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DNA sequence analysis of a small cryptic plasmid from *Lactococcus lactis* subsp. *lactis* M14

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Abstract

A small plasmid designated pAR141 was isolated from *Lactococcus lactis* subsp. *lactis* M14 and its complete 1594 base pair nucleotide sequence was determined. Analysis of the sequence indicated that this plasmid does not carry any industrially important determinants besides the elements involved in plasmid replication and control. The transcriptional repressor CopG and replication initiation protein RepB appeared as a single operon. A small countertranscribed RNA (ctRNA) coding region was found between the *copG* and *repB* genes. The double strand origin (*dso*) and single strand origin (*sso*) of rolling circle replicating (RCR) plasmids were also identified in pAR141, suggesting that this plasmid replicates by rolling circle (RC) mode. This observation was supported by S1 nuclease and Southern hybridization analyses.

Keywords: Cryptic plasmids; *Lactococcus lactis*; Replication; Rolling circle replicating plasmid

1. Introduction

Lactococcus lactis is a mesophilic lactic acid bacterium (LAB) widely used in the dairy industry. It obtained the GRAS (generally regarded as safe) status due to its long safe use as starter culture in dairy fermentation for the manufacture of cheese, butter milk and other products. It has been known that some properties important to the food industry such as lactose fermentation, proteinase activity, citrate metabolism, bacteriophage resistance, and bacteriocin production, are spontaneously lost during consecutive subculturing. This ultimately led to the discovery of plasmids in these bacteria (Davey, 1984; Kuhl et al., 1979; McKay and Baldwin, 1975; Otto et al., 1982). Most lactococcal strains harbour between 4 and 7 plasmid molecules, with sizes ranging from 2 to 80 kb. The phenotype-encoding plasmids are relatively large. Some other smaller plasmids encode no recognizable phenotype besides their replicative functions. These plasmids are known as cryptic plasmids (Sánchez and Mayo, 2003). Both

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types of plasmids can be made into valuable tools for DNA cloning, gene expression, and other biotechnological applications. However, extensive molecular studies on them are required before they can be used in genetic manipulation. In this study, a small cryptic plasmid was isolated from *L. lactis* subsp. *lactis* and its sequence analyzed.

Rolling circle replicating (RCR) plasmids are ubiquitous in Gram-positive bacteria, although they have been reported in many Gram-negative bacteria and archea (Khan, 2005). RCR plasmids are usually small in size (less than 10 kb), have multiple copies, and are tightly organized. Three important elements, a gene encoding the initiator (Rep) protein, the double strand origin (*dso*) and the single strand origin (*sso*), are contained in all RCR plasmids (Khan, 2005). RCR plasmids can be grouped into several families (http://www.essex.ac.uk/bs/staff/osborn/DPR_home.htm), for example, pT181, pC194, pSN2, pMV158, based on the sequence similarities in their *dso*s and Rep proteins (Khan, 2005). RC replications are generally divided into two stages, leading strand replication and lagging strand replication (del Solar et al., 1998; Khan, 1997, 2000, 2005). The RC replication initiates with specific binding of the plasmid-encoded Rep protein to the binding (*bind*) region of the cognate *dso* and the assembly of initiation complex by other host replication proteins. The Rep protein generates a strand- and site-specific nick within the *dso* nick (*nic*) region of the leading strand. The free 3′-OH end of the nick site serves as the primer for the initiation of DNA replication. Host proteins-like DNA polymerase III, DNA helicase and single-stranded DNA binding (SSB) proteins, are involved in the elongation of the new leading strand and the displacement of the parental strand. The leading strand replication ends by the DNA strand transferase activity of the Rep protein. The Rep protein nicks and closes plasmid DNA in a type I topoisomerase-like fashion, when the *dso* is regenerated. By this, a single-stranded (ss) circular plasmid consisting of the parental leading strand and a double-stranded (ds) circular plasmid containing the newly synthesized leading strand and the parental lagging strand are produced. The lagging strand replication of the RCR plasmids started at the *sso* sites, utilizing host proteins. An RNA primer is synthesized by the host RNA polymerase, and extended by DNA polymerase I and DNA polymerase III subsequently. The dsDNA products of RC replication are converted to the supercoiled form by host DNA gyrase. RCR plasmids of different families may replicate with slightly varied steps. To know the mode of replication for our cryptic plasmid, S1 nuclease and Southern blot analysis was carried out in this study.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactococcus lactis subsp. *lactis* M14 was cultured in M17 medium (Oxoid) containing 0.5% (w/v) glucose (GM17) at 30 °C for ~16 h as stand-culture (Terzaghi and Sandine, 1975). *Escherichia coli*, which was used as transformation host, was grown overnight in Luria Bertani (LB) broth at 37 °C with vigorous shaking at 200 rpm (Sambrook and Russell, 2001). Ampicillin was added to a final concentration of 50 μg/mL when appropriate. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 0.004% (w/v) for screening of recombinants.

2.2. Plasmid isolation

Overnight culture of *L. lactis* M14 was used for plasmid extraction through the alkaline lysis method as described by Sambrook and Russell (2001) with minor modifications. Lysozyme was added to a final concentration of 5 mg/mL to weaken the cell wall of Gram-positive lactococci prior to the cell lysis step. The extracted plasmid DNA was subjected to 0.7% (w/v) agarose gel electrophoresis analysis. The plasmid band corresponding to pAR141 was then eluted from the gel with Qiagen Gel Extraction Kit (Qiagen) for further study. Plasmid DNAs from *E. coli* were extracted by alkaline lysis method (Sambrook and Russell, 2001).

2.3. Cloning and sequencing of pAR141

The isolated plasmid pAR141 was treated with several commonly used restriction enzymes (REs) to generate a putative RE map. The RE-digested fragments were then cloned into pUC19 and transformed into competent *E. coli* TOP10 cells (Sambrook and Russell, 2001). The REs and T4 DNA ligase used in this study were purchased from Fermentas and used as recommended by the supplier.

The 1.6 kb pAR141 fragment cloned into pUC19 was used as template for sequence determination using the universal sequencing primers, M13F and M13R. The nucleotide and deduced amino acid sequences were analysed with BLAST program (Altschul et al., 1997) on the EMBL/GenBank databases. Computer scanning of the promoter was carried out with the Neural Network Promoter Prediction at the Internet site http://www.fruitfly.org/seq_tools/promoter.html. Conserved domains of the putative protein