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Feld, Louise; Bielak, Eliza Maria; Hammer, Karin; Wilcks, Andrea

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1 **Characterisation of a small erythromycin resistance plasmid pLFE1 from the**
2 **food-isolate *Lactobacillus plantarum* M345**
3

4 Louise Feld[1], Eliza Bielak[1], Karin Hammer[2] and Andrea Wilcks[1*]
5 [1]Department of Microbiology and Risk Assessment, National Food Institute, Technical University
6 of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark
7 [2]Center for Systems Microbiology, Department of Systems Biology, Technical University of
8 Denmark, DK-2800 Lyngby, Denmark
9

10 **Keywords:** erythromycin resistance, plasmid, host-range, mobilisation, *Lactobacillus plantarum*
11

12 ***Correspondence:**
13 Andrea Wilcks
14 Department of Microbiology and Risk Assessment
15 National Food Institute
16 Technical University of Denmark
17 Mørkhøj Bygade 19
18 DK-2860 Søborg
19 Tel.: +45 7234 7185
20 E-mail: anwi@food.dtu.dk
21
22
23
24

Abstract

This paper reports the complete 4031 bp nucleotide sequence of the small erythromycin resistance plasmid pLFE1 isolated from the raw-milk cheese isolate *Lactobacillus plantarum* M345. Analysis of the sequence revealed the coding regions for the erythromycin resistance determinant Erm(B). A replication initiation protein RepB was identified belonging to the RepB proteins of the pMV158 family of rolling-circle replicating plasmids. The transcriptional repressor protein CopG and a small counter transcribed RNA, two elements typically involved in replication control within this family were also found. A putative replication initiation site including a single-strand origin (*sso*) -like region succeeded by a characteristic pMV158 family double-strand origin (*dso*) was located upstream of the replication region. An open reading frame following a typical origin of transfer (*oriT)* site and coding for a putative truncated mobilisation (Mob) protein with a size of 83 aa was detected. The product of the putative *mob* gene showed large similarity to the N-terminal region of the pMV158 family of Pre/Mob proteins, but was much smaller than other proteins of this family. We therefore suggest that the Mob function in pLFE1 is supplied in *trans* from another plasmid present in *L. plantarum* M345.

Filter mating experiments showed that pLFE1 has a broad host-range with transconjugants obtained from *Lactobacillus rhamnosus, Lactococcus lactis, Listeria innocua*, the opportunistic pathogen *Enterococcus faecalis* and the pathogen *Listeria monocytogenes*.

Keywords: plasmid, sequence, *Lactobacillus plantarum*, erythromycin resistance gene, mobilisation

1 **1. Introduction**

2 *Lactobacillus plantarum* is a species of substantial industrial importance and has been used for
3 centuries as starter culture in the manufacture of a broad selection of food products both for human
4 and animal consumption. In addition, many strains of *L. plantarum* are recognized to have a general
5 beneficial health effect, and are thus used as probiotics. *L. plantarum* is often well adapted to
6 survive passage through the upper gastrointestinal tract and has frequently been shown to be one of
7 the dominating *Lactobacillus* species in the human small intestine (Ahrne et al., 1998; Johansson et
8 al., 1993).
9 Natural resident plasmids are common in *L. plantarum* and several papers have reported the
10 nucleotide sequence of these: pM4 (Yin et al., 2008), pWCFS101, pWCFS102, pWCFS103 (van
11 Kranenburg et al., 2005), p256 (Sorvig et al., 2005), pPB1 (de las Rivas et al., 2004), pMD5057
12 (Danielsen, 2002), pPLA4 (van Reenen et al., 1998), pA1 (Vujcic and Topisirovic, 1993), pLB4
13 (Bates and Gilbert, 1989) and pC30il (Skaugen, 1989). However, most plasmids in lactobacilli are
14 small rolling-circle replicating (RCR) cryptic plasmids without any assigned function besides their
15 own replication apparatus and occasionally mobilisation genes (Pouwels and Leer, 1993). Some
16 documents exist on the occurrence of antibiotic resistance genes harboured on *Lactobacillus*
17 plasmids, however, the number is relatively limited (Axelsson et al., 1988; Danielsen, 2002; Fons et
18 al., 1997; Gevers et al., 2003; Lin et al., 1996; Tannock et al., 1994; Vescovo et al., 1982). Due to
19 the wide environmental distribution of *L. plantarum*, the presence of antibiotic resistance plasmids
20 in this species can be a potential health hazard. If mobile, the plasmids may be transferred to other
21 bacteria within the food chain and in worst-case reach potentially pathogenic bacteria invading the
22 consumer. Transfer of different *L. plantarum* plasmids harbouring resistance genes towards
23 erythromycin and tetracycline has been documented *in vitro* to *Enterococcus faecalis* and

1 *Lactococcus lactis* (Gevers et al., 2003) and *in vivo* to *E. faecalis* using gnotobiotic rats as a model

2 of the human intestine (Jacobsen et al., 2007).

3 The present paper reports on the small *L. plantarum* erythromycin resistance plasmid pLFE1,

4 previously shown to transfer at high frequency to *E. faecalis* in the gnotobiotic rat model, but not in

5 the more complex streptomycin-treated mice model (Feld et al., 2008). The whole nucleotide

6 sequence of pLFE1 was determined and analysis was carried out in order to learn more about the

7 mechanistic basis for mobilisation, potential host-range, incompatibility grouping and expression of

8 antibiotic resistance.

9

1 **2. Materials and methods**

2 *2.1 Bacterial strains, plasmids and culture conditions*

3 The raw-milk cheese isolate *L. plantarum* M345 (Feld et al., 2008) previously shown to harbour the
4 erythromycin resistance plasmid pLFE1 was used for plasmid preparation of pLFE1 and as donor in
5 *in vitro* conjugation assays. Strain M345 was grown anaerobic at 37 °C for 24-48 h in de Man,
6 Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, UK) supplemented with erythromycin
7 when appropriate (for concentrations, see below).
8 Bacterial strains used as recipients in the conjugation study are listed in Table 1. *E. faecalis* was
9 grown on Brain Heart Infusion (BHI) medium (Oxoid), *L. lactis* on GM17 media (M17 (Oxoid) +
10 10% glucose), *Bacillus* on Bacillus Cereus Selective Agar (Oxoid) and *Listeria* on Palcam (Oxoid).
11 When appropriate, antibiotics were added to the media at the following final concentrations: 100 μg
12 ml^{-1} or 500 μg ml^{-1} streptomycin (str) for selection of *Bacillis* sp. and *E. faecalis*, respectively, 500
13 μg ml^{-1} spectinomycin (spec), 25 μg ml^{-1} tetracycline (tet), 100 μg ml^{-1} rifampicin (rif) and 100 μg
14 ml^{-1} nalidixic acid (nal). Erythromycin was applied at 16 or 50 μg ml^{-1} for selection of
15 transconjugants and donors, respectively.
16

17 *2.2 Sequencing of pLFE1 plasmid*

18 The *erm*(B) gene harboured on plasmid pLFE1 from *L. plantarum* M345 was analysed by
19 sequencing standard PCR products. Plasmids were extracted from the strain as earlier described
20 (Jacobsen et al., 2007) and subsequently used as template in PCR amplification of the *erm*(B) gene,
21 as previously described (Jacobsen et al., 2007). Eight PCR reactions were set up, and the eight
22 products pooled and purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany).
23 The size of the PCR product (424 bp) was confirmed by gel electrophoresis and the sample
24 subsequently sequenced using the ermB-F and ermB-R primers (Jensen et al., 1999). The remaining

1 part of the *L. plantarum* M345 *erm*(B) plasmid was sequenced by primer walking using the plasmid
2 preparation of strain M345 as template. Initially, the primers Lor1 (5'-AGA TGA CTG TCT AAT
3 TCA ATA G-3') and Lor3 (5'-TGC ACA CTC AAG TCT CGA-3') targeting the *erm*(B) gene and
4 reading outwards, were applied. All sequencing reactions were performed at GATC Biotech
5 (Konstanz, Germany).
6
7 *2.3 Verification of pLFE1 structure*
8 A restriction map of pLFE1 was made by digestion with the restriction enzymes *Xcm*I, *Xmn*I, *Ase*I,
9 *Hinc*II, *Bss*SI and *Nde*I. As template, plasmid extraction from a pLFE1-carrying transconjugant
10 isolate previously obtained by conjugation between *L. plantarum* M345 and *E. faecalis* JH2SS was
11 used (Feld et al., 2008). Analysis of the plasmid profile showed that this isolate contained the
12 pLFE1 plasmid solely. Restriction analysis was performed using restriction enzymes from New
13 England Biolab (Herts, UK) according to the description of the manufacturer. Digested DNA was
14 run by gel electrophoresis on a 1% agarose gel and subsequently stained with ethidium bromide.
15 The structure of pLFE1 was further confirmed by PCR using primers mob_1F (5'- TGG GTC AAT
16 CGA GAA TAT C-3') binding at position 1213-1231, mob_2F (5'-GAT TTG GTC AAT CGG ACA
17 G-3') binding at position 3444-3462 and mobR (5'-GAA CGC AAA TAT GAG CTT C-3') binding
18 at position 3729-3711 and 1623-1605. PCR mixtures were prepared using 5 µl DNA template, 1
19 PuReTaq Ready-To-Go PCR bead (Amersham Biosciences, Buckinghamshire, UK) and 20 pmol of
20 each primer in a total volume of 25 µl. Amplification using the primers mob_1F + mobR or mob_2F +
21 mobR should result in a 286 bp and a 411 bp PCR product, respectively. PCR was carried out by
22 initial denaturation at 95 ºC for 10 min followed by 30 cycles of 95 ºC for 45 s, 55 ºC for 45 s and
23 72 ºC for 2 min. A final extension step at 72 ºC for 10 min ended the programme.
24

2.4 In vitro mating experiment

The ability of plasmid pLFE1 to be transferred to and replicate in different Gram-positive species was assessed using a filter-mating assay. The donor and recipient strains were grown in broth supplemented with the respective antibiotics to an OD_{600} = 0.7-1.0. The bacteria were mixed in equal volumes and vacuum filtered onto sterile filters (HAWP04700, Millipore, Bedford, MA). The filters were placed on agar mating plates and incubated overnight at conditions optimal for the recipients. For *L. lactis* mating, GM17 media was used, for the rest BHI medium was used as mating media. All matings were performed at 37°C under aerobic conditions, except the matings with *L. lactis* and *Bacillus* as recipients, where the matings were performed at 30°C. After overnight incubation, the bacteria were washed off the filters and suitable dilutions prepared on plates selective for donor, recipients and transconjugants. Transconjugants were selected at conditions identical to their respective recipients except that erythromycin was added to the plates and the plates were incubated for 36h. Controls of separate donor and recipient plates were also prepared. The experiment was performed in triplicates.

2.5 Exclusion of transfer via transformation and transduction

Transformation was excluded by adding DNase I to the mating plates at a concentration of 100 μg/ml, and performing a filter mating experiment between *L. plantarum* M345 and *E. faecalis* JH2SS as described above. No difference in transfer frequency was detected (data not shown), indicating that pLFE1 is not transferred via transformation.

Two experiments were conducted to exclude that pLFE1 is transferred via transduction. In the first one, supernatant from a *L. plantarum* M345 culture was used in a filter mating experiment with *E. faecalis* JH2SS. In the second experiment, the donor and recipient were filtered on two separate filters that were placed upside down of each other, so that there was no direct contact between the

1 two strains. In both cases no transconjugants were detected, excluding the possibility of
2 transduction as a mechanism of pLFE1 transfer (data not shown).
3
4 *2.6 Verification of transconjugants*
5 In order to verify that colonies isolated from selective agar plates were true transconjugants and not
6 mutants of donor or recipient, specific PCR and fingerprinting assays were performed. The presence
7 of the *erm*(B) gene was verified by PCR using primers specific for *erm*(B) as earlier described
8 (Jacobsen et al., 2007). Donor and recipients were included as positive and negative controls,
9 respectively. Random amplified polymorphic DNA (RAPD) technique was used to show similar
10 fingerprints of recipients and transconjugants and differentiate them from the fingerprint of the
11 donor. Template DNA of donor, recipients and transconjugants was prepared as previously
12 described (Jacobsen et al., 2007). The PCR reaction mixtures contained 5 µl DNA template, 1
13 PuReTaq Ready-To-Go PCR bead and 20 pmol of primer OPA-02 (Operon Biotechnologies,
14 Cologne, Germany) in a total volume of 25 µl. The PCR programme was as follows; initial
15 denaturation 94 ºC for 4 min followed by 40 cycles of 94 ºC for 1 min, 32 ºC for 1 min and 72 ºC
16 for 2 min. The program was ended by extension at 72 ºC for 5 min. Isolates of *Listeria*
17 *monocytogenes* transconjugants were further tested by PCR using primers IntA_C_up (5'-TAG
18 AAG TAG TGT AAA GAG CTA GAT G-3') and IntA_C_down (5'-ATA TAA AGC TTG CGG
19 CCG CTT CTG CAA AAG CAT CAT CTG GAA AA-3') amplifying a 260 bp sequence of the
20 virulence gene *Int*A coding for Internalin A. PCR reaction mixtures contained 5 µl DNA template, 1
21 PuReTaq Ready-To-Go PCR bead and 20 pmol of each primer IntA_C_up and IntA_C_down in a
22 total volume of 25 µl. The PCR programme was as follows; initial denaturation at 95 ºC for 3 min
23 followed by 30 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s and finally extension at 72
24 ºC for 3 min. The donor and recipient were included as negative and positive controls, respectively.

1 All PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Bio-
2 Rad, Waltham, MA, USA). The PCR products were run on a 1.5% agarose gel for 1h and visualized
3 by ethidium bromide staining.
4
5 *2.7 Stability of pLFE1 in donor and transconjugants*
6 The donor strain *L. plantarum* M345 and the obtained transconjugants from mating with *E. faecalis*,
7 *B. subtilis, L. lactis, L. monocytogenes* and *L. innocua* were inoculated from selective (containing
8 erythromycin) agar plates to selective liquid media and grown until exponential phase was reached
9 (OD_{600} in the range of 0.8-1.2). The cultures were diluted 100 times by transferring 200 μl of the
10 culture into 20 ml non-selective media appropriate for the strain (Table 1) and incubated until
11 stationary phase was reached. The cycle of growth and dilution in non-selective broth was repeated
12 8 times, which yielded approximately 53 generations according to the following equation:
13 $g=\ln F/\ln 2$, where g is the number of generation in each cycle, and F is the dilution factor.
14
15 *2.8 Erythromycin-clindamycin double-disk test*
16 Overnight cultures of *L. plantarum* M345 were diluted 100 times, and 100 μl of the dilution was
17 spread on MRS agar plates. An erythromycin disk (15 μg/ml, Oxoid) was placed 5-10 mm from a
18 clindamycin disk (10 μg/ml, Oxoid). The plates were incubated overnight under anaerobic
19 conditions at 37 °C. Blunting of the clindamycin proximal to the erythromycin disk was classified
20 as D-zone test–positive (inducible resistance), whereas no blunting of the zone was classified as D-
21 zone test–negative (no induction).
22 Two *Streptococcus thermophilus* strains E2 and E18 (Tosi et al., 2007) were used as controls for
23 inducible and constitutive resistance, respectively.

1

2 *2.9 Nucleotide sequence analysis*

3 Analysis of the complete nucleotide sequence for open reading frames (ORF) was performed using
4 the web-based NCBI ORF Finder programme. Database searches and comparisons of DNA
5 sequences or DNA-derived protein sequences were carried out using BLASTN, BLASTP and
6 BLASTX programmes (Altschul et al., 1990). Multiple sequence alignments were made with the
7 ClustalW2 program (Larkin et al., 2007) and alignments of conserved domains of proteins encoded
8 on pLFE1 was retrieved from the Conserved Domain Database (CDD) (Marchler-Bauer et al.,
9 2007). Secondary structure and calculation of free energy for hairpin formations was determined
10 using the Mfold programme (Zuker, 2003).

11

12 *2.10 Nucleotide sequence accession number*

13 The complete nucleotide sequence of pLFE1 has been deposited in the GenBank database under the
14 Accession No. **FJ374272**.

15

16

1 **3. Results and Discussion**

2 *3.1 General features of pLFE1 sequence*

3 The complete nucleotide sequence of plasmid pLFE1 was determined to be 4031 bp in length. The
4 correctness of the deduced pLFE1 nucleotide sequence was verified with single digests using the
5 restriction enzymes shown in Fig. 1 and gel electrophoresis, which confirmed the predicted
6 nucleotide band sizes (data not shown). The average G + C content of pLFE1 is 34.4%, which is
7 lower than that of the *L. plantarum* chromosome average of 44.5% (Kleerebezem et al., 2003), but
8 within the range described for other small *L. plantarum* plasmids such as pA1 (34.9%) (Vujcic and
9 Topisirovic, 1993), pPB1 (37.4%) (de las Rivas et al., 2004), p256 (37%), pWCFS101 (39.5%) and
10 pWCFS102 (34.3%) (van Kranenburg et al., 2005). Analysis of the whole nucleotide sequence
11 revealed 16 putative ORFs of at least 33 aa in length and one ORF smaller than 33 aa (Table 2). In a
12 region of 3-12 nucleotides upstream of each of the ORFs, analysis was carried out for detection of
13 ribosome-binding sites (RBS) using the *L. plantarum* RBS sequence GGAGG as consensus
14 (Pouwels and Leer, 1993). However, in many cases either no or only poor potential RBS sites could
15 be detected and translational coupling to other putative proteins did not seem plausible, and these
16 ORFs were considered artefacts.
17
18 *3.2 Replication initiator (RepB) protein*

19 A putative replication initiator (RepB) protein was identified at position 2421-3059 (212 aa) (Fig. 1
20 + Fig. 2), showing high similarity to the RepB proteins of the *L. plantarum* rolling-circle replicating
21 (RCR) plasmid pLB4 (80% identity over 211 aa) (Bates and Gilbert, 1989), RCR plasmid pLF1311
22 from *Lactobacillus fermentum* (82% identity over 206 aa) (Aleshin et al., 1999) and RCR plasmid
23 pBM02 (71% identity over 205 aa) from *L. lactis* subsp. *cremoris* (Sanchez and Mayo, 2003). The
24 putative RepB protein showed a conserved domain architecture corresponding to that of Rep_2

1 plasmid replication proteins (pfam01719) in the Conserved Domain Database (CDD). The sequence
2 included five motifs (Fig. 2 + Fig. 3) typical for plasmid family pMV158 (del Solar et al., 1993).
3 These motifs include the putative binding site for Mg^{++} (motif R-III), which is thought to be
4 required for nicking activity and the active tyrosine residue (motif R-IV and/or motif R-V) involved
5 in the catalytic attack of the plasmid DNA during initiation of replication (del Solar et al., 1993; del
6 Solar et al., 1998; Dyda and Hickman, 2003; Ilyina and Koonin, 1992).
7
8 *3.3 Plasmid copy number control*
9 A putative gene coding for CopG (59 aa) previously referred to as RepA was found at position
10 2118-2297 bp (Fig. 1 + Fig. 2), showing high similarity to RepA (70% identity over 51 aa) of pLB4
11 (Bates and Gilbert, 1989), RepA (70% identity over 51 aa) of pLF1311 (Aleshin et al., 1999), RepA
12 (68% identity over 51 aa) of the *L. lactis* plasmid pAR141 (Raha et al., 2006) and RepA (69%
13 identity over 46 aa) of pBM02 (Sanchez and Mayo, 2003). The putative CopG protein revealed a
14 conserved sequence structure similar to the RHH_1 ribbon-helix-helix family of CopG repressor
15 proteins (pfam01402 in the Conserved Domain Database) (Fig. 4).
16 CopG is a transcriptional repressor protein that regulates the plasmid copy number in the pMV158
17 derivative plasmid pLS1 (del Solar et al., 1995; del Solar and Espinosa, 1992). By binding to the
18 single *copG-repB* promoter region, CopG prevents host RNA polymerase binding and thus
19 represses the synthesis of the replication initiation protein RepB as well as itself (del Solar et al.,
20 1990). A structure of the replication region comparable to pLS1 has also been revealed in other
21 plasmids belonging to the pMV158 family suggesting that they are controlled in a similar fashion
22 (Bates and Gilbert, 1989; Cocconcelli et al., 1996; Raha et al., 2006; Vujcic and Topisirovic, 1993).
23 Sequence analysis of pLFE1 indicated a homologous architecture with a putative promoter 18
24 nucleotides upstream of the *copG* start codon with a –35 (TTGTAT) and –10 (TATAAT) sequence

1 in a distance of 17 base pairs and typical RBS sites located upstream each gene, *copG* and *repB*
2 (Fig. 2). However, no sequence matching the criteria of a 13-bp pseudo symmetric element
3 overlapping the -35 *copG-repB* promoter box could be found. This element was demonstrated to be
4 the target binding site of CopG in pLS1 (del Solar et al., 1990) and similar sequences have been
5 detected in other pMV158 family plasmids, although their function have not been elucidated (del
6 Solar et al., 2002).
7 The active DNA binding unit of CopG from plasmid pMV158 has been shown to display a
8 characteristic ribbon-helix-helix (RHH) motif, which is built up around a glycine residue mediating
9 a turn between two α-helices. At conserved positions of the α-helices specific residues are required
10 to maintain the hydrophobic core of the motif (Gomis-Ruth et al., 1998). Within the putative *copG*
11 gene of pLFE1 the presence of a glycine residue and specifically positioned hydrophobic residues
12 were established (Fig. 4).
13 Apart from CopG, a small countertranscribed RNA (ctRNA) of approximately 50 nucleotides in
14 size has been shown to control the plasmid copy number of pLS1 (del Solar et al., 1995; del Solar et
15 al., 1997). The ctRNA is transcribed in the opposite direction of the mRNA encoding the RepB
16 protein and thus inhibits translation of RepB by binding to a region of the *copG-repB* mRNA,
17 which includes the RBS of *repB* (del Solar et al., 1997). Similar genetic organizations of ctRNA are
18 present in other members of the pMV158 family. Thus, the coordinate action of the two plasmid-
19 encoded elements CopG and ctRNA has therefore been suggested to be a common mode of plasmid
20 copy number regulation in the pMV158 plasmid family (del Solar and Espinosa, 1992). Indeed,
21 sequence analysis of pLFE1 revealed a putative ctRNA encoded on the complementary strand in the
22 region between *copG* and *repB* (Fig. 1 + Fig. 2). Within the amino-terminal region of *repB* a
23 putative promoter consisting of less conserved –10 (TTTCAT) and –35 (TAGGCA) boxes in a
24 distance of 18 nucleotides was detected. A possible transcriptional terminator was found

overlapping the carboxyl-terminal end of *copG.* This plausible terminator could be a *rho*-independent site, containing an inverted repeat configuring a potential hairpin structure followed by a T-stretch (depicted by an A-stretch at the complementary strand in Fig. 2).

3.4 Origin of replication

In RCR plasmids the double-strand origin (*dso*) is the initiation site of leading-strand synthesis. The *dso* contains a *nic* locus, which generally is well conserved within each plasmid family and a binding site (the *bind* locus) showing larger sequence variation (Khan, 1997). Within pLFE1 a region of 22 bp (5'-atGGGGGcACTACGACaCCCCC-3') showing high similarity (four mismatches) to the pMV158 family *nic* loci (del Solar et al., 1998) was found 162 bp upstream of *copG* at position 1934-1955 bp (Fig. 1 + Fig. 2). This region showed 100% similarity to the *dso* of both *L. fermentum* (Aleshin et al., 1999) and the shuttle vector pLF14 (unpublished, Accession No. **X85436.1**). The putative nick sequence and the corresponding nick site (5'-cACTACG/AC-3') corresponds well (one mismatch) with the previously defined consensus sequence for the pMV158 family (Moscoso et al., 1995). The nick sequence of pLFE1 is contained within an inverted repeat (IR5 with a calculated free energy dG = -8.4 kcal/mol) and flanked by a three direct repeats identical to those composing the Proximal Direct Repeats (PDR) of the *nic* locus from plasmid pE194 (Ruiz-Maso et al., 2007) (Fig. 2). Direct repeats constituting the Distal Direct Repeats (DRR) or *bind* locus identified in several RCR plasmids, but not all, of the pMV158 family (Ruiz-Maso et al., 2007) could not be identified on pLFE1. In other RCR plasmids, binding and interaction with RepB has been shown to change the characterised secondary structure resulting in creation of a hairpin, which in turn exposes the nick sequence and thus initiates replication (Khan, 1997).

A region of 266 nucleotides immediately upstream of the putative *dso* showed a large potential of palindromic structures (dG = -50.6 kcal/mol) (Fig. 1 + Fig. 2). We propose that this region constitutes the single-strand origin (*sso*) of pLFE1 from where lagging-strand synthesis is initiated. *Sso*s are by substantial secondary structure, but the sequence similarity may be very low even between plasmids belonging to the same family (Gruss and Ehrlich, 1989; Khan, 1997). Comparison of the putative *sso* sequence of pLFE1 with four types of *sso*s (*sso*A, *sso*U, *sso*T and *sso*W) previously described (Kramer et al., 1999) showed no general sequence similarity (data not shown). However, an RS_B (recombination site) or RS_B-like site (5'-TTTTCGTCGGCATAA-3') was recognized at position 1669-1683 (Fig. 2) at the basis of the stem of a putative hairpin structure (dG for IR1 = -33 kcal/mol). This site has apart from being a recombination site been proposed to be involved with host RNA polymerase binding to the *sso.* The RS_B may therefore be critical for the synthesis of a primer RNA that can be used to initiate lagging-strand replication. The RS_B was first recognized in *sso*A origins, but homologues have been detected in the other types of *sso*s as well (Kramer et al., 1999).
A second region of plasmid pLFE1 potentially containing an origin of replication was found in the non-coding area downstream the putative *mob* gene. A sequence of 20 bp (5'-TtcTTCTTATCTTGATAcTA-3'), which showed high similarity (three mismatches) with pC194 family *dso*s was found at position 4009-4028 (Fig. 1) (del Solar et al., 1998; Wu et al., 2007). This sequence showed 100% identity with the putative *dso* of the *L. plantarum* plasmids pLP1 (Bouia et al., 1989) and pC30il (Skaugen, 1989) and the *L. lactis* plasmid pWC1 (Pillidge et al., 1996).
Upstream of the putative *dso* another potential *sso* region of 240 nucleotides (Fig. 1), which showed high potential of palindromic structures (dG = -63.2 kcal/mol) was detected. This region contained an RS_B-like sequence 100% identical to the one described above, which also was placed at the basis of the stem of a potential hairpin structure (dG = -35.2 kcal/mol). The sequences pursuing these two

1 identical RS_B-like sequences were 75% identical with the similarity discontinuing upstream of the
2 putative *dso*s (data not shown). Thus, despite the lack of overall sequence similarity to any
3 previously known *sso*s, we suggest the presence of two *sso*s within pLFE1. The existence of two
4 functionally intact *sso*s simultaneously present on a single plasmid has earlier been reported for
5 pMV158, which has been shown to contain both an *sso*A (del Solar et al., 1987) and *sso*U type (van
6 der Lelie et al., 1989).
7
8 *3.5 Erythromycin resistance*
9 A putative antibiotic resistance gene *erm*(B) conferring resistance to macrolide, lincosamides and
10 streptogramin (MLS) antibiotics was located at position 625-1362. The *erm*(B) gene encodes a
11 protein 100% identical to the erythromycin ribosome methylase Erm(B) of *Enterococcus faecium*
12 (De Leener et al., 2005). The group of Erm(B) proteins is well-conserved showing 98-100%
13 similarity at the nucleotide level (Roberts et al., 1999). Erm(B) enzymes confer resistance by adding
14 one or more methyl groups to a specific adenine residue located in the 23S rRNA subunit thereby
15 preventing binding of the antibiotic (Weisblum, 1995a).
16 A small region (27 aa) was identified 124 nucleotides upstream the *erm*(B) start codon, which at its
17 full length showed 100% identity with MLS leader peptides from a range of mobile elements
18 harboured in different bacterial species. The functional role of MLS leader peptides is to regulate
19 expression of the erythromycin methylase by conformational changes of the secondary structure of
20 the mRNA, which affects the neighbouring region upstream of the methylase (Weisblum, 1995b).
21 In the absence of erythromycin, translation of the leader peptide is active. Hereby, the mRNA
22 assumes a configuration that positions the RBS site and initial codons of the methylase within the
23 stem of a hairpin structure, thus preventing its translation. However, when erythromycin is present,
24 it binds to and suspends ribosomes involved in translation of the leader peptide. This in turn results

in naked mRNA, encoding the remainder of the leader ORF, this additional free RNA gives rise to another conformation of RNA stem-loops than when translation of the leader peptide occurs and thereby exposes the RBS site of the methylase thereby promoting its translation (Min et al., 2008a; Weisblum, 1995b).

Several isolates have been reported constitutively to produce erythromycin methylase although putative MLS leader peptides were detected (Gfeller et al., 2003; O'Connor et al., 2007). However, the putative MLS leader peptide of pLFE1 is identical to leader peptides from strains of *Streptococcus pneumoniae*, which have been reported functional (Okitsu et al., 2005). Compared to the leader peptide in the *erm*(B) gene of Tn*917* (Shaw and Clewell, 1985) the peptide in pLFE1 is only 27 aa i.e. 9 aa shorter due to a single mutation resulting in an earlier stop codon. A previous study suggested that a similar mutation caused an increased level of methylase translation, resulting in an apparently constitutive resistant phenotype. The mutation increased the basal methylase activity by approximately 3.8 fold and the induced activity by approximately 4.9 fold (Oh et al., 1998). However, there are also reports where this shortening in the length of the leader peptide has no influence on the expression of *erm*(B), i.e. the leader peptide is still functional (Min et al., 2008b; Min et al., 2008a). Using the erythromycin-clindamycin double-disk test, the strain *L. plantarum* M345 was assigned to the constitutive resistance phenotype (data not shown). This constitutive expression may be caused by a change in nucleotide just after RBS for *erm*(B). Compared to the sequence of the Tn*917*-like transposon LP-1 from *Streptoccous pneumoniae* (Okitsu et al., 2005) harbouring an inducible phenotype, there is a shift from GGAGTG to GGAGAG. Min and co-workers (Min et al., 2008a) showed that a shift in this sequence resulted in destabilization of a stem loop resulting in an increased basal level of expression. Using the Mfold programme, the RBS for *erm*(B) was fully exposed, confirming the constitutive resistance phenotype.

3.6 Mobilisation region

A truncated recombinase/mobilisation (*pre/mob*$_1$) gene (Fig. 1) was found at position 1386-1606, which showed 100% nucleotide identity with the carboxyl-terminal end of the *Lactobacillus sakei* plasmid pYS18 *pre/mob* gene (unpublished, Accession No. **EU185047**) and 94% nucleotide identity with the *pre/mob* gene of *L. lactis* subsp. *lactis* plasmid pK214 (unpublished, Accession No. **YP_001429536**). However, despite the presence of an ATG start codon, no potential RBS or transcriptional coupling could be detected (Table 2) thus rendering translation of this protein unlikely.

In a different region of pLFE1 a second truncated *pre/mob*$_2$ gene was found at position 3312-3563 bp. A strong RBS site (AGGAG) was located seven nucleotides upstream of the ATG start codon and a possible promoter was found with a -35 box (TTACGA) and a -10 box (TATACT) in a distance of 17 nucleotides (Fig. 5). The *pre/mob*$_2$ gene showed 94% nucleotide identity with the amino-terminal end of the *pre/mob* gene of pK214 and 81% nucleotide identity with the *pre/mob* gene of pYS18. Sixty-one nucleotides downstream of the *pre/mob*$_2$ stop codon a fragment showing 93% nucleotide identity with the carboxyl-terminal end of the pK214 *pre/mob* gene and 96% nucleotide identity with the *pre/mob* gene of pYS18 was found (Fig. 5). However, no start codon or putative RBS could be identified for this small fragment.

The odd sequence structure of pLFE1 with several truncated *pre/mob* genes or gene fragments was verified by PCR using primers mob$_1$F, mob$_2$F and mobR (data not shown). The mob$_2$F + mobR primers were specifically designed to confirm the short distance of 61 nucleotides and thus lack of a middle region of the *pre/mob* gene between the amino-terminal *pre/mob*$_2$ gene and the carboxyl-terminal *pre/mob* fragment. Mob$_1$F + mobR were designed to confirm the spatial separation of *pre/mob*$_1$ and *pre/mob*$_2$ with the former placed downstream of *erm*(B).

1 The Pre/Mob proteins of pK214 and pYS18 belong to the pMV158 family of Pre/Mob proteins that
2 functions both in mobilisation and recombination (Pre for plasmid recombination enzyme) (Francia
3 et al., 2004). The Pre/Mob family contains within the amino-terminal region three highly conserved
4 motifs considered to form part of the catalytic centre of the relaxase enzyme. In the *pre/mob*$_2$ region
5 of pLFE1 only two of the three motifs could be identified (Fig. 5). Additionally, the size of the
6 putative Pre/Mob$_2$ is only 83 aa in contrast to other considerably larger Pre/Mob proteins
7 (approximately 350-500 aa) belonging to this family (de las Rivas et al., 2004; Gennaro et al., 1987;
8 Josson et al., 1990; Somkuti and Steinberg, 2007).
9 Mobilisation proteins normally demonstrate relaxase activity and are thus essential in preparation of
10 the plasmid for transfer (Francia et al., 2004). In plasmid pMV158 the Pre/Mob protein has been
11 shown to nick supercoiled plasmid DNA at the origin of transfer (*oriT*) (Guzman and Espinosa,
12 1997) and without the presence of an intact Pre/Mob protein conjugal transfer was suspended
13 (Priebe and Lacks, 1989; van der Lelie et al., 1990). Since pLFE1 has been shown transferable
14 (Feld et al., 2008) but does not seem to contain an intact Pre/Mob protein, we suggest that it must
15 be supplied in *trans* in *L. plantarum* M345. This organization has been shown functional in
16 *Bacillus*, where transfer-deficient Mob$^-$ hybrid plasmid constructs of pUB110 and pBC16 could be
17 mobilised by complementation of a Mob protein in *trans* (Selinger et al., 1990). It was excluded by
18 *in vitro* transfer experiments that pLFE1 is transferred via transformation or transduction (data not
19 shown). Apart from relaxase activity, which could be provided in *trans*, the presence of an *oriT*
20 region in *cis* has been shown to be a minimal requirement for mobilisation of plasmids such as
21 pUB110 and pBC16 (Selinger et al., 1990). The *oriT* site is very similar among members of the
22 pMV158 family and is located upstream of the *mob* gene, overlapping its promoter. The *oriT* has a
23 conserved hairpin structure with an IR of 7-10 nucleotides forming the stem and a loop of usually 6
24 nucleotides presenting the nick sequence (Francia et al., 2004; Guzman and Espinosa, 1997). A

1 putative *oriT* site matching these criteria was identified in pLFE1 at position 3257-3276, 34
2 nucleotides upstream of the *pre/mob*$_2$ start codon. A putative –10 promoter box was embedded
3 within this sequence and an IR consisting of 8 nucleotides interrupted by 6 nucleotides was also
4 recognized (Fig. 5).
5
6 *3.7 Plasmid recombination*
7 Apart from being the origin of transfer, *oriT* also functions as a putative recombination site (RS_A).
8 The plasmid-encoded Pre/Mob protein mediates site-specific recombination at RS_A in *trans*
9 (Gennaro et al., 1987). Analysis of several plasmids has shown a marked sequence divergence at the
10 RS_A site with the one side showing significant similarity to one plasmid and the other side showing
11 similarity to a second plasmid – thus indicating development by recombination of the plasmids at
12 their respective RS_A sites (Hauschild et al., 2005; van der Lelie et al., 1989). In pLFE1 a similar
13 organization was detected where the sequence including and upstream RS_A exhibits 96% nucleotide
14 identity with a region of the *L. lactis* plasmid pK214, yet the sequence immediately downstream
15 RS_A presents no significant similarity to pK214 but 96% identity with regions of the *L. plantarum*
16 plasmids pPLA4 (van Reenen et al., 1998) and pPB1 (de las Rivas et al., 2004) (data not shown).
17 However, pLFE1 has a very unusual sequence structure amongst others with several interrupted
18 *pre/mob* regions suggesting that pLFE is the product of several relatively recent recombination
19 events. The presence of *pre/mob*$_1$ could be the result of recombination with pYS18, since a fragment
20 of 308 nucleotides including *pre/mob*$_1$ shows 100% nucleotide identity with pYS18. The similarity
21 starts with G in the putative ATG start codon and the complete identity continues ten nucleotides
22 downstream of the RS_B recombination site (data not shown).
23

1 *3.8 Host range and stability of pLFE1*

2 A filter-mating assay was used to assess the ability of pLFE1 to be mobilised to and replicate in
3 different Gram-positive bacteria. Potential pLFE1-carrying transconjugants were isolated on
4 selective agar plates from matings with *E. faecalis*, *Bacillus subtilis*, *L. rhamnosus*, *L. lactis*,
5 *Listeria monocytogenes* and *Listeria innocua* recipients (Table 3). These isolates were confirmed to
6 be true transconjugants by PCR using specific primers, RAPD fingerprinting and plasmid analysis
7 (data not shown). No transfer was detected to the recipient *B. thuringiensis* under the experimental
8 conditions applied (Table 3). However, transfer and maintenance of pLFE1 in this species cannot be
9 excluded from the present results, but may be possible under different conditions.
10 The stability of pLFE1 in its original host and the obtained transconjugants was tested. During
11 growth for 53 generations without selective pressure, pLFE1 was 100% stable in all species tested,
12 except *L. monocytogenes* where 93% of the tested cells retained the plasmid (data not shown).
13 The *in vitro* mating experiment included in this study established the ability of a broad range of
14 Gram-positive species to function as hosts of pLFE1 and to stably maintain the plasmid. An
15 important factor for the stable maintenance of RCR plasmids in a host is the presence of a
16 compatible *sso*, since lack of this will result in accumulation of single-stranded plasmid DNA (Leer
17 et al., 1992; van der Lelie et al., 1989) and subsequently segregation (Gruss and Ehrlich, 1989). The
18 efficiency of the host RNA polymerase to bind to the plasmid *sso* promoter sequence has earlier
19 been suggested as a key factor deciding the compatibility between the host and the plasmid (Kramer
20 et al., 1998; Kramer et al., 1999). In pLFE1, the observed broad host-range could imply the
21 presence of an *sso*U type of *sso*, since the other types of *sso*s seem to function optimally only in
22 their natural hosts, possibly due to a more specific binding site. In contrast, *sso*U has been isolated
23 from a broad range of Gram-positive bacteria such as *B. subtilis*, *Staphylococcus aureus*, *S.*
24 *pneumoniae* and *L. lactis* (Kramer et al., 1999).

1 Analysis of the pLFE1 nucleotide sequence failed to detect the presence of a typical *mob* gene, but
2 rather revealed a number of truncated genes or fragments coding for proteins with similarity to the
3 pMV158 family of recombination and mobilisation proteins. Mobilisation of members of this
4 family can be facilitated by a wide range of helper plasmids such as the Gram-positive conjugative
5 plasmids pIP501, pAMβ1, pLS20, pXO11, pXO12 and pAD1 as well as Gram-negative broad host-
6 range plasmids belonging to incompatibility group IncW, IncF and IncP and conjugative
7 transposons like Tn*916*, Tn*925* and Tn*1545* (for a review, see (Francia et al., 2004). However,
8 whether the *mob* genes found in pLFE1 encode functional Mob proteins and can provide the
9 relaxase activity needed to initiate plasmid transfer is uncertain and requires further study.
10 However, we suggest that the *mob* function of pLFE1 is supplied in *trans* from another plasmid
11 present in the *L. plantarum* M345 host. If a functional *mob* gene is absent in pLFE1, it significantly
12 reduces the probability of dissemination of pLFE1 from hosts other than *L. plantarum* M345.
13 Hence, in order to sustain transfer, both a *mob* gene compatible with the pMV158 type of *oriT* and
14 several *tra* genes facilitating the mating channel between the new host and recipient will be required
15 in *trans.*
16
17

1 **4. Conclusion**

2 In this paper we have analysed the nucleotide sequence of plasmid pLFE1 and found two regions
3 containing a putative double-strand origin and single-strand origin suggesting that pLFE1 replicates
4 via a rolling-circle mechanism. Furthermore, analysis of the replication region places pLFE1 in the
5 pMV158 family of RCR plasmids. We have shown that pLFE1 has a broad-host range and is stably
6 maintained in its new hosts, however the presence of a typical *mob* gene could not be confirmed.
7 Instead, several truncated genes encoding proteins with similarity to the pMV158 family of
8 Pre/Mob proteins were detected, and analysis of the Mob function in pLFE1 requires further study.
9

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14

1 **Legends to figures**

2 Fig. 1

3 Map of plasmid pLFE1 showing elements described in the text and restriction sites for enzymes
4 used for nucleotide sequence verification. (black arrows: ORFs; grey arrow: ctRNA; grey boxes:
5 *ori T*, *sso*s and *dso*s.

6

7 Fig. 2

8 Detailed DNA sequence of pLFE1 replication region. Inverted repeats (IR) are underlined and
9 proximal direct repeats (PDR) are double underlined. The RS_B-region of the putative *sso* is shown
10 in bold and the *dso* nick site is indicated by an arrow. The deduced amino acid sequences of CopG
11 and RepB are shown and putative –35 and –10 promoter boxes and ribosome binding sites (RBS)
12 are underlined with a slashed line. The start and stop codons are marked in bold as are the five
13 conserved motifs of repB. The putative promoter and terminator regions of the ctRNA are shaded.
14 Finally the five conserved motifs (see Fig. 3) of Rep proteins from the pMV158 family are marked
15 in bold.

16

17 Fig. 3

18 Multiple sequence alignment of RepB proteins belonging to Rep_2 plasmid replication proteins
19 (pfam01719) in the conserved domain database (CDD). Motifs typical of plasmid family pMV158
20 are shown according to (del Solar et al., 1993). Numbers to the left indicate the position of the
21 motifs in each plasmid.

22

23 Fig. 4

1 Multiple sequence alignment of CopG proteins belonging to RHH_1 ribbon-helix-helix CopG

2 repressor proteins (pfam01402) in the conserved domain database (CDD). The glycine residues

3 mediating the turn between the two helices are framed. Conserved positions with hydrophobic

4 residues are marked with grey, as are the highly conserved Thr/Ser residues in the turn between

5 helix A and helix B (after (Gomis-Ruth et al., 1998). Numbers to the left and right indicate the

6 aligned start and end position of the protein sequences.

7

8 Fig. 5

9 Sequence analysis of the putative *pre/mob$_2$* region of pLFE1. The deduced amino acid sequence and

10 the two conserved motifs are indicated as is the amino acid sequence corresponding to the small

11 *pre/mob* carboxyl-terminal fragment. The putative RBS is indicated in small letters and predicted –

12 10 and –35 promoter box sequences are underlined. The nucleotide sequence written in bold is the

13 potential *oriT* region, which includes the IR forming the hairpin structure and the nick

14 site, indicated in italic and with an arrow, respectively.

15

1

2 **References**

3

4 Ahrne, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A.E., Molin, G., 1998. The normal
5 Lactobacillus flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. 85, 88-94.

6 Aleshin, V.V., Semenova, E.V., Doroshenko, V.G., Jomantas, Y.V., Tarakanov, B.V., Livshits,
7 V.A., 1999. The broad host range plasmid pLF1311 from *Lactobacillus fermentum* VKM1311.
8 FEMS Microbiol. Lett. 178, 47-53.

9 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search
10 tool. J. Mol. Biol. 215, 403-410.

11 Axelsson, L.T., Ahrne, S.E., Andersson, M.C., Stahl, S.R., 1988. Identification and cloning of a
12 plasmid-encoded erythromycin resistance determinant from *Lactobacillus reuteri*. Plasmid 20, 171-
13 174.

14 Bates, E.E., Gilbert, H.J., 1989. Characterization of a cryptic plasmid from *Lactobacillus*
15 *plantarum*. Gene 85, 253-258.

16 Bouia, A., Bringel, F., Frey, L., Kammerer, B., Belarbi, A., Guyonvarch, A., Hubert, J.C., 1989.
17 Structural organization of pLP1, a cryptic plasmid from *Lactobacillus plantarum* CCM 1904.
18 Plasmid 22, 185-192.

19 Cocconcelli, P.S., Elli, M., Riboli, B., Morelli, L., 1996. Genetic analysis of the replication region
20 of the *Lactobacillus* plasmid vector pPSC22. Res. Microbiol. 147, 619-624.

1 Danielsen, M., 2002. Characterization of the tetracycline resistance plasmid pMD5057 from
2 *Lactobacillus plantarum* 5057 reveals a composite structure. Plasmid 48, 98-103.

3 de las Rivas, B., Marcobal, A., Munoz, R., 2004. Complete nucleotide sequence and structural
4 organization of pPB1, a small *Lactobacillus plantarum* cryptic plasmid that originated by modular
5 exchange. Plasmid 52, 203-211.

6 De Leener, E., Martel, A., De Graef, E.M., Top, J., Butaye, P., Haesebrouck, F., Willems, R.,
7 Decostere, A., 2005. Molecular analysis of human, porcine, and poultry *Enterococcus faecium*
8 isolates and their *erm*(B) genes. Appl. Environ. Microbiol. 71, 2766-2770.

9 del Solar, G.H., Perez-Martin, J., Espinosa, M., 1990. Plasmid pLS1-encoded RepA protein
10 regulates transcription from repAB promoter by binding to a DNA sequence containing a 13-base
11 pair symmetric element. J. Biol. Chem. 265, 12569-12575.

12 del Solar, G.H., Puyet, A., Espinosa, M., 1987. Initiation signals for the conversion of single
13 stranded to double stranded DNA forms in the streptococcal plasmid pLS1. Nucleic Acids Res. 15,
14 5561-5580.

15 del Solar, G., Acebo, P., Espinosa, M., 1995. Replication control of plasmid pLS1: efficient
16 regulation of plasmid copy number is exerted by the combined action of two plasmid components,
17 CopG and RNA II. Mol. Microbiol. 18, 913-924.

18 del Solar, G., Acebo, P., Espinosa, M., 1997. Replication control of plasmid pLS1: the antisense
19 RNA II and the compact rnaII region are involved in translational regulation of the initiator RepB
20 synthesis. Mol. Microbiol. 23, 95-108.

1 del Solar, G., Espinosa, M., 1992. The copy number of plasmid pLS1 is regulated by two trans-
2 acting plasmid products: the antisense RNA II and the repressor protein, RepA. Mol. Microbiol. 6,
3 83-94.

4 del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., Diaz-Orejas, R., 1998. Replication
5 and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev 62, 434-464.

6 del Solar, G., Hernandez-Arriaga, A.M., Gomis-Ruth, F.X., Coll, M., Espinosa, M., 2002. A
7 genetically economical family of plasmid-encoded transcriptional repressors involved in control of
8 plasmid copy number. J. Bacteriol. 184, 4943-4951.

9 del Solar, G., Moscoso, M., Espinosa, M., 1993. Rolling circle-replicating plasmids from Gram-
10 positive and Gram-negative bacteria: a wall falls. Mol. Microbiol. 8, 789-796.

11 Dyda, F., Hickman, A.B., 2003. A mob of reps. Structure 11, 1310-1311.

12 Feld, L., Schjorring, S., Hammer, K., Licht, T.R., Danielsen, M., Krogfelt, K., Wilcks, A., 2008.
13 Selective pressure affects transfer and establishment of a *Lactobacillus plantarum* resistance
14 plasmid in the gastrointestinal environment. J. Antimicrob. Chemother. 61, 845-852.

15 Fons, M., Hege, T., Ladire, M., Raibaud, P., Ducluzeau, R., Maguin, E., 1997. Isolation and
16 characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance.
17 Plasmid 37, 199-203.

18 Francia, M.V., Varsaki, A., Garcillán-Barcia, M.P., Latorre, A., Drainas, C., de la Cruz, F., 2004. A
19 classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol. Rev. 28, 79-
20 100.

1 Franke, A.E., Clewell, D.B., 1981. Evidence for a chromosome-borne resistance transposon
2 (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a
3 conjugative plasmid. J. Bacteriol. 145, 494-502.

4 Gennaro, M.L., Kornblum, J., Novick, R.P., 1987. A site-specific recombination function in
5 *Staphylococcus aureus* plasmids. J. Bacteriol. 169, 2601-2610.

6 Gevers, D., Huys, G., Swings, J., 2003. In vitro conjugal transfer of tetracycline resistance from
7 *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiol. Lett. 225, 125-130.

8 Gfeller, K.Y., Roth, M., Meile, L., Teuber, M., 2003. Sequence and genetic organization of the
9 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum*
10 ROT1. Plasmid 50, 190-201.

11 Gomis-Ruth, F.X., Sola, M., Acebo, P., Parraga, A., Guasch, A., Eritja, R., Gonzalez, A., Espinosa,
12 M., del, S.G., Coll, M., 1998. The structure of plasmid-encoded transcriptional repressor CopG
13 unliganded and bound to its operator. EMBO J. 17, 7404-7415.

14 Gruss, A., Ehrlich, S.D., 1989. The family of highly interrelated single-stranded deoxyribonucleic
15 acid plasmids. Microbiol. Rev. 53, 231-241.

16 Guzman, L.M., Espinosa, M., 1997. The mobilization protein, MobM, of the streptococcal plasmid
17 pMV158 specifically cleaves supercoiled DNA at the plasmid *oriT*. J. Mol. Biol. 266, 688-702.

18 Hauschild, T., Luthje, P., Schwarz, S., 2005. Staphylococcal tetracycline-MLSB resistance plasmid
19 pSTE2 is the product of an RSA-mediated in vivo recombination. J. Antimicrob. Chemother. 56,
20 399-402.

1 Ilyina, T.V., Koonin, E.V., 1992. Conserved sequence motifs in the initiator proteins for rolling
2 circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and
3 archaebacteria. Nucleic Acids Res. 20, 3279-3285.

4 Jacobsen, L., Wilcks, A., Hammer, K., Huys, G., Gevers, D., Andersen, S.R., 2007. Horizontal
5 transfer of *tet*(M) and *erm*(B) resistance plasmids from food strains of *Lactobacillus plantarum* to
6 *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnotobiotic rats. FEMS Microbiol. Ecol.
7 59, 158-166.

8 Jensen, G.B., Wilcks, A., Petersen, S.S., Damgaard, J., Baum, J.A., Andrup, L., 1995. The genetic
9 basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large
10 conjugative plasmid pXO16. J. Bacteriol. 177, 2914-2917.

11 Jensen, L.B., Frimodt-Moller, N., Aarestrup, F.M., 1999. Presence of *erm* gene classes in gram-
12 positive bacteria of animal and human origin in Denmark. FEMS Microbiol. Lett. 170, 151-158.

13 Johansson, M.L., Molin, G., Jeppsson, B., Nobaek, S., Ahrne, S., Bengmark, S., 1993.
14 Administration of different *Lactobacillus strains* in fermented oatmeal soup: *in vivo* colonization of
15 human intestinal mucosa and effect on the indigenous flora. Appl. Environ. Microbiol. 59, 15-20.

16 Josson, K., Soetaert, P., Michiels, F., Joos, H., Mahillon, J., 1990. *Lactobacillus hilgardii* plasmid
17 pLAB1000 consists of two functional cassettes commonly found in other gram-positive organisms.
18 J. Bacteriol. 172, 3089-3099.

19 Khan, S.A., 1997. Rolling-circle replication of bacterial plasmids. Microbiol. Mol. Biol. Rev 61,
20 442-455.

1 Kleerebezem, M., Boekhorst, J., van, K.R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R.,
2 Peters, S.A., Sandbrink, H.M., Fiers, M.W., Stiekema, W., Lankhorst, R.M., Bron, P.A., Hoffer,
3 S.M., Groot, M.N., Kerkhoven, R., de, V.M., Ursing, B., de Vos, W.M., Siezen, R.J., 2003.
4 Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci., 1990-
5 1995.

6 Kramer, M.G., Espinosa, M., Misra, T.K., Khan, S.A., 1998. Lagging strand replication of rolling-
7 circle plasmids: specific recognition of the *ssoA*-type origins in different gram-positive bacteria.
8 Proc. Natl. Acad. Sci. 95, 10505-10510.

9 Kramer, M.G., Espinosa, M., Misra, T.K., Khan, S.A., 1999. Characterization of a single-strand
10 origin, ssoU, required for broad host range replication of rolling-circle plasmids. Mol. Microbiol.
11 33, 466-475.

12 Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H.,
13 Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G.,
14 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

15 Leer, R.J., van, L.N., Posno, M., Pouwels, P.H., 1992. Structural and functional analysis of two
16 cryptic plasmids from *Lactobacillus pentosus* MD353 and *Lactobacillus plantarum* ATCC 8014.
17 Mol. Gen. Genet. 234, 265-274.

18 Lin, C.F., Fung, Z.F., Wu, C.L., Chung, T.C., 1996. Molecular characterization of a plasmid-borne
19 (pTC82) chloramphenicol resistance determinant (*cat-TC*) from *Lactobacillus reuteri* G4. Plasmid
20 36, 116-124.

1 Marchler-Bauer, A., Anderson, J.B., Derbyshire, M.K., Weese-Scott, C., Gonzales, N.R., Gwadz,
2 M., Hao, L., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Krylov, D., Lanczycki, C.J., Liebert, C.A.,
3 Liu, C., Lu, F., Lu, S., Marchler, G.H., Mullokandov, M., Song, J.S., Thanki, N., Yamashita, R.A.,
4 Yin, J.J., Zhang, D., Bryant, S.H., 2007. CDD: a conserved domain database for interactive domain
5 family analysis. Nucleic Acids Res. 35, D237-D240.

6 Min, Y.H., Kwon, A.R., Yoon, E.J., Shim, M.J., Choi, E.C., 2008a. Translational attenuation and
7 mRNA stabilization as mechanisms of *erm*(B) induction by erythromycin. Antimicrob. Agents
8 Chemother. 52, 1782-1789.

9 Min, Y.H., Kwon, A.R., Yoon, J.M., Yoon, E.J., Shim, M.J., Choi, E.C., 2008b. Molecular analysis
10 of constitutive mutations in *ermB* and *ermA* selected in vitro from inducibly MLSB-resistant
11 enterococci. Arch. Pharm. Res. 31, 377-380.

12 Moscoso, M., del Solar, G., Espinosa, M., 1995. In vitro recognition of the replication origin of
13 pLS1 and of plasmids of the pLS1 family by the RepB initiator protein. J. Bacteriol. 177, 7041-
14 7049.
15
16 Neve, H., Geis, A., Teuber, M., 1984. Conjugal transfer and characterization of bacteriocin
17 plasmids in group N (lactic acid) streptococci. J. Bacteriol. 157, 833-838.

18 O'Connor, E.B., O'Sullivan, O., Stanton, C., Danielsen, M., Simpson, P.J., Callanan, M.J., Ross,
19 R.P., Hill, C., 2007. pEOC01: a plasmid from *Pediococcus acidilactici* which encodes an identical
20 streptomycin resistance (*aadE*) gene to that found in *Campylobacter jejuni*. Plasmid 58, 115-126.

21 Oh, T.G., Kwon, A.R., Choi, E.C., 1998. Induction of *ermAMR* from a clinical strain of
22 *Enterococcus faecalis* by 16-membered-ring macrolide antibiotics. J. Bacteriol. 180, 5788-5791.

1 Okitsu, N., Kaieda, S., Yano, H., Nakano, R., Hosaka, Y., Okamoto, R., Kobayashi, T., Inoue, M.,
2 2005. Characterization of *ermB* gene transposition by Tn*1545* and Tn*917* in macrolide-resistant
3 *Streptococcus pneumoniae* isolates. J. Clin. Microbiol. 43, 168-173.

4 Pillidge, C.J., Cambourn, W.M., Pearce, L.E., 1996. Nucleotide sequence and analysis of pWC1, a
5 pC194-type rolling circle replicon in *Lactococcus lactis*. Plasmid 35, 131-140.

6 Pouwels, P.H., Leer, R.J., 1993. Genetics of lactobacilli: plasmids and gene expression. Antonie
7 Van Leeuwenhoek 64, 85-107.

8 Priebe, S.D., Lacks, S.A., 1989. Region of the streptococcal plasmid pMV158 required for
9 conjugative mobilization. J. Bacteriol. 171, 4778-4784.

10 Raha, A.R., Hooi, W.Y., Mariana, N.S., Radu, S., Varma, N.R., Yusoff, K., 2006. DNA sequence
11 analysis of a small cryptic plasmid from *Lactococcus lactis* subsp. *lactis* M14. Plasmid 56, 53-61.

12 Roberts, M.C., Sutcliffe, J., Courvalin, P., Jensen, L.B., Rood, J., Seppala, H., 1999. Nomenclature
13 for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob.
14 Agents Chemother. 43, 2823-2830.

15 Ruiz-Masó, J.A., Lurz, R., Espinosa, M., del Solar, G., 2007. Interactions between the RepB
16 initiator protein of plasmid pMV158 and two distant DNA regions within the origin of replication.
17 Nucleic Acids Res. 35, 1230-1244.

18 Sanchez, C., Mayo, B., 2003. Sequence and analysis of pBM02, a novel RCR cryptic plasmid from
19 *Lactococcus lactis* subsp *cremoris* P8-2-47. Plasmid 49, 118-129.

1 Selinger, L.B., McGregor, N.F., Khachatourians, G.G., Hynes, M.F., 1990. Mobilization of closely
2 related plasmids pUB110 and pBC16 by Bacillus plasmid pXO503 requires trans-acting open
3 reading frame beta. J. Bacteriol. 172, 3290-3297.

4 Shaw, J.H., Clewell, D.B., 1985. Complete nucleotide sequence of macrolide-lincosamide-
5 streptogramin B-resistance transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. 164, 782-796.

6 Skaugen, M., 1989. The complete nucleotide sequence of a small cryptic plasmid from
7 *Lactobacillus plantarum*. Plasmid 22, 175-179.

8 Somkuti, G.A., Steinberg, D.H., 2007. Molecular organization of plasmid pER13 in *Streptococcus*
9 *thermophilus*. Biotechnol. Lett. 29, 1991-1999.

10 Sorvig, E., Skaugen, M., Naterstad, K., Eijsink, V.G., Axelsson, L., 2005. Plasmid p256 from
11 *Lactobacillus plantarum* represents a new type of replicon in lactic acid bacteria, and contains a
12 toxin-antitoxin-like plasmid maintenance system. Microbiology 151, 421-431.

13 Tannock, G.W., Luchansky, J.B., Miller, L., Connell, H., Thode-Andersen, S., Mercer, A.A.,
14 Klaenhammer, T.R., 1994. Molecular characterization of a plasmid-borne (pGT633) erythromycin
15 resistance determinant (*ermGT*) from *Lactobacillus reuteri* 100-63. Plasmid 31, 60-71.

16 Tomich, P.K., An, F.Y., Clewell, D.B., 1980. Properties of erythromycin-inducible transposon
17 Tn*917* in *Streptococcus faecalis*. J. Bacteriol. 141, 1366-1374.

18 Tosi, L., Berruti, G., Danielsen, M., Wind, A., Huys, G., Morelli, L., 2007. Susceptibility of
19 *Streptococcus thermophilus* to antibiotics. Antonie Van Leeuwenhoek 92, 21-28.

1 van der Lelie, D., Bron, S., Venema, G., Oskam, L., 1989. Similarity of minus origins of replication
2 and flanking open reading frames of plasmids pUB110, pTB913 and pMV158. Nucleic Acids Res.
3 17, 7283-7294.

4 van der Lelie, D., Wosten, H.A., Bron, S., Oskam, L., Venema, G., 1990. Conjugal mobilization of
5 streptococcal plasmid pMV158 between strains of *Lactococcus lactis* subsp. *lactis*. J. Bacteriol.
6 172, 47-52.

7 van Reenen, C.A., Dicks, L.M., Chikindas, M.L., 1998. Isolation, purification and partial
8 characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. J. Appl.
9 Microbiol. 84, 1131-1137.

10 van Kranenburg, R., Golic, N., Bongers, R., Leer, R.J., de Vos, W.M., Siezen, R.J., Kleerebezem,
11 M., 2005. Functional analysis of three plasmids from *Lactobacillus plantarum*. Appl. Environ.
12 Microbiol. 71, 1223-1230.

13 Vescovo, M., Morelli, L., Bottazzi, V., 1982. Drug resistance plasmids in *Lactobacillus acidophilus*
14 and *Lactobacillus reuteri*. Appl. Environ. Microbiol. 43, 50-56.

15 Vicente, M.F., Baquero, F., Pérez-Díaz, J.C., 1985. Cloning and expression of the *Listeria*
16 *monocytogenes* haemolysin in *E. coli*. FEMS Microbiol. Lett. 30, 77-79.

17 Vujcic, M., Topisirovic, L., 1993. Molecular analysis of the rolling-circle replicating plasmid pA1
18 of *Lactobacillus plantarum* A112. Appl. Environ. Microbiol. 59, 274-280.

19 Weisblum, B., 1995a. Erythromycin resistance by ribosome modification. Antimicrob. Agents
20 Chemother. 39, 577-585.

1 Weisblum, B., 1995b. Insights into erythromycin action from studies of its activity as inducer of
2 resistance. Antimicrob. Agents Chemother. 39, 797-805.

3 Wu, E., Jun, L., Yuan, Y., Yan, J., Berry, C., Yuan, Z., 2007. Characterization of a cryptic plasmid
4 from *Bacillus sphaericus* strain LP1-G. Plasmid 57, 296-305.

5 Yin, S., Hao, Y., Zhai, Z., Li, R., Huang, Y., Tian, H., Luo, Y., 2008. Characterization of a cryptic
6 plasmid pM4 from *Lactobacillus plantarum* M4. FEMS Microbiol. Lett. 285, 183-187.

7 Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic
8 Acids Res. 31, 3406-3415.

9

Table 1

Strains used

Strains	Characteristics*	Source or reference
Lactobacillus plantarum M345	Plasmid pLFE1, *erm*(B)	Feld et al., 2008
Enterococcus faecalis OG1SS	Str^{r}, spec^{r}	Franke and Clewell, 1981
E. faecalis JH2SS	Str^{r}, spec^{r}	Tomich et al., 1980
Lactobacillus rhamnosus VTT E-042730	Tet^{r}	VTT
Lactococcus lactis BU2-60	Str^{r}, Rif^{r}	Neve et al., 1984
Listeria monocytogenes LO28	Nal^{r}	Vicente et al., 1985
Listeria innocua DSM 20649	Nal^{r}	DSMZ
Bacillus subtilis AW59	Str^{r}	Wilcks, A., unpublished
Bacillus thuringiensis GBJ01	Str^{r}	Jensen et al., 1995

* The r indicates resistance to the following antibiotics streptomycin (str), spectinomycin (spec), tetracycline (tet), rifampicin (rif), nalidixic acid (nal). DSMZ: German Collection of Microorganisms and Cell Cultures. VTT Culture Collection, Finland.

Table 2

Description of putative ORFs in pLFE1. ORFs marked in bold have a high probability of translation as predicted from RBS sites and potential translational coupling.

Name	strand	Size (aa)	Position (bp)	RBS	% aa identity	Best BLAST match	Accession no.
***Erm*(B) leader peptide**	**+**	**27**	**417-500**	**GGAGG**	**100 (27 aa)**	**MLS leader peptide, *Streptococcus pneumoniae*, transposon Tn6003**	**AM410044**
***Erm*(B)**	**+**	**245**	**625-1362**	**GGAGA**	**100 (245 aa)**	**ErmB, Erythromycin ribosome methylase of *E. faecium* clone 1**	**AAX12187**
ORF 3	+	34	1166-1270	–		no significant similarity	
ORF 4	+	64	1307-1501	GTACC	73 (34 aa)	hypothetical protein, *Streptococcus cristatus*	**AAY63933**
pre/mob$_1$	+	73	1384-1605	–	100 (72 aa)	Mob, mobilization protein of *Lactobacillus sakei* plasmid pYS18	**ABW71679**
CopG	**+**	**59**	**2118-2297**	**AGAGA**	**70 (51 aa)**	**RepA, plasmid copy number control protein of *L. plantarum* RCR plasmid pLB4**	**P20044**

ORF 7	+	42	2272-2400	CAAGC		no significant similarity	
RepB	**+**	**212**	**2421-3059**	**GGAAG**	**80 (211 aa)**	**RepB, replication initiation protein of *L. plantarum* RCR plasmid pLB4**	**P20045**
ORF 9	+	33	3077-3178	–		no significant similarity	
pre/mob$_2$	**+**	**83**	**3312-3563**	**AGGAG**	**95 (83 aa)**	**Mob, mobilization protein of *L. lactis* subsp. *lactis* plasmid pK214**	**YP_001429536**
ORF 11	+	33	3442-3543	–		no significant similarity	
ORF 12	+	35	3800-3907	AGCGT		no significant similarity	
ORF 13	–	59	338-159	TAAGA	31 (61 aa)	C-terminal domain containing protein, *Tetrahymena termophila*	**XP_001013113**
ORF 14	–	72	1560-1342	_	33 (56 aa)	hypothetical protein, *Paramecium tetraurelia*	**XP_001449055**
ORF 15	–	41	1735-	GCAAG		no significant similarity	

			1610				
ORF 16	–	36	1763-1653	GCAAA		no significant similarity	
ORF 17	–	65	3176-2979	AGCGC	34 (58 aa)	rCG54873, isoform CRA_a, *Rattus norvegicus*	**EDL98520**

Table 3

Mobilisation of pLFE1 from *Lactobacillus plantarum* M345 to various Gram-positive recipients

Recipient	Transfer frequency* (Transconjugants/recipient)
E. faecalis JH2SS	9.8×10^{-9} (1.3×10^{-8})
E. faecalis OGISS	1.6×10^{-7} (1.0×10^{-7})
L. lactis BU2-60	2.4×10^{-8} (9.2×10^{-10})
B. subtilis AW59	3.4×10^{-9} (3.4×10^{-9})
B. thuringiensis GBJ01	$<1.0 \times 10^{-9}$
Listeria innocua BSM 20649	7.1×10^{-8} (2.5×10^{-8})
Listeria monocytogenes LO28	3.6×10^{-6} (2.8×10^{-6})

* Results are average from triplicates and standard deviations are given in brackets.

Figure 1

Figure 1

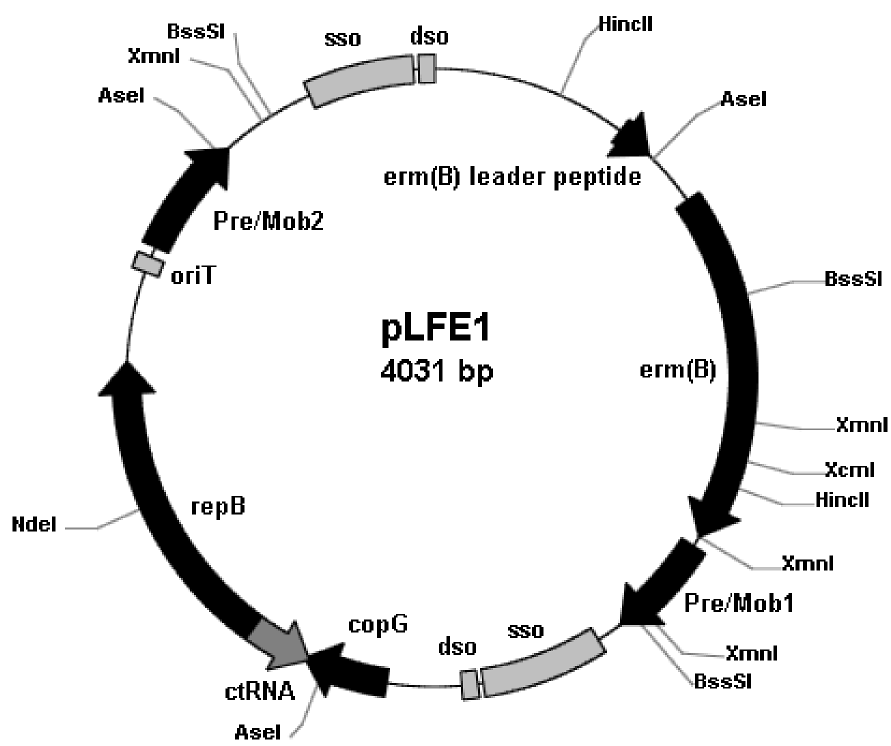

Figure 2

```
1561   AGAAATGAAACTCGAACACGAGATAGAGATGAATTAAGTCGTTAGAAGCTCATATTTGCG

                                                                RS_B
1621   TTTTAAGGCGTTTTAAATCAATTCGAGTATATTTATACCTGTTTGGTCTTTTCGTCGGCA

                       IR1
1681   TAAGCGTTAAAAATGGCAATAGAAAAAGCAGCAAGGGCGAACGTAGTGAGTGCATTTACC

                       IR1
1741   CTTGCTGCTTTTTTTGTTGTCATTTAGCTTTTGCATAGACGACGGAAAGCCAACAGGTGT

                                                IR2       IR2          IR3
1801   GAATGTTTAGCTACTTTTCAGAGGCATGATCAAACGCATTGCGTTTGTGGGTGTGGGCAA

         IR3                                                     IR4
1861   CGCCCGCGGTTTTATTAGTTTTTAGGAATTGGGAAGCAAACTGGGGGGAGATTTTGAAAA

         IR4           IR5         nick       IR5           PDR
1921   AATCGACCAACTCATGGGGGCACTACGACACCCCCCATGTGTCCATTGTCCATTGTCCAA
                                     ↑
1981   ATAGATAAAAAGATTAAATGTCTTGTAAATACCTTTATATGAACGTTTTAAGCACCTATT

                                               -35                 -10
2041   TCGAGGTGAAAAAAAGTTTTTTGTAAAATCTTGTATTCCTGTCGTATGTATAATATAATT

                    RBS    CopG
2101   ACAGTGTAAAAGAGATTTTGAAGAGAATTGAGGTGTATTTTGTGGTTGAAGTTGAGAAGA
                              M  K  R  I  E  V  Y  F  V  V  E  V  E  K

2161   AAAAAGTGACTTTATCTTTACCTGTTGAATCGAATGACAAACTAGAAAAAATGGCACAAA
       K  K  V  T  L  S  L  P  V  E  S  N  D  K  L  E  K  M  A  Q

2221   AATATGGAATGACTAAATCAGGTTTGGTTACATTTTTAATTAATCAAGCTGATGATAAGG
       K  Y  G  M  T  K  S  G  L  V  T  F  L  I  N  Q  A  D  D  K
                                IR6           IR6
2281   GGACTATTTTTAAATAAAAAAAGCCCTGCTAGGAAGATAGCAAGGGCTCGCAAGATTTGG
       G  T  I  F  K  *

2341   TTTTCTTTGATTTCGCAACTCAAGTTTATCAAATTTTCCCTTGTTTTCCTAGTAGAGTAA

          RBS              repB     -10                      -35
2401   CTAGGAAGGTGTAGAAAAATATGGAAAATGAAAATAGAACAGATTGGACATTGCCTAGAA
                                 M  E  N  E  N  R  T  D  W  T  L  P  R

2461   AGAATTTAAATCCAAAAACTAAACAGCCTTATAAGCGAGGTCGTAACTGGTGGATTGTTG
       K  N  L  N  P  K  T  K  Q  P  Y  K  R  G  R  N  W  W  I  V

2521   TTTATCCTGAAAGTTTGCCAGAGAATTGGAAAGAGATTATTTCAACTGAACCTGTGGCAA
       V  Y  P  E  S  L  P  E  N  W  K  E  I  I  S  T  E  P  V  A

2581   TTAGTCCATTACATGATAAAGATGTTAATGCAGACGGAACAAAGAAAAAGCCGCATTATC
       I  S  P  L  H  D  K  D  V  N  A  D  G  T  K  K  K  P  H  Y

2641   ATATAGTTTTTAATTACAAGGGCAATAAATCATTTGAACAAATGGACGAAATGGCAAGAG
```

```
      H I V F N Y K G N K S F E Q M D E M A R

2701  CTTTAAGGGCACCGATTCCTGAAAGAATAAGTGGTTTAACTGGTGCTGTTAGATATTTAA
      A L R A P I P E R I S G L T G A V R Y L

2761  CGCATATGGATAATCCTGAAAAGTATCAATACGATAATACAGAAATACAAGTGTTTGGTG
      T H M D N P E K Y Q Y D N T E I Q V F G

2821  GATTTGACCTTGAAAGTTGTTTAGCGTTATCTACTGGTGATAAAAGACAAGCGTTAAAAG
      G F D L E S C L A L S T G D K R Q A L K

2881  AAATGCTTGGTTTTATTTCGGATAACAATATTATGCATTTAAAAGATTTTGCTGATTATT
      E M L G F I S D N N I M H L K D F A D Y

2941  GTATGTCTGACCGAGCTCCTGCTGGTTGGTTCGAATTGCTAACAGAGAGGAATACTCTTT
      C M S D R A P A G W F E L L T E R N T L

3001  TTATAAAAGAGTACATAAAATCGAATTGGCAAAAAGAAAACCAAGTTTATAAAGAGTGAG
      F I K E Y I K S N W Q K E N Q V Y K E *
```

Figure 3

Figure 3

	Motif R-I		Motif R-II		Motif R-III		Motif R-IV		Motif R-V	
pLFE1	29	WWIVVYPESL	52	AISPLHDKD	68	KKPHYHIVFNY	102	ISGLTGAVRYLTH-MDN	121	YDN
pLB4	29	WGIVVYPESL	52	AVSPLHDKD	68	KKSHYHLVLNY	102	ISSLTGAVRYLTH-MDN	123	YDN
pWV01	10	FGFLLYPDSI	35	AVSPLHDMD	64	KKPHYHVI--Y	102	LDYIKGSYEYLTHESKD	127	YDK
pCI411	19	WSWIVYPESA	42	IESPLHDKD	59	KKPHWHII-IS	93	VASLQGSVQYLWHR-NN	114	YDK
pLA106	13	WTFVLYPESA	38	VESPLHDKD	54	KKPHWHIA-MF	94	IENAKGMIRYFAH-MDN	107	YQY
pE194	19	WTFVLYPESA	44	ALSPLHDKD	60	KKEHYHILVMY	95	AGSVKGLVRYMLH-MDD	115	YQK
pHPK255	27	FGFIIYPESA	53	ALSPLHDKD	69	KKPHFHAI-IV	107	NERVKGAYEYFTH-SNP	130	YDK
pPSC22	10	FGFLLYPDSI	35	AVSPLHDMD	64	KKPHYHVI--Y	102	LDYIKGSYEYLTHESKD	127	YDP
pLS1	8	FTFLLYPESI	33	AISPLHDKD	51	KKAHYHVL--Y	89	VLNVENMYLYLTHESKD	114	YDK
pJB01	8	FTFLLYPESI	33	AVSPLHDKD	51	KKAHYHVI--Y	90	STSMENMYLYLTHESKD	115	YSK

Figure 4

Figure 4

```
                         strand I           helix A         turn      helix B

pLFE1-copG      12  VEKKKVTLSLPVESNDKLEKMAQKY GMT KSGLVTFLINQADDKG 63
pLB4-repA        4  VEKKKITLSIPVETNGKLEELAQKY GMT KSGLVNFLVNQVAEAG 55
pMV158-copG      1   MKKRLTITLSESVLENLEKMAREM GLS KSAMISVALENYKKGQ 51
pHD2-ORFB        1    MVRVNTRISKKLNDWLDEYSKES GVP KSTLVHLALENYVNQK 50
pCB101-repC      1     MRVNISIPDEVKQFFEDYSKKT GVP QSSLMALALSEYKDKI 49
pFX2-copX        5  ESKKRVMISLTKEQDKKLTDMAKQK GFS KSAVAALAIEEYA-RK 57
pHT926-unknown   5  QERERMQIRLSKTNMQRLQDMAGRY GMS ANSLVSYILGQWLDNN 56
pRN2-copG        3  STKPNVHIRLREEERKLLKEIAQKY DIS ESDVVKIALKKLAREL 54
pE194-cop6       8  EKKVAVTLRLTTEENEILNRIKEKY NIS KSDATGILIKKYAKEE 59
pA1-repA         1  MERVKVGITLTEDTLARLEEICKEM GLS KSQALSMLVNKEYLEK 52
pLA106-repA      2  TEKKRLTVSFSHKIANQLEELAKDQ GLT KSGLLTVLISKEIERK 53
```

Figure 5

1 Figure 5

2

```
        -35           .      ↓ . -10      .         .         .
3241 CCACCTTTACGAAGTAAAGTATAGTGGGTTATACTTTGCATGGAAGCTGTCCCGAAGTTa
     ============================================================
     GGTGGAAATGCTTCATTTCATATCACCCAATATGAAACGTACCTTCGACAGGGCTTCAAT

               .         .         .         .         .         .
3301 ggagTGTTTATATGTCATTTGTAGTGGCGAGAATGCAGAAGGTAAAATCAGGAAATTTAG
                 M  S  F  V  V  A  R  M  Q  K  V  K  S  G  N  L
     ============================================================
     CCTCACAAATATACAGTAAACATCACCGCTCTTACGTCTTCCATTTTAGTCCTTTAAATC

               .      Motif I       .         .         .         .
3361 TTGGGGTAGGTAATCATAATCAGAGAAATACAGACAATCATTCCAACAAAGATATTGATG
     V  G  V  G  N  H  N  Q  R  N  T  D  N  H  S  N  K  D  I  D
     ============================================================
     AACCCCATCCATTAGTATTAGTCTCTTTATGTCTGTTAGTAAGGTTGTTTCTATAACTAC

               .         Motif II .         .         .         .
3421 TTGAACGGTCACATTTAAATTATGATTTGGTCAATCGGACAGAAAATTATAAACGAGATA
     V  E  R  S  H  L  N  Y  D  L  V  N  R  T  E  N  Y  K  R  D
     ============================================================
     AACTTGCCAGTGTAAATTTAATACTAAACCAGTTAGCCTGTCTTTTAATATTTGCTCTAT

               .         .         .         .         .         .
3481 TTGAGCAATTTATTAACGACAACAAATCAAGTAGTCGTGCTGTCAGAAAAGATGCTGTAT
     I  E  Q  F  I  N  D  N  K  S  S  S  R  A  V  R  K  D  A  V
     ============================================================
     AACTCGTTAAATAATTGCTGTTGTTTAGTTCATCAGCACGACAGTCTTTTCTACGACATA

               .         .         .         .         .         .
     TAATAAACGAAAGAGTACTTTAAAGATTCTAAGCAAGTTACAACTGAACAGGTTAAAACG
3541 L  I  N  E  R  V  L  *
     ============================================================
     ATTATTTGCTTTCTCATGAAATTTCTAAGATTCGTTCAATGTTGACTTGTCCAATTTTGC

               .         .         .         .         .         .
     CTTGTTAGTGGCGTTTTAGACAAAGTAAAAGAATTTGTAAAAGGTGGAGAATTTGAAAAA
3601                           V  K  E  F  V  K  G  G  E  F  E  K
     ============================================================
     GAACAATCACCGCAAAATCTGTTTCATTTTCTTAAACATTTTCCACCTCTTAAACTTTTT

               .         .         .         .         .         .
     ATTCATAGAAATGAAACTCAAACACGAGATAGAGATGAATTAAGTCGTTAGAAGCTCATA
3661  I  H  R  N  E  T  Q  T  R  D  R  D  E  L  S  R  *
     ============================================================
     TAAGTATCTTTACTTTGAGTTTGTGCTCTATCTCTACTTAATTCAGCAATCTTCGAGTAT
```

3