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Sequence analysis of plasmid pIR52-1 from *Lactobacillus helveticus* R0052 and investigation of its origin of replication

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ABSTRACT

Lactobacillus helveticus R0052 is a bacterium used in commercial probiotic preparations. R0052 contains a small, cryptic plasmid comprised of eight open reading frames, four of which encode proteins of unknown function. Based on the sequence of the replication initiation protein RepA, pIR52-1 is a member of the recently described RepA_N family of Gram-positive theta-replicating plasmids. The *rep*A gene of pIR52-1 is the minimal origin of replication for *L. helveticus* and other *Lactobacillus* hosts. Additionally, pIR52-1 belongs to a subgroup of the RepA_N plasmid family which have RepA proteins of high amino acid identity and a conserved, non-coding element upstream of *rep*A which, in pIR52-1, is responsible for the control of plasmid copy number and contributes to plasmid maintenance.

1. Introduction

Plasmids are extrachromosomal elements which can replicate autonomously from the cellular chromosome of their host bacterium. *Lactobacillus* plasmids were first identified in *Lactobacillus casei* by Chassy et al. (1976) and rolling circle and theta-replicating plasmids have since been observed in many species of lactobacilli. Sequencing of the genomes of several lactobacilli has also yielded sequences for a number of novel plasmids (Makarova et al., 2006; Kleerebezem et al., 2003; van Kranenburg et al., 2005; Claesson et al., 2006; Fang et al., 2008; Desmond et al., 2005).

Rolling circle replicating plasmids in *L. helveticus* have been observed in a variety of strains, and have been characterized from strains SBT2161 (plasmid LJ1, Takiguchi et al., 1989), ATCC 15009^T (pLH1, pLH2 and pLH3, Fortina et al., 1993) LBL4 (pLH4, Pridmore et al., 1994), strain S 36.2 (pLJH1, de Rossi et al., 1989) and CP53 (plasmid pCP53, Yamamoto and Takano, 1996). In 1999, the complete sequence of plasmid pLH1 was obtained (Thompson et al., 2001). pLH1 was the first plasmid observed in *L. helveticus* to replicate via the theta mechanism (Thompson et al., 2001). The gene encoding the putative replication protein (ORF-351) was observed to contain a variety of stem loops and direct repeats of 37, 10 and 7 bp, which may serve as the binding sites for a Rep protein that is involved in theta replication (Thompson et al., 2001). Plasmids pLH2, pLH3 and pLH4 have been shown to be rolling circle replicating plasmids, as the replication protein genes are similar to those in the Lactococcal rolling circle replicating plasmid pWV01 (Pridmore et al., 1994). Few phenotypic characteristics have been associated with *L. helveticus* plasmids to date. de Rossi et al. (1989) observed a drop in proteolytic activity when plasmid pLHJ1 was cured from *L. helveticus* S 36.2. Fortina and Silva (1996)

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observed two *L. helveticus* plasmids in strain ILC 54 to be associated with lactate production and proteolytic activity and de Los Reyes-Gavilan et al. (1990) found a restriction/modification system associated with a plasmid from *L. helveticus* CNRZ1094.

In 2006, Ricci et al. (2006) performed a survey of the plasmid content from *L. helveticus* cheese isolates. Three subtypes of plasmids were observed: those similar in synteny to pLH3 and with Rep proteins homologous to that of pLH3; those homologous to pLH2; and plasmid pLHp1 in a Provolone cheese isolate which appeared quite different from the previously characterized plasmids (Ricci et al., 2006). They confirmed the previous observation that an intergenic region between ORFs 1 and 2 of pLH2 is frequently conserved among small *L. helveticus* plasmids (Ricci et al., 2006).

Few attempts have been made to use small plasmids from *L. helveticus* as cloning vectors. Some preliminary experiments detailing the electrotransformation of *L. helveticus* strains with plasmid pCP53 have been performed (Yamamoto and Takano, 1996), although the plasmid has not been used to introduce novel genes into *L. helveticus*. Hashiba et al. (1992) used a 1.5 kb fragment of plasmid pLJ1 (containing the plasmid's only gene) to generate a shuttle vector capable of replication in *Escherichia coli* and *L. helveticus*. Bhowmik and Steele (1994) used pSA3, an *E. coli*/Gram + shuttle vector derived from *Streptococcus* (Dao and Ferretti, 1985) to inactivate the chromosomal D(−)LDH gene in *L. helveticus* CNRZ32. Thompson et al. (2001) were able to transfer several genes in various *L. helveticus* strains through mobilization with a pIP501-derived vector, although they were not stable during repeated sub-culturing.

Lactobacillus helveticus R0052 was isolated from a dairy starter culture in March 1990 and since that time it has been used in a number of commercially available probiotic formulations. It contains a small cryptic plasmid known as pIR52-1. This plasmid is maintained in the host strain without selective pressure. In order to ensure consumer safety and to investigate possible genes useful for cloning vectors or metabolic analyses it was necessary to identify any potential antibiotic resistance genes in the plasmid as well as to investigate the plasmid's origin of replication, maintenance systems, and metabolic genes. We have therefore completely sequenced and annotated pIR52-1 and identified the minimal origin of replication. By doing so, it was possible to generate vectors which replicate in both *E. coli* and *L. helveticus* and may therefore be useful as shuttle vectors for *L. helveticus* and other *Lactobacillus* species.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lactobacillus helveticus R0052 and CNRZ32 (a plasmid-free strain), *L. casei* ATCC 334, and *L. plantarum* ATCC 4008 were propagated at 37 °C in MRS broth or on MRS agar and glycerol stocks were maintained at −80 °C. R0052 was grown anerobically whereas CNRZ32, ATCC 334, ATCC 4008, and their transformants were grown aerobically. *E. coli* was grown in LB broth, SOB broth or on LB agar.

2.2. Detection of plasmid DNA

Plasmid DNA from *L. helveticus* R0052 was isolated using a modified protocol based on the procedure described by Walker and Klaenhammer (1994). Briefly, the glycerol stock of R0052 was streaked on MRS agar and a single colony was used to inoculate 40 mL MRS broth. After overnight (18 h) growth, the entire volume was used to inoculate 200 mL of pre-warmed MRS broth. The culture was grown for 2 h (until the OD_{600} was 0.3) and the bacteria were collected by centrifugation (3000*g* for 5 min at 4 °C). The bacteria were washed twice in half a volume of cold sterile MilliQ water, resuspended in 20 mL freshly prepared cell suspension buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 8% [w/v] sucrose, 10 mg/mL lysozyme, 0.1 mg/mL RNAse A) and incubated on ice for 1 h. The treated bacteria were centrifuged for 2 min at 3000*g* at 4 °C to create a loose pellet. The supernatant was carefully discarded and the pellet was resuspended in 10 mL freshly prepared lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 3% [w/v] SDS, 0.105 N NaOH, 0.1 mg/mL RNAse A) and incubated at 65 °C for 30 min. The tubes were cooled at room temperature for 10 min and then 5 mL high salt solution (3 M KOAc, 5% [w/v] glacial acetic acid) were added. The debris was cleared by centrifugation at 9000*g* for 5 min at 4 °C and the supernatant was collected and subjected to the same centrifugation. The resulting supernatant was passed through a sterile 0.22 μm vacuum filter (Millipore Cat. No.: SCGPU01RE, Billerica, Massachusetts) and then applied to a pre-equilibrated QIAGEN-tip 100 (QIAGEN Plasmid Midi kit Cat. No.: 12143, Mississauga, Ontario). The plasmid preparation was washed, eluted and concentrated as described by the manufacturer's protocol. The resulting DNA was resuspended in 10 mM Tris–HCl, pH 8.0, and analyzed on a 0.6% [w/v] agarose gel.

2.3. Subcloning and sequencing of pIR52-1

pIR52-1 was digested with EcoRI and EcoRV (New England Biolabs) at 37 °C for 4 h. Single-stranded ends generated by EcoRI were filled in using an excess of dNTPs (1 mM each) and T4 DNA polymerase (New England Biolabs) for 40 min. The pUC19 cloning vector (New England Biolabs) was digested with SmaI (New England Biolabs) and ligated with blunt-ended pIR52-1 using T4 ligase. Two microlitres of the ligation mix were transformed into *E. coli* UltraMAX DH5α-FT (Invitrogen) or JM109 (Promega) according to the manufacturer's protocols. Clones were grown in a microtitre plate and plasmid DNA was extracted from each clone using the alkaline lysis method followed by PEG precipitation (Sambrook et al., 1989). Plasmid DNA preparations were screened for inserts and partially sequenced with the M13F and M13R primers (Table 1) at the Biotechnology Research Institute DNA sequencing facility according to the facility protocols. Primer walking was performed to sequence the fragments of pIR52-1 using custom oligonucleotides from Invitrogen.