Plasmid xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Characterization of pR18, a novel rolling-circle replication plasmid from *Lactobacillus plantarum*

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ARTICLE INFO

Article history:
Received 22 December 2013
Accepted 19 April 2014
Available online xxxx
Communicated by F. de la Cruz

Keywords:
Lactobacillus plantarum
Cryptic plasmid
Rep
linA
qPCR

ABSTRACT

Lactobacillus plantarum PA18, a strain originally isolated from the leaves of *Pandanus amaryllifolius*, contains a pR18 plasmid. The pR18 plasmid is a 3211 bp circular molecule with a G + C content of 35.8%. Nucleotide sequence analysis revealed two putative open reading frames, ORF1 and ORF2, in which ORF2 was predicted (317 amino acids) to be a replication protein and shared 99% similarity with the Rep proteins of pLR1, pLD1, pC30il, and pLP2000, which belong to the RCR pC194/pUB110 family. Sequence analysis also indicated that ORF1 was predicted to encode linA, an enzyme that enzymatically inactivates lincomycin. The result of Southern hybridization and mung bean nuclease treatment confirmed that pR18 replicated via the RCR mechanism. Phylogenetic tree analysis of pR18 plasmid proteins suggested that horizontal transfer of antibiotic resistance determinants without genes encoding mobilization has not only occurred between *Bacillus* and *Lactobacillus* but also between unrelated bacteria. Understanding this type of transfer could possibly play a key role in facilitating the study of the origin and evolution of *lactobacillus* plasmids. Quantitative PCR showed that the relative copy number of pR18 was approximately 39 copies per chromosome equivalent.

1. Introduction

Lactobacillus plantarum is a Gram-positive bacterium found in a variety of environments ranging from cheese, dairy, meat, fermented vegetables, and plant material, as well as the human gastrointestinal tract. *L. plantarum* was the first of the *Lactobacillus* spp. to have its genome sequenced (Kleerebezem et al., 2003). Analysis of whole genome sequences make *L. plantarum* applicable for industrial plasmid-encoded capacity such as bacteriocin synthesis, lactose fermentation, bacteriophage resistance, and proteinase activity (Roland, 2011). *L. plantarum* is widely used as a natural or added starter microorganism in food fermentation. Because of the importance of *lactobacillus* as a starter culture and their fermentative capacities, the development of food-grade cloning vectors derived from LAB plasmids would be particular interest of benefit to the food industry (Shareck et al., 2004).

The wealth of naturally occurring plasmids in LAB would offer endless opportunities to analyze their genetic relationship to host cells, their evolutionary origins, and putative horizontal gene transfer between bacteria (Francia et al., 2004). Many species of the genus

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http://dx.doi.org/10.1016/j.plasmid.2014.04.004
0147-619X/

Lactobacillus, such as *L. plantarum*, contain plasmids that can vary in size (Mayo et al., 1989). The plasmids of *L. plantarum*, including pYS18 (Zhai et al., 2009), pLP1 (Bouia et al., 1989), pLP2000 (Daming et al., 2003), and pLR1 (Li et al., 2009), have been completely sequenced and analyzed. Up to the present, numerous small plasmids have been shown to replicate via a rolling-circle (RC) mechanism. In general, all rolling-circle-replicating (RCR) plasmids are composed of a Rep protein, a double-stranded origin (dso), and a single-stranded origin (sso). Based on sequences encoded by the rep gene and dso, RCR plasmids can be grouped into several major families, e.g., pT181, pE194/pLS1, pC194/pUB110, and pSN2 (del Solar et al., 1993).

In the RCR plasmid initiation step, Rep introduces a specific nick in the double-stranded origin (dso) and cleaves the dso at the conserved nick site, recruiting replication proteins from the host to synthesize the leading strand. After leading strand synthesis is completed, a circular single-stranded DNA (ssDNA) intermediate is converted to double-stranded DNA (dsDNA) utilizing the single-stranded origin (sso) and host proteins (Khan, 2005)

In the last two decades, resistance to Macrolides, lincosamides, and streptogramins (MLS) has been reported in approximately half of the *Lactobacillus* spp. These bacteria have been shown to transfer to other commensal bacteria or pathogens present in the gastrointestinal tract (GIT) due to transposases, integrons, and other potential mobile elements (Cauwerts et al., 2006). To date, very little is known about lincosamide resistance plasmids in lactobacilli. Only two linA (lincosamide O-nucleotidyltransferase) -carrying *lactobacillus* plasmids pLR585 (*Lactobacillus reuteri*) and pYS18 (*Lactobacillus sakei*) have been sequenced completely.

In this paper, an entirely new plasmid pR18 isolated from *L. plantarum* PA18 was sequenced and characterized. For the first time, the linA gene and conferring resistance to lincomycin was identified in *L. plantarum*. The copy number and mode of replication for this plasmid were identified by PCR and Southern hybridization analysis. The plasmid is 3211 bp in length, and the backbone can potentially be developed for use as a cloning vector in biotechnology applications (Shareck et al., 2004).

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The *lactobacillus* used in this study was isolated from the tropical plant *Pandanus amaryllifolius*, which is commonly used in Southeast Asian cuisines, using standard microbiological procedures (van den Berg et al., 1993). For identification and characterization purposes, the isolate was grown on MRS medium and incubated at 30 °C.

From the isolate identified at the genus level, fermentation of 49 sugars and poly-alcohols (control) was carried out using the API 50 CH system (bioMérieux, Montalieu–Vercieu, France) at 30 °C. *Escherichia coli* TOP10 cells were grown with shaking at 250 rpm in Luria–Bertani (LB) broth at 37 °C. When appropriate, ampicillin at a final concentration 100 μg/ml was added to the medium (Sambrook and Russell, 1989). The plasmid pGEM-T easy vector (Promega Co., USA) and pUC19 (Invitrogen, Carlsbad, CA) were used as the subcloning vectors for sequencing.

2.2. DNA isolation

L. plantarum cultures were incubated at 30 °C in MRS solid or liquid media without agitation. Plasmids DNA of *lactobacillus* was extracted according to the method described by O'Sullivan and Klaenhammer (1993). Briefly, plasmids were extracted from overnight cultures of *L. plantarum* PA18 at OD600 nm 0.8–1.0 by alkaline lysis. Cells were harvested by centrifugation and washed in TES buffer (50 mM Tris–HCl, 30 mM EDTA, 25% sucrose, pH 8.0), and then lysozyme was added at a final concentration of 30 mg/ml. The suspension was incubated at 37 °C for 1 h. Plasmid DNA from *E. coli* was isolated using a HighYield Plasmid Mini Kit (Yeastern Biotech Co., Taiwan). The extracted plasmid DNAs were separated by electrophoresis on 0.8% (w/v) agarose gel. Total genomic DNA was extracted and purified using the Master Pure Gram Positive DNA Purification Kit (Epicenter Biotechnologies, USA). Then, the genomic DNAs were subjected to 1% (w/v) agarose electrophoresis analysis.

2.3. 16S rDNA analysis of L. plantarum PA18

The 16S rDNA gene fragments were amplified from *L. plantarum* PA18 genomic DNA using universal primers close to the 3′ and 5′ ends (Table 1) in a PCR Master Cycler (Eppendorf, Germany) (Weisburg et al., 1991). The PCR products of the 16S rDNA were purified using Wizard SV Gel and the PCR Clean-Up System Kit (Promega Co., USA). Next, the products were ligated into a pGEM-T easy vector (Promega Co., USA), transformed into TOP10 chemically competent *E. coli* cells (Invitrogen, USA), and sequenced (Bioneer Co., Korea).

2.4. pR18 plasmid sequencing and analysis

The plasmid DNA was excised from 0.8% agarose gel and recovered using Wizard SV Gel and the PCR Clean-Up System Kit (Promega Co., USA). Purified pR18 plasmid was digested with commonly used restriction enzymes (REs) HindIII, XhoI, EcoRV, EcoRI, SmaI and PaeI to determine an appropriate RE site. A 584-bp of pR18 as an EcoRI–HindIII fragment was cloned into pUC19. General cloning procedures were performed as described previously (Sambrook and Russell, 1989). The complete nucleotide sequence of pR18 was obtained through primer walking method. Sequencing was done with universal M13 primers and with specific primers derived from sequences of the cloned plasmid fragments. Sequence similarity and database searches of DNA sequences or DNA-derived protein sequences were carried out using BLASTN, BLASTP, and BLASTX programs provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990). After the BLASTN and BLASTP searches were carried out, sequences receiving the top scores were retrieved from the GenBank database. The

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Table 1
Primers used in this study.

Target	Location	Name	Sequence 5′ → 3′	Amplicon size (bp)
16S	Ribosome	16S-F 16S-R	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	1500
ldh	Chromosome	ldh-F ldh-R	AACACCGTCTTCTAACTTGGCT ATCCTCGTTCCGTTGATGCTT	117
RepA	pR18	rep-F rep-R	CGGGCACATGATGTACGGGG GCTCTCTTGCAACTGCTTCCGC	187

ORF finder program was used to analyze the complete nucleotide sequences of predicted ORFs. Alignments of conserved domains of proteins were retrieved from the Conserved Domain Database (CDD) at the NCBI (Marchler-Bauer et al., 2007). Multiple sequence alignments were performed using ClustalW accessed on the European Bioinformatics Institute website (http://www.ebi.ac.uk/-clustalw/) (Thompson et al., 1994). Repetitions within the nucleotide sequence (e.g., direct and inverted repeats) were detected by the Clone Manager 9.0 professional edition software (Scientific & Educational Software, Cary, NC). Promoter prediction programs (PPPs) were used to identify promoter regions (http://www.fruitfly.org/-seq-tools/promoter.html). For phylogenetic analysis, the MAFFT 6.0 aligner (Katoh and Toh, 2008) and MEGA 4.1 package (Tamura et al., 2007) were used to construct the neighbor-joining phylogenetic tree. The sequences were edited using the BioEdit software (Hall, 1999).

2.5. Southern hybridization and detection of single-stranded intermediates

Southern blot hybridization for the verification of ssDNA replication intermediates was performed as described by Te Riele et al. (1986), using pR18 plasmid as a hybridization probe (Holtwick et al., 2001). In brief, *L. plantarum* PA18 was grown in MRS medium to an OD600 nm of 0.6–0.7 with or without rifampicin (Sigma) and total DNA was extracted. Rifampicin was applied to the cultures at final concentration of 100 μg/ml for 30 min. Total DNA from culture without rifampicin treatment, was incubated with mung bean nuclease at 37 °C for 30 min. DNA was then electrophoresed on a 0.8% (w/v) agarose gel and transferred to a nylon membrane without denaturation treatment. Labeling was performed using the Biotin DecaLable DNA Labeling Kit, and labeled DNA was detected with the Biotin Chromogenic Detection Kit (Fermentas Lifesciences).

2.6. Copy number determination by qPCR

The relative copy number of pR18 was assessed by quantitative PCR (qPCR). Amplification and detection were carried out in a Bio-Rad real-time PCR detection system (Bio-Rad) using Brilliant III Ultra-Fast SYBR Green PCR Master Mix (Agilent Technologies, Santa Clara, CA). PCR primers were designed using the software Primer Premier 5.0. A chromosomally single copy of the *L-lactate dehydrogenase* gene (*ldhL*) in *L. plantarum* WCFS1 was used as the reference gene (GenBank accession No. AL935255). A 181-bp fragment of the *rep* gene was amplified with the primers Rep-F and Rep-R, and a 155-bp fragment of the *ldhL* gene was amplified with the primers ldh-F and ldh-R (Table 1). The reaction conditions for amplification of DNA were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. After completion of the amplification reaction, a melt-curve was performed for each amplification to determine the specificity of the target genes. The pR18 relative copy number to the chromosome was calculated using $\mathrm{PCN} = (1+E)^{-\Delta C_T}$ (Lee et al., 2006), where E and ΔC_T represented the PCR amplification efficiency and the difference between the threshold cycle number (C_T) of the *ldhL* and *rep* reactions, respectively. All qPCR reactions were run in triplicate, and the average results reported.

2.7. Nucleotide sequence accession number

The complete DNA sequence of pR18 has been deposited in GenBank under the accession number JN601038.2.

3. Results

3.1. Isolation and characterization of L. plantarum PA18

The LAB strain from *P. amaryllifolius* leaves was identified as *L. plantarum* PA18 based on direct sequences of the 16S rDNA gene as determined using a blast search and a carbohydrate fermentation pattern with similarity index of 99.9%. *L. plantarum* PA18 was able to be distinguished from other major bacteria in the same genus by comparing the 16S rDNA gene sequences. In the phylogenetic analysis, *L. plantarum* PA18 was found to be closely related to *L. plantarum* WCFS1 (Fig. 1A). Plasmid extraction results showed that *L. plantarum* PA18 harbored more than one plasmid (Fig. 2A). The study of the antibacterial susceptibility profiles has been achieved using the discolor diffusion method. A certain resistance has been noted to the 15 μg Lincomycin, in comparison to a plasmid-free *L. plantarum* reference strain, and confirmed that this resistance is functional (data not shown). There were no data available on the occurrence of linA resistance among *L. plantarum* isolates in the ARDB (Antibiotic Resistance Data Base). Resistance to lincomycin in lactobacilli is due to a plasmid carrying an inactivating enzyme that disrupts the structure of the antibiotic (Roberts, 2011).

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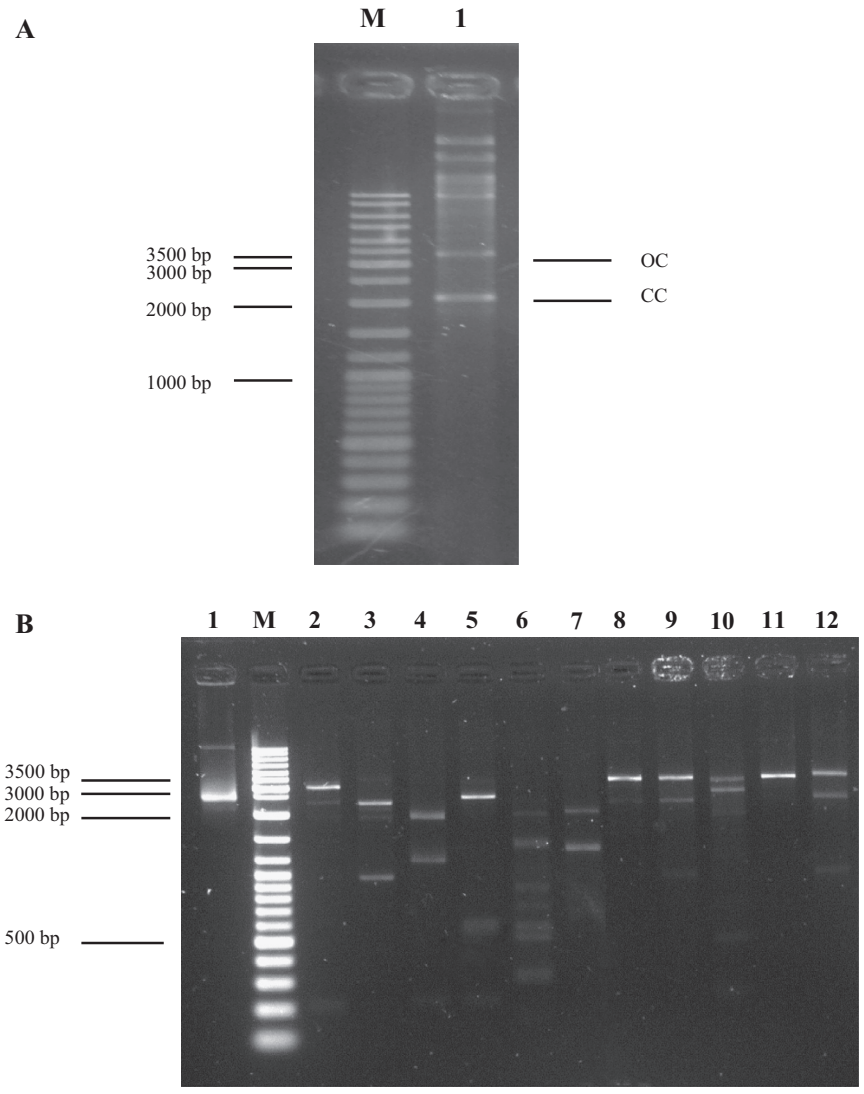

Fig. 1. (A) Agarose electrophoresis of the pR18 plasmid from *Lactobacillus plantarum* PA18. Plasmid DNA was isolated using a High Yield Plasmid Mini Kit and electrophoresed on a 0.8% (w/v) agarose gel. (1) Bands of open circular (oc) and closed circular (cc) forms of pR18 are visible (Heinl et al., 2011). (B) A restriction fragment length polymorphism (RLFP) profile of purified pR18 plasmid (Lane 1) obtained by double digestion with *HindIII–XhoI* (Lane 2), *XhoI–EcoRV* (Lane 3), *HindIII–EcoRV* (Lane 4), *HindIII–EcoR*I (Lane 5), *EcoRI–XhoI* (Lane 6), *EcoRI–EcoRV* (Lane 7), *SmaI–XhoI* (Lane 8), *SmaI–EcoRV* (Lane 9), *PaeI–HindIII* (Lane 10), *PaeI–XhoI* (Lane 11) and *PaeI–EcoRV* (Lane 12). The 584-bp *HindIII–EcoRI* (Lane 5) digested fragment of the pR18 plasmid was cloned into the *EcoRI–HindIII* sites of pUC19. (M) GeneRuler DNA Ladder Mix (Thermo Scientific) used as a molecular size marker.

3.2. Sequence analysis of the pR18 plasmid

A small ~3 kb plasmid was isolated from *L. plantarum* PA18 and identified for further analysis. Restriction endonuclease mapping revealed that pR18 contained a single site for EcoRI and two sites for HindIII (Fig. 2B). The 584-bp EcoRI–HindIII fragment of the pR18 plasmid was sequenced on both strands to identify the nucleotide sequence of pR18 using the primer-walking method with an automated DNA sequencer (Table 1) (1th BASE, Malaysia).

The sequencing results showed that pR18 was a circular DNA molecule of 3211 bp in length (Fig. 3) with a G + C content of 35.8%. Plasmid pR18 harbors eleven putative proteins encoding ORFs of more than 100 nt in length, of which seven were in the same orientation and four were encoded on the opposite DNA strand. All ORFs were scored according to how closely they match the model of a real gene by using Glimmer 3.2 (Delcher et al., 1999). Only five ORFs with patterns similar to the real genes were considered (Table. 2); however, only two ORFs were predicted to produce protein products. The two ORFs predicted to produce protein products were located at positions 73–558 and 1677–2630 and designated as linA and Rep proteins, respectively, based on protein sequence similarity.

The linA protein encoded by ORF1 displayed extensive protein sequence similarity (98–100% identity) to the sequenced linA proteins encoded by staphylococcal plas-

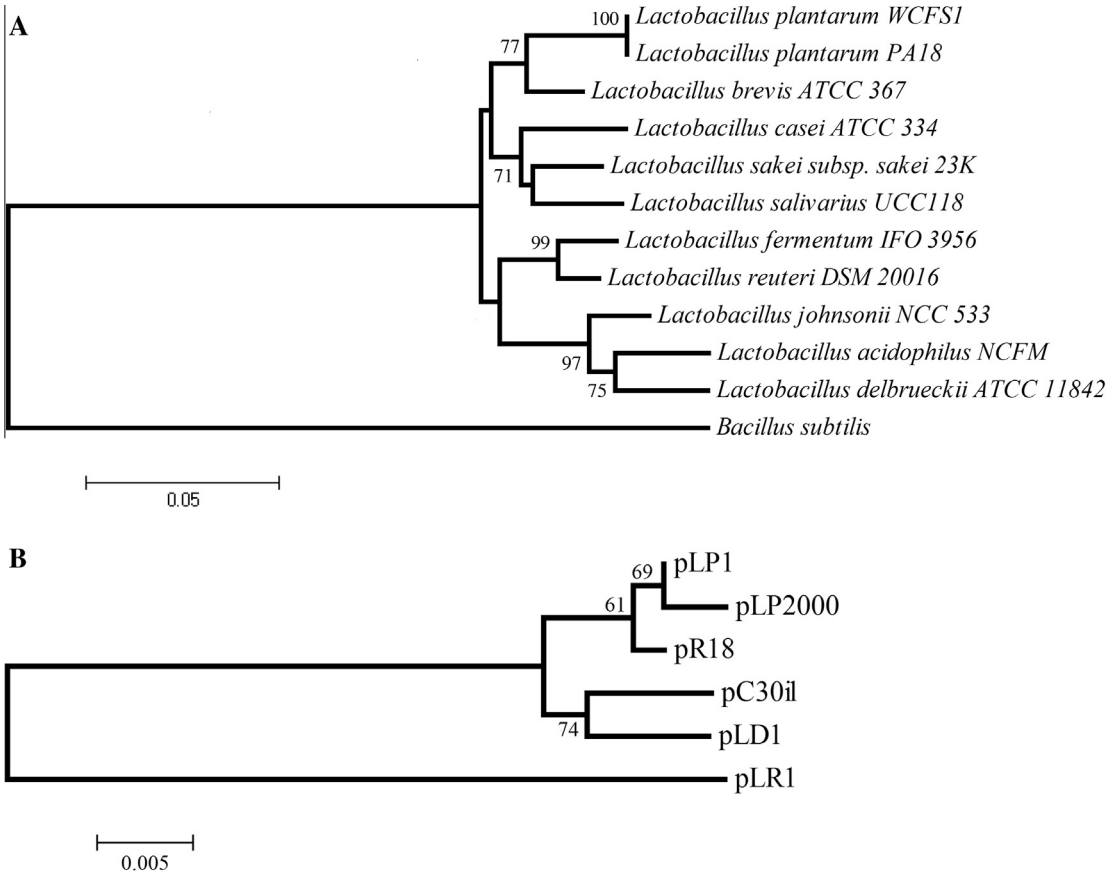

Fig. 2. A phylogenetic tree was constructed with the MEGA version 4.1 program using 16S rDNA gene sequences (A) and six RepA proteins (B). Data for 16S rDNA phylogenetic analysis were obtained from the Genbank nucleotide sequence database for the following strains: *Lactobacillus sakei* subsp. *sakei* 23k (GenBank accession No. NC_007576), *Lactobacillus casei* ATCC 334 (GenBank accession No. CP000423), *Lactobacillus salivarius* UCC 118 (GenBank accession No. NC_007929), *Lactobacillus acidophilus* NCFM (GenBank accession No. NC_006814), *Lactobacillus plantarum* WCSF1 (GenBank accession No. AL935263), *Lactobacillus reuteri* JCM 1112 (GenBank accession No. NC_009513), *Lactobacillus brevis* ATCC 367 (GenBank accession No. NC_008497), *Lactobacillus fermentum* IFO 3956 (GenBank accession No. NC_010610), *Lactobacillus reuteri* DSM 20016 (GenBank accession No. NC_009513), *Lactobacillus johnsonii* NCC 533 (GenBank accession No. NC_005362), and *Lactobacillus delbruekii* ATCC 11842 (GenBank accession No. NC_008054). *Bacillus subtilis* NCDO 1769 (GenBank accession No. NC_000964) was used as an out-group organism. To construct the phylogenetic tree based on RepA, top hits for RepA protein sequences from *L. plantarum* plasmids replicating by RCR after BLASTP similarity searches were clustered, and the relationships among the proteins were determined. The bar indicates the number of nucleotide (A) and amino acid (B) substitutions per site. The robustness of the NJ tree was tested by bootstrapping with 1000 replicates of data, and percentages are reported at the nodes (only values above 50% are reported).

mids pLNU4, pLNU7, pLNU5, and pLNU6 (Luthje et al., 2007).

The 954 bp ORF2 encoded a protein of 317 amino acids, which was identified as RepA, and was the largest ORF in the pR18 plasmid. Sequence analysis showed that ORF2 shared 99% similarities with the Rep proteins of plasmids pLR1, pLD1 (Du et al., 2010), pC30il (Skaugen, 1989), and pLP2000, which belong to the RCR plasmid family (Khan, 2005). Construction of a phylogenetic tree based on RepA revealed a clear relation between this protein and several *lactobacillus* replication initiation proteins from the same family (Fig. 1B).

In addition, based on our understanding of the mechanism of the RCR plasmid and comparing the sequences of known RCR initiator proteins, three highly conserved motifs were detected in the PR18 Rep protein. The metal binding (QHLHVLVF: motif II) and catalytic (TAKYEVKSAD: motif III) motifs, both present in the Rep proteins of the *lactobacillus* RCR plasmids, were found in pR18. The motif I region FLTLTVKN is suggested to recognize the dso (Fig. 4) (Ilyina and Koonin, 1992).

Downstream of the rep gene, inverted repeats followed by a stretch of Ts were found forming a possible transcription terminator from positions 2641 to 2685. The dso showed homology with the dso of the RCR pC194 plasmids (Li et al., 2009) with 35 bp conserved sequence 5′-CGCTTTCTTCTTATCTTGATACTATTAGCAACAAC-3′ that is located 309 bp upstream of the rep start codon. As in the case of pR18, downstream of the dso conserved sequence, there were two direct repeats clusters designated as DRIII and DRIV. DRIII (5′-GGTTATAA-3′) and DRIV (direct repeats of 5′-CACGTG-3′) are predicted to form stable stem-loop structures (Fig. 4). The nic site (TTG↓ATA) is located in this stem-loop structure. The replication of the lagging strand of RC plasmids initiated from their sso, which was generally located at the 5′ of the dso and exposed after the syn-

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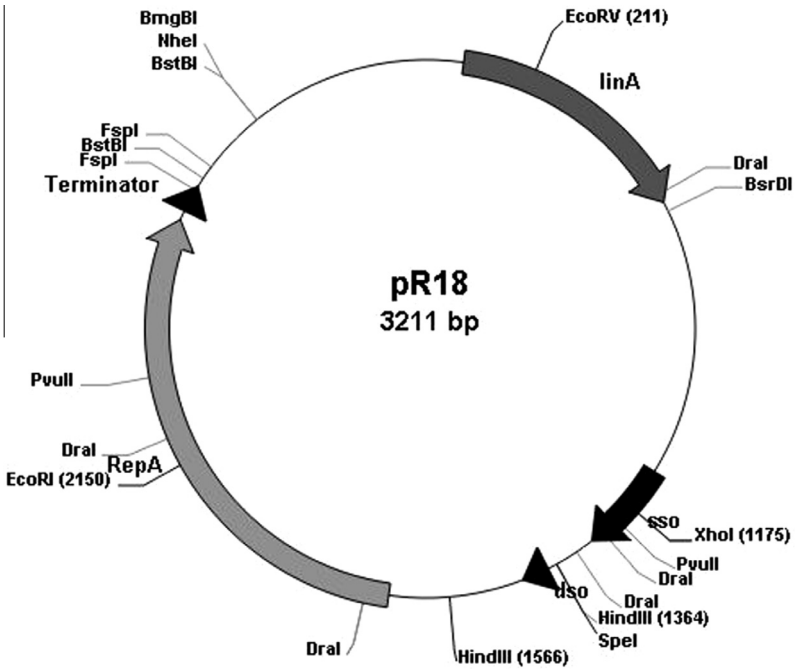

Fig. 3. A physical map of the pR18 plasmid from *Lactobacillus plantarum* PA18. The plasmid origins of replication, *dso* and *sso*, are shown as black closed arrows. The gray closed arrows indicate *repA* and *linA* and their direction of transcription. Common restriction enzyme sites are also shown.

Table 2
Similarity of amino acid sequence encoded by open reading frames identified on pR18 with proteins reported in the GenBank database.

Orf name	Size (aa)	Position (bp)	Best match in BLAST and % aa identity	Accession No.
Rep	317	1677–2630	Replication protein, *Lactobacillus plantarum* pLP1 (99%)	AAA98164.1
LinA	161	73–558	*Staphylococcus chromogenes* pLNU4 (100%)	YP_473357.1
ORF3	87	2949–3210	Hypothetical protein, *Indibacter alkaliphilus* (32%)	WP_016254708.1
ORF4	53	909–1070	Hypothetical protein, *Culex quinquefasciatus* (40%)	XP_001846097.1
ORF5	60	2900–2718	Hypothetical protein, *Lactobacillus plantarum* WCFS1-pWCFS101 (83%)	YP_133709.1

thesis of the leading strand. The sso of pR18, a 162 nucleotide sequence corresponding to ordinate 1115 to 1275 of the pR18 sequence, was 98% identical to the putative sso of pLAC1 (Asteri et al., 2010; Khan, 2000). The sso has several inverted repeats that could generate stem-loop structures, which are known to be important in lagging strand initiation similar to those in the isomeric *E. coli* phages a3, G4, and St-1 (Li et al., 2009).

In a number of prokaryotic plasmids that replicate by a RCR type of mechanism, replication and copy number are controlled by small divergently synthesized Rep antisense transcripts, which act by binding to counter-transcribed RNAs (CT-RNA) involved in the initiation of replication (del Solar et al., 1998). Computer promoter predictions revealed a sequence (nucleotides 1720–1675) that could function as a promoter for the synthesis of the antisense strand, which was complementary to the untranslated RepA leader mRNA. No ORF could be identified preceding this sequence. Interestingly, a transcriptional terminator loop is also located on the opposite strand at position 1559–1602. Accordingly, the RepA mRNA and its antisense transcript overlap one another by 127 nucleotides, consisting of 118 nucleotides 5′ upstream of the RepA translational start and 9 nucleotides of the RepA coding sequence.

3.3. Determination of pR18 copy number

The relative Plasmid copy number (PCN) was determined using the equation: PCN $= E^{-\Delta C_t}$, considering amplification efficiencies (E) and C_t values for the two amplicons (chromosome-c and plasmid-p). A 10-fold serial dilution of the total DNA from *L. plantarum* PA18 was prepared to construct a relative standard curve for both *repA* and *ldh* (Table 1). The accuracy of the standard curve was determined from five dilutions and in conditions in which $r^2 \geqslant 0.99$. In this work, the slopes obtained for *repA* and *ldhL* were 3.401 and 3.546, respectively (Lee et al., 2006; Providenti et al., 2006). Analysis of the results revealed that the relative copy number was approximately 39.6 ± 0.4 copies per chromosome equivalent. Plasmid copy numbers of rolling-circle plasmids in lactobacilli cover the range of approximately 25–142 copies/genome as defined in the recent literature (Yin et al., 2008; Heinl et al., 2011).

3.4. Detection of ssDNA intermediates

Generation of *ssDNA* intermediate is a structural hallmark of the RCR mechanism (del Solar et al., 1998). Southern blot hybridization was performed for the verification of

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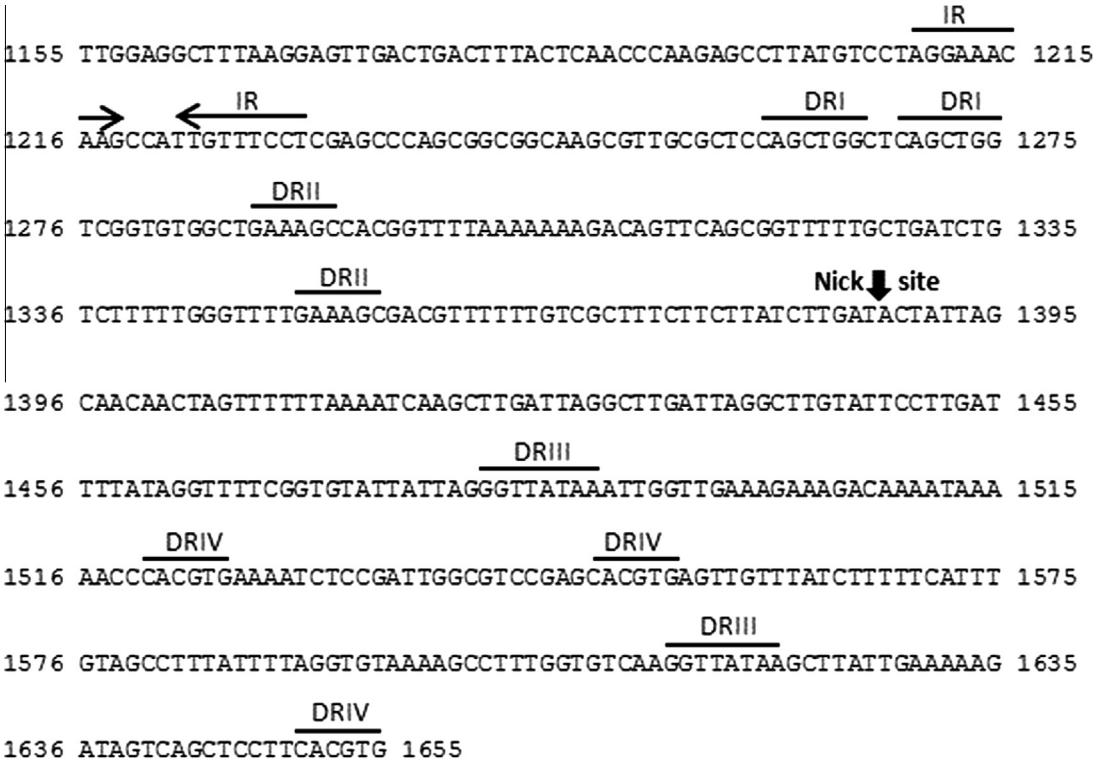

Fig. 4. The sequence of the predicted origin of replication for the pR18 plasmid is shown. An upright arrowhead indicates the conserved nick site. An inverted repeat (IR) is indicated by a small horizontal arrow, and direct repeats (DRs) are marked with Roman numerals.

ssDNA replication intermediates using *EcoRV* linearized plasmid sample as a probe to confirm the plasmid replication mechanism as was used by Yang et al. (2009) and Holtwick et al., (2001). The pR18 plasmid samples with or without pretreatment with mung bean nuclease were transferred to a nylon membrane under non-denaturing conditions. Under these conditions, *ssDNA* and *dsDNA* would be transferred to the membrane. Te Riele et al. (1986) found that "20% of hybridization with a probe that contained both pC194 strands occurred at the position of the gel where single-stranded DNA would migrate; allowing for the fact that a double-stranded molecule gives a hybridization signal twice as intense as a single-stranded molecule". Based on the previous studies, *dsDNA* of pR18 was also detected above its single strand due to the use of large fragment probe (Holtwick et al., 2001; Loeza-Lara et al., 2005; Yang et al., 2009 (Fig. 5-lane 2). Positive signals were observed in the sample without mung bean nuclease treatment, while *ssDNA* just below the *dsDNA* disappeared almost completely after in the correspondingly treated sample due to its specificity for *ssDNA* (Fig. 5-lane 1). Cells were grown in medium with rifampicin treatment to prevent the conversion of *ssDNA* to *dsDNA* due to its inhibition of the host RNA polymerase (del Solar et al., 1998). In most cases, the *ssDNA* intermediate was barely detectable in extracts from cells grown in the absence of rifampicin (Park et al., 2004). Together, these results confirmed that pR18 replicated via the RCR mechanism.

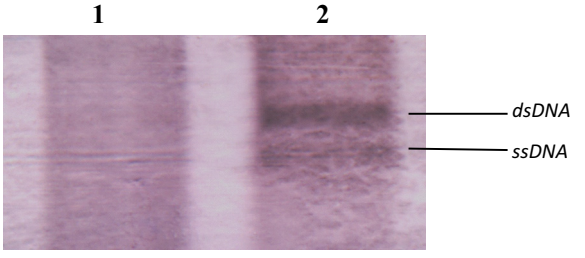

Fig. 5. Detection of single-stranded pR18 was performed by Southern blot hybridization. Cells were cultured in MRS medium with rifampicin, the pR18 samples was treated with (lane 1) or without (lane 2) mung bean nuclease before electrophoresis and transferred to a nylon membrane without denaturation. *dsDNA* of pR18 was also detected above its single strand (Holtwick et al., 2001).

4. Discussion

In this study, we reported the characterization of the new RCR pR18 plasmid isolated from *L. plantarum* PA18. Sequence analysis revealed two major ORFs on the same strand that encode putative lincomycin resistance and replication proteins. The enzyme that disrupts the structure of lincomycin was found in *L. plantarum* for the first time, while it was previously reported in *L. reuteri* SD2112 and *L. sakei* YS18 among *Lactobacillus* spp.

It is important, while the replication backbone of pR18 was highly conserved, BLASTN queries returned only 58–59% coverage with pLP2000, pC30il, pLD1and pLR1 plasmids over the entire plasmid sequence. Among the top BLASTN hits, *linA* of pR18 displayed 100% homology with *linA* gene of pLNU4 plasmid isolated from *Staphylococcus chromogenes* (100% query coverage and 100% identity). It is important to note, pR18 plasmid shared 17–19% of homology with *linA*-carrying plasmids pLR585 (*L. reuteri*), pYS18 (*L. sake*i) and pLNU4 only in the linA region.

Sequence homologies of pR18, along with low diversity of the *linA* gene among *Lactobacillus* spp., could indicate that the *linA* gene on the pR18 plasmid may not have originated from closely related strains. To our knowledge, *linA* had previously only been found in staphylococci, streptococci, enterococci, and lactobacilli of animal origin and in staphylococci isolated from humans (Bozdogan et al., 1999). Where resistance has been acquired by a strain belonging to a taxonomic group usually susceptible to an antibiotic, the spread of resistance gene is generally considered to be substantially greater than that associated with intrinsic resistance. Inter-plasmid exchange of the *linA* gene seems to occur easily, as illustrated by its presence in different plasmid backbones. This diversity may facilitate the acquisition of *linA*-carrying plasmids and their stable maintenance in a new bacterial host (Luthje et al., 2007).

In multiple alignment analysis, the replication module of the pR18 plasmid was found to contain elements typical of plasmids that replicate via RCR, including three conserved amino acid motifs and two untranslated sequences, *dso* and *sso*. A sequence analysis of putative DNA binding sequences suggested that motif I is involved in the *dso* binding region specificity (Ilyina and Koonin, 1992). The two His residues of motif II are highly conserved in Rep proteins of RC plasmids. These histidine residues may act as ligands for the Mg^{2+} and Mn^{2+} ions which are required for Rep function (del Solar et al., 1998). Motif III included one active DNA-linking Tyr residue, and through this residue, Rep protein interacts with the plasmid and becomes covalently attached to the 5′-Pi of *dso* at the nick sequence (Ilyina and Koonin, 1992).

Mechanisms controlling replication have been studied in various systems, and several types of inhibitors have been detected: (i) antisense RNA (ColE1, R1, and pT181); (ii) both an antisense RNA and a protein (pMV158 and pIP501); and (iii) DNA sites for binding initiator proteins (F, P1, RK2, and R6K) (del Solar et al., 1998). The pR18 plasmid contains a replication protein that has strong sequence similarity to the replication proteins of the pUB110 and pC194 plasmids. The available data and literatures suggested that synthesis of CT-RNA from plasmids pUB110 and pC194 is initiated downstream from the translation initiation codon. Consequently, CT-RNA of these plasmids is complementary to a region, which includes both the Shine-Dalgarno region and the ATG start codon (Holtwick et al., 2001). Since no other open reading frames coding for plasmid-encoded control proteins have been observed, control of pR18 replication does not involve a repressor protein, as was found for the pMV158 plasmid.

Indeed, sequence analysis of pR18 indicated the possible synthesis of an antisense RNA containing approximately 127 nucleotides that overlap the translational start of the *repA* transcript. The size of the overlap corresponds with other RCR plasmids, ranging from 40 nucleotides in pJDB21 from *Selenomonas ruminantium* (Zhang and Brooker, 1993) up to 148 nucleotides in pLAB1000 of *Lactobacillus hilgardii* (Josson et al., 1990). In addition, the pR18 plasmid forms a *ssDNA* intermediate via the RCR mechanism, and this mode of replication was confirmed through Southern hybridization.

5. Conclusion

To date, several *lactobacillus* plasmids have been sequenced, and have provided a vast array of genetic elements for vector construction; in comparison with *Lactococcus*, the molecular tools for the genetic manipulation of lactobacilli are still not sufficient.

We found that notwithstanding the overall similarity in *rep* and *linA* sequences, the pR18 backbone is sufficiently different from the backbones existing in databases. However, the use of new generation of plasmid backbones with GRAS origin devoid of antibiotic resistance gene marker increased our interest in possibly of removing undesirable vector elements from pR18 and thereby developing valuable *lactobacillus* food-grade vectors for application in food industry.

6. Uncited reference

Koonin and Ilyina (1993). Q2

Acknowledgments

We take this opportunity to thank the Malaysian Ministry of Science, Technology and Innovation, and the Malaysian Genome Institute (MGI) for funding this study under grant number 5487732. Special thanks to the members of the Microbial Biotech Laboratory, UPM for their ideas and support.

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Please cite this article in press as: Jalilsood, T., et al. Characterization of pR18, a novel rolling-circle replication plasmid from *Lactobacillus plantarum*. Plasmid (2014), http://dx.doi.org/10.1016/j.plasmid.2014.04.004