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ORIGINAL ARTICLE

Complete sequence analysis of two cryptic plasmids from *Bifidobacterium kashiwanohense* JCM 15439 (type strain) isolated from healthy infant feces

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ABSTRACT

Bifidobacterial plasmids reported so far are derived from a limited number of strains and plasmids of bifidobacterial type strains isolated from humans are unknown. We found that *Bifidobacterium kashiwanohense* JCM 15439 (type strain) isolated from a healthy infant contained two cryptic plasmids, designated pBBKW-1 and pBBKW-2. We determined and analyzed the complete sequences of both plasmids. pBBKW-1 (7716 bp) was predicted to replicate by a rolling-circle mechanism and encode six protein-coding genes, two of which are putative replication proteins. pBBKW-1 seems to be a cointegrate plasmid containing two copies of the plasmid pMG1 from *Bifidobacterium longum*. pBBKW-2 (2920 bp) was predicted to encode six protein-coding genes and be a theta-type replicating plasmid, which has been reported to be more stable than a rolling circle-type replicating plasmid frequently found in bifidobacteria. Our finding will provide new insights into safe recombinant plasmid constructions for humans.

Key words: Bifidobacterium, *human origin, plasmid.*

INTRODUCTION

Bifidobacterium is a Gram-positive, anaerobic, branched rod-shaped bacterium, and is frequently isolated from the human intestine. Bifidobacteria have health-promoting effects through interaction with their hosts. According to the definition by the World Health Organization, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (Picard *et al*. 2005). Several bifidobacterial strains have for long been used as probiotics in a wide range of different probiotic products, marketed in many countries. Bifidobacteria isolated from the large intestine of healthy humans are more likely to be safe. If it is possible to insert useful genes into bifidobacteria, we may be able to use bifidobacteria as safe and inexpensive vaccines and gene therapy agents, but many extensive genetic manipulation technologies are required to achieve these purposes.

Genetic manipulation of bifidobacteria has detected plasmids from four bifidobacteria species (Sgorbati *et al*. 1982), and then other plasmids have also been found from bifidobacteria (Kiewiet *et al*. 1993; Rossi *et al*. 1996; O'Riordan & Fitzgerald 1999; Park *et al*. 1999; Corneau *et al*. 2004; Tanaka *et al*. 2005; Lee & O'Sullivan 2006; Guglielmetti *et al*. 2007; Sangrador-Vegas *et al*. 2007). Most bifidobacterial plasmids are cryptic and thus it is necessary to add a selection marker to insert plasmids into a bifidobacterial cell. Materials combining bifidobacterial plasmid pMB1 (Matteuzzi *et al*. 1990), a plasmid from *Escherichia coli*, and the *spectinomycin resistance* gene from *Enterococcus faecalis* provide a new shuttle vector (Missich *et al*. 1994), and were transformed into *Bifidobacterium longum*. Many plasmid vectors for bifidobacteria have been constructed, such as cloning and expression vectoring, incorporating a drug-resistant gene (Nakamura *et al*. 2002).

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In this study, identification of bifidobacterial cryptic plasmids was initially conducted by screening plasmid-bearing strains, and the profile showed that bifidobacterial strains harboring cryptic plasmids were both rare and limited to specific species. We have deposited a novel species candidate, *Bifidobacterium* sp. HM2-2 into the Japan Collection of Microorganisms (JCM) as a type strain, and this strain, *Bifidobacterium* sp. HM2-2, is recognized as a novel species (Morita *et al*. 2011) with JCM 15439 as a JCM accession number. We identified two cryptic plasmids, pBBKW-1 and pBBKW-2, from *B. kashiwanohense* JCM 15439 (type strain), which were isolated from healthy infant feces, and then determined the complete nucleotide sequences of both plasmids. We will provide new insights into plasmids of bifidobacteria from humans.

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions

The bifidobacterial strains, *B. angulatum* JCM 7096 (feces of human subjects), *B. bifidum* JCM 1255 (feces of human infant), *B. breve* JCM 1192 (feces of human infant), *B. catenulatum* JCM 1194 (feces of human subjects), *B. dentium* JCM 1195 (human dental caries), *B. longum* JCM 1217 (feces of human adult), *B. pseudocatenulatum* JCM 1200 (feces of human infant) and *B. scarcovii* JCM 12489 (human blood), were obtained from the JCM. We had isolated *B. kashiwanohense* JCM 15439 from healthy infant feces (Morita *et al*. 2011). All these strains were type strains and of human origin. Blood liver (BL) agar plates (Eiken Chemical, Tokyo, Japan) and anaerobic bacterial culture medium (ABCM broth; Eiken Chemical) were used for cell cultures. These bifidobacterial strains were anaerobically cultured at 37°C in ABCM broth and on BL agar plates for 24 h and 72 h, respectively.

Plasmid profile analysis of bifidobacterial type strains

Plasmid DNA was purified from cultured cells of these strains as previously described (Lee & O'Sullivan 2006). The plasmid profiles of these strains were compared by agarose gel electrophoresis of purified plasmids in 0.8% agarose gels in 1 × TAE 1 x TAE (40 mmol/L Tris, 20 mM acetic acid, 1 mmol/L ethylenediaminetetraacetic acid, pH 8.5). Plasmid DNA was visualized in the BioDoc-It™ Imaging System (UVP, Upland, CA, USA) after gels were stained using an ethidium bromide solution (10 μg/mL).

Sequencing and informatics

Randomly sheared plasmid DNA as a library was constructed using the HydroShear DNA shearing machine (Genomic Solutions, Ann Arbor, MI, USA). Sequencing of library clones was performed using a Big-Dye terminator and an ABI Prism 3100 Auto sequencer (Applied Biosystems, Foster City, CA, USA). DNASTAR (DNASTAR, Inc., Madison, WI, USA) was used for assembly of contigs, and the remaining gaps were filled by primer walking. The multiple sequence alignments were performed using ClustalW (Larkin *et al.* 2007). Predicted protein-coding genes were identified using Glimmer 3.0 (Delcher *et al.* 2007). Protein domains were identified using the Pfam database (Punta *et al.* 2012).

Southern hybridization and single-stranded DNA (ssDNA) detection

The ssDNA was detected by a previously described method (Lee & O'Sullivan 2006). Southern hybridization was performed using the DIG DNA Labeling and Detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The accumulation of ssDNA intermediates, indicative of rolling-circle replication, was evaluated by the effect of S1 nuclease (Takara Bio, Otsu, Japan) digestion on *B. kashiwanohense* JCM15439 plasmids.

Nucleotide sequence accession numbers

The sequence data have been deposited in DNA Databank of Japan (DDBJ)/GenBank/EMBL (European Molecular Biology Laboratory) under accession numbers AB713428 (pBBKW-1) and AB713429 (pBBKW-2).

RESULTS AND DISCUSSION

Among nine type strains of bifidobacterial species from humans, only *B. kashiwanohense* JCM 15439 contained two cryptic plasmids (pBBKW-1 and pBBKW-2) (data not shown), but its role is not yet clear. We determined the complete nucleotide sequences of both plasmids. pBBKW-1 consisted of 7716 base pairs (bp), with a G/C content of 65%. It was predicted to encode six protein-coding genes (Fig. 1A). The gene sets of pBBKW-1 were almost identical to those of the plasmid pMG1 of *B. longum* (3682 bp, accession no. AY210701). Region-1 (35–500), region-2 (1970–4065) and region-3 (4066–7716) of pBBKW-1 showed 95–98% nucleotide sequence identity to pMG1, suggesting that pBBKW-1 consists of a combination of two pMG1 (Fig. 1A). The cryptic plasmid pDOJH10L of *B. longum* DJO10A is the fused form of the two plasmids (pKJ50 and pNAC2) derived from two different bifidobacteria (Lee & O'Sullivan 2006). There has been no other report on the fusion of two bifidobacterial plasmids and it may not be a frequent event.

pBBKW-1 encoded two genes for Rep proteins (P1-0002 and P1-0005). At the upstream of duplication origin of pMG1, a 22-bp iteron sequence is tandemly repeated four and a half times (Park *et al*. 2003). Iteron structures serve as binding sites for replication proteins, and may be involved in copy-number control and incompatibility of plasmids (Frey *et al*. 1992). pBBKW-1 also contained these iteron structures and DnaA box for the binding of DNA polymerase at the upstream of P1-0005 (Fig. 2A). P1-0002 showed 85% amino acid identity to the replication protein of pNAC3_p1 from *B. longum* (accession no. AAM66775). There were also three contiguous repetitive sequences approximately 180 bp upstream of P1-0002. These sequences seem to contain the iteron sequence, but

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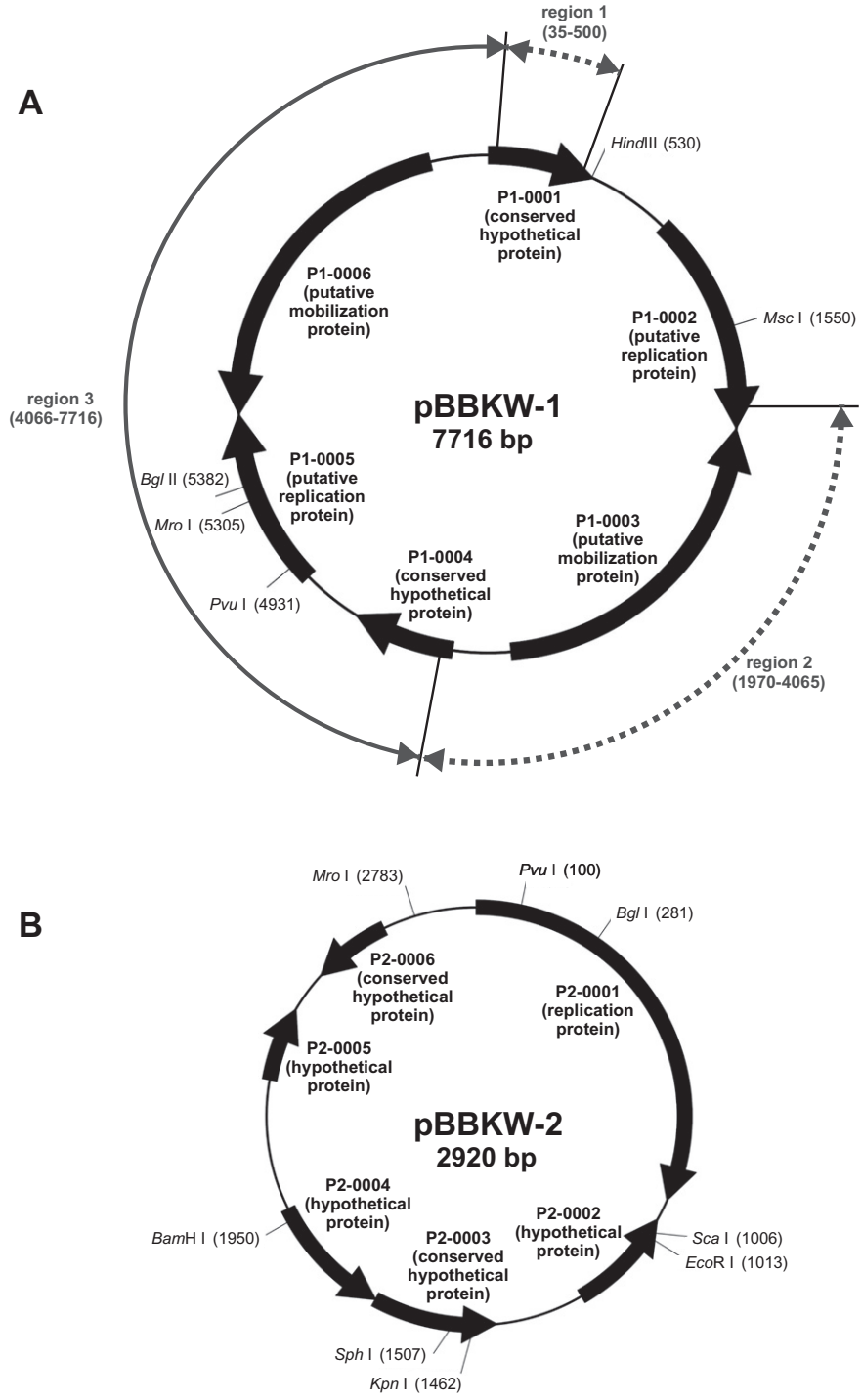

Figure 1 Circular representations of pBBKW-1 (A) and pBBKW-2 (B). Predicted protein-coding genes are marked as arrows in the direction of transcription. Outer arrows indicate regions of high sequence identity between pBBKW-1 and pMG1.

two bases were deleted in the third repetitive sequence (Fig. 2B). P1-0003 and P1-0006 were predicted to encode mobilization protein, were the same length, and shared 100% amino acid sequence identity. Both contained a conserved sequence motif (Pfam PF03389), which is found in mobilization proteins. pBBKW-1 contained a putative *oriT* structure upstream of P1-0003 and P1-0006. The putative *oriT* consists of an inverted repeat and a conserved DNA sequence (5'-TAAGTGCGCCCT-3'), and was identical to several other mobilizable plasmids (Cook & Farrand 1992; Wang & Macrina 1995; Climo *et al*. 1996; Park

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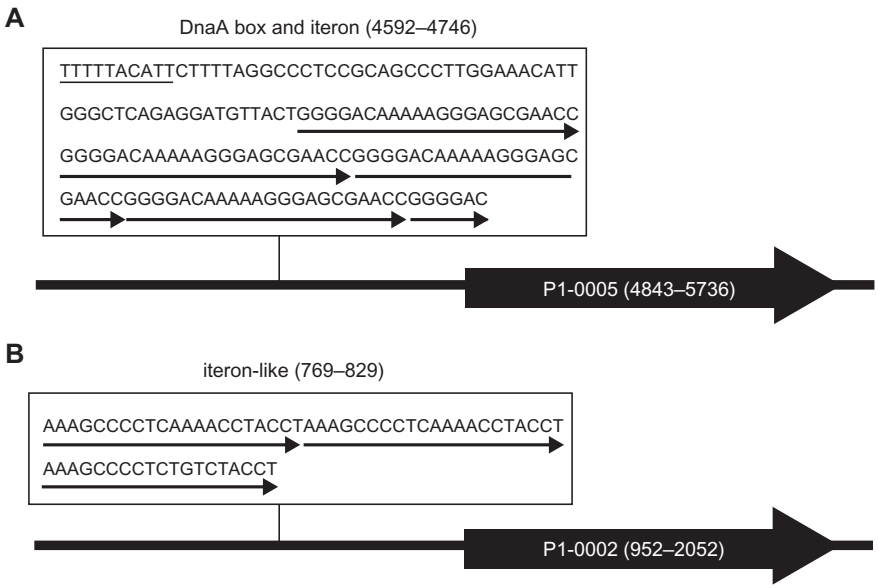

Figure 2 Proposed functional *ori* regions of P1-0005 (A) and P1-0002 (B). The sequences of putative DnaA box (underlined) and iteron region (single arrow) are shown.

pBBKW-1 (region 2)	AATGCAACCTCCGGTTGCATG**TAAGTGCGCCC**TAATC
pBBKW-1 (region 3)	AATGCAACCTCCGGTTGCATG**TAAGTGCGCCC**TAATC
pMG1	AATGCAACCTCCGGTTGCATG**TAAGTGCGCCC**TAATC
pGO1	CACGCGAACGGAACGTTCGCA**TAAGTGCGCCC**TTAC
pTiC58	CAAGGCGTCGCGTCAGCGACGTA**TAATTGCGCCC**TTG
pIP501	ATACGAAGTAACGAAGTTACTGCGTA**TAAGTGCGCCC**TTAGT
pKJ50	TGAATGTTACCACCGGTAACATG**TAAGTGCGCCC**TCAAT
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Figure 3 Alignment of the *oriT* region in plasmids. The sequences of the putative two *oriT* sites of pBBKW-1 are aligned with those in other plasmids: pMG1 (accession no. AY210701), pGO1 (FM207042), pTiC58 (AJ237588), pIP501 (L03355) and pKJ50 (U76614). Inverted repeat sequences are marked by underlines. The asterisks and bold characters indicate sequences conserved among the seven *oriT* regions.

et al. 1999) (Fig. 3). P1-0001 and P1-0004 had conserved sequences with unknown function, and shared 97% amino acid sequence identity with each other, suggesting that both would encode proteins with similar function.

pBBKW-2 consists of 2920 bp with a G/C content of 63%, and was predicted to encode six protein-coding genes (Fig. 1B). It contained sequences homologous with pBC1 (a 2.5-kbp cryptic plasmid of *Bifidobacterium catenulatum* L48) (accession no. DQ011664). P2-0001 showed 84% amino acid identity with RepB of pBC1. pBC1 has a region rich in secondary structures such as inverse repeats (IRs) and direct repeats (DRs) upstream of the *repB* gene (Álvarez-Martin *et al*. 2007), whereas IR and DR sequences were absent in the corresponding region of pBBKW-2. P2-0003 showed 59% amino acid identity to CopG-like protein of pBC1. P2-0003 may be involved in the stability of pBBKW-2, because it has been reported that truncating the *copG-like* gene of pBC1 causes a decrease in the stability and copy number of plasmids (Álvarez-Martin *et al*. 2007).

Bifidobacterial plasmids have two replication mechanisms, rolling circle (RC) and theta-type replication (Khan 1997; del Solar *et al*. 1998). Theta-type plasmids have been reported to be stable in lactic acid bacteria, whereas RC replicating plasmids are likely to drop out from the host with lactic acid bacteria (Kiewiet *et al*. 1993). pMG1 from *B. longum* have been shown to follow RC replication (Park *et al*. 2003), and thus pBBKW-1 seems to be a RC-type plasmid because pBBKW-1 was very similar to pMG1 (Fig. 1A). On the other hand, pBBKW-2 has no structure found in RC replication plasmids, such as a conserved DnaA box in an AT-rich region and iteron structures. P2-0001 showed 84% and 50% amino acid identity with RepB of pBC1 and RepA of pDOJH10S in *B. longum* DJO10A, respectively. pBC1 and pDOJH10S are known as

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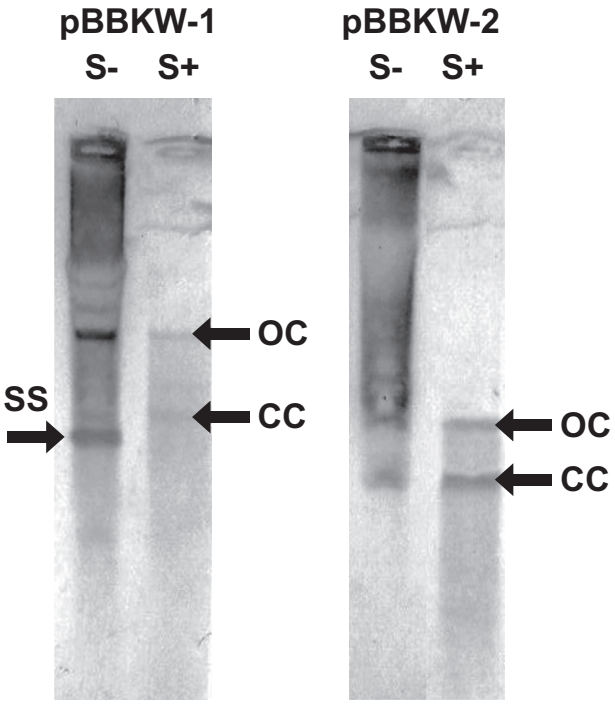

Figure 4 S1 nuclease analysis of pBBKW-1 and pBBKW-2. After agarose gel electrophoresis, the plasmids, pBBKW-1 and pBBKW-2, from *B. kashiwanohense* JCM 15439 were treated with each probe for Southern hybridization, respectively. S-, not treated with S1 nuclease; S+, treated with S1 nuclease; CC, closed circular plasmid DNA; OC, open circular plasmid DNA; SS, single-stranded DNA intermediates.

theta-type replicating plasmids (Lee & O'Sullivan 2006; Álvarez-Martin *et al*. 2007), and thus pBBKW-2 seems to a theta-replication plasmid. Several *E. coli–Bifidobacterium* shuttle vectors have been constructed, and almost all of these are based on RC replication plasmids from bifidobacteria (Matteuzzi *et al*. 1990; Missich *et al*. 1994; Matsumura *et al*. 1997). To verify that pBBKW-1 and pBBKW-2 replicate via RC- and theta-type replication respectively, plasmids from *B. kashiwanohense* JCM15439 were subjected to S1 nuclease digestion, and followed by Southern hybridization with the pBBKW-1 and pBBKW-2 probes (Lee & O'Sullivan 2006; Tauch 2010). A single-stranded DNA band disappeared in pBBKW-1, strongly suggesting that pBBKW-1 was a RC-type plasmid. In contrast, pBBKW-2 had no single-stranded intermediates, showing that pBBKW-2 was not a RC-type plasmid (Fig. 4). pBBKW-2, probably a theta-type replicating plasmid, is derived from bifidobacteria isolated from healthy human intestine, and encodes no mobilization protein. Thus, pBBKW-2 may be a good candidate for safe and stable cloning and expression vectoring.

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