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Isolation and Characterization of a Theta-Type Cryptic Plasmid from *Bifidobacterium longum* FI10564

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A number of bifidobacterial species of human origin were screened for the presence of cryptic plasmids. One strain, *Bifidobacterium longum* FI10564, harbored plasmids of approximately 2.2 kb, 3.6 kb, and 4.9 kb in size. The smallest plasmid, pFI2576 (2,197 bp), was studied in detail and its complete nucleotide sequence was determined. Computer-assisted analysis of this novel plasmid (G+C content 62%) identified 9 putative open reading frames (*orfs*), 3 of which were shown to be probable genes. These putative genes are arranged in an operon-like structure, in which the overlapping *orfs* 1 and 2 encode putative Rep proteins and are highly homologous to the *rep* genes of the *B. longum* plasmid pMB1 (1,847 bp). The mechanism of replication of pFI2576 was investigated using Southern blot analysis of whole cell lysates, with and without S1 nuclease treatment, and atomic force microscopy (AFM). The results indicate that pFI2576 is likely to use the theta mode of replication.

Keywords: Cryptic plasmid, *Bifidobacterium longum*, theta replication

Bifidobacterium species are important members of the human gastrointestinal tract. They are regarded as beneficial bacteria for human health [4] and are therefore supplemented as probiotics in health-promoting foods [8, 17]. The beneficial attributes of these species include inhibition of potential pathogens [10], stimulation of the host immune response [26], reduction in serum cholesterol [6], and anticarcinogenic properties [22]. In addition these bacteria are considered to be potential hosts for delivery of oral vaccines and other biologically active proteins such as cholesterol oxidase and bile salt hydrolase. Despite their importance, to date, few genetic tools have been developed to study these microorganisms. Although endogenous plasmids have been described in *Bifidobacterium* species since 1982, approximately 19 plasmids have been completely sequenced and characterized [1, 5, 9, 12, 13, 18, 21, 23]. These plasmids vary in size from 1.8 kb to 10.2 kb. Some of these plasmids have provided the basis for construction of *E. coli-Bifidobacterium* spp. shuttle vectors [5, 9, 14, 18, 21] or have been developed as vectors for heterologous expression of some interesting foreign genes in *Bifidobacterium* spp. [11, 16, 19, 21]. An example of this is the heterologous expression and secretion of a recombinant pediocin PA-1 from a *B. longum* strain using the signal sequence of bifidobacterial α-amylase. This resulted in a recombinant strain that efficiently inhibited the growth of *Listeria monocytogenes* [11].

In general, plasmids from *Bifidobacterium* spp. use the rolling circle mode of replication (RCR) and carry *mob* genes that enable plasmid transfer [1, 9, 12, 13]. Vectors based on plasmids that employ the RCR mechanism are often intrinsically unstable. Furthermore, the carriage of mobilization genes is not a favorable feature for cloning vectors, because of the implications for biosafety [7, 23, 25]. To develop improved bifidobacterial cloning vectors, one strategy would be to use theta-replicating plasmids that lack mobilization genes. In this study, we report the isolation and characterization of a novel theta-type plasmid without a *mob* gene, derived from *Bifidobacterium longum*.

Materials and Methods

Bacterial Strains and Growth Conditions

Several bifidobacterial species including *B. longum*, *B. bifidum*, *B. adolescentis*, *B. lactis*, and *B. animalis* were screened for the presence of plasmids. The strains were obtained from the in-house culture collection of the Institute of Food Research. They were originally isolated from stool samples of healthy human volunteers. The identities of the bifidobacterial strains were confirmed by DNA sequencing of

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the 16S rRNA gene using primers 8F (5'-AGAGTTTGATCCTGGCTCA G-3') and 1401R (5'-CGGTGTGTACAAGACCC-3'). All bifidobacterial strains were cultured in BHI broth (Oxoid, Basingstoke, Hampshire, U.K.) at 37°C under anaerobic conditions. *Escherichia coli* was cultured in L-broth at 37°C on a shaking incubator and *E. coli* transformants were selected on L agar plates supplemented with 100 μg/ml of ampicillin or 50 μg/ml of kanamycin depending on the selection marker.

Plasmid Purification

In order to purify the plasmid DNA from *E. coli* and *Bifidobacterium* spp., the Qiagen plasmid prep kit (Qiagen, Hilden, Germany) was used, with a minor modification, where 30 mg/ml of lysozyme was added to buffer P1 and the bacterial suspension was incubated at 37°C for 1 h prior to the addition of buffer P2. The purified plasmid DNA was run on 0.8% agarose gels and visualized by ethidium bromide staining.

DNA Sequencing and Analysis

The plasmid DNA of pFI2576 was gel-eluted, restriction-digested at a unique BamHI site, and subsequently cloned into the BamHI site of the *E. coli* cloning vector pUC19. Sequencing of the resulting plasmid pFI2577 was performed using an *in vitro* transposon insertion method. A small library of 13 randomly chosen transposon-containing pFI2577 derivatives was generated using an EZ::TN <R6Kγ*ori*/KAN-2> Insertion Kit (CAMBIO, Cambridge, U.K.) following the manufacturer's guidelines. Each derivative carried a copy of the <R6Kγ*ori*/KAN-2> transposon inserted at random. The transposon carries the R6Kγ*ori* and a kanamycin resistance marker for propagation and selection in *E. coli* EC100D *pir*-116. Sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (ABI, Warrington, U.K.). Samples were analyzed on an ABI3100-Avant Genetic Analyzer (Applied Biosystems, Foster, CA, U.S.A.). Most of the sequence could be obtained using universal primers and the primers supplied with the transposon insertion kit. The final gap was closed by primer walking and the sequence data were deposited in the GenBank database (Accession No. DQ452864). Basic sequence analysis was carried out with the computational molecular biology software provided by the BBSRC BioScience IT Services (U.K.). Homology searches were done at the BLAST server of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/).

Southern Blot Analysis

To confirm if *B. longum* FI10564 produces single-stranded DNA (ssDNA) intermediates of pFI2576, a Southern blot analysis was performed. Whole cell lysate from a culture, grown until the OD_{600} reached approximately 1.0, was treated with 50 μg/ml of RNase (Sigma, Gillingham, U.K.) and divided into two aliquots, one of which was treated with 16,000 U/ml of S1 nuclease (Promega, Southampton, U.K.) [3]. Samples of the aliquots were run on a 0.8% gel at 20 V overnight. The DNA was transferred to a Hybond N+ Nylon membrane (Amersham, Little Chalfont, U.K.) by capillary blotting [20]. Subsequently, hybridization with a PCR product representing a fragment of the *rep* gene of pFI2576 that had been labeled using the ECL direct labeling kit (Amersham) was carried out. The bound probe was detected according to the manufacturer's instructions.

Atomic Force Microscopy

For AFM imaging, plasmids were purified by ethidium bromide/cesium chloride (EtBr-CsCl) density gradient centrifugation [20]. The plasmid samples were immobilized on mica treated with poly-L-lysine (Sigma, Poole, U.K.) as follows: A freshly cleaved mica sheet was incubated for 10 min in a 0.001% solution of poly-L-lysine made up shortly before with pure water from a 0.1% stock solution. Subsequently, the mica was rinsed off with ultrapure water (5×1 ml) and blown dry with nitrogen. Finally, a 10-μl drop of the plasmid solution (1 μg/ml) was incubated on the treated mica for 1 min before rinsing it off with ultrapure water (5×1 ml). The mica was blown dry once again with nitrogen prior to imaging. AFM imaging was carried out under redistilled butanol, using an ECS AFM instrument (Cambridge, U.K.) operating in the "tapping mode" using 100-μm-long oxide-sharpened nanoprobe levers (NP-S, Santa Barbara, CA, U.S.A.) with a quoted force constant of $k = 0.38\ Nm^{-1}$. The levers were driven close to their fundamental resonance frequency (11–13 kHz) by applying a small sinusoidal drive signal to the Z axis of the piezoelectric scanner. The liquid in the cell, which sits on top of the scanner, transmits this oscillation to the cantilever by viscous coupling to produce an amplitude of cantilever oscillation around 10 nm. In the so-called "tapping modes" of imaging, the AFM tip is in transient contact with the sample surface. In fact, the tip touches the surface only at the end of each cycle, producing dramatically less shear force between the tip and the surface than the conventional contact mode imaging process, leading to better images of the rather small sized plasmids examined in this study. The imaging force was minimized by operating at a set point at which the tip just tracked the surface in a stable manner. This corresponded to about 5% damping of the amplitude of oscillation of the cantilever.

Results and Discussion

Plasmid Profile of *Bifidobacterium longum* FI10564

The plasmid content of 12 bifidobacterial strains spanning 7 different species was screened. Amongst them, only one strain, *B. longum* FI10564, contained plasmids of 2.2, 3.6, and 4.9 kb, respectively (Fig. 1). Except for pMB1 (1,847 bp)

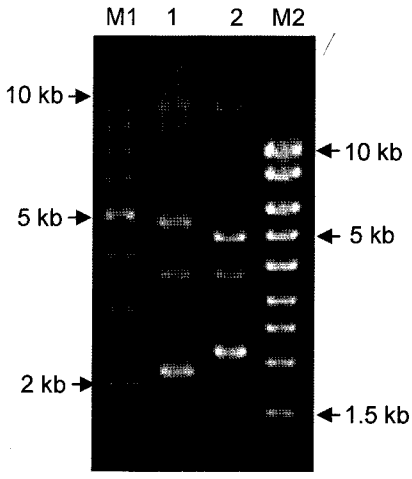

Fig. 1. Plasmid profile of *Bifidobacterium longum* FI10564.
Lane M1, supercoiled DNA ladder (Promega); lane 1, covalently closed circular DNA ; lane 2, plasmid preparation treated with AcI I; lane M2, hyperladder I (Bioline, London, U.K.).

[18], pFI2576 is the smallest of the bifidobacterial plasmids isolated so far. Since the whole plasmid can be used as a replicon, it is more convenient for the construction of cloning and expression vectors. Therefore, this small plasmid pFI2576 was selected for sequencing and detailed characterization.

Sequencing of pFI2576

To identify a unique restriction enzyme site, several enzymes were tested. Among them, BamHI was shown to cut pFI2576 only once. The plasmid was digested with this enzyme and subsequently cloned into the BamHI site of pUC19. The integrity of the recombinant plasmid pFI2577 (4.9 kb) was confirmed by restriction analysis (data not shown) and DNA sequencing was performed as described in Materials and Methods.

Bioinformatic Analysis of pFI2576

The whole nucleotide sequence of pFI2576 was analyzed. The plasmid is 2,197 bp in size and has an average G+C content of 62%. The nucleotide sequence of pFI2576 shared extensive similarities with other bifidobacterial plasmids: pMB1 (62.5% identity; GenBank Accession No., X84655), pBC1 (43.9% identity; DQ011664), and pNAC1 (22.4% identity; AY112724). Sequence analysis revealed that pFI2576 contains 9 potential open reading frames (*orfs*). The relevant *orfs* (*orf1-3*) and restriction sites are shown in Fig. 2. All potential *orfs* were assessed using the following criteria. First, that an *orf* consists of at least 100 codons preceded by a potential Shine-Dalgarno (SD) sequence with an appropriate distance (2-9 nt) from the translational start site (AUG, GUG or UUG) and displays a GC bias in the third codon position.

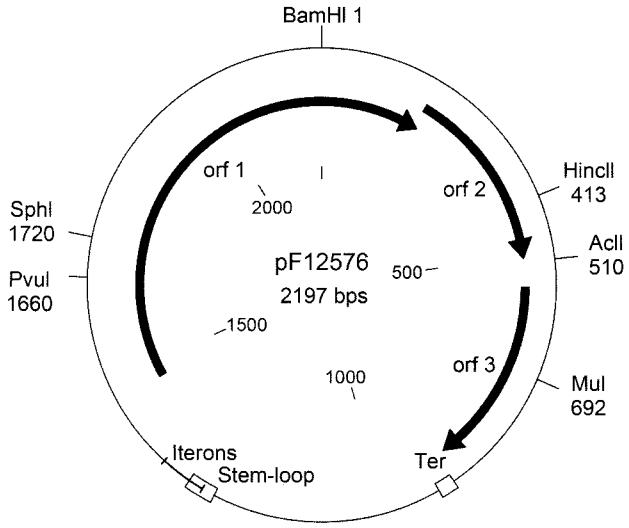

Fig. 2. Physical map and genetic organisation of pFI2576.
Arrows indicate the direction of transcription. Putative terminator (Ter) and stem-loop structure as well as iterons are shown on the map.

Although *orf1* is not preceded by a typical SD sequence, we nevertheless identified it as an *orf* on the basis of the GC bias in its third codon position. Furthermore, its gene product matches the Pfam replicase family (PF03090) and shares an overall sequence identity of 90% with Orf2 of the *B. longum* plasmid pMB1. This Rep protein also has reduced homology to the putative replicase of pDOJH10S from a *B. longum* (GenBank Accession No., AF538869), or potential replicase of pBC1 from a *B. catenulatum* (GenBank accession No., DQ011664) (Fig. 3). We concluded that *orf1* is the

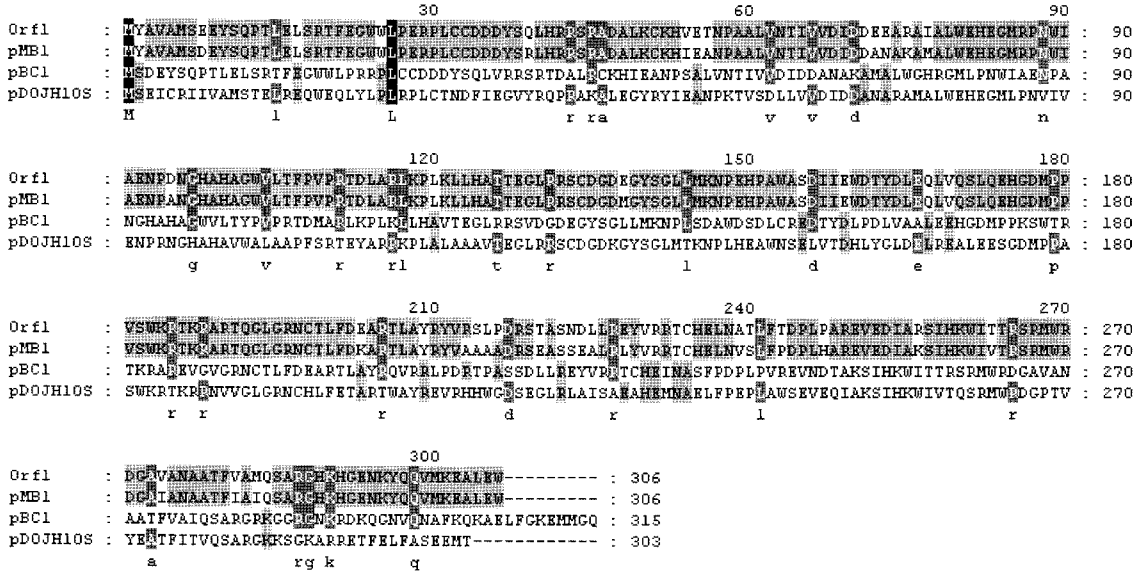

Fig. 3. Alignment of the Rep protein (Orf1) of pFI2576 with those from other bifidobacterial plasmids: pMB1 (GenBank Accession No. X84655), pBC1 (DQ011664), and pDOJH10S (AF538869).
The protein sequences were aligned using ClustalX [24]. Shading indicates the extent of sequence conservation (black 100%, dark grey 75%, and light grey 50%).

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GAAGGGCGCTGGAGGTGCTGCGTTCGGTTCATGTACATGAACCGTCTAGCTTGCTTACCT   1260

TCGATTTGATGGGCATCTCCATGTGGATGTCCATGGAGATGTCCATCGAGATATCCATAG   1320

GGTGTCCATGGAGATGTCCATGGAGATGTTCATGGAGATGTCCATCTGAGCGCACAGGAA   1380

GCCGGGGCGGTGGGCTGGGTTCAGCCGACGCTTTGCTGGGCTGAACTATCTGACTTGGTT   1440

                                  M  Y  A  V  A  M  S  E  E  Y  S
CCCGCGTATTTGTTCACTGTACAAATACGATGTATGCTGTAGCTATGTCTGAAGAGTATT   1500
                                  orf1
```

Fig. 4. The putative origin of replication of pFI2576.
The suspected *ori* region consists of 6 DNA iterons (represented by black arrows) and two inverted repeats (represented by dotted arrows above the sequence).

DNA replication initiator protein in pFI2576. Gene *orf2* is preceded by an SD sequence (GGAGG) and together with *orf1* and *orf3* forms a possible operon, in which *orf1* and *orf2* overlap by 7 bp. Similar overlap arrangements have been observed for *orf1* and *orf2* in pMB1 (7 bp) and for the *repB* gene and *orfX* in pBC1 (4 bp). The gene product of *orf2* is almost identical (98%) to protein Orf1 from pMB1. Gene *orf3* is preceded by an SD sequence (GGAGG) and shows a clear GC bias in its third codon position, but its gene product does not show similarity to any protein from the public databases. A rho-independent terminator (dG –19.4 kcal/mol) that terminates the operon is identified 15 nt downstream of *orf3*. The suspected origin region of pFI2576 is located approximately 100 bp upstream of *orf1*. It comprises two inverted repeats, one of which is predicted to form a stem-loop structure that overlaps with the first two copies of a series of 6 consecutive 12 bp iterons (Fig. 4). Iterons have been described for plasmids replicating *via* RCR as well as the theta mechanism of replication [2]. Therefore, we decided to look for the presence of single-stranded replication intermediates.

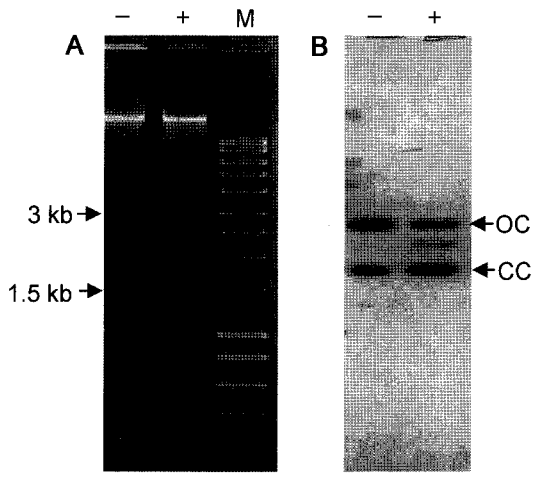

Fig. 5. Southern blot analysis for the detection of ssDNA intermediates from pFI2576.
A. Electrophoresis gel; **B.** Exposed x-ray film. Plus (+) and minus (–) indicate whether or not the samples were treated with S1 nuclease prior to electrophoresis. OC and CC indicate open circular and closed circular plasmid forms, respectively.

Detection of ssDNA Intermediates

In order to establish whether pFI2576 uses the RCR mode, a Southern blot analysis was performed. We were unable to detect any ssDNA intermediates (Fig. 5), indicating that pFI2576 does not replicate *via* this mechanism. As previously mentioned, the putative Rep protein (Orf1) of pFI2576 showed high similarity (90% identity) to the replicase of pMB1 (GenBank Accession No. X84655). The replication mode for pMB1 has not been established yet. However, it was interesting to note that based on the amino acid sequence homology of the Rep protein, Corneau *et al.* [1] attempted to organize all the bifidobacterial plasmids into groups and had placed pMB1 in group 5. Within this group, the family members showed similarity to ColE2, a theta-replication plasmid from *E. coli*. To date, the replication mechanism of plasmids from bifidobacteria has been experimentally analyzed in 9 plasmids: eight plasmids, pKJ50, pCIBb1, pKJ36, pMG1, pNAC1, pDOJH10L, pNAL8L, and pASV479, replicate *via* RCR [1, 5, 9, 12, 13, 14, 15, 21] and one plasmid, pDOJH10S, replicates *via* the theta-type mechanism [9].

Atomic Force Microscopy

To our knowledge, this is the first example where AFM microscopy has been used to study plasmid replication mechanisms and our results indicate that this is a useful method for such analysis. We were able to obtain images for the possible intermediate (Fig. 6A) and termination (Fig. 6B) steps in theta replication, providing further indication that pFI2576 is a theta-type replicon.

Today, bifidobacteria are recognized as one of the important groups of bacteria in the GI tracts of humans. A

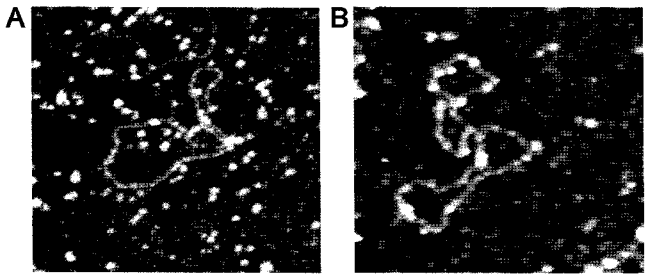

Fig. 6. AFM images of plasmid DNA pFI2576.
A. Intermediate stage of theta replication (scan size: 400×400 nm, Z range 4.9 nm); **B**. Termination stage of theta replication (300×300 nm, Z range 7.2 nm).

wide range of probiotic properties have been ascribed to these bacteria and hence they have significant potential for use in functional foods. They are already added as probiotics in large numbers of commercial dairy and fermented food products. Fundamental studies aimed at understanding these microorganisms, their interactions with the human host, and their potential to serve as probiotics require molecular tools that are still less developed than in other Gram-positive bacteria such as lactic acid bacteria or *Streptomyces*, which like bifidobacteria are members of the actinomycetes with high G+C content. Plasmids play a critical role in molecular studies as they provide the basis for vectors required for cloning, gene deletion, and gene expression. In this study, we have described a novel cryptic plasmid from a *B. longum* strain, which is only the second example of a bifidobacterial plasmid isolated so far that replicates *via* the theta mode. In addition, there is no evidence for the presence of plasmid transfer genes (*mob*), therefore making it an ideal plasmid to be used as a replicon for the construction of safe and stable bifidobacterial cloning and expression vectors.

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