobacteria (being members of the *Actinobacteria* phylum) in the gut has been correlated with particular health-promoting effects, such as enforcement of the intestinal barrier, activation/modulation of the host's immune response and protection against particular infections (4), and for this reason representatives of this genus have been included as active ingredients in functional foods (5).

Since the first genome sequence of *B. longum* subsp. *longum* NCC2705 was released in 2002, further sequencing endeavors have produced additional bifidobacterial genome sequences in an effort to elucidate the mode of action of their beneficial effects. In this respect genome sequencing and subsequent functional analyses have revealed that members of this genus possess genetic features that support the process of colonization and adhesion to the intestinal mucosa (6), evasion/stimulation of the host's immune system (7), and a metabolic ability to use host-derived glycans, all of which are believed to contribute to competitive exclusion of pathogens (4) and perhaps other beneficial activities.

The presence of plasmids among representatives of the genus *Bifidobacterium* has been reported for certain bifidobacterial species (e.g., *Bifidobacterium pseudolongum* subsp. *globosum*, *Bifidobacterium indicum*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium catenulatum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium kashiwanohense*, *Bifidobacterium breve*, *Bifidobacterium longum* subsp. *longum*, and *Bifidobacterium longum* subsp. *infantis*) (8–11). Typically, when plasmids are found, just a single plasmid per isolate is present, with some notable exceptions: *B. kashiwanohense* JCM 15439, *B. longum* subsp. *longum* DJO10A, *B. longum* subsp. *infantis* 157F, and *B. longum* subsp. *longum* KACC 91563, which each harbor two such genetic elements (2, 8). Among the identified bifidobacterial plasmids, most have been isolated from strains of *B. longum* subsp. *longum* (8). The bifidobacterial plasmids that have been charac-

terized thus far exhibit a size not exceeding 10.5 kb and the possible presence of extrachromosomal genetic elements larger than 100 kb, which are designated megaplasmids (12), in *Bifidobacterium* has only been suggested for two porcine cecal isolates (13).

Bacterial conjugation is one of the possible means of horizontal transfer of genetic material and it has been intensively studied in Gram-negative bacteria, in particular in the plant pathogen *Agrobacterium tumefaciens* due to its pathogenic and biotechnological relevance (e.g., tumor-inducing plasmid Ti) (14–16). Conjugation has been described for Gram-positive bacteria, with the difference that a somewhat simpler conjugative machinery appears to be required due to the fact that DNA traverses just a single membrane layer (17). With regard to bifidobacteria, recent genome analyses for members of this genus has indeed provided evidence of horizontal transfer events (3, 6, 18–20), where prophages are believed to be one of the principal vehicles of such DNA transfer phenomena (21).

The presence of (conjugative) megaplasmids in representatives of the phylum *Actinobacteria* has thus far only been observed for members of the *Gordonia*, *Rhodococcus*, *Brevibacterium*, *Nocardiosis*, and *Mycobacterium* spp. (with sizes ranging between 100 and 700 kb) (12, 22), of which p1CP from *Rhodococcus opacus* PD630 (23), pREA400, pREA250, and pREA100 from *Rhodococcus erythropolis* AN12 (24), and pKB1 from *Gordonia westfalica*

Received 3 September 2014 Accepted 13 October 2014

Accepted manuscript posted online 17 October 2014

Citation Bottacini F, O'Connell Motherway M, Casey E, McDonnell B, Mahony J, Ventura M, van Sinderen D. 2015. Discovery of a conjugative megaplasmid in *Bifidobacterium breve*. *Appl Environ Microbiol* 81:166–176.
doi:10.1128/AEM.02871-14.

Editor: M. W. Griffiths

Address correspondence to Douwe van Sinderen, d.vansinderen@ucc.ie. F.B. and M.O.M. are co-senior authors.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.02871-14>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.02871-14

Kb1 (25) have conclusively been shown to be conjugative. With regard to conjugative transfer in bifidobacteria, only a recent study proposed a new shuttle vector pDOJHR-WD2 based on the conjugative machinery of *Escherichia coli* WM3064(pBB109), which was successfully used for DNA transfer between *E. coli* and different *Bifidobacterium* species (26); however, no functional, native bifidobacterial conjugation system has as yet been identified.

In this study we present the sequence and *in silico* annotation of a megaplasmid present in *B. breve* JCM 7017. This 190.17-kb plasmid, designated pMP7017, the first and largest megaplasmid to be identified among a member of the genus *Bifidobacterium* and actinobacterial gut commensals in general, is able to transfer to other bifidobacteria by an apparent conjugative mechanism. Interestingly, and consistent with the horizontal transfer ability of pMP7017, we also detected homologs of this megaplasmid in three representatives of *B. longum* subsp. *longum*.

MATERIALS AND METHODS

Sequencing and annotation. DNA sequencing of pMP7017 was performed using protocols developed by the sequencing provider Macrogen (Seoul, Republic of Korea) and employing a combination of two next-generation sequencing (NGS) platforms (see below). An initial 454 run using a Roche genome sequencer FLX Titanium instrument employing a long-tag, paired-end library (average read length of 400 bp) resulted in the construction of an initial scaffold, which was subsequently improved with an additional Illumina Hiseq 2000 paired-end sequencing run with an average read length of 101 bp.

The resulting sequences were then assembled in a hybrid 454-Illumina approach using a combination of Newbler v2.9 (98.83% of aligned reads and 0.04% inferred read error) (454 Sequencing; Roche) for long reads and Abyss v1.3.4 (<http://www.bcgsc.ca/>) for short reads, resulting in a high-quality and ungapged final consensus sequence, where the average sequence coverage of each base pair was >200-fold.

Replicon analyses and bioinformatic analysis. Open reading frame (ORF) prediction was performed with a combined approach of the tool Prodigal v2.0 (<http://prodigal.ornl.gov>). Functional assignment of the predicted ORFs was first performed on the basis of BLASTP v2.2.26 (27) searches against the nonredundant protein database curated by the National Centre for Biotechnology Information (<ftp://ftp.ncbi.nih.gov/blast/db/>) and further refined, when necessary, employing BLASTX v2.2.26 searches (27).

Artemis v.14 (<http://www.sanger.ac.uk/resources/software/artemis/>) was used to inspect the results of the ORF prediction and used for manual editing, if necessary, of the start codon of a predicted gene. Gene annotations were further integrated with the information retrieved from alternative databases, e.g., protein family (Pfam) (<http://pfam.sanger.ac.uk>).

TRNA genes were identified using tRNAscan-SE v1.4 (28) and the presence of Restriction-Modification (R-M) systems was investigated using BLASTP (27) alignments against the downloaded REBASE database (<http://rebase.neb.com/rebase/rebase.html>; cutoff E value of 0.0001 and showing at least 30% similarity across at least 80% of the complete length of the deduced amino acid sequence).

The presence of CRISPR loci was inspected using the CRISPR finder tool (<http://crispr.u-psud.fr>), while codon usage and amino acid frequency were computed using the GCUA (29) tool and visualized with scatter3D module of the statistical package R (<http://www.r-project.org/>). Sequence comparisons at the protein level were performed using an bidirectional BLASTP alignment (27) (cutoff E value of 0.0001, with at least 50% identity across at least 50% of either protein sequence).

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in the present study are listed in Table 1. Bifidobacterial strains were routinely cultured in reinforced clostridial medium (RCM [Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom]) or modified de Man, Rogosa, and Sharpe medium (mMRS) prepared from first princi-

TABLE 1 Bacterial strains and plasmids used in this study^a

Strain or plasmid	Relevant feature(s) ^b	Source or reference
Strains		
<i>Escherichia coli</i>		
<i>E. coli</i> EC101	Cloning host, <i>repA</i> ⁺ Km ^r	53
<i>Bifidobacterium breve</i>		
JCM 7017	Isolate from human feces	JCM
JCM 7017-199	pORI19-tet-0199 insertion mutant of pMP7017 in JCM7017	This study
UCC2003	Isolate from nursing stool	19
JCM 7019	Isolate from infant feces	JCM
NCFB 2258	Isolate from infant intestine	NCFB
UCC2003-TC	<i>B. breve</i> UCC2003 transconjugant harboring pMP7017_199	This study
JCM 7019-TC	<i>B. breve</i> JCM7019 transconjugant harboring pMP7017_199	This study
NCFB 2258-TC	<i>B. breve</i> NCFB 2258 transconjugant harboring pMP7017_199	This study
<i>B. longum</i> subsp. <i>longum</i>		
NCIMB 8809	Isolate from nursing stool	NCIMB
1-6B	Isolate from 6-year-old child	51
2-2B	Isolate from 6-year-old child	51
44B	Isolate from infant at 1 year	51
35B	Isolate from infant at 1 year	51
NCIMB 8809-TC	<i>B. longum</i> NCIMB 8809-TC transconjugant harboring pMP7017_199	This study
Plasmids		
pAM5	pBC1-puC19-Tc ^r	54
pORI19	Em ^r ; $\Delta repA$; ori ⁺ , cloning vector	53
pORI19-tet-0199	Internal 937-bp fragment of pI_0199 and <i>tetW</i> cloned in pORI19	This study

^a JCM, Japan Collection of Microorganisms; NCFB, National Collection of Food Bacteria; NCIMB, National Collection of Industrial and Marine Bacteria.

^b Em^r, erythromycin resistance.

ples (30). Prior to inoculation, mMRS was supplemented with cysteine-HCl (0.05% [wt/vol]) and lactose (1.0% [wt/vol]) (Sigma). Bifidobacterial cultures were incubated at 37°C under anaerobic conditions, which were maintained using an anaerobic hood (Davidson and Hardy, Belfast, Ireland). *E. coli* was cultured in Luria-Bertani (LB) broth (31) at 37°C with agitation. Where appropriate, growth media contained tetracycline (10 $\mu\text{g ml}^{-1}$), ampicillin (100 $\mu\text{g ml}^{-1}$ for *E. coli*), erythromycin (100 $\mu\text{g ml}^{-1}$ for *E. coli*), or kanamycin (50 $\mu\text{g ml}^{-1}$ for *E. coli*). Recombinant *E. coli* cells containing pORI19 were selected on LB agar containing erythromycin and supplemented with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) at 40 $\mu\text{g ml}^{-1}$ and 1 mM IPTG (isopropyl- β -D-galactopyranoside).

DNA manipulations. Chromosomal DNA was isolated from *B. breve* JCM 7017 as previously described (32). Minipreparation of plasmid DNA from *E. coli* was achieved using a QIAprep spin plasmid miniprep kit (Qiagen GmbH, Hilden, Germany). Single-stranded oligonucleotide primers (see Table S1 in the supplemental material) used in the present study were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs were performed using TaqPCR mastermix (Qiagen), whereas high-fidelity PCR was achieved using KOD polymerase (Novagen, Darmstadt, Germany). *B. breve* colony PCRs were performed as described previously (33). PCR fragments were purified using the Qiagen PCR purification kit. Electroporation of plasmid DNA into *E. coli* was performed as described by Sambrook et al. (31), and into *B. breve* JCM 7017 as described by Maze et al. (34).

TABLE 2 Conjugation between *B. breve* JCM 7017 and representative *B. breve* and *B. longum* subsp. *longum* strains

Donor	Carbohydrate selection for donor	Recipient	Carbohydrate selection for recipient	Conjugation efficiency ^a
<i>B. breve</i> JCM 7017_199	Sorbitol	<i>B. breve</i> UCC2003	Ribose	5.1×10^{-9}
<i>B. breve</i> JCM 7017_199	Sorbitol	<i>B. breve</i> NCFB 2258	Ribose	1.3×10^{-8}
<i>B. breve</i> JCM 7017_199	Cellobiose	<i>B. breve</i> JCM 7019	Melezitose	1.33×10^{-6}
<i>B. breve</i> JCM 7017_199	Sorbitol	<i>B. longum</i> NCIMB 8809	Arabinose	4.7×10^{-8}

^a Expressed as the average number of transconjugants per recipient from triplicate conjugation experiments.

Antibiotic marking of pMP7017 through creation of an insertion mutant of pMP7017_0199. An internal 937-bp fragment of ORF pMP7017_0199 was amplified by PCR using *B. breve* JCM 7017 chromosomal DNA as a template and primer pairs IM199F and IM199R (see Table S1 in the supplemental material). The insertion mutation in *B. breve* JCM 7017 was generated essentially as described previously (35) to produce *B. breve* JCM 7017-199. Site-specific recombination in tetracycline-resistant isolates was confirmed by colony PCR using the primer combination tetWFw and tetWRv to verify *tetW* gene integration and the primer combination 0199-FW (positioned upstream of the selected internal fragment of pMP7017_0199) and pORI19For to confirm integration at the correct genetic location.

Conjugal matings. Conjugations were performed by inoculating overnight cultures of donor and recipient at 1% into mMRS broth supplemented with cysteine-HCl (0.05% [wt/vol]) and lactose (1.0% [wt/vol]) and growing at 37°C under anaerobic conditions for 7 h to late log phase. Next, 1 ml of each culture (donor and recipient) was mixed and concentrated by centrifugation, and the resultant pellet was resuspended in 200 μ l of RCM prior to spread plating on reinforced clostridial agar (RCA) plates. RCA plates were incubated overnight at 37°C under anaerobic conditions. Subsequently, the conjugation mixture was recovered from the plates and resuspended in 2 ml of phosphate-buffered saline and serially diluted. Donor and recipient cells in each conjugation mixture were enumerated based on selective carbohydrate utilization by the donor or recipient strain on mMRS agar, whereas transconjugants were isolated by adopting the appropriate carbohydrate to select for recipient cells in combination with tetracycline (10 μ g ml⁻¹) (Table 2).

PFGE plug preparation. Agarose gel plugs of high-molecular-weight DNA for pulsed-field gel electrophoresis (PFGE) were prepared according to previously described protocols (36, 37), with minor modifications, as outlined below. All bifidobacterial strains were grown in mMRS broth supplemented with 0.05% cysteine and 1% (wt/vol) lactose to early stationary phase. A volume of culture containing $\sim 10^9$ bacteria (equivalent to an optical density at 600 nm of 2.0) was centrifuged (5,000 \times g for 1 min), washed once with 1 ml of NT buffer (1 M NaCl, 10 mM Tris-HCl [pH 7.6]), and repelleted (5,000 \times g for 1 min). The cell pellet was resuspended in 300 μ l of NT buffer, to which an equal volume of melted 2% (wt/vol) low-melting-point (LMP) agarose, prepared in 0.125 M EDTA (pH 7.6) and maintained at 50°C, was added. The cell suspension and LMP agarose were mixed carefully without introducing bubbles. Gel plugs were formed by pipetting 300- μ l volumes into plug molds and allowed to solidify at 4°C for 20 min. Subsequently, two plugs per strain were added to 2 ml of EC buffer (1 M NaCl, 6 mM Tris-HCl, 100 mM EDTA [pH 7.6]) containing 1% (wt/vol) Sarkosyl, 10 mg ml⁻¹ lysozyme, and 40 U ml⁻¹ mutanolysin, followed by incubation at 37°C for 24 h. The lysozyme solution was replaced with 5 ml of 0.5 M EDTA (pH 8.0) containing 1% (wt/vol) Sarkosyl and 0.5 mg/ml proteinase K and then incubated at 37°C for 24 h. Plugs were then washed with 5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) containing 1 mM PMSF (phenylmethylsulfonyl fluoride; freshly prepared) at 37°C for 1 h to inactivate the proteinase K. This was followed by three 30-min incubations in 5 ml of TE buffer at room temperature to remove the PMSF. Plugs were stored in 10 mM Tris-HCl–100 mM EDTA (pH 8.0) at 4°C.

Treatment with S1 nuclease or restriction endonucleases. Single slices (2 mm by 2 mm) were soaked in either 200 μ l of S1 buffer (50 mM

NaCl, 30 mM sodium acetate [pH 4.5], 5 mM ZnSO₄) or an appropriate restriction buffer at room temperature for 30 min. The S1 buffer was replaced with another 200 μ l of S1 buffer containing 1 unit of *Aspergillus oryzae* S1 nuclease and then incubated at 37°C for 45 min. The restriction buffer was replaced with fresh restriction buffer containing 15 U of the appropriate restriction enzyme (SpeI and AscI), followed by incubation at 37°C for 16 h. Reactions were stopped by suspending the treated plugs in 200 μ l of 0.5 M EDTA (pH 8.0) and held at room temperature for 10 min. The 0.5 M EDTA was replaced with 200 μ l of TE and left at room temperature for at least 30 min before loading onto a gel.

PFGE. Plug slices were loaded directly into the wells of a 1% (wt/vol) PFGE agarose gel melted in 0.5 \times TBE (89 mM Tris-borate, 2 mM EDTA [pH 8.3]) buffer. The wells were sealed with molten agarose in 0.5 TBE buffer. DNA fragments were resolved using a CHEF-DR III pulsed-field system (Bio-Rad Laboratories, Hercules, CA) at 6 V/cm for 18 h with 0.5 \times TBE running buffer maintained at 14°C. Linear ramped pulse times were selected depending on the size of DNA fragments to be resolved; for the S1 nuclease-treated samples, a linear ramped pulse time of 3 to 50 s was used; for SpeI- and AscI-digested samples, a linear ramped pulse time of 1 to 15 s was used. A molecular size Chef DNA lambda ladder was included in each gel (number 170-3635; Bio-Rad Laboratories). The gels were stained in distilled water containing ethidium bromide for 120 min under light-limited conditions and destained in distilled water for 60 min. The gels were visualized, and images were captured under UV light.

Nucleotide sequence accession numbers. The sequences generated in this study have been submitted to GenBank database with the accession number (GenBank accession no. [KM406416](#)). All of the sequences used for our analysis have been retrieved from NCBI database with the following accession numbers: *B. breve* UCC2003 (GenBank accession no. [NC_020517](#)), *B. breve* JCM 7017 (GenBank accession no. [CP006712](#)), *B. breve* NCFB 2258 (GenBank accession no. [CP006714](#)), *B. breve* JCM 7019 (GenBank accession no. [CP006713](#)), *B. longum* subsp. *longum* 1-6B (GenBank accession no. [AJTF00000000](#)), *B. longum* subsp. *longum* 2-2B (GenBank accession no. [AJTJ00000000](#)), *B. longum* subsp. *longum* 35B (GenBank accession no. [AJTI00000000](#)), and *B. longum* subsp. *longum* 44B (GenBank accession no. [AJTM00000000](#)).

RESULTS AND DISCUSSION

Sequence analysis and general features of pMP7017. During the genome sequencing and assembly of the *Bifidobacterium breve* strain JCM 7017 (3), a sequence scaffold of 190,178 bp was detected, corresponding to a DNA element that was not part of the chromosome, thereby providing preliminary evidence for the presence of a megaplasmid in this strain. Sequence analysis of this pMP7017 replicon showed an overall G+C mol% of 57.43, comparable with the G+C content of its host (58.65 G+C mol%) and of bifidobacterial genomes in general (9). However, the fact that a slightly different G+C content was observed for about half of its sequence, roughly corresponding to nucleotide coordinates 22,000 to 125,000 (Fig. 1), suggests that this megaplasmid is the result of cointegration of two originally separate modules (having a G+C mol% of either 60.1 or 55.3) (Fig. 1). The pMP7017 plasmid harbors 228 ORFs, of which 70% do not have significant hits

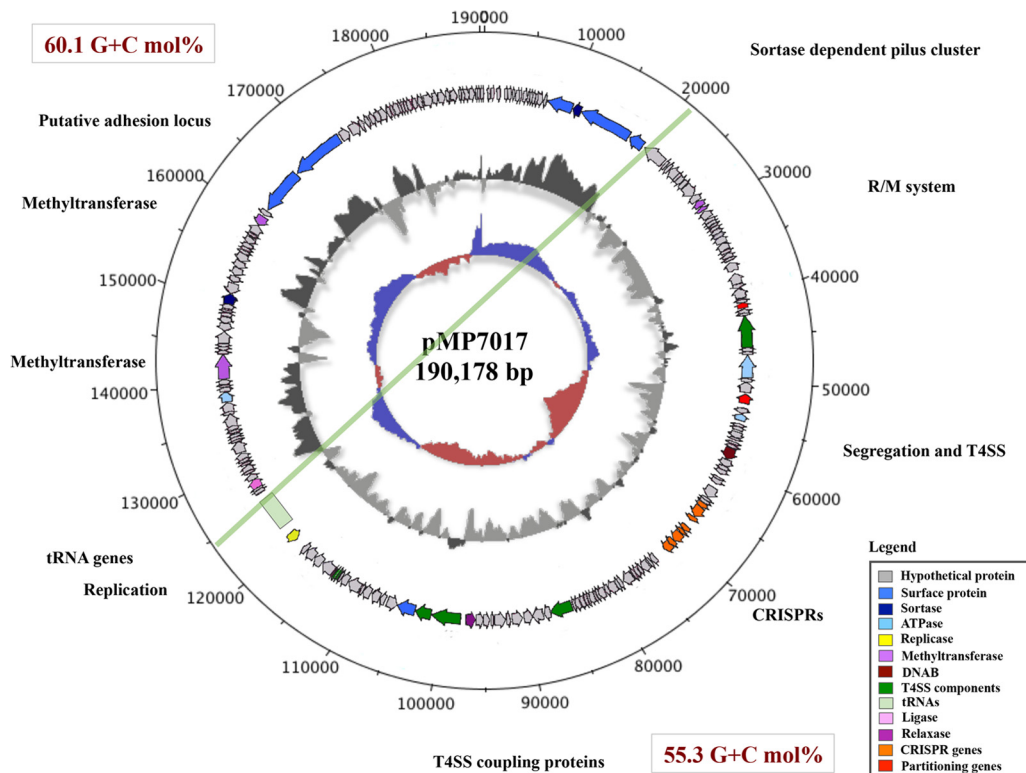


FIG 1 pMP7017 general features. Genome atlas representing the circular sequence of the megaplasmid pMP7017 with the relative ORFs organization is shown. Highlighted are the four main functional regions (REG1 to -4) with relative genetic features. A green line also divides pMP7017 into two putative cointegrates.

in database searches, thus representing hypothetical genes which is a common genetic feature of plasmids, while the remaining ORFs are associated with predicted functions that are principally involved in DNA replication, DNA methylation, partitioning, DNA transfer, and colonization (Fig. 1).

The first (arbitrary) half of the pMP7017 sequence (corresponding to locus tag names pMP7017_0001 through to pMP7017_0100) contains ORFs which, among others, represent a sortase-dependent pilus cluster (cluster I) encoding a predicted sortase (pMP7017_0021) and three putative surface-anchored proteins (pMP7017_0022-24). Interestingly, this cluster shows significant homology (above 50% of similarity in a BLASTP alignment) with predicted pilus clusters of particular *B. longum* strains, though it is not significantly similar to those identified in other bifidobacteria (19, 38, 39). This portion of pMP7017 also contains two genes encoding a potential type II R/M system (pMP7017_0033-34), followed by genetic functions putatively involved in plasmid segregation and DNA transfer, encompassing a predicted chromosome partitioning genes *parA* (pMP7017_0053), as well as adjacent genes predicted to encode a mating-pair formation complex (Mpf) for DNA translocation represented by two VirD4-B4 ATPases (pMP7017_0059 and pMP7017_0062, respectively) and a transmembrane pore-forming VirB6 (pMP7017_0056) (Table 3). The VirD4-B4 and VirB6 proteins in the plant pathogen *Agrobacterium tumefaciens* are known to form the structural scaffold and motor of a type IV secretion system (T4SS) that acts as a DNA secretion channel (40–42). The observed similarity may thus indicate a similar role in the conjugal transmission of pMP7017 between bifidobacterial species (see below). Finally, two adjacent

CRISPR/Cas loci (corresponding to locus tags pMP7017_0080-85 and pMP7017_0086-90) are present in this section of the megaplasmid, which are known to provide immunity against invading phages and plasmids in bacteria (43, 44). Further discussion on these CRISPR/Cas systems will follow below.

The second half of the replicon (locus tags pMP7017_0101 through to pMP7017_0146) encodes the presumed replicative apparatus consisting of a possible relaxase (pMP7017_0124) and a predicted replication protein (pMP7017_0146) (Pfam PF04796.5), as well as several genes that specify coupling proteins (CP) of a type IV secretion system associated with the Mpf complex directing DNA translocation (40–42): FtsK/SpoIIIE family proteins (pMP7017_0114-0116 and pMP7010_0125-26) and a DivIVA domain-containing protein (pMP7017_0134) (Table 3). This second portion of the megaplasmid also specifies biosynthetic functions for a putative adhesion locus encoding two predicted surface proteins (pMP7017_0199-0200), of which the corresponding genes are located in the vicinity of a sortase-encoding gene (pMP7017_0184), as well as two genes that each encode a putative methyltransferase (pMP7017_0174/0197); this region also harbors the largest density of hypothetical genes.

Interestingly, the pMP7017 replicon also specifies three predicted toxin-antitoxin (T-A) systems (pMP7017_0014-15, pMP7017_101-102 and pMP7017_164-165), as well as a putative antidote protein (pMP7017_0019). Such T-A systems are known to be involved in ensuring plasmid maintenance (45).

Finally, downstream of the gene encoding the predicted replication protein (pMP7017_0146), a cluster of 14 tRNA genes (base

TABLE 3 Segregation and conjugal transfer genes in pMP7017

Cellular process	ORF	Annotation	Pfam no.
Segregation DNA translocation	pMP7017_0053	Chromosome partitioning protein <i>parA</i>	PF01656
	pMP7017_0062	VirB4	PF01656
	pMP7017_0056	VirB6	PF04610
	pMP7017_0059	VirD4	PF10412
	pMP7017_0136	Protein translocase subunit <i>secE</i>	PF00584
	pMP7017_0138	Protein translocase subunit <i>secG</i>	PF03840
Conjugal transfer coupling proteins (CP)	pMP7017_0114	Ftsk/SpoIIIE family protein	PF01580
	pMP7017_0115	CHAP possible extracellular subunit	
	pMP7017_0116	Hypothetical protein with CHAP domain	PF05257
	pMP7017_0125	Ftsk/SpoIIIE family protein	PF01580
	pMP7017_0126	Ftsk/SpoIIIE family protein	PF01580
	pMP7017_0127	Hypothetical protein with flagellar basal body-associated domain (putative VirB10)	PF03739
	pMP7017_0134	DivIVA domain containing protein (putative VirC1)	PF05103
Replication and DNA processing	pMP7017_0124	Putative relaxase	PF04796
	pMP7017_0073	Replicative DNA helicase <i>dnaB</i>	PF03796
	pMP7017_0108	Dna polymerase III alpha subunit <i>dnaE</i>	PF00929
	pMP7017_0146	Predicted <i>repA</i> replicase protein	PF04796

positions 121532 to 125960) is present, and their possible role will be discussed below.

Host adaptation: codon usage and amino acid frequency.

The evolutionary process that drives codon usage bias across a genome and indirectly determines the overall tRNA abundance in a given organism is believed to be mediated by natural selection in favor of those codons for which sufficient cognate tRNA is present in order to optimize protein translation efficiency (46, 47).

In order to explore the possible relationship between the *B. breve* JCM 7017 chromosome and its coexisting megaplasmid pMP7017 a multivariate analysis on codon usage by plasmid and chromosome-encoded proteins was performed (see Fig. S1a in the supplemental material). The resulting tridimensional plot demonstrated that the pMP7017 replicon possesses a codon bias that is compatible with that of the chromosome, while it also revealed that plasmid-borne ORFs with a deviating codon bias mostly specify hypothetical proteins (see Table S2 in the supplemental material). This result indicates that pMP7017 and its host DNA currently coexist following convergent evolution that has led to optimal production of chromosome- and plasmid-encoded functions.

The presence of 14 clustered tRNA genes on pMP7017 suggests a correspondence between codon usage and tRNA abundance. In order to investigate this notion, amino acid frequencies for pMP7017 and *B. breve* JCM 7017-encoded ORFs were computed and the resulting heatmap, based on hierarchical clustering, showed that concordance exists between frequently used codons in the plasmid and the host and that this is consistent with the tRNA genes present in the plasmid (Fig. 2a and b). For this reason is tempting to argue that pMP7017 followed an evolutionary process which made its codon usage fitting the one of the host allowing efficient protein translation, but also that its maintenance and expression is supported by a selected set of tRNA genes that provides translational backup to the host.

Protection against DNA invasion: CRISPRs. Plasmid pMP7017 is predicted to encode several methyltransferases (pMP7017_0033-34, pMP7017_0174, and pMP7017_0197) which may be part of R-M

systems, while it also encompasses two adjacent CRISPR/Cas systems (pMP7017_0080-85 and pMP7017_0086-90) (Fig. 1). These features may promote the exclusion of (other) invading genetic elements in concert with the chromosomally encoded protection systems (constituted by two R-M systems and a CRISPR/Cas locus) (3), thus providing mutual benefit.

Comparative analysis of the three type CRISPR/Cas systems encoded by *B. breve* JCM 7017 revealed a subtype I-C system present on the JCM 7017 chromosome and two subtype I-E systems harbored by pMP7017, and named B7017-CRISPR1, pMP7017-CRISPR1, and pMP7017-CRISPR2, respectively. *In silico* analysis of these three type I CRISPR/Cas systems allowed the identification of 74 nonredundant spacer sequences (Fig. 3a and b), which together are believed to constitute a genetic memory of previous challenges by phages (or other invading DNA elements) mounted against this *B. breve* strain or against previous hosts of the conjugative pMP7017 megaplasmid.

Of these three systems, B7017-CRISPR1 located on the *B. breve* JCM 7017 chromosome (~18 kb in size with gene coordinates 1632794 to 1650595) possesses a total of 49 nonredundant spacers, of which the distal end (spacers 27 to 49) returned several hits with CRISPR-associated regions of other *B. breve* representatives (in particular *B. breve* S27, *B. breve* ACS-071-V-Sch8b, and *B. breve* NCFB 2258), while the proximal end (spacers 1 to 26) appears to be more variable, suggesting that this CRISPR/Cas system was originally acquired by certain representatives of *B. breve* and diversified over time as a result of subsequent phage infections and consequent spacer acquisition events (Fig. 3b).

The presence of at least one *cas-1* and *cas-2* gene couple in the B7017-CRISPR1 and pMP7017-CRISPR2, being a signature of an active CRISPR/Cas system (48), suggests that they constitute fully functional CRISPR systems with proto-spacer processing/acquisition activity, as a crucial part of the adaptation stage. In contrast, the pMP7017-CRISPR1 locus only appears to encode endonuclease activity and may therefore be active solely in the CRISPR/Cas-mediated immunity phase (49, 50) or rely on the *cas-1* and *cas-2* activities of the other two systems. Interestingly, BLASTN-

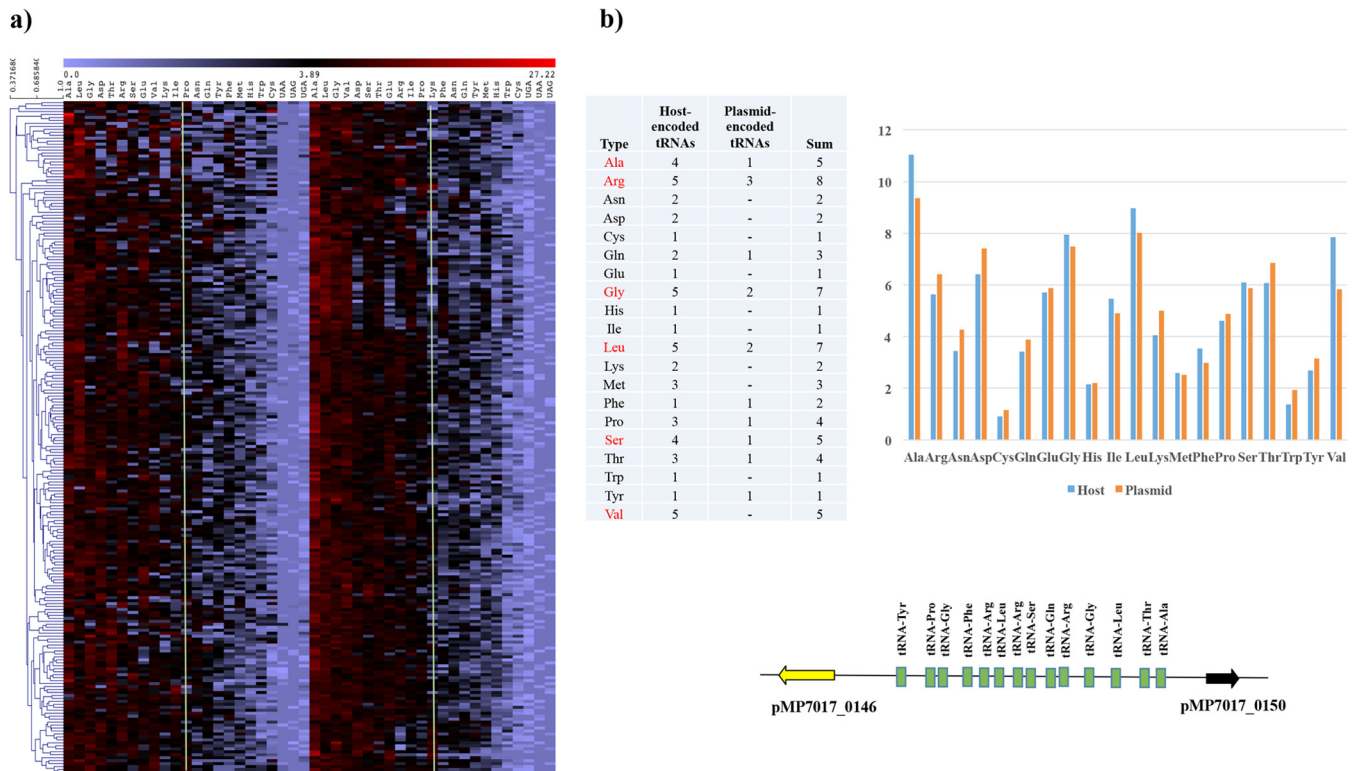


FIG 2 Amino acid usage comparison and tRNA genes. (a) Heatmap and hierarchical clustering showing the amino acid frequencies computed for the predicted proteome of pMP7017 and the host. For representative reasons, a sample of equal numbers of 227 randomly selected ORFs of *B. breve* JCM 7017 and pMP7017 are displayed in this heatmap. (b) Bidimensional barplot representing the cumulative amino acid frequency in pMP7017 and the host with locus map indicating the 14 consecutive tRNA genes encountered in pMP7017 sequence. A table with the total number of tRNA in the plasmid and chromosome is also included.

based searches of the identified spacer sequences against the National Center for Biotechnology Information (NCBI) nonredundant (nr) database returned a number of significant hits against phage-related sequences (i.e., located in predicted bifidobacterial prophage regions) and other CRISPR-associated spacers, as well as against sequences that are associated with horizontal gene transfer (HGT) (such as portions of putative integrative conjugative elements or integrated plasmids) of various bifidobacteria, mostly those that are commonly found in the infant gut (e.g., *B. breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *B. bifidum*) (48) (Fig. 3b). These results support the notion that CRISPR/Cas systems not only constitute a barrier to phage infection but also to other DNA invasion events that promote HGT mediated by mobilizable elements (44).

Presence of a megaplasmid in *B. breve* JCM 7017 and strains of *B. longum* subsp. *longum*. The process of gene annotation, followed by an *in silico* comparative analysis conducted on the ORFeome of pMP7017, allowed the identification of 90 to 100 kb of homologous sequences in the draft genomes (deposited as a varying number of contigs) of *B. longum* subsp. *longum* strains 1-6B, 2-2B, and 44B (51). These homologous sequences (when translated) display an overall conservation of BLASTP similarity between 60 and 100%, whereas at nucleotide level (BLASTN), high levels of identity (80 to 100%) were only observed for portions of these contigs. In order to investigate synteny between the pMP7017 plasmid sequence and the above-mentioned *B. longum* subsp. *longum* contigs, a dot plot alignment was performed, showing that a degree of synteny is observed for the pMP7017 region

corresponding to the approximate nucleotide coordinates 20,000 to 125,000 (Fig. 4a), which also specifies the predicted conjugative apparatus (Fig. 1) and which supports the above-mentioned notion that this megaplasmid represents a cointegrate of two plasmids.

The comparative analysis at protein level showed that ca. 70% of the pMP7017 ORFs returned significant similarities to proteins encoded by *B. longum* subsp. *longum* (Fig. 4b). The ORFs that were shown to be similar between pMP7017 and the draft genome sequence of one or more of these three *B. longum* subsp. *longum* strains include those that encode sortase-dependent pilus clusters I and II, the ORFs predicted to be involved in plasmid replication and conjugation, and the characteristic 14 clustered tRNA genes and CRISPR/cas genes, the latter showing 80 to 100% similarity at amino acid level (see Fig. S2 in the supplemental material) and identical repeats (see Table S3 in the supplemental material), while the spacers appeared different. However, the draft nature of these *B. longum* subsp. *longum* sequences prevented a full-length alignment of these contigs with pMP7017, while it was also not clear whether these sequences were associated with a megaplasmid. For this reason, in order to not only verify the presence of pMP7017 in *B. breve* JCM 7017 but also evaluate the presence of a megaplasmid in the three aforementioned strains of *B. longum* subsp. *longum*, S1 nuclease PFGE was performed (Fig. 4c). This indeed confirmed the presence of pMP7017 in *B. breve* JCM 7017 and also revealed the presence of a megaplasmid, of equivalent size, in *B. longum* subsp. *longum* 1-6B, *B. longum* subsp. *longum* 2-2B and *B. longum* subsp. *longum* 44B. In contrast, the negative

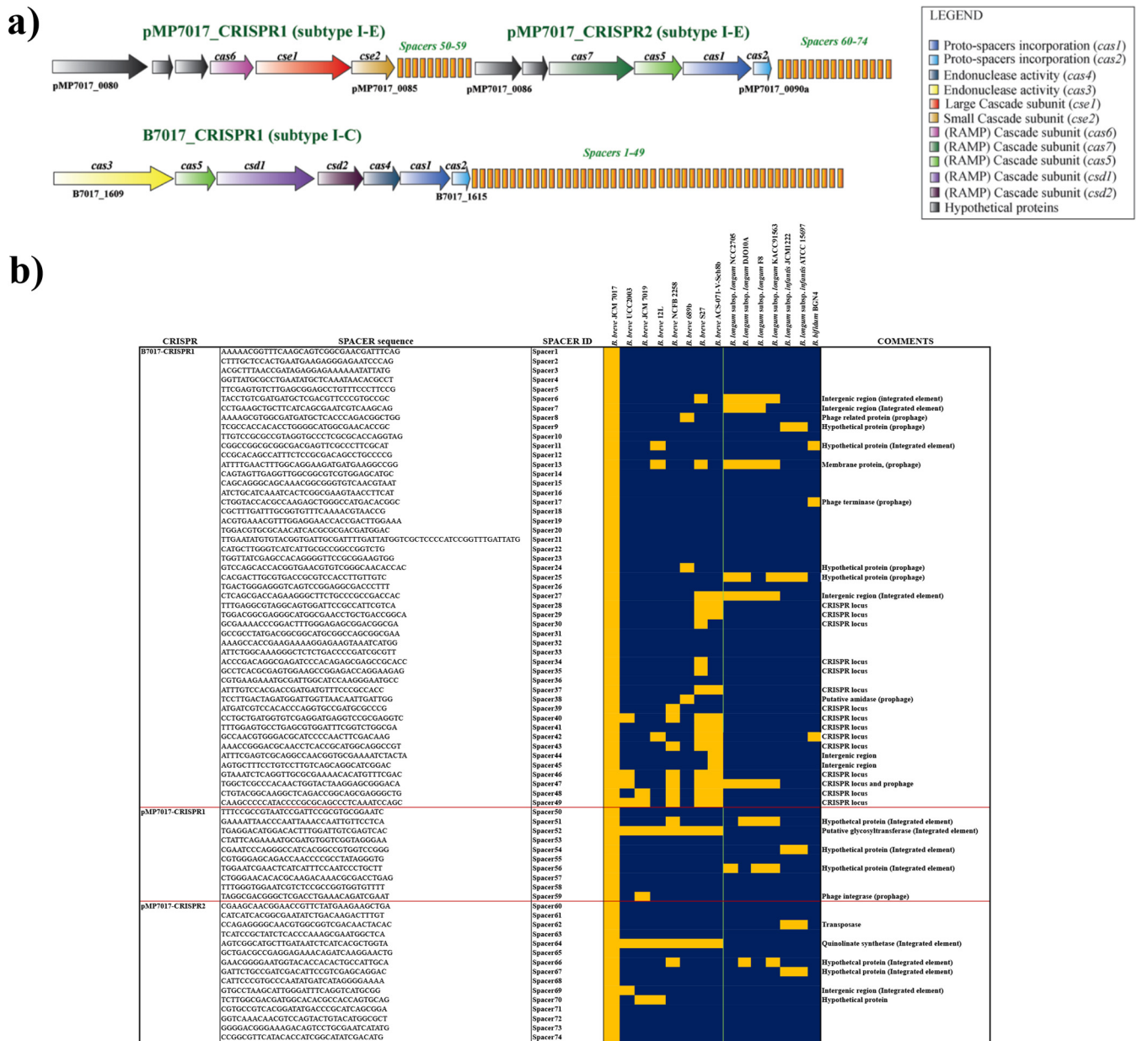


FIG 3 CRISPR/Cas systems. (a) Locus map showing the organization of the CRISPR/cas locus in *B. breve* JCM 7017 chromosome and in the megaplasmid. (b) Heatmap showing the BLASTP analysis of the 74 spacer regions found in the CRISPR/cas systems of the *B. breve* JCM 7017 chromosome and relative megaplasmid, aligned against NCBI nonredundant (nr) database. A green line also indicates matches within representatives of *B. breve* species.

controls, *B. breve* UCC2003 and *B. longum* subsp *longum* 35B, did not appear to contain a megaplasmid, concordant with what was expected from the *in silico* comparative analysis (Fig. 4b).

Conjugal transfer of pMP7017 to representative *B. breve* and *B. longum* strains. *In silico* characterization of pMP7017 (see above) suggests that this plasmid specifies a DNA conjugation mechanism involving the concerted action of an indigenous partitioning, replication, membrane fusion and DNA translocation system, not previously described for bifidobacteria (Table 3).

In order to establish if pMP7017 is able to transfer to other strains by conjugation, a derivative strain, designated *B. breve* JCM 7017-199, was constructed which carries the megaplasmid in

which pMP7017_0199, encoding a predicted surface protein (with no predicted function in conjugation), was interrupted by site-specific homologous recombination, with the resultant megaplasmid being marked with a gene that confers tetracycline resistance. Conjugation experiments were performed using *B. breve* JCM 7017-199 as a donor and strains *B. breve* UCC2003, *B. breve* JCM 7019, *B. breve* NCFB 2258, *B. longum* subsp. *longum* NCIMB 8809 as recipients with selection of transconjugants based on selective carbohydrate utilization by each recipient strain and tetracycline resistance (see Materials and Methods and Table 2). Transconjugants were obtained for each recipient strain at frequencies ranging from approximately 10^{-9} to 10^{-6} . The presence

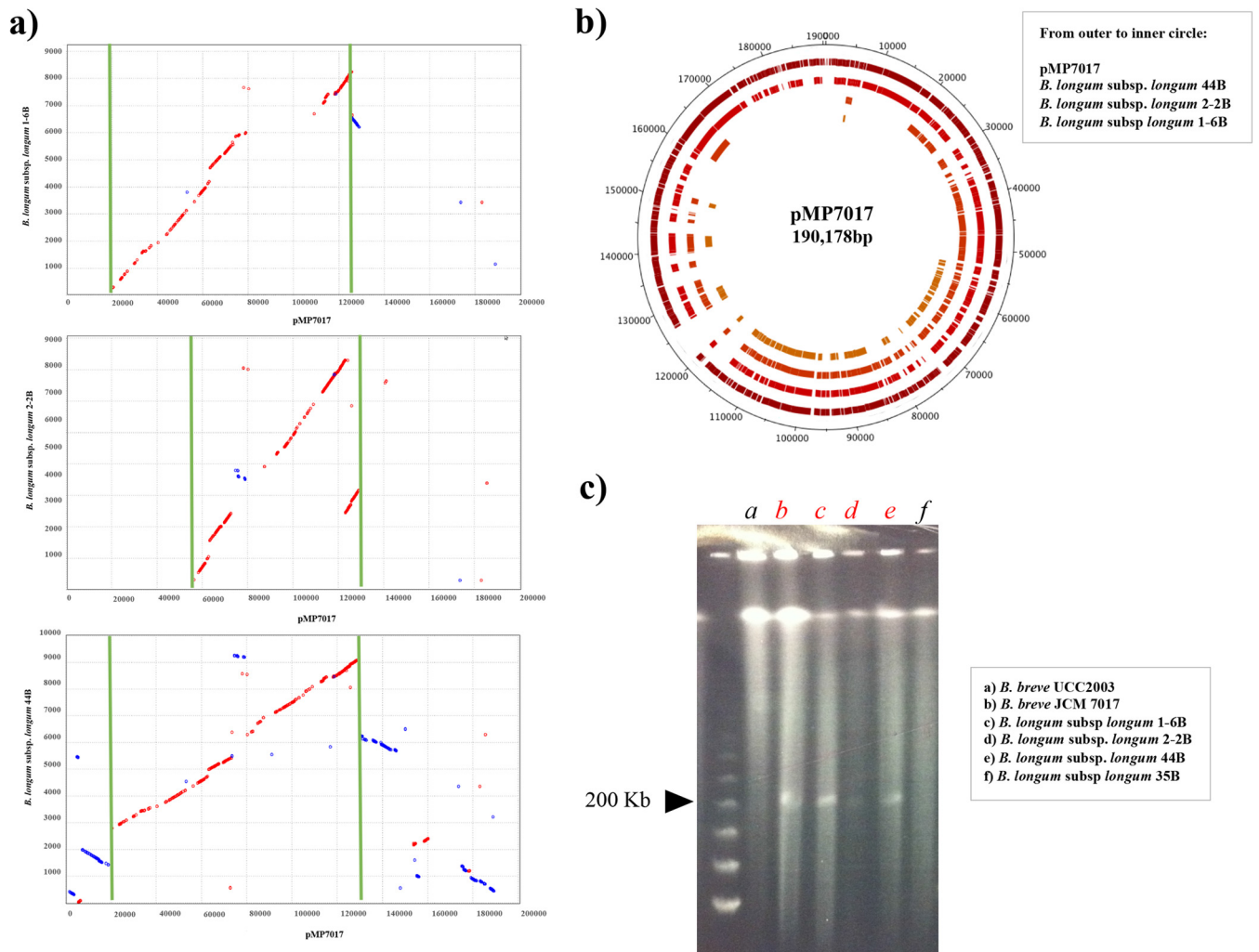


FIG 4 Presence of pMP7017 in *B. breve* and *B. longum* subsp. *longum*. (a) Dot plot alignment showing the comparison of pMP7017 megaplasmid with the contigs of *B. longum* subsp. *longum* of which ORFs displayed significant match in BLASTP alignment (e.g., *B. longum* subsp. *longum* 1-6B, *B. longum* subsp. *longum* 2-2B, and *B. longum* subsp. *longum* 44B). Delimited by green bars are the regions displaying the most of synteny. (b) BLAST-based genome atlas showing the comparative analysis conducted on pMP7017 against the draft sequence of *B. longum* subsp. *longum* 1-6B, *B. longum* subsp. *longum* 2-2B, and *B. longum* subsp. *longum* 44B. From the outer to the inner circle and shaded in gradient from dark red to orange: *B. breve* JCM 7017 pMP7017, *B. longum* subsp. *longum* 44B, *B. longum* subsp. *longum* 2-2B, and *B. longum* subsp. *longum* 1-6B. (c) PFGE experiment showing the presence of a megaplasmid of a size comparable to that of pMP7017 (about 200 kb) also in *B. longum* subsp. *longum* 1-6B, *B. longum* subsp. *longum* 2-2B, and *B. longum* subsp. *longum* 44B, but absent in *B. longum* subsp. *longum* 35B and *B. breve* UCC2003 as a negative control.

of pMP7017_199 in the transconjugants was confirmed by PCR targeting genes on the megaplasmid and, for each recipient strain, genes for selective carbohydrate utilization. Furthermore, additional primer pairs based on *B. breve* JCM 7017 chromosomal genes were also included and as expected, these gave no PCR product for the transconjugant strains (see Fig. S3 in the supplemental material). To further verify conjugative transfer of pMP7017_199 to *B. breve* and *B. longum* PFGE of restricted total DNA was performed on representative *B. breve* UCC2003_pMP7017_199 and *B. longum* NCIMB 8809_pMP7017_199 transconjugants. Adopting the restriction enzymes *AscI* for *B. breve* UCC2003 and *SpeI* for *B. longum* subsp. *longum* NCIMB 8809, we clearly demonstrate the presence of expected pMP7017_199 restriction fragments in the presumed transconjugants and also the correct recipient strain restriction profile thereby confirming the conjugal transfer of

pMP7017_199 from *B. breve* JCM 7017_199 to strains of *B. breve* and *B. longum* (Fig. 5).

Conclusions. The presence of plasmids does not represent a typical feature of *B. breve* and *B. longum* genomes and all currently available bifidobacterial plasmids are considered cryptic as their encoded functions (besides, in some cases, those involved in replication) are unknown. In this respect the pMP7017 megaplasmid, identified in *B. breve* JCM 7017, represents an exceptional bifidobacterial plasmid, which not only represents the largest reported conjugative plasmid identified thus far in the genus *Bifidobacterium* and actinobacterial gut commensals (at >190 kb) but also possesses a number of genomic features indicating the presence of parasite-benefactor balance reached over time through convergent evolution with the host. In addition, it also provides two CRISPR/Cas systems to counter phage infections and other invad-

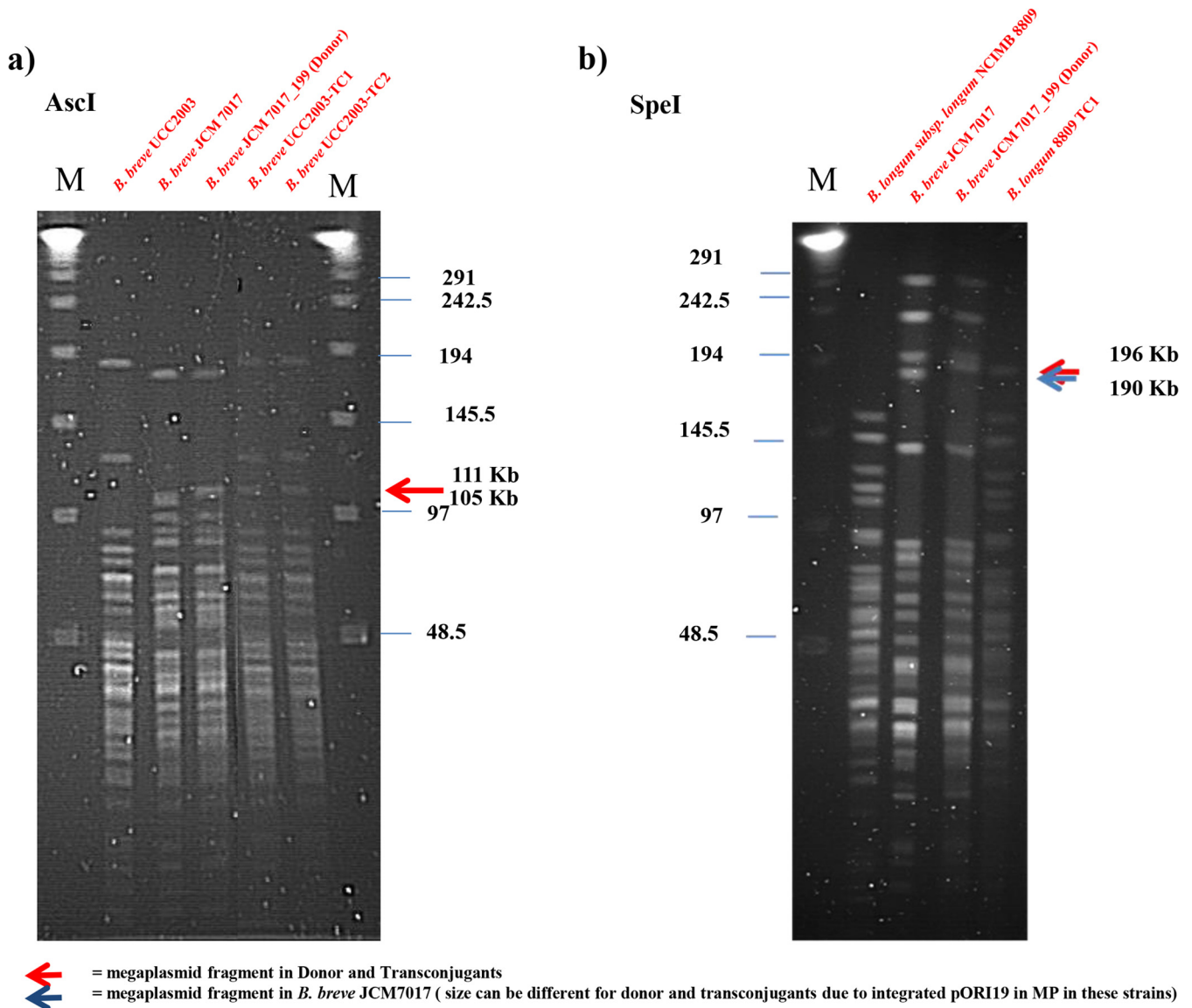


FIG 5 Conjugal transfer of pMP7017. PFGE experiments for *B. breve* UCC2003 or *B. longum* NCIMB 8809 transconjugants conducted with *AsclI* (a) and *SpeI* (b) enzymes, respectively. The presence of megaplasmid in the parent strain, *B. breve* JCM 7017, and the donor strain, *B. breve* JCM 7017_199, is depicted in each panel. The presence of pMP7017 in *B. breve* UCC2003 transconjugants is evidenced by a 111-kb fragment in total DNA digests with *AsclI*, while the megaplasmid presence in *B. longum* NCIMB 8809 transconjugants is verified by the presence of a 196-kb fragment.

ing DNA elements. Plasmid pMP7017 also possesses a number of genes whose products are similar to proteins that are known to be part of a conjugative machinery present in both Gram-positive and Gram-negative bacteria (40–42).

The capacity of this element of being horizontally transferable between different species was first suspected due to the finding that elements similar to pMP7017 of *B. breve* JCM 7017 were also present in several members of the *B. longum* subsp. *longum* taxon.

Plasmid-mediated conjugation in other bacteria is known to involve the transfer of a protein-single-strand DNA complex (called the relaxosome) pumped through an aqueous pore of a type IV secretion system from the donor to the recipient cell (40–42). In bifidobacteria, a gene specifying a protein similar to the type IV secretion protein has been observed in the chromosome of *B. bifidum* MIMBb75 (52). The structural scaffold of such DNA

transfer system in pMP7017 is believed to be represented by a VirB6 structural scaffold, which is coupled to aVirD4-B4 ATPases, to provide the energy for DNA translocation. The functionality of the predicted conjugative machinery in pMP7017 megaplasmid was conclusively demonstrated by conjugation experiments showing that this element is capable to spread and persist in different bifidobacterial strains and species.

One of the conditions for initiating a conjugative transfer is the establishment of cell-to-cell contact, which in Gram-negative bacteria is accomplished by proteinaceous filaments called sex pili, while in Gram-positive bacteria such structures have not yet been clearly identified. The fact that pMP7017 is predicted to encode two different sortase-dependent pili (pMP7017_0021 to pMP7017_0024 and pMP7017_0184-pMP7017_0199-pMP7017_0200) which have been shown to

promote bifidobacterial coaggregation (39) suggests a role in bacterial conjugation.

B. breve and *B. longum* subsp. *longum* both represent common inhabitants of the infant gut (1), which supports the hypothesis that the cooccurrence of apparent homologs of this conjugative element in both of these species may be a result of previous DNA exchanges, followed by diversification and stable maintenance of this element over generations. The fact that pMP7017 represents the first native plasmid capable of conjugal transmission between bifidobacterial species is highly relevant as it may confer this replicon a relevant biotechnological potential for improving the future genetic manipulation of members of this genus.

ACKNOWLEDGMENTS

This study was supported by a Science Foundation Ireland grant (SFI/12/RC/2273) and an HRB postdoctoral fellowship (grant PDTM/2011/9) awarded to M.O.M.

We declare that we have no competing interests.

REFERENCES

- Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M. 2012. Diversity of bifidobacteria within the infant gut microbiota. *PLoS One* 7:e36957. <http://dx.doi.org/10.1371/journal.pone.0036957>.
- Takahata M, Toh H, Nakano A, Takagi M, Murakami M, Ishii Y, Takizawa T, Tanabe S, Morita H. 2014. Complete sequence analysis of two cryptic plasmids from *Bifidobacterium kashiwanohense* JCM 15439 (type strain) isolated from healthy infant feces. *Anim Sci J* 85:158–163. <http://dx.doi.org/10.1111/asj.12095>.
- Bottacini F, O'Connell Motherway M, Kuczynski J, O'Connell KJ, Serafini F, Duranti S, Milani C, Turroni F, Lugli GA, Zomer A, Zhurina D, Riedel C, Ventura M, van Sinderen D. 2014. Comparative genomics of the *Bifidobacterium breve* taxon. *BMC Genomics* 15:170. <http://dx.doi.org/10.1186/1471-2164-15-170>.
- Ventura M, Turroni F, Lugli GA, van Sinderen D. 2014. Bifidobacteria and humans: our special friends, from ecological to genomics perspectives. *J Sci Food Agric* 94:163–168. <http://dx.doi.org/10.1002/jsfa.6356>.
- Stanton C, Ross RP, Fitzgerald GF, Van Sinderen D. 2005. Fermented functional foods based on probiotics and their biogenic metabolites. *Curr Opin Biotechnol* 16:198–203. <http://dx.doi.org/10.1016/j.copbio.2005.02.008>.
- Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sanchez B, Bidossi A, Ferrarini A, Giubellini V, Delle Donne M, Henrissat B, Coutinho P, Oggioni M, Fitzgerald GF, Mills D, Margolles A, Kelly D, van Sinderen D, Ventura M. 2010. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc Natl Acad Sci U S A* 107:19514–19519. <http://dx.doi.org/10.1073/pnas.1011100107>.
- Fanning S, Hall LJ, van Sinderen D. 2012. *Bifidobacterium breve* UCC2003 surface exopolysaccharide production is a beneficial trait mediating commensal-host interaction through immune modulation and pathogen protection. *Gut Microbes* 3:420–425. <http://dx.doi.org/10.4161/gmic.20630>.
- Lee JH, O'Sullivan DJ. 2010. Genomic insights into bifidobacteria. *Microbiol Mol Biol Rev* 74:378–416. <http://dx.doi.org/10.1128/MMBR.00004-10>.
- Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D. 2007. Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* 71:495–548. <http://dx.doi.org/10.1128/MMBR.00005-07>.
- Cronin M, Knobel M, O'Connell-Motherway M, Fitzgerald GF, van Sinderen D. 2007. Molecular dissection of a bifidobacterial replicon. *Appl Environ Microbiol* 73:7858–7866. <http://dx.doi.org/10.1128/AEM.01630-07>.
- Mayo B, van Sinderen D. 2010. *Bifidobacteria: genomics and molecular aspects*. Caister Academic Press, Norfolk, United Kingdom.
- Schwartz E. 2009. *Microbial megaplasmids*. Springer, Berlin, Germany.
- Simpson PJ, Stanton C, Fitzgerald GF, Ross RP. 2003. Genomic diversity and relatedness of bifidobacteria isolated from a porcine cecum. *J Bacteriol* 185:2571–2581. <http://dx.doi.org/10.1128/JB.185.8.2571-2581.2003>.
- Piper KR, Beck von Bodman S, Farrand SK. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* 362:448–450. <http://dx.doi.org/10.1038/362448a0>.
- Lang S, Gruber K, Mihajlovic S, Arnold R, Gruber CJ, Steinlechner S, Jehl MA, Rattei T, Frohlich KU, Zechner EL. 2010. Molecular recognition determinants for type IV secretion of diverse families of conjugative relaxases. *Mol Microbiol* 78:1539–1555. <http://dx.doi.org/10.1111/j.1365-2958.2010.07423.x>.
- Wetzel ME, Kim KS, Miller M, Olsen GJ, Farrand SK. 2014. Quorum-dependent mannopine-inducible conjugative transfer of an *Agrobacterium* opine-catabolic plasmid. *J Bacteriol* 196:1031–1044. <http://dx.doi.org/10.1128/JB.01365-13>.
- Grohmann E, Muth G, Espinosa M. 2003. Conjugative plasmid transfer in gram-positive bacteria. *Microbiol Mol Biol Rev* 67:277–301. <http://dx.doi.org/10.1128/MMBR.67.2.277-301.2003>.
- Ventura M, Turroni F, Zomer A, Foroni E, Giubellini V, Bottacini F, Canchaya C, Claesson MJ, He F, Mantzourani M, Mulas L, Ferrarini A, Gao B, Delle Donne M, Henrissat B, Coutinho P, Oggioni M, Gupta RS, Zhang Z, Beighton D, Fitzgerald GF, O'Toole PW, van Sinderen D. 2009. The *Bifidobacterium dentium* Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PLoS Genet* 5:e1000785. <http://dx.doi.org/10.1371/journal.pgen.1000785>.
- O'Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Munoz JA, Kearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van Sinderen D. 2011. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc Natl Acad Sci U S A* 108:11217–11222. <http://dx.doi.org/10.1073/pnas.1105380108>.
- Bottacini F, Milani C, Turroni F, Sanchez B, Foroni E, Duranti S, Serafini F, Viappiani A, Strati F, Ferrarini A, Delle Donne M, Henrissat B, Coutinho P, Fitzgerald GF, Margolles A, van Sinderen D, Ventura M. 2012. *Bifidobacterium asteroides* PRL2011 genome analysis reveals clues for colonization of the insect gut. *PLoS One* 7:e44229. <http://dx.doi.org/10.1371/journal.pone.0044229>.
- Ventura M, Lee JH, Canchaya C, Zink R, Leahy S, Moreno-Munoz JA, O'Connell-Motherway M, Higgins D, Fitzgerald GF, O'Sullivan DJ, van Sinderen D. 2005. Prophage-like elements in bifidobacteria: insights from genomics, transcription, integration, distribution, and phylogenetic analysis. *Appl Environ Microbiol* 71:8692–8705. <http://dx.doi.org/10.1128/AEM.71.12.8692-8705.2005>.
- Dib JR, Wagenknecht M, Hill RT, Farias ME, Meinhardt F. 2010. Novel linear megaplasmid from *Brevibacterium* sp. isolated from extreme environment. *J Basic Microbiol* 50:280–284. <http://dx.doi.org/10.1002/jobm.200900332>.
- Konig C, Eulberg D, Groning J, Lakner S, Seibert V, Kaschabek SR, Schlomann M. 2004. A linear megaplasmid, p1CP, carrying the genes for chlorocatechol catabolism of *Rhodococcus opacus* 1CP. *Microbiology* 150:3075–3087. <http://dx.doi.org/10.1099/mic.0.27217-0>.
- Yang JC, Lessard PA, Sengupta N, Windsor SD, O'Brien XM, Bramucci M, Tomb JF, Nagarajan V, Sinskey AJ. 2007. TraA is required for megaplasmid conjugation in *Rhodococcus erythropolis* AN12. *Plasmid* 57:55–70. <http://dx.doi.org/10.1016/j.plasmid.2006.08.002>.
- Broker D, Arenskotter M, Steinbuchel A. 2008. Transfer of megaplasmid pKB1 from the rubber-degrading bacterium *Gordonia westfalica* strain Kb1 to related bacteria and its modification. *Appl Microbiol Biotechnol* 77:1317–1327. <http://dx.doi.org/10.1007/s00253-007-1262-8>.
- Dominguez W, O'Sullivan DJ. 2013. Developing an efficient and reproducible conjugation-based gene transfer system for bifidobacteria. *Microbiology* 159:328–338. <http://dx.doi.org/10.1099/mic.0.061408-0>.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403–410. [http://dx.doi.org/10.1016/S0022-2836\(05\)80360-2](http://dx.doi.org/10.1016/S0022-2836(05)80360-2), <http://dx.doi.org/10.1006/jmbi.1990.9999>.
- Lowe TM, Eddy SR. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* 25:955–964. <http://dx.doi.org/10.1093/nar/25.5.0955>.
- McInerney JO. 1998. GCUA: general codon usage analysis. *Bioinformatics* 14:372–373. <http://dx.doi.org/10.1093/bioinformatics/14.4.372>.
- De Man JC, Rogosa M, Sharpe ME. 1960. A medium for the cultivation

- of lactobacilli. *J Appl Bacteriol* 23:130–135. <http://dx.doi.org/10.1111/j.1365-2672.1960.tb00188.x>.
31. Sambrook J, Russell DW, Fritsch EF, Maniatis T. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 32. O’Riordan K, Fitzgerald GF. 1998. Evaluation of bifidobacteria for the production of antimicrobial compounds and assessment of performance in cottage cheese at refrigeration temperature. *J Appl Microbiol* 85:103–114. <http://dx.doi.org/10.1046/j.1365-2672.1998.00474.x>.
 33. Motherway MO, Fitzgerald GF, Neiryneck S, Ryan S, Steidler L, van Sinderen D. 2008. Characterization of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* 74:6271–6279. <http://dx.doi.org/10.1128/AEM.01169-08>.
 34. Maze A, O’Connell-Motherway M, Fitzgerald GF, Deutscher J, van Sinderen D. 2007. Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* 73:545–553. <http://dx.doi.org/10.1128/AEM.01496-06>.
 35. Motherway MO, O’Driscoll J, Fitzgerald GF, van Sinderen D. 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. *Microb Biotechnol* 2:321–332. <http://dx.doi.org/10.1111/j.1751-7915.2008.00071.x>.
 36. Li Y, Canchaya C, Fang F, Raftis E, Ryan KA, van Pijkeren JP, van Sinderen D, O’Toole PW. 2007. Distribution of megaplasmids in *Lactobacillus salivarius* and other lactobacilli. *J Bacteriol* 189:6128–6139. <http://dx.doi.org/10.1128/JB.00447-07>.
 37. Wall R, Fitzgerald G, Hussey S, Ryan T, Murphy B, Ross P, Stanton C. 2007. Genomic diversity of cultivable *Lactobacillus* populations residing in the neonatal and adult gastrointestinal tract. *FEMS Microbiol Ecol* 59:127–137. <http://dx.doi.org/10.1111/j.1574-6941.2006.00202.x>.
 38. Foroni E, Serafini F, Amidani D, Turrone F, He F, Bottacini F, O’Connell Motherway M, Viappiani A, Zhang Z, Rivetti C, van Sinderen D, Ventura M. 2011. Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb Cell Fact* 10(Suppl 1):S16. <http://dx.doi.org/10.1186/1475-2859-10-S1-S16>.
 39. Turrone F, Serafini F, Foroni E, Duranti S, Motherway MO, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sanchez B, Santoni A, Gioiosa L, Ferrarini A, Delle Donne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M. 2013. Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host interactions. *Proc Natl Acad Sci U S A* 110:11151–11156. <http://dx.doi.org/10.1073/pnas.1303897110>.
 40. Jakubowski SJ, Krishnamoorthy V, Cascales E, Christie PJ. 2004. *Agrobacterium tumefaciens* VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion system. *J Mol Biol* 341:961–977. <http://dx.doi.org/10.1016/j.jmb.2004.06.052>.
 41. Christie PJ, Cascales E. 2005. Structural and dynamic properties of bacterial type IV secretion systems. *Mol Membr Biol* 22:51–61. <http://dx.doi.org/10.1080/09687860500063316>.
 42. Kahrstrom CT. 2014. Structural biology: solving the T4SS structural mystery. *Nat Rev Microbiol* 12:312. <http://dx.doi.org/10.1038/nrmicro3254>.
 43. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315:1709–1712. <http://dx.doi.org/10.1126/science.1138140>.
 44. van der Oost J, Jore MM, Westra ER, Lundgren M, Brouns SJ. 2009. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem Sci* 34:401–407. <http://dx.doi.org/10.1016/j.tibs.2009.05.002>.
 45. Goeders N, Van Melderen L. 2014. Toxin-antitoxin systems as multilevel interaction systems. *Toxins* 6:304–324. <http://dx.doi.org/10.3390/toxins6010304>.
 46. Ikemura T. 1985. Codon usage and tRNA content in unicellular and multicellular organisms. *Mol Biol Evol* 2:13–34.
 47. Akashi H. 1994. Synonymous codon usage in *Drosophila melanogaster*: natural selection and translational accuracy. *Genetics* 136:927–935.
 48. Makarova KS, Haft DH, Barrangou R, Brouns SJJ, Charpentier E, Horvath P, Moineau S, Mojica FJM, Wolf YI, Yakunin AF, van der Oost J, Koonin EV. 2011. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* 9:467–477. <http://dx.doi.org/10.1038/nrmicro2577>.
 49. Dong HJ, Nilsson L, Kurland CG. 1996. Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates. *J Mol Biol* 260:649–663. <http://dx.doi.org/10.1006/jmbi.1996.0428>.
 50. Novoa EM, de Pouplana LR. 2012. Speeding with control: codon usage, tRNAs, and ribosomes. *Trends Genet* 28:574–581. <http://dx.doi.org/10.1016/j.tig.2012.07.006>.
 51. Shkorporov AN, Efimov BA, Khokhlova EV, Chaplin AV, Kafarskaya LI, Durkin AS, McCorison J, Torralba M, Gillis M, Sutton G, Weibel DB, Nelson KE, Smeianov VV. 2013. Draft genome sequences of two pairs of human intestinal *Bifidobacterium longum* subsp. *longum* strains, 44B and 1-6B and 35B and 2-2B, consecutively isolated from two children after a 5-year time period. *Genome Announce* 1:e00234-13. <http://dx.doi.org/10.1128/genomeA.00234-13>.
 52. Guglielmetti S, Balzaretto S, Taverniti V, Miriani M, Milani C, Scarafoni A, Corona S, Ciranna A, Arioli S, Santala V, Iametti S, Bonomi F, Ventura M, Mora D, Karp M. 2014. TgaA, a VirB1-like component belonging to a putative type IV secretion system of *Bifidobacterium bifidum* MIMBb75. *Appl Environ Microbiol* 80:5161–5169. <http://dx.doi.org/10.1128/AEM.01413-14>.
 53. Law J, Buist G, Haandrikman A, Kok J, Venema G, Leenhouts K. 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol* 177:7011–7018.
 54. Alvarez-Martin P, O’Connell-Motherway M, Van Sinderen D, Mayo B. 2007. Functional analysis of the pBC1 replicon from *Bifidobacterium catenulatum* L48. *Appl Microbiol Biotechnol* 76:1395–1402. <http://dx.doi.org/10.1007/s00253-007-1115-5>.