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Characterization of a Region of Plasmid pBL1 of *Brevibacterium lactofermentum* Involved in Replication via the Rolling Circle Model

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The minimal region for autonomous replication of pBL1, a 4.5-kb cryptic plasmid of *Brevibacterium lactofermentum* ATCC 13869 that has been used to construct a variety of corynebacterium vectors, was shown to be contained on a 1.8-kb *Hin*dII-*Sph*I DNA fragment. This region contains two open reading frames (ORFs) (ORF1 and ORF5) which are essential for pBL1 replication in *B. lactofermentum*. Accumulation of single-strand intermediates in some of the constructions indicates that plasmid pBL1 replicates via the rolling circle replication model; its plus strand and minus strand were identified by hybridization with two synthetic oligonucleotide probes complementary to each pBL1 strand. ORF1 seems to encode the Rep protein and showed partial homology with sequences for Rep proteins from *Streptomyces* plasmids which replicate via rolling circle replication such as pIJ101, pSB24, and pJV1.

pBL1 is a multicopy plasmid isolated from *Brevibacterium lactofermentum* ATCC 13869 (19) and later described as pAM330 (14), pWS101 (26), pEX1910 (22), and pX19 (25). An apparently identical plasmid, pBL70 (18), was reported by Sandoval et al. from a different *B. lactofermentum* CECT70 isolate. Plasmids pBL1 and pAM330 and the cryptic plasmid described by Filpula et al. (6) have the same restriction maps, and their nucleotide sequences are almost identical. Plasmid pBL1 is one of the most frequently used coryneform replicons for construction of cloning vectors, shuttle vectors, or promoter-probe vectors (3, 13, 21, 22, 25). In our laboratory, several antibiotic resistance genes have been successfully used for developing a pBL1-based cloning system for *B. lactofermentum*: the kanamycin resistance gene (*kan*) from Tn*5*, the hygromycin resistance gene (*hyg*) from *Streptomyces hygroscopicus*, and the chloramphenicol resistance gene (*cat*) from *Streptomyces acrimycini* (20, 21). Cadenas et al. (2) described the efficient expression in *B. lactofermentum* in pBL1-derived vectors of a heterologous gene coding for an α-amylase from *Streptomyces griseus*. Other groups have described the use of *Escherichia coli* and *Bacillus subtilis* phenotypic markers in corynebacteria (14, 16, 22).

Only a few aspects of the molecular biology of the corynebacterial plasmids have been described. Santamaría et al. (19) and Yeh et al. (25) suggested that the *Bcl*I site of pBL1 is in a region essential for plasmid replication. Yamaguchi et al. (24) proposed a relationship between ORF1 and the replication functions of pBL1 by analyzing its nucleotide sequence. Trautwetter and Blanco (23) and Archer and Sinskey (1a) described the minimal regions for autonomous replication of *Corynebacterium glutamicum* plasmids pCG100 and pSR1, respectively.

Some pBL1 derivatives are known to be unstable. Santamaría et al. (20) described the construction of plasmid pUL61, able to replicate in *E. coli*, *B. lactofermentum*, and *Streptomyces lividans*. pUL61 was unstable in *B. lactofermentum*, and two stable deletion derivatives (pUL330 and pUL340) appeared after *B. lactofermentum* had been transformed with pUL61. A similar instability has also been observed in most other small plasmids from gram-positive bacteria. The main characteristic of plasmids showing instability is their mode of replication via the rolling circle replication (RCR) mechanism. RCR plasmids were classified into four families according to homologies of their replication proteins (Rep) and the position of the double-stranded origin (DSO) (7). RCR plasmids have the information to synthesize their own Rep protein. The Rep protein initiates replication by nicking the DNA at the DSO sequence, after which replication of the plus strand begins. The newly generated single-stranded DNA (ssDNA) intermediates (plus strand) serve as a template for minus strand synthesis. The conversion of the ssDNA intermediates to duplex forms requires the recognition of another sequence, different and separated from the DSO sequence, called single-stranded origin (SSO). The absence or nonfunctionality of SSO results in the accumulation of ssDNA. The SSO sequence is rather specific, and it is usually recognized inefficiently in hosts different from the parental one (7, 15).

We describe in this paper the identification and characterization of the region involved in replication functions of pBL1. Our results indicate that pBL1 belongs to the group of plasmids which replicate via the RCR mechanism.

MATERIALS AND METHODS

Plasmids, bacterial strains, and transformation conditions. Plasmids used in this study are listed in Table 1. *B. lactofermentum* BL31 (20), a pBL1-cured strain, and *C. glutamicum* ATCC 13032 were grown in Trypticase soy broth (TSB) medium and transformed by electroporation as described by Dunican and Shivnan (5). *E. coli* DH5α was grown in L broth and transformed by the method of Hanahan (8). *Rhodococcus*

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TABLE 1. Plasmids used in this study

Plasmid(s)	Description	Reference and/or figure in text
pBL1	4.5-kb cryptic plasmid isolated from *B. lactofermentum* ATCC 13869	19, Fig. 1
pIJ2921	pUC18 derivative	10, Fig. 1
pULMJ8	pBR322 derivative containing the *kan* gene from Tn*5*	Fig. 1
pULMJ20	pIJ2921 derivative with *kan* instead *bla*	Fig. 1
pULMJ201 and pULMJ202	pBL1 (*Hin*dIII) in pULMJ20 *Hin*dIII site (two orientations)	Fig. 1
pULMJ207	3.3-kb *Hin*dIII (filled-in)-*Sca*I* fragment of pBL1 in pULMJ20 *Hin*dII site	Fig. 2
pULMJ208	3.5-kb *Sph*I*-*Hin*dIII (filled-in) fragment of pBL1 in pULMJ20 *Hin*dII-*Sph*I sites	Fig. 2 and 5
pULMJ209	2.4-kb *Sca*I*-*Sph*I* fragment of pBL1 in pULMJ20 *Hin*dIII-*Sph*I sites	Fig. 2
pULMJ203	1.8-kb *Hin*dII*-*Sph*I* fragment of pBL1 in pULMJ20 *Hin*dII-*Sph*I sites	Fig. 2
pULMJ204	1.5-kb *Sph*I*-*Xba*I fragment of pBL1 in pULMJ20 *Sph*I-*Xba*I sites	Fig. 2
pULMJ205	1.4-kb *Fsp*I-*Hin*dII* fragment of pBL1 in pULMJ20 *Hin*dII site	Fig. 2
pULMJ206	1.1-kb *Bst*EII (filled-in)-*Hin*dII* fragment of pBL1 in pULMJ20 *Hin*dII site	Fig. 2
pULMJ600	1.4-kb *Hin*dII fragment of pULMJ8 containing the *kan* gene from Tn*5* replacing the small *Sca*I fragment of pBL1	Fig. 2
pULCF200	1.4-kb *Hin*dII fragment of pULMJ8 (*kan* gene) replacing the 1.7-kb *Hin*dII fragment of pBL1	Fig. 2
pULCF280	1.4-kb *Hin*dII fragment of pULMJ8 (*kan* gene) in pBL1 *Sph*I* (filled-in)-*Hin*dIII (filled-in) sites	Fig. 2
pULCF230	1.4-kb *Hin*dII fragment of pULMJ8 (*kan* gene) in pBL1 *Hin*dII*-*Sph*I (filled-in) sites	Fig. 2 and 5
pULCF21	Deletion derivative of pULMJ203 containing ORF1 and ORF5	Fig. 5
pULCF23	1.4-kb *Hin*dII fragment of pULMJ8 (*kan* gene) in pULCF21 *Hin*dII site	Fig. 5

fascians D1885 was transformed as described by Crespi et al. (4).

Nocardia lactamdurans LC411 was transformed with pBL1 or pBL1 derivatives by a specific procedure developed for this strain (11a).

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* according to the method of Birnboim and Doly (1b) and from corynebacteria and nocardioform bacteria as described by Kieser (11). For the isolation of single-stranded plasmid DNA, cells were grown in TSB medium to an optical density at 595 nm of 1.5 to 2. Ten milliliters of culture was harvested, and the cells were resuspended in lysis buffer (0.3 M sucrose, 25 mM Tris [pH 8], 25 mM EDTA) containing 10 mg of lysozyme per ml. After 1 h of incubation at 37°C, 25 μl of 20% sodium dodecyl sulfate (SDS) was added and the sample was mixed by a vortex for 1 min and extracted with neutral phenol and chloroform-isoamyl alcohol.

For nuclease S1 digestion, 10 μl of 10× S1 buffer (50 mM Na acetate [pH 4.7], 3 M NaCl, and 100 mM Zn acetate) and 1,600 U of nuclease S1 (Boehringer) per ml were added to 100 μl of lysate and the mixture was incubated for 20 min at 37°C. The reaction mixture was loaded on two Tris-borate-EDTA (TBE) agarose (0.8%) gels containing ethidium bromide (0.5 μg/ml). Electrophoresis was conducted at constant voltage (1 V/cm) for 18 h. The two gels were transferred to nitrocellulose filters (12), one of them without prior denaturation with alkali. The plasmid pBL1, labelled by nick translation, was used as a radioactive probe. Filters were hybridized at 42°C overnight in 150 μl of hybridization solution (50% [vol/vol] formamide, 5× SCC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% [wt/vol] SDS, 5× Denhardt's solution, and 500 μg of denatured salmon sperm DNA per ml) per cm^2. Membranes were removed and rinsed briefly with 2× SSC and then washed twice at 42°C with 2× SSC–0.1% (wt/vol) SDS and 0.2% SSC–0.1% (wt/vol) SDS, respectively. After the washes, the membranes were exposed to Fuji RX X-ray film.

DNA sequencing of pULMJ230 and pULCF23 to analyze the extent of the deletion was carried out by the dideoxy nucleotide chain termination method of Sanger et al. (18a). A synthetic oligonucleotide (5′-CCTGAGTGCTTGCGGCAG-3′) complementary to the 5′ end of the Tn*5* fragment carrying the *kan* gen was used as the primer.

Oligonucleotide hybridizations. To determine the plus strand of pBL1, two oligonucleotides, oligo(−), 5′-CTTTCTT CCACTGAGATGTT-3′; and oligo(+), 5′-AACATCTCAGT GGAAGAAAG-3′, were designed according to the published pBL1 sequence (24) and labelled with T4 polynucleotide kinase (Promega). The filters were hybridized at 50°C. The hybridization solution contained 5× Denhardt's solution, 0.5% Nonidet P-40, 100 μg of denatured salmon sperm DNA per ml, and 6% NET (3 M NaCl, 0.6 M Tris HCl [pH 8.0], and 20 mM EDTA [pH 8.0]). The membranes were washed as described above.

Fragment purification was carried out by using a Gene Clean kit (Bio 101, La Jolla, Calif.). Restriction enzymes were purchased from Boehringer, Promega, or Biolabs. Computer analysis was performed by using DNASTAR computer programs (DNAstar, Inc., London, United Kingdom).

RESULTS

Localization of a region involved in pBL1 replication. To locate the region(s) involved in pBL1 replication (*ori*), the suicide plasmid pULMJ20 (an *E. coli* plasmid that does not replicate in *B. lactofermentum* but contains the *kan* gene for selection in *B. lactofermentum*) was constructed (Fig. 1).

Santamaría et al. (20) and Yeh et al. (25) suggested that the *Bcl*I site of pBL1 was located in a region essential for plasmid replication. Several fragments from pBL1, all containing the *Bcl*I site, were cloned into pULMJ20. Plasmid pULMJ20 was used as a negative control, and pULMJ20 plasmids carrying the whole pBL1 digested with *Hin*dIII in both orientations (pULMJ201 and pULMJ202) (Fig. 2) served as positive controls. Plasmid DNA isolated from *E. coli* was used to transform *B. lactofermentum*, and four different plasmids (pULMJ202, pULMJ207, pULMJ208, and pULMJ209) were isolated by their ability to replicate in *B. lactofermentum* (Fig. 2), suggesting that the minimal region required for pBL1 replication is in a *Hin*dII*-*Sph*I* (asterisks denote sites used for cloning) fragment of 1,813 bp. Computer analysis of the pBL1 sequence

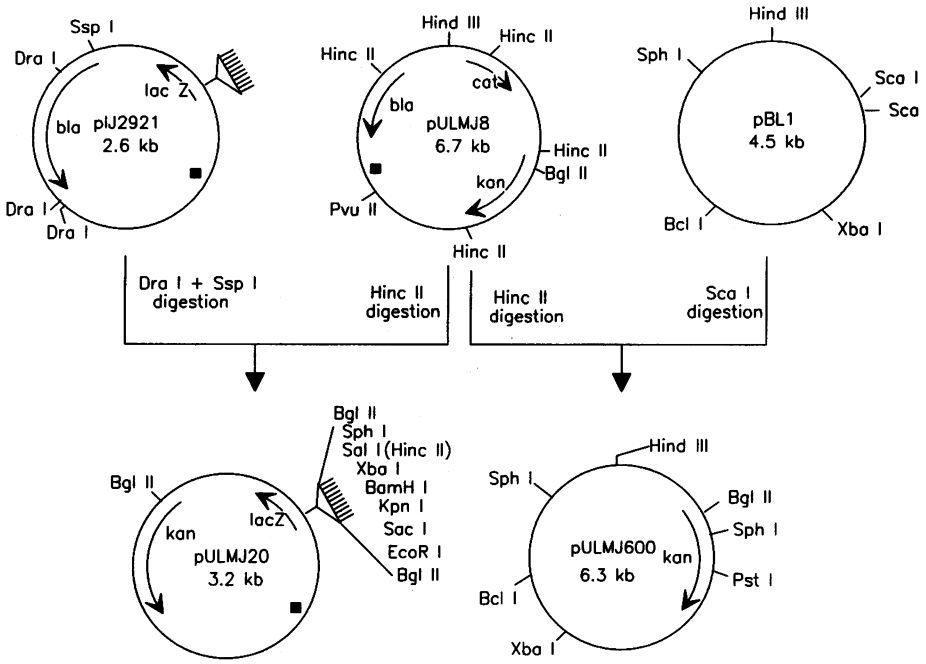

FIG. 1. (Left) Construction of the suicide plasmid pULMJ20. Since the ampicillin resistance marker of pIJ2921 (10) is not expressed in coryneform bacteria (19), it was replaced by the kanamycin resistance gene (*kan*) from Tn*5* isolated as a 1.4-kb *Hinc*II fragment from pULMJ8. (Right) Construction of pULMJ600 by replacement of the small *Sca*I fragment of pBL1 by the 1.4-kb *Hinc*II fragment from pULMJ8 carrying the *kan* gene. ■, ColE1 origin of replication.

(6, 24) showed that there are at least two open reading frames (ORFs) (ORF1 and ORF5) in this region. Deletion of either ORF (pULMJ204, pULMJ205, and pULMJ206) led to the inability to transform *B. lactofermentum*, suggesting that they may be involved in plasmid replication.

Hybrid plasmids were stable in *E. coli*, but when they were isolated from *B. lactofermentum*, they gave rise to a variety of deletions of different sizes (except for pULMJ201, pULMJ202, and pULMJ207, which were stable in *B. lactofermentum*). As can be observed in Fig. 2, all the unstable plasmids (pULMJ203, pULMJ208, and pULMJ209) lacked the 870-bp *Hin*dIII-*Sph*I* fragment of pBL1, which contained the ORF described by Yamaguchi et al. (24) as ORF4. These results also suggest that the 1,100-bp *Hin*dIII-*Sca*I* fragment seems to be

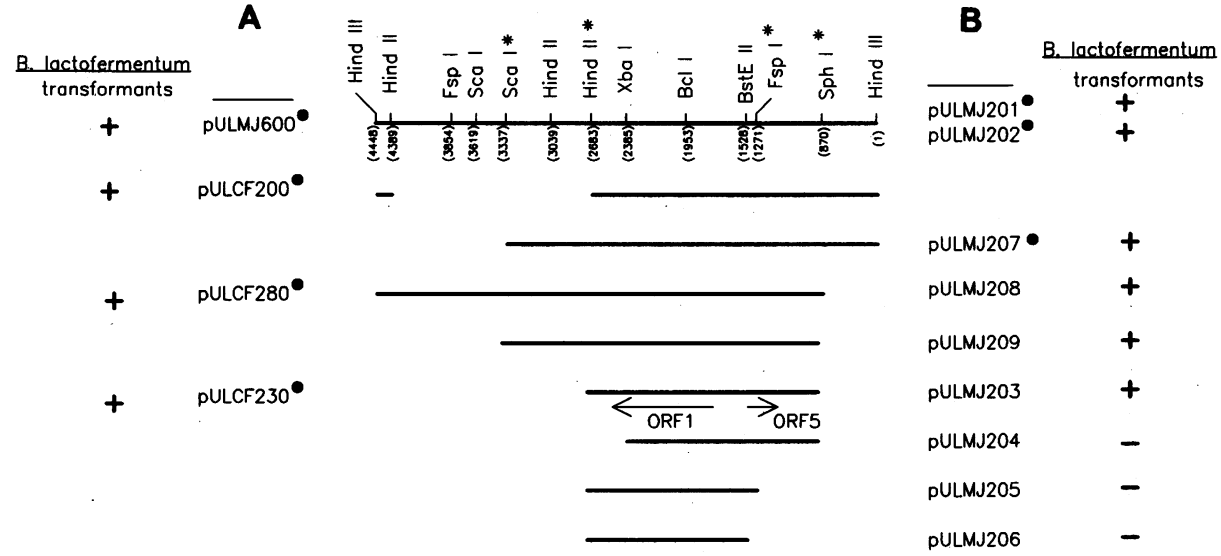

FIG. 2. Mapping of the minimal region of pBL1 required for replication. (A) pBL1 derivatives obtained by insertion of the *kan* gene. (B) Bifunctional constructions obtained by cloning pBL1 fragments into the suicide plasmid pULMJ20. For details of the various constructions, see Table 1. Numbers in parentheses represent nucleotides in the pBL1 sequence (24). Symbols: ●, plasmid structurally stable in *B. lactofermentum*; *, sites used for cloning; +, *B. lactofermentum* transformant; −, not *B. lactofermentum* transformant.

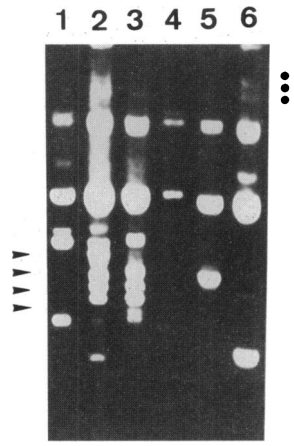

FIG. 3. Agarose (0.8%) gel electrophoresis of plasmid DNAs from various *B. lactofermentum* (lanes 1, 2, 3, 5, and 6) and *E. coli* (lane 4) transformants carrying plasmid pULMJ203. Note that pULMJ203 is unstable in *B. lactofermentum*, giving rise to a variety of natural deletions (arrowheads) and high-molecular-weight plasmid multimers (filled circles).

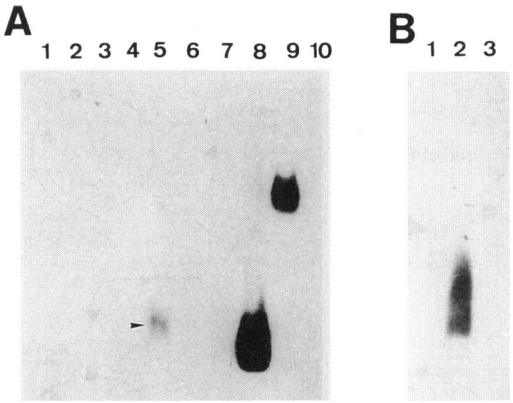

FIG. 4. (A) Single-stranded plasmid DNA generated in *B. lactofermentum* harboring pULMJ600 (lane 1), pULMJ600 treated with S1 nuclease (160 U/ml) (lane 2), pULMJ202 (lane 3), pULMJ202 treated with S1 nuclease (lane 4), pULMJ203 (lane 5), pULMJ203 treated with S1 nuclease (lane 6), pULMJ208 treated with S1 nuclease (lane 7), pULMJ208 (lane 8), pULMJ209 (lane 9), and pULMJ209 treated with S1 nuclease (lane 10). Filters were hybridized to pBL1 labelled by nick translation (see Materials and Methods). The arrowhead shows the ssDNA from pULMJ203 (lane 5). (B) Single-stranded plasmid DNA accumulated in *B. lactofermentum* harboring pULCF230 (lane 1) and pULCF23 (lane 2) and in *R. fascians* harboring pULCF230 (lane 3).

a dispensable DNA fragment in relation to stability and replication.

Figure 3 shows the generation of a variety of deletions of pULMJ203 in *B. lactofermentum* (arrowheads) and high-molecular-weight plasmid multimers that may be responsible for the plasmid's structural instability (6a). When this population of plasmids was used to retransform *E. coli*, only the original plasmid, pULMJ203, was recovered, suggesting that *B. lactofermentum* eliminates those segments of the DNA derived from the *E. coli* plasmid.

Mode of replication of pBL1. To avoid the possibly negative effect of the *E. coli* plasmid sequences on the stability of the hybrid plasmids in *B. lactofermentum*, several monofunctional constructions were made by inserting the *kan* gene from Tn*5* into pBL1 or pBL1 fragments. All of the constructions (pULMJ600, pULCF200, pULCF280, and pULCF230) replicated and were stable in *B. lactofermentum* (Fig. 2). The bifunctional plasmid pULMJ208 is unstable, whereas its analogous monofunctional plasmid pULCF280 is fully stable in *B. lactofermentum*. Similar results were obtained with pULMJ203 and pULCF230, indicating that the lack of stability of the plasmids is probably due to the presence of foreign sequences in pBL1, and not to the absence of ORF4, as previously thought. This behavior of pBL1 resembles the behavior of those small plasmids of gram-positive bacteria which replicate by the RCR mechanism (7, 17). To determine if pBL1 replicates via RCR, several bifunctional constructions were tested for their ability to accumulate ssDNA as intermediates during replication. Cell lysates from *B. lactofermentum* transformed with plasmids pULMJ600, pULMJ202, pULMJ203, pULMJ 208, and pULMJ209 were tested for accumulation of ssDNA as described in Materials and Methods. ssDNA intermediates in cell lysates could be detected easily as circular molecules which run faster than covalently closed circular DNA forms in agarose gels containing ethidium bromide. They are able to bind to nitrocellulose filters without previous denaturation and are sensitive to nuclease S1 digestion. A long exposure time was needed to see ssDNA on the filter corresponding to nondenatured plasmid pULMJ600, but clear positive hybridization bands were found for nondenatured plasmids pULMJ203, pULMJ208, and pULMJ209. However, the size of the ssDNA detected (pULMJ203, pULMJ208, and pULMJ 209) was smaller than that expected for the parental plasmids, which suggests that spontaneous deletions have occurred because of the instability of these plasmids in *B. lactofermentum* (Fig. 3). Some of the natural deletions generated after *B. lactofermentum* had been transformed with bifunctional plasmids might be responsible for the accumulation of ssDNA (Fig. 4A), since these plasmids may have lost the SSO sequence. The smallest deletion plasmid able to accumulate ssDNA and to replicate in *B. lactofermentum* was found to be a pULMJ208 transformant. This deleted plasmid (pULCF21; 1.8 kb) was mapped and lacks part of pBL1 and all of the pULMJ20 moiety. The *kan* resistance gene from Tn*5* was cloned into the unique *Hin*dII site of pULCF21, giving rise to plasmid pULCF23 (Fig. 5). This construction is stable in *B. lactofermentum*, accumulates ssDNA, and contains the two ORFs (ORF1 and ORF5) required for pBL1 replication. In contrast, pULCF230 does not accumulate ssDNA (Fig. 4B). The physical maps of pULCF23 and pULCF230 are almost identical (Fig. 5), suggesting that the SSO could be located on a small DNA fragment present in pULCF230 and absent in pULCF23. The study of the sequences of both plasmids allows us to identify the SSO of pBL1 by comparison with the SSO sequence of pIJ101 (28). The putative pBL1 SSO sequence TGTCGT is located in a loop structure found in pULCF230 and absent in pULCF23 (Fig. 6). This is in agreement with the fact that only pULCF23 accumulates ssDNA. The rest of the monofunctional plasmids should have SSO, because no ssDNA was observed (data not shown).

Another strategy to demonstrate that pBL1 replicated via ssDNA was to introduce pBL1 derivatives into heterologous hosts to look for accumulation of ssDNA intermediates present because of the reported inefficient recognition of the SSO sequence by heterologous hosts (7). It has been reported previously that pBL1 can replicate in *C. glutamicum* (20), but we tried to find an alternative host similar to the coryneform group of bacteria. Plasmids pULMJ600 and pULCF230 were

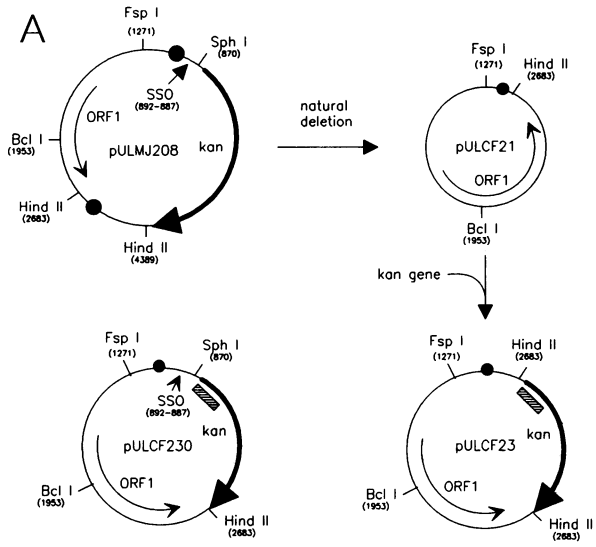

FIG. 5. (A) Plasmid pULCF23 was constructed by inserting the 1.4-kb *Hin*dII fragment of pULMJ8 carrying the *kan* gene into the unique *Hin*cII site of pULCF21, a naturally deleted derivative of pULMJ208. Filled circles represent the homologous zones found in pBL1 which could be responsible for the pULMJ208 recombination event leading to pULCF21, numbers in parentheses represent nucleotides in the pBL1 sequence (24), and hatched boxes represent the oligonucleotide used for the sequencing of pULCF23 and pULCF230 to localize the SSO (nt 892 to 887). (B) Nucleotide sequence of the relevant regions in plasmids pULMJ208 and pULCF23. The sequence across the deletion giving rise to pULCF21, the in vivo derivative of pULMJ208, revealed that the deletion occurred at or around nt 2761 and 969 of the pBL1 sequence (24). Filled circles indicate identical nucleotides.

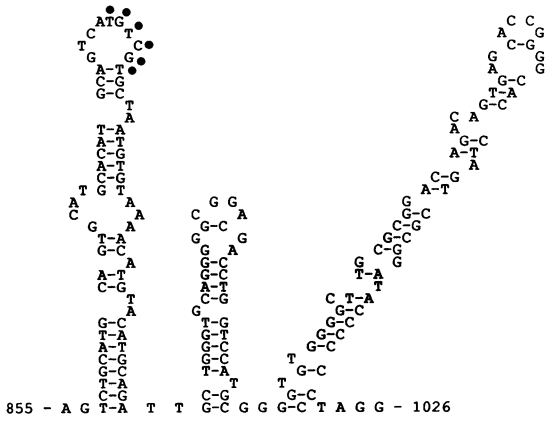

FIG. 6. Potential secondary structure between nt 855 and 1026 on the pBL1 sequence (24) containing the putative SSO. Filled circles indicate bases that are homologous to the TAGCGT consensus sequence found in SSO regions of several plasmids and phages (27).

introduced into *R. fascians*, and kanamycin-resistant transformants were selected. Plasmid DNA in these transformants was detected only by hybridization; the DNA was of the expected size and was able to replicate when transformed back into *B. lactofermentum*. The reason for the low copy number of these plasmids in *R. fascians* is not yet clear, but no ssDNA was detected (Fig. 4B). Plasmids pULMJ600 and pULCF230 were also used to transform *N. lactamdurans*, but no kanamycin-resistant transformants were found after several transformation attempts.

Cell extracts from *B. lactofermentum* and *C. glutamicum* containing pULMJ600 and pULMJ208 were tested for accumulation of ssDNA. As shown in Fig. 7, ssDNA is observed more clearly in *C. glutamicum* than in *B. lactofermentum*, indicating that the SSO may not be recognized efficiently by the heterologous host *C. glutamicum*. To confirm that the hybridizing bands observed were ssDNA molecules, the samples were treated with 160 U of nuclease S1 per ml as described in Materials and Methods. The results clearly demonstrated that the radioactive band disappeared after treatment with S1 nuclease (data not shown).

In all of the plasmids which replicate via the RCR mechanism, the strand accumulating as circular intermediates of ssDNA is the plus strand. If pBL1 replicates via RCR, the ssDNA accumulated should correspond to the released strand. Two oligonucleotides, complementary to each strand, were synthesized (see Materials and Methods) and used for hybridization to DNA from cell lysates of *B. lactofermentum* transformed with pULCF23. As positive controls, two ssDNA plasmids (pULCF1+ and pULCF2−) were constructed by cloning the *Sph*I*-*Hin*dII* fragment of pBL1 into pBluescript KS+ and KS−, respectively (Fig. 8). As shown in Fig. 9, only one of the oligonucleotides hybridized with the ssDNA, suggesting that there is only one strand of the pBL1 derivatives in

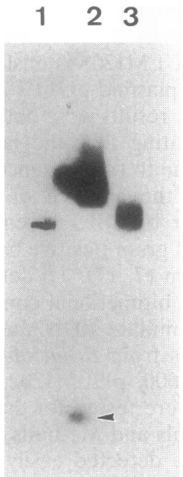

FIG. 7. Southern hybridizations from *B. lactofermentum* harboring pULMJ600 (lane 1) and pULMJ208 (unstable plasmid) (lane 2) and *C. glutamicum* harboring pULMJ600 (lane 3). Samples were blotted without previous denaturation and hybridized to plasmid pBL1 labelled by nick translation (see Materials and Methods). ssDNA molecules are accumulated by two different deletions of the unstable derivative pULMJ208 in *B. lactofermentum*. The arrowhead shows the ssDNA molecules derived from pULMJ208.

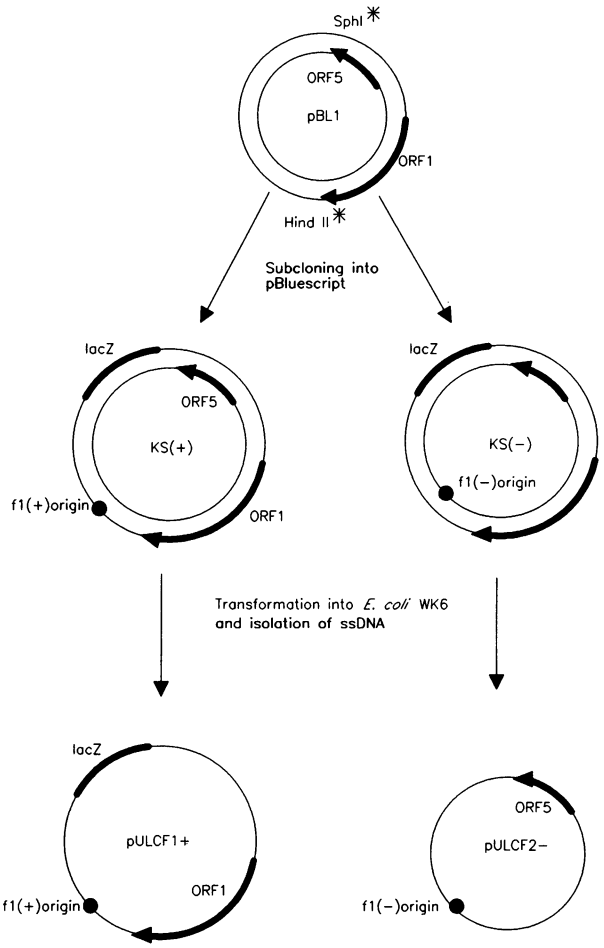

FIG. 8. Construction of pULCF1+ and pULCF2− (see text for details).

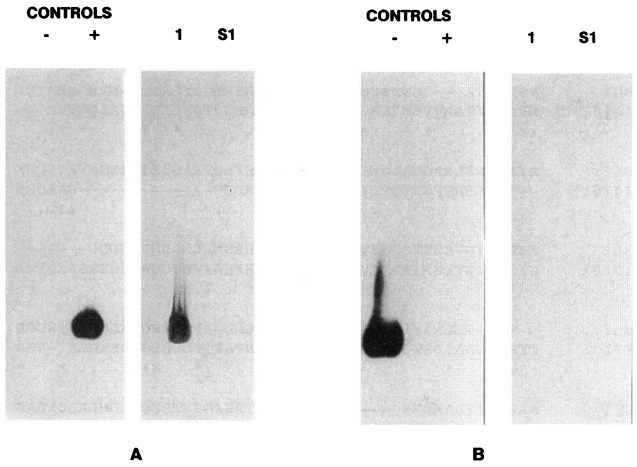

FIG. 9. ssDNA intermediates of pBL1. Cell lysates from *B. lactofermentum* harboring pULCF23 (lane 1) were run and transferred without denaturation to two nitrocellulose filters; samples in the lanes marked S1 were treated with S1 nuclease. pULCF1+ (+) and pULCF2− (−) were used as positive controls. For this experiment, two different oligonucleotides were designed, one specific for each strand of pBL1. Each oligonucleotide hybridizes specifically with the complementary single-stranded pBluescript plasmid. Note that the pULCF23 single-stranded plasmid hybridizes to only one of the oligonucleotides. (A) Filter hybridized to oligo(−); (B) filter hybridized to oligo(+).

the cytoplasm of *B. lactofermentum* and that this strand is sensitive to S1 nuclease digestion. This result allowed us to identify the plus strand of pBL1 as the strand which encodes ORF1 according to the Yamaguchi sequence (24).

The putative DSO of pBL1 was found by comparing the minimal replicative region of pBL1 (*Sph*I*-*Hin*dII*) and the sequences flanking the nick sites of plasmids pIJ101, pSB24, and pJV1 (21a) (Fig. 10).

Rep protein. Since two ORFs (ORF1 and ORF5) seem to be required for the replication of pBL1, one of these ORFs should code for the Rep protein. The amino acid sequence of the protein encoded by the larger one (ORF1) (6, 24) was compared with sequences in the EMBL data bank. A partial homology was found with the putative Rep protein of the *S. lividans* plasmid pIJ101, which accumulates ssDNA (17, 27, 28); with the Rep protein of plasmid pSB24 from *Streptomyces cyanogenus* (9); and with the Rep protein of plasmid pJV1 (21a). A comparison of the amino acid sequence of the protein encoded by ORF1 of pBL1 with the putative Rep protein of pIJ101 is shown in Fig. 11. Ilyina and Koonin (9) found three conserved motifs in all the described Rep proteins from RCR plasmids. One of them included a putative DNA-linking Tyr residue(s) (see Discussion). This motif is found in the protein encoded by the pBL1 ORF1; in the putative Rep proteins from pIJ101, pSB24, pJV1, and ISS108; and in the rest of the RCR Rep proteins (Fig. 12A). The amino acid comparison between the four Rep proteins shows other possible active sites, which contain a tyrosine residue (Fig. 12B). Therefore, these *Streptomyces* plasmids, together with pBL1, might form a new family of RCR plasmids.

The predicted amino acid sequence of the ORF5 protein (the second protein encoded by the indispensable DNA region of pBL1) was compared with protein sequences in the EMBL data bank, and no significant homologies were found. We do not yet know the function of ORF5, but a promoter for this gene (P5) was isolated and its transcription-initiation site was determined, suggesting that the ORF5 region is transcribed in *B. lactofermentum* and could be involved in transcription control of the *rep* gene.

DISCUSSION

Twenty-five percent of the genetic information of plasmid pBL1 from *B. lactofermentum* (1.1 kb) seems to be dispensable for its stability and replication in *B. lactofermentum*. The minimal region involved in replication lies in a 1.8-kb *Hin*dII-*Sph*I fragment and contains at least two ORFs (ORF1 and ORF5) according to the published pBL1 sequences (6, 24).

```
                                        C A
pBL1    1303 AACACTGAAATAGAAGTGAACACCTTAGGAACCGCAGGTCAATGAGG
             | ||   | ||| |||||||||| ||||   |   ||  |
pSB24   1237 CCGCCCGAGGCAAAAGCGAACACCTTGGGAAAGAAACAACAGAGTTT
             ||||||||||||||||||||||||||||||||||||||||||||||
pIJ101  1690 CCGCCCGAGGCAAAAGCGAACACCTTGGGAAAGAAACAACAGAGTTT
             ||||||  |||||||  | ||  ||| ||  ||||     |||||||||
pJV1    1662 CCGCCCCTGGCAAAAAGGGACGCCTAGGTAAAGGGTTGACAGAGTTT
                *      * **  * **  *** *  **        **  *
```

FIG. 10. Homology between pIJ101, pSB24, and pJV1 regions flanking the nick site (DSO) and the minimum replicative region of pBL1. Asterisks indicate conserved nucleotides, and the two filled boxes show the position of the nick site in pJV1 (21a).

```
pBL1      VNTSKEPQVNEGSKVTR--------ARAWRRQNVMYKITNSKALAGCHRW
pIJ101    MDPASGVIVAQTAAGTSVVLGLMRCGRIW-----LCPVCAATI-----RH
          .. .   * . .  *          .* *     .  .  .       *

pBL1      RRDEAVA---VSWSSNGASQFEGLQNSHSRWGSPLAEL--EVMGERRIEL
pIJ101    KRAEEITAAVVEWIKRGGTAYLVTFTARHGHTDRLADLMDALQGTRK---
          .* * ..   * *   *.. .      ..      **.*   . * *.

pBL1      AIATKNHLAAGGALMMFVGTVRHNRSQSFAQVEAGIKTAYSSMVKTSQWK
pIJ101    --TPDSPRRPGAYQRLITGGTWAGRRAKDGH-------------RAADRE
            .  .   .*.   .. *  .  *    ..               .....

pBL1      KERARYGVEHTYSDYEVTDSWANGWHLHRNMLLFLDRPLSDD--------
pIJ101    GIRDRIGYVGMIRATEVTVGQINGWHPHIHAIVLVGGRTEGERSAKQIVA
            * * *         *** .  **** * . .....    ..

pBL1      -------ELKAFEDSMFSRWSAGVVKAGMD-APLREHGVKLDQVSTWGGD
pIJ101    TFEPTGAALDEWQGHWRSVWTAALRKVNPAFTPDDRHGVDFKRLET-ERD
                *   ..   * *.*.. *     .*   *** . .. *   *

pBL1      AAKMATYLAKGMSQ-----ELTGSATKTASKGSYTPFQMLDMLADQSDAG
pIJ101    ANDLAEYIAKTQDGKAPALELARADLKTATGGNVAPFELLGRIGDLTGGM
          *  .* *.**          **. .  ***. *. .**..*. ..* ...

pBL1      EDMDAVLVA-------RWREYEVGSKNLRS-SWSRGAKRALGIDYIDADV
pIJ101    TEDDAAGVGSLEWNLSRWHEYERATRGRRAIEWTRYLRQMLGLDGGDTE-
           . **  *.        **.*** ...  *.  *.*  .. **.*  *..

pBL1      RREMEEELYKLAGLEAPERVESTRVAVALVKPDDWKLIQS---DFAVRQY
pIJ101    ----ADDLDLL--LAADADGGELRAGVA-VTEDGWHAVTRRALDLEATRA
               ..*  *  * *       * .** *  *.*  .     *.   .

pBL1      VLDCVDKAKDVAAAQRVANEVLASLGVDSTPCMIVMDDV---R---DLDAVL
pIJ101    AEGKDGNEDPAAVGERV-REVLALADAADTVVVLTAGEVAEAYADMLAAL
            .  ..    * ..**  ****  .   *  ..  ..*     *. * *

pBL1      PTH-----------------GDATKRDLNAAVFAGNEQTILRTH
pIJ101    AQRREEATARRRREQDDDQDDDADDRQERAARHIARLASGPTSH
          . .                  .**  *.  **   .   .   .*
```

FIG. 11. Comparison of the pBL1 ORF1 protein amino acid sequence with the putative Rep protein sequence of *S. lividans* plasmid pIJ101. Asterisks indicate identical amino acid residues, and dots indicate similar amino acid residues.

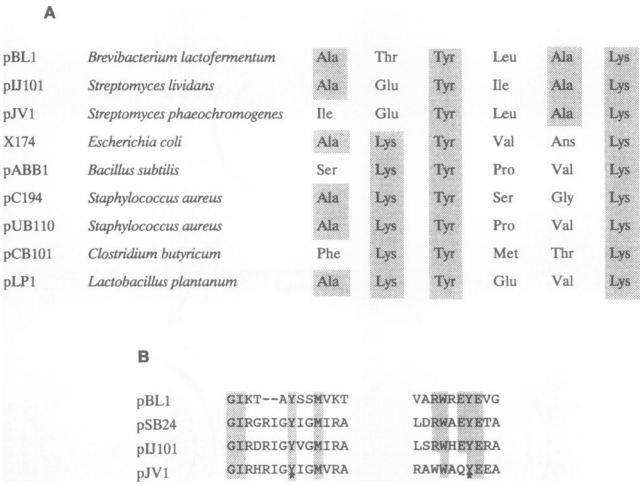

FIG. 12. (A) Conserved DNA-linking motif of the Rep proteins of different ssDNA plasmids and phage X174. (B) Possible sequences involved in the DNA-linking process during the replication of pBL1, pIJ101, pJV1, and pSB24. Asterisks indicate Tyr residues.

This result agrees with those of Santamaría et al. (20) and Yeh et al. (25), who indicated that the *BclI* site in pBL1 was located in a region essential for replication.

The instability of hybrid plasmids between pULMJ20 and pBL1 or of pBL1 fragments when introduced into corynebacteria was initially thought to be due to the requirement of the ORF4 gene product for plasmid stability. This hypothesis was based on the observation that bifunctional constructions pULMJ203, pULMJ208, and pULMJ209 (Fig. 2) were unstable in *B. lactofermentum* and all lacked an 870-bp *Hin*dIII-*Sph*I* fragment from pBL1, which includes ORF4. Although we cannot exclude this hypothesis, it was shown that monofunctional constructions pULMJ230 and pULMJ280 lacking ORF4 were stable in *B. lactofermentum*. A possible function for the 870-bp *Hin*dIII-*Sph*I* fragment is to stabilize bifunctional constructions by avoiding readthrough from the *E. coli* vector into the Rep region. The finding of three terminators in this region of pBL1 confirms this hypothesis.

The instability of some pBL1 derivatives might be due to the replication of pBL1 via the RCR mechanism, as occurs with several other small plasmids of gram-positive bacteria. Plasmids that replicate via the RCR mechanism frequently accumulate ssDNA when the single-strand origin is not functional or is inefficiently recognized. Indeed, ssDNA was found in some unstable bifunctional pBL1 derivatives, pULMJ203, pULMJ208, and pULMJ209 (Fig. 4A). The size of the ssDNA does not correspond to the size of the parental plasmid, suggesting that only some of the deletion plasmids are responsible for the accumulation of ssDNA. Plasmid pULCF23 was constructed from the smallest deletion plasmid (pULCF21) originating from pULMJ208. pULCF23 is unstable in *B. lactofermentum* and accumulates ssDNA. The molecular mechanism of the deletion (Fig. 5B) can be explained by a recombination event between short directed repeats located around nucleotides (nt) 761 and 969 of pBL1 (24). In vivo deletion resulting in the novel expression of genes by recombination between short directed repeats has already been described (1a). Comparison of the sequence of the smallest plasmid which accumulates ssDNA (pULCF23) with the map of pULCF230, which replicates stably, allowed us to identify the SSO by homology with the SSO of pIJ101 (28). The putative DSO of pBL1 was also identified by comparison with the DSOs of pJV1, pIJ101, and pSB24 (21a).

The pBL1 plus strand has been identified by using specific probes, suggesting that the plus strand of pBL1 corresponds to the sense strand encoding ORF1; this should be the template to synthesize the minus strand, which encodes ORF5. Computer analysis of ORF1 confirmed that this ORF may code for a Rep protein analogous to Rep proteins from plasmids that replicate by RCR, such as pIJ101 from *S. lividans* (17), pJV1 from *Streptomyces phaerochromogenes* (21a), and pSB24 from *S. cyanogenus* (9) (Fig. 11). pIJ101, pJV1, and pSB24 might form a separate family of RCR plasmids that includes pBL1, according to the evidence shown in this article. Comparison of the amino acid sequence of the ORF1 protein of pBL1 with the putative Rep protein of pIJ101 (Fig. 11) showed a DNA-linking motif typical of Rep proteins of RCR plasmids (9) (Fig. 12). Some RCR Rep proteins appear to have only one Tyr residue which binds to the nicked DNA. Two other sequences present in the Rep proteins of pBL1, pIJ101, pJV1, and pSB24 could be involved in the DNA-linking process because a tyrosine residue is found in them (Fig. 12B). In the A protein from phage X174, two Tyr residues, separated by three amino acids, are involved in binding to the 5′ end of the nicked viral DNA. The results from this comparison allowed the prediction of the amino acid sequence involved in DNA linking which is required for the initiation of RCR.

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