Sequence analysis of pLBB1, a cryptic plasmid from *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Abstract: The first report of the complete nucleotide sequence of a cryptic plasmid from *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) is presented. The plasmid pLBB1 consists of 6127 bp with a GC content of 44.8%. No ssDNA was detected by hybridization experiments, which is consistent with the notion that pLBB1 does not replicate by a rolling circle mechanism. A putative replication region of pLBB1 was cloned and found to be functional in *Lactobacillus johnsonii* and *Lactococcus lactis*. Plasmid pLBB1 showed significant DNA sequence identity with plasmid pLL1212 from *Lactobacillus delbrueckii* subsp. *lactis* (*Lactobacillus lactis*) CRL1212 (GenBank accession No. AF109691). Four open reading frames (ORFs) larger than 100 amino acids were identified. ORFA shared similarity with a putative primase–helicase system, and ORFB and ORFC exhibited limited identity with a mobilization protein and a transposase, respectively. Curing experiments did not allowed us to assign a function to the ORFs.

Key words: *Lactobacillus*, cryptic plasmid, sequence analysis.

Résumé : Nous présentons la première séquence complétée d'un plasmide cryptique de *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*). Le plasmide pLBB1 est composé de 6127 pb ayant un contenu en CG de 44,8 %. Aucun ADN simple brin n'a été détecté par l'expérience d'hybridation, ce qui est conforme avec la notion que pLBB1 ne se réplique pas par un mécanisme de cercle roulant. Une éventuelle région de réplication de pLBB1 a été clonée et s'est avérée fonctionnelle chez *Lactobacillus johnsonii* et *Lactococcus lactis.* Le plasmide pLBB1 a démontré une similitude significative avec le plasmide pLL1212 de *Lactobacillus delbrueckii* subsp. *lactis* (*Lactobacillus lactis*) CRL1212 (n°. d'identification GenBank AF109691). Quatre cadres de lecture ouverts (CLOs) plus grands que 100 acides aminés ont été identifiés. ORFA ressemblait à un système de primase–hélicase putatif, et ORFB et ORFC présentaient des identités limitées avec une protéine de mobilisation et une transposase, respectivement. Des expériences de cure du plasmide n'ont pas permis de décerner des fonctions aux ORFs.

Mots clés : *Lactobacillus*, plasmide cryptique, analyse de séquence.

[Traduit par la Rédaction]

Introduction

Lactic acid bacteria are widely used in the production of fermented dairy foods, in particular, the thermophilic starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) are important in the manufacture of yogurt and Italian and Swiss-style cheeses. The development of gene transfer systems for *Lactobacillus bulgaricus* is necessary for the application of recombinant DNA technology to the practical improvement of this bacterium. In this sense, plasmids play a central role in gene technology, and the study of *Lactobacillus bulgaricus* plasmid DNA is a prerequisite to using these molecules as vectors to deliver genes.

Plasmids are common in *Lactococcus lactis* (*Lc. lactis*) and in some species of lactobacilli and pediococci, but they are not frequently present in *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, or the intestinal lactobacilli (Davidson et al. 1996). So far, there have been no reports describing the complete nucleotide sequence of any plasmids of *Lactobacillus bulgaricus*. In the present study, 48 *Lactobacillus bulgaricus* strains were screened for the presence of plasmids. The first report of the entire nucleotide sequence of a small cryptic plasmid from this subspecies is presented.

Materials and methods

Bacterial strains and growth conditions

The 48 strains of *Lactobacillus bulgaricus* screened for plasmid DNA in this study and *Lactobacillus casei* CRL705 were obtained from our culture collection (Centro de

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Table 1. Primers used in this study.

Primer	Sequence
UBC4	5′-CCTGGGCTGG-3′
1817I	5′-GTCTAAGAAGGCAACGCTGAG-3′
362I	5′-GAAAATCTCTGCTGTTGTCTG-3′
262F	5′-CTGGCATTGTCGTTAGACC-3′
PLB16	5′-AGAGTTTGATCCTGGCTCAG-3′
MLB16	5′-GGCTGCTGGCACGTAGTTAG-3′

Referencia para Lactobacilos, CERELA, Tucumán, Argentina). These strains have been isolated from commercial and regional fermented products. *Lactobacillus johnsonii* NCK65 (Muriana and Klaenhammer 1991) and *Lactococcus lactis* IL1403 (Langella and Chopin 1989) are plasmid-free strains from the NCK collection (North Carolina State University, N.C., U.S.A.) and the Institut National de la Recherche Agronomique (Jouy-en-Josas, France), respectively. *Lactobacillus* strains were grown at 37°C in MRS broth (De Man et al. 1960). *Lactococcus lactis* was grown at 30°C in M17 medium (Terzhagi and Sandine 1975) containing 0.5% glucose. *Escherichia coli* DH5α served as the host strain for plasmid manipulations and was grown at 37°C in Luria-Bertani medium (Sambrook et al. 1989). The media were solidified with 1.5% agar (Britania, Argentina), when necessary. For selection of transformants, the media were supplemented with the following appropriate antibiotics using the following concentrations ($\mu g \cdot mL^{-1}$): ampicillin, 100; chloramphenicol, 10; tetracycline, 10; and erythromycin, 2.

Isolation and characterization of a plasmid-free derivative of strain B1

Lactobacillus bulgaricus B1 cells were treated with ethidium bromide (10–200 $\mu g \cdot mL^{-1}$) for 16 h (Crossa et al. 1994), and several colonies randomly isolated were analyzed by plasmid DNA content. The range of carbohydrates dissimilated by strains B1 and C1, a B1 plasmid-free derivative, was determined using API 50-CHL strips (BioMérieux, France). Production of antimicrobial compounds and resistance to the bacteriocin Lactocin 705 were analyzed by well diffusion assay (Vignolo et al. 1993). Sensitivity to bacteriophages mv4, LL-H, and lb539 was determined by standard techniques (Auad et al. 1997). Penicillin G, oxacilin, cephalotin, vancomycin, clyndamicin, erythromycin, rifampicin, pephloxacin, ampicillin, neomycin, ciprophloxacin, chloramphenicol, norphloxacin, colistin, gentamycin, furazolidon (Britania, Argentina), nalidixic acid, streptomycin, kanamycin, tetracycline, and spectinomycin (Sigma, St. Louis, Mo., U.S.A.) (in a range of 2–100 $\mu g \cdot mL^{-1}$) were used to investigate the response to antibiotics. RAPD (Random Amplification of Polymorphic DNA) was accomplished under standard PCR conditions using primer UBC4 (Table 1). SDS-PAGE analysis of whole-cell proteins was performed according to Laemmli (1970). The variable (V1) region of the 16S ribosomal RNA gene from strain B1 and its plasmid-free derivative, C1, was amplified as described by Kullen et al. (2000), using primers PLB16 and MLB16 (Table 1).

Recombinant plasmids and DNA manipulation techniques

General molecular DNA techniques were used according to Sambrook et al. (1989). Primers were synthesized by The Great American Gene Company (Ramona, Calif., U.S.A.). Rapid isolation of plasmid DNA from *E. coli* was accomplished by the alkaline lysis method of Birnboim and Doly (1979). Isolation of plasmid DNA from lactic acid bacteria was performed by an alkaline lysis protocol (Muriana and Klaenhammer 1991). *Escherichia coli* were transformed according to Joerger and Klaenhammer (1990). *Lactococcus* cells were prepared and electrotransformed as described by Le Loir et al. (1994). Electroporation of *Lactobacillus* was performed as described by Walker et al. (1996). The electrocompetent cells were incubated for 16 h after the electroporation pulse to allow expression of the erythromycin resistance marker.

The complete nucleotide sequence of plasmid pLBB1 was determined by combining shotgun cloning with a primer walking strategy. For sequence analysis, the *Bgl*II fragments of pLBB1 were ligated into the *Bam*HI site of plasmid pBlueScript SKII (Stratagene, La Jolla, Calif., U.S.A.). Nucleotide sequencing of pLBB1 was carried out by the North Carolina State University Sequencing Facility. The nucleotide data reported in this manuscript have been deposited with the GenBank database under accession No. AF236060. Potential coding regions and DNA sequence information were analyzed through the National Center for Biotechnology Information (NCBI) by using the BLAST network service to search GenBank (Altschul et al. 1997). Putative promotors were detected using the Promoter Prediction by Neural Network (http://www.fruitfly.org/seq_tools/promoter.html). Protein motifs were analyzed through the Conserved Domain Database at NCBI (http://www.ncbi.nl.nih.gov./structure/cdd).

The origin-probe plasmid pSA34 (Sanders and Shultz 1989) was used to identify the replication origin of pLBB1. To identify the replication region in pLBB1, plasmid DNA was linearized with *Eco*RV and cloned into the *Eco*RV site of pSA34, generating plasmid pSB26 (Fig. 1). Two PCR fragments of pLBB1, 4.08 and 0.96 kb (Fig. 1), were also amplified under PCR standard conditions, using *Pfu*I DNA Polymerase (Promega) and primers 1817I/362I and 262F/362I (Table 1), respectively, and were cloned into the *Eco*RV site of pSA34, generating plasmids pB2 and pSS1 (10.12 and 7.01 kb, respectively; see Fig. 1). The recombinant plasmids were identified by insertional inactivation of the tetracycline resistance marker. The plasmids were amplified in *E. coli* and used to transform *Lactobacillus* and *Lactococcus* strains.

Detection of ssDNA in whole-cell lysates of *Lactobacillus bulgaricus* and inhibition of RNA polymerase by rifampicin

The detection of ssDNA in whole-cell lysates of *Lactobacillus bulgaricus* and the inhibition of RNA polymerase by rifampicin were performed as described by Leenhouts et al. (1991), with minimal modifications. Cells were grown until an optical density (OD_{600}) of 0.6–0.8 was achieved. Rifampicin (Sigma) was added to a concentration of 100 $\mu g \cdot mL^{-1}$. After 2 h of incubation, cells were treated with 10 $mg \cdot mL^{-1}$ lysozyme (Sigma) and no mutanolysin was

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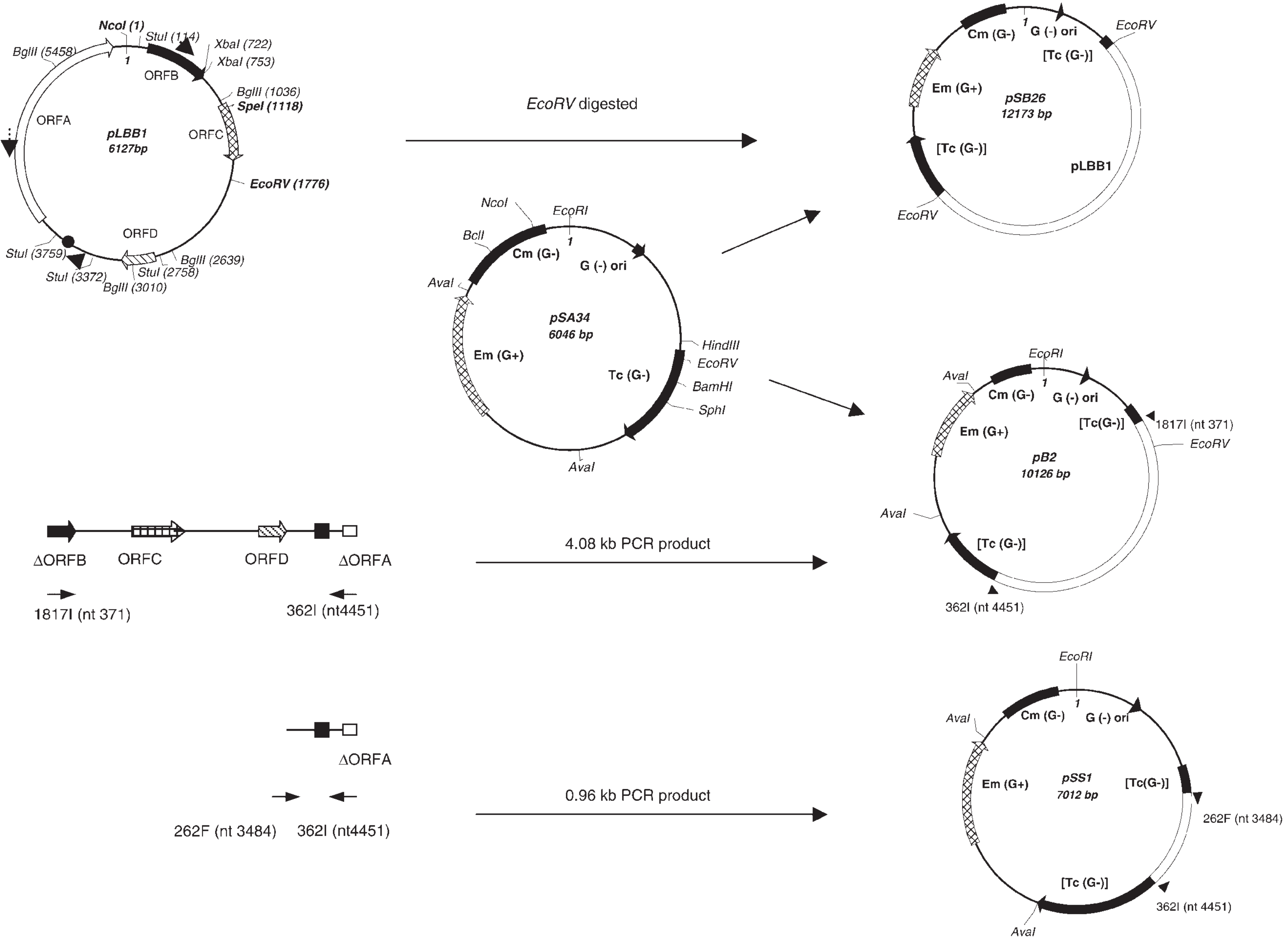

Fig. 1. Restriction map of pLBB1 (GenBank accession No. AF236060). The locations of ORFs A–D are indicated. Plasmid pLBB1, linearized with *Eco*RV, and the 4.08- and 1-kb PCR products were cloned into the *Eco*RV of the origin-probe vector, pSA34, generating plasmids pSB26, pB2, and pSS1, respectively. The PCR products from pLBB1 were amplified as indicated in Materials and methods. Nucleotides 371, 3484, and 4451 (in parenthesis) indicate the 5′-end of primers 1817I-forward, 262F-forward, and 362I-reverse, respectively, and their positions in the restriction map of pLBB1 are indicated by arrowheads. ●, the putative ori region of pLBB1. G+ and G–, gram positive and gram negative, respectively.

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Table 2. Open reading frames (ORFs) identified in pLBB1.

Gene	Location	%G+C	Putative protein (according to BLAST)	No. of amino acids	RBS[a]	Putative promoter[b]
ORFA	3939–6005	46.93	Primase–helicase	688	GAGG	AGTTTCACTTGTTAATAGTTTAATAGTATTAAAA GCAACG**G**CACAGCTTG
ORFB	198–776	46.80	Mobilization	192	GAGGAGG	GTAGACATTTAATTCGTTTTTGGGCGTGTCATA ATCCGGA**C**TTTTTCTTT
ORFC	1077–1601	44.38	Transposase	174	GAAAGAGA	GCGGCTTTACAATGACACTAGATCTACACTATA ATTACAG**C**AGAAAGAGA
ORFD	2804–3109	48.37	Unknown	101	GAAGAGAA	CAACCATTGTCAGAAGGTCATAAGAAGCGCAT TATGGCGG**C**GATTAAACA

Note: RBS, ribosome binding sequence.
[a]Nucleotides complementary to the 3′-end of *Lactobacillus delbrueckii* 16S rRNA (N_xUCCUCCU).
[b]According to the Promoter Prediction by Neural Network. The potential transcriptional start point is indicated. The –35 and –10 hexamers are underlined (Matern et al. 1994).

Fig. 2. Primase–helicase conserved motifs detected in ORFA according to the Conserved Domain Database at NCBI. (A) Alignment with the Zinc-finger domain present in a primase from *Pseudomonas aeruginosa* PA01 (Stover et al. 2000). (B) Alignment of C-terminal of ORFA with a replicative helicase from *Helicobacter pylori* (accession No. AE001551). Purine nucleoside triphosphate (NTP)-binding patterns, identical residues, and conservative replacements are indicated. I, II, and III indicate other conserved motifs in replicative helicases (Ilyna et al. 1992).

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A)

L. bulgaricus  33   FSCLNPNHPDKHPSMCVDRNHPQYVHCFSCGVSYD  67

P. aeruginosa  1    YSACCPFHKEKTPSFTVSPDK-QFYYCFGCGAGGN  34


B)
                              purine NTP-binding pattern          I
ORFA       359 IPTGFKNLDDELDGGLQPKLYVLGAVSSLGKTTFALNVADNLAKQGRHVFFFSMESSKRE 418
               IPTGF  LD+   G  Q  L +LGA  S+GKT+  +N+  +     R V  FS+E S  +
H. pylori  179 IPTGFVQLDNYTSGFNQGSLVILGARPSMGKTSLMMNMVLSALNDDRGVAVFSLEMSAEQ 238

ORFA       419 VTDKLLSRASCLSNGHKWTQLQVNRGAWLNNAEDKEKFDGLFKAFSRYQRFLHIYD-NRV 477
               +  + LS  + + N H     +++   W N A   + FD L       Q+ L  YD + V
H. pylori  239 LALRALSDLTSI-NMHDLESARLDDDQWENLA---KCFDHL------SQKKLFFYDKSYV 288

                              purine NTP-binding pattern          II
ORFA       478 KASQVKDLVNGWLDNHPDEKKPLVVVDYLQILQAEQDNVTDKAKVTDSVSVLSELTKQAE 537
               +  Q++  +      H  ++  +  +DYLQ++   +       ++ +    L  L ++ E
H. pylori  289 RMDQIRLQLRKLKSQH--KELGIAFIDYLQLMSGNKATKERHEQIAEISRELKTLARELE 346

                                                 III
ORFA       538 VPVLVISSLNRASYWQDVS---FESFKESGEIEYSADVMLGL---------------EFA 579
               +P++ +  LNR+   +D         K+SG IE  AD++L L               +
H. pylori  347 IPIIALVQLNRSLENRDDKRPILSDIKDSGGIEQDADIVLFLYRGYIYQMRAEDNKIDKL 406

ORFA       580 HREEYITVKGNGHVELNKEKFDQRKQEVPRRVEMVILKNRTGKTGGHIFFKYNAMFNSYQ 639
                +E  +      H+++N+E+   ++       E+++ KNR G T G ++ ++NA F  Y+
H. pylori  407 KKEGKVEEAQELHLKVNEERRIHKQNGSIEEAEIIVAKNRNGAT-GTVYTRFNAPFTRYE 465
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used. Subsequent steps were carried out as described by Leenhouts et al. (1991).

Results and discussion

Detection of plasmid DNA in *Lactobacillus bulgaricus* strains and characterization of *Lactobacillus bulgaricus* B1

Plasmids are not often detected in *Lactobacillus bulgaricus* (Davidson et al. 1996). This fact was confirmed in our laboratory; from a total of 48 strains of *Lactobacillus bulgaricus* screened by small-scale lysis procedures for the presence of extrachromosomal elements, only a small plasmid in strain B1 was detected (data not shown). The plasmid, designated pLBB1, was estimated by restriction enzyme analysis to be 6.1 kb in size. Strain B1, isolated from an Italian hard cheese, was confirmed as *Lactobacillus bulgaricus* based on phenotypic and biochemical tests and on partial sequencing of its 16S rRNA. For curing, B1 cells were grown in MRS broth containing ethidium bromide at 10 μg·mL^{-1}. After 16 h of incubation, cells were plated on MRS, and several colonies were analyzed for plasmid con-

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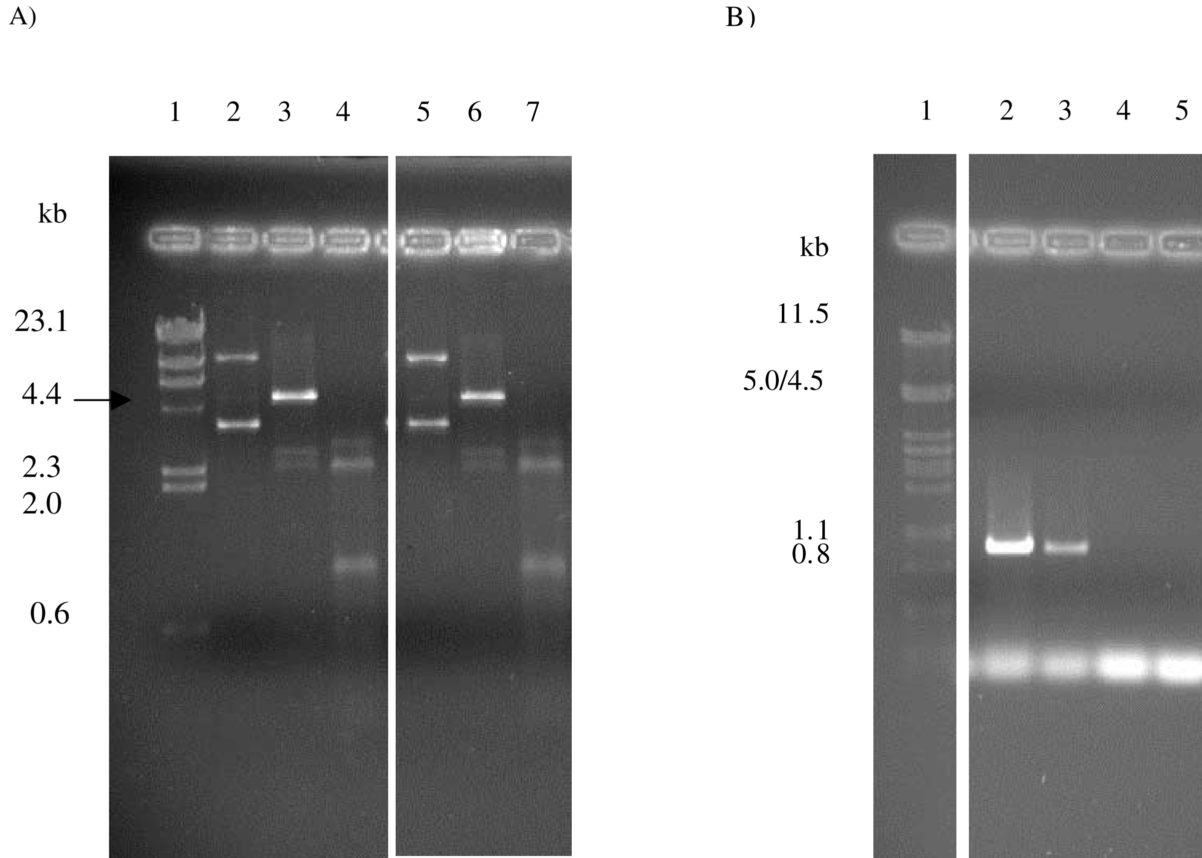
Fig. 3. (A) Agarose gel electrophoresis of recombinant plasmid pSS1 isolated from *Escherichia coli* cells and from electrotransformants of *Lactobacillus johnsonii* NCK65. Lanes: 1, λ *Hin*dIII digested; 2–4, plasmid pSS1 isolated from *E. coli*, uncut and digested with *Nae*I and *Sau*3AI; 5–7, plasmid pSS1 isolated from *L. johnsonii* NCK65, uncut and digested with *Nae*I and *Sau*3AI. (B) PCR analysis of plasmid pSS1 isolated from *E. coli* (lanes 2 and 4) and *L. johnsonii* NCK65 (lanes 3 and 5) cells. Lanes: 1, λ *Pst*I digested; 2 and 3, PCR reactions, run with primers 262F and 362I, confirm the presence of the 0.96-kb fragment containing the iterons from pLBB1; 4 and 5, PCR reactions, run with primers 1817I and 362I (negative controls).



tent. Approximately 3% (1 out of 32 colonies) of the population had lost plasmid pLBB1. The loss of pLBB1 in a cured derivative, strain C1, was confirmed by dot blot experiments, indicating that the plasmid pLBB1 was not inserted into the chromosome (data not shown). Strains B1 and C1 were compared by sequencing of their 16S rRNA V1 region, by RAPD techniques, and by SDS-PAGE of the whole-cell proteins (data not shown), and they were examined for phenotypic traits, which included antibiotic resistance, metabolism of different carbon sources, sensitivity to bacteriocin, and sensitivity to the phages specific for *Lactobacillus bulgaricus*, mv4, LL-H, and lb539. No differences were detected between the parent and the cured variant, and therefore, plasmid pLBB1 remains cryptic.

Nucleotide sequence analysis

Plasmid pLBB1 had a size of 6127 bp and a calculated GC content of 44.8%, which is lower than the GC content of *Lactobacillus delbrueckii* chromosomal DNA (50%). The single *Nco*I site was arbitrarily taken as position 1 (Fig. 1). A nucleotide homology search revealed similarity with the plasmid pLL1212 (accession No. AF109691), a 8713-bp plasmid from *Lactococcus lactis* CRL1212. No similarity was observed with other plasmids from lactic acid bacteria. The high similarity found between pLBB1 and pLL1212 (over 85%) might indicate a common origin for these plasmids. The highest level of identity was detected in the DNA sequence that contains the putative open reading frame A (ORFA). The identity at the nucleotide level reaches a 94% similarity over 1905 nucleotides (nt 4141–6046).

Sequence analysis of open reading frames (ORFs)

Analysis of the plasmid pLBB1 DNA sequence revealed the presence of four putative ORFs (Table 2; Fig. 1). These were identified on the basis that an ORF consisted of at least 100 codons preceded by a Shine-Dalgarno (SD) sequence at an appropriate distance (7–11 nt) from the start codon (AUG). The four ORFs showed sequences for a putative promoter, as compared with the canonical *E. coli* promoter. ORFA encoded a potential primase–helicase; the amino acid end of the deduced protein (aa 10–331) showed identity to DNA primases, including those from *Thermotoga maritima* (accession No. AE001796; 36% similarity and 25% identity) and *Chlamydia pneumoniae* (accession No. AE001674; 34% similarity and 24% identity). A putative Zn^{2+}-binding domain was found in the amino terminal of ORFA (Fig. 2A).

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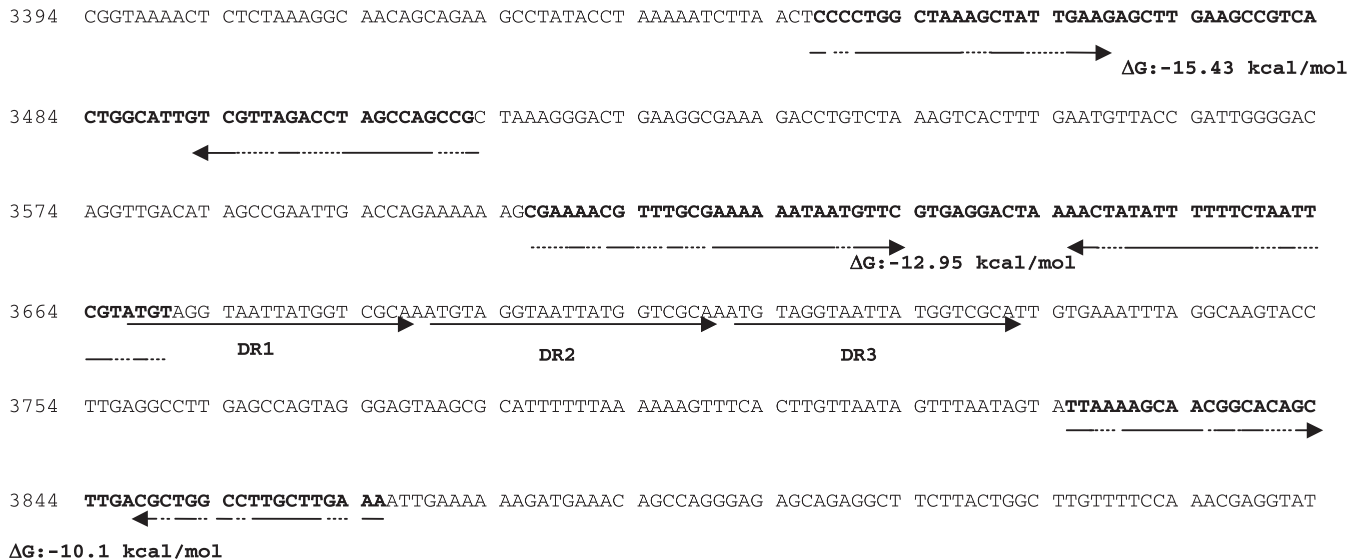

Fig. 4. Sequence analysis of the region containing the three 21-bp direct repeats (DR1–DR3) in plasmid pLBB1 that resembles the origin of replication consistent with an iteron mechanism of replication. Imperfect inverted repeats and their calculated free energy (in kcal/mol) are also indicated with broken arrows. Nucleotide numbers are indicated on the left.

Zn^{2+}-binding motifs are implicated in DNA identification, although it is not the exclusive determinant for DNA sequence recognition (Bird et al. 1998). The carboxyl terminal end of ORFA showed significant identity (in about 300 aa) to several replicative helicases (see Fig. 2B), such as those from *Helicobacter pylori* (accession No. E001551; 45% similarity and 25% identity), *Thermotoga maritima* (accession No. AE001812; 46% and 27%), *Borrelia burgdorferi* (accession No. AE001123; 46% and 24%), *Aquifex aeolicus* (accession No. AE000742; 45% and 28%), and *Bacillus stearothermophilus* (accession No. E106032; 46% and 28%). Analysis of the C-terminal region of ORFA showed conserved motifs identified by Ilyna et al. (1992), including the purine nucleoside triphosphate binding pattern in the 33-kDa tryptic fragment of DnaB (Fig. 2B).

ORFB encoded a putative polypeptide of 192 amino acids (21.8 kDa). A BLASTP homology search showed significant identity with (*i*) (from aa 10–157) a protein of 19.7 kDa of no known function, encoded by plasmid pIP404 from *Clostridium perfringens* (accession No. YPI6_CLOPE; 44% similarity and 25% identity), and (*ii*) (from aa 17–192) a peptide of 192 amino acids encoded by plasmid pLH2 from *Lactobacillus helveticus* (accession No. Q48563; 41% similarity and 25% identity). All these proteins showed limited similarity with the mobilization protein encoded by the plasmid pBI143 from *Bacteroides fragilis* (accession No. AAB39964.1; 46% similarity and 25% identity) and with a mobilization protein from *Bacillus subtilis* (accession No. AAC44416.1; 51% similarity and 29% identity). The predicted translation product of ORFB was shorter (192 aa) than the mobilization protein MobA encoded by pBI143 (367 aa; Smith et al. 1995).

The predicted translation product of ORFC showed 44% similarity and 30% identity with a protein of 159 aa of no known function encoded by *Bacillus halodurans* (accession No. GI10176158) and showed 36% similarity and 25% identity (from aa 16–89) with a transposase from *Streptococcus pyogenes* (accession No. AF082865). The transposase from *S. pyogenes* was encoded by the insertion sequence IS*1239*, which is homologous to IS*30* from *E. coli* and is widely distributed in the group A of streptococci (Kapur et al. 1994). However, in contrast to the transposase from IS*1239* (338 aa), the polypeptide encoded by ORFC was 174 aa. The analysis of a translation product of ORFC showed the absence of conserved domains present in bacterial transposases. However, in the protein encoded by *Bacillus halodurans*, a conserved domain, also present in the insertion element IS*2A* from *E. coli* (accession No. GI6176586), was detected from aa 10–84.

A BLASTP homology search did not identify any known proteins in the GenBank database giving identity with the translation product of ORFD.

Replication of pLBB1

Rolling circle (RC) and theta replication are the two main mechanisms of plasmid DNA replication described in gram-positive bacteria. RC replicons encode a specific replication protein, which initiates DNA synthesis after nicking its target site at the double-stranded origin of replication. RC plasmids are distinguished by the formation of single-stranded DNA intermediates (ssDNA) (Del Solar et al. 1998). No ssDNA was detected in *Lactobacillus bulgaricus* B1 by Southern hybridization (data not shown). Furthermore, the deduced products of ORFs A–D do not share homology with known replication proteins. These data indicated that the replication of pLBB1 might not be directed by a RC mechanism.

Experiments designed to identify replication functions of pLBB1 were conducted with plasmids pSB26, pB2, and pSS1 (Fig. 1). A few transformants (<10) per microgram of DNA of *Lactobacillus johnsonii* NCK65 and *Lactococcus lactis* IL1403 were obtained with pB2 and pSS1, but no transformants were obtained with the plasmid containing the complete molecule of pLBB1. Analysis of plasmid content of the transformants containing pSS1 showed the presence

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of a plasmid with the expected size and restriction enzymes pattern (Fig. 3A). The presence of the 0.96-kb fragment from pLBB1 was also confirmed by PCR (Fig. 3B). However, analysis of the transformants containing pB2 showed the presence of a plasmid smaller than the expected one (data not shown). All attempts to transform strains B1; its plasmid-cured derivative, C1; and *Lactobacillus casei* CRL705 with plasmids pB2 and pSS1 were unsuccessful. Also, despite numerous attempts, no transformants of *Lactobacillus johnsonii* or *Lactococcus lactis* cells with plasmid pSB26 were obtained. The larger size of this plasmid and the overall low efficiency of transformation might explain these results.

Sequence analysis revealed that the predicted products of pLBB1 do not share homology with known replication proteins associated with plasmids (Table 2). Moreover, the putative primase–helicase of pLBB1 is absent in pSS1 and ORFB, indicating that the peptides encoded by these ORFs are not essential for the replication of pSS1 in *Lactobacillus johnsonii* and *Lactococcus lactis*. Interestingly, however, three 21-bp repeats (iterons) were found between nucleotides 3668 and 3730 (Fig. 4) and resemble the origin of replication consistent with an iteron mechanism of replication. The presence of this region that contains the putative iterons of pLBB1 was also confirmed by PCR to be present in transformants containing deleted pB2 (data not shown). Analysis of this region showed also the presence of three imperfect inverted repeats with the capacity to form secondary structures and a slightly lower content of GC (40.7%) when compared with the overall molecule (44.8%). These data might indicate that this region is involved in plasmid replication. Iterons may be the binding sites for plasmid-encoded Rep proteins and are key elements for the control of plasmid replication. These sequences have been described in plasmids that replicate by the theta mechanism, the strand-displacement mechanism, and the rolling circle mechanism (Del Solar et al. 1998).

This is the first nucleotide sequence of a plasmid from *Lactobacillus bulgaricus* to be reported. Although further characterization of the mode of replication of pLBB1 is needed, our data should be useful for the development of pLBB1-based cloning vectors for this subspecies.

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