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Characterization and Sequence Analysis of a Stable Cryptic Plasmid from *Enterococcus faecium* 226 and Development of a Stable Cloning Vector†

HERB A. WYCKOFF,[1]‡ MARY BARNES,[2]§ KEVIN O. GILLIES,[3] AND W. E. SANDINE[1]*

Department of Microbiology, Oregon State University, and Oregon Agricultural Experiment Station, Corvallis, Oregon 97331-3804[1]; 1404 Girard Street, San Marcos, Texas 78666[2]; and Rhone-Poulenc Dairy Ingredients, Madison, Wisconsin 53711-1075[3]

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A small cryptic plasmid, pMBB1, isolated from *Enterococcus faecium* 226 was characterized. The plasmid contained an extremely stable replicon which has limited homology to the lactococcal plasmid pCI305. Sequence analysis of the replicon detected one open reading frame of 822 bp capable of encoding a 32-kDa protein. No detectable single-stranded intermediates were found for the replicon, suggesting that pMBB1 may be included in the same family as pCI305, although pCI305 exhibits a more narrow host range. A small stably maintained vector able to replicate in a variety of lactic acid bacteria, containing a large multiple cloning region, was constructed by using the pMBB1 replicon.

Most vectors for lactic acid bacteria are rolling-circle replicating plasmids which replicate by using single-stranded intermediates (17, 27). While these plasmids have been indispensable in development of gene cloning systems in lactic acid bacteria, problems with the stability of plasmids which replicate by a single-stranded intermediate are well documented (2, 8, 15, 19, 21). These problems include segregation instability, production of high-molecular-weight plasmid multimers, and decreasing plasmid copy numbers with increasing size of cloned inserts (15).

Cloning vectors that replicate without the use of a single-stranded intermediate are beneficial as shown by derivatives of pAMβ1 (4), another enterococcal plasmid which replicates without the use of a single-stranded intermediate (13). The replication of pAMβ1 and other enterococcal plasmids is included in the review by Jannière et al. (14).

Preliminary examination of the small cryptic plasmid of *Enterococcus faecium* 226 indicated that it contained a replicon capable of stable maintenance. The bacterium of unknown origin was deposited in the Department of Microbiology Culture Collection and designated a *Leuconostoc* species; during this work it proved to be a strain of *Enterococcus faecium*. Further characterization of the plasmid located the replicon on a 1.9-kb region and led to the development of a small stable cloning vector. The nucleotide sequence of the replication region is presented, and relationship to the lactococcal plasmid pCI305 is discussed.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani broth (18) at 37°C, lactococci were grown in M17G broth (M17 containing 0.5% glucose) (24) at 30°C, and *Enterococcus faecium* was grown in MRS broth (Difco) at 30°C. Ampicillin (50 μg/ml) for *E. coli*), kanamycin (70 μg/ml for *E. coli*), and chloramphenicol (20 μg/ml for *E. coli* and 5 μg/ml for lactococci) were added to media when required. The identity of *Enterococcus faecium* 226 was determined by analysis of cellular fatty acids by MicroCheck Inc. (Northfield, Va.) and confirmed by growth at 45°C within 24 h (20).

Plasmid DNA was purified from *E. coli* strains by using Qiagen (Chatsworth, Calif.) plasmid columns per the manufacturer's instructions. The procedure of Anderson and McKay (1) was used to isolate plasmid DNAs from lactococci and *Enterococcus faecium* 226. Single-stranded DNA was purified as described by Maniatis et al. (18). A Geneclean kit (Bio 101, Inc., La Jolla, Calif.) was used to purify DNA from agarose. For detection of single-stranded replicative forms, DNA was purified and fractionated from whole-cell lysates by the procedure of Leenhouts et al. (17).

Restriction enzymes, ligase, and Klenow enzyme were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.) and were used according to the manufacturer's instructions. *E. coli* cultures were transformed by the $CaCl_2$ protocol (18), while lactococci were transformed by electroporation (5) using a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Reference or source
E. coli	
JM107	28
DH5α MCR	BRL[a]
MC1061	3
E. faecium 226	OSU[b]
L. lactis LM0230	6
pCI341	12
pBluescript II KS−	Stratagene
pUC118	26
pUC119	26
pGK12	16
pGK13	Jan Kok

[a] BRL, Bethesda Research Laboratories.
[b] OSU, Oregon State University.

* Corresponding author.

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‡ Present address: ARS/USDA National Center for Agricultural Utilization Research, Peoria, IL 61604-3999.

§ Present address: Department of Biology, Southwest Texas State University, San Marcos, TX 78666.

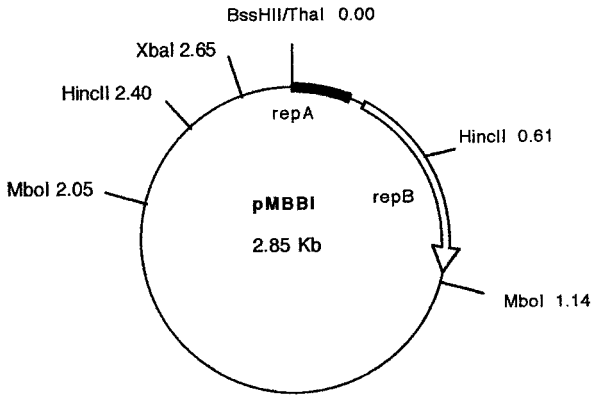

FIG. 1. Restriction map of the cryptic plasmid pMBB1 from *Enterococcus faecium* 226. The unique *Bss*HII restriction site is designated map position 0.

Isolated DNA fragments were cloned into vectors pUC118 and pUC119 in *E. coli* JM107 and were sequenced by the dideoxy chain termination method with the Sequenase kit (United States Biochemical, Cleveland, Ohio). Production of single-stranded DNA was initiated by using the helper phage M13K07 (26). Universal primers as well as primers synthesized on an ABI 380B synthesizer (Applied Biosystems, Foster City, Calif.) were used. The sequences of both strands were determined. DNA sequence analyses were carried out with IntelliGenetics (Mountain View, Calif.) programs.

Whole-cell lysates were electrophoresed on an agarose gel and transferred to a nylon membrane (Zeta-probe; Bio-Rad) by using a vacuum blotter (Hoefer Scientific Products, San Francisco, Calif.) according to the membrane manufacturer's protocol. Hybridizations were carried out in a Hybrid-Ease hybridization chamber (Hoefer Scientific Products). The hybridization conditions of Hardy et al. (9) were used. Radiolabeled probes were generated by using a random priming kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions to incorporate [α-^{32}P]dCTP (Amersham).

TABLE 2. Stabilities of pMBB1 derivatives during extended growth under nonselective conditions

Plasmid	% *L. lactis* LM0230 isolates with pMBB1 derivatives[a]	
	50 generations	100 generations
pHW112	98.5	95.2
pHW700	72.5	18.8
pHW800	99.5	100

[a] Average for duplicate experiments.

To determine the stability of the pMBB1 replicon, *Lactococcus lactis* cultures containing derivatives with antibiotic resistance markers (pHW112, pHW700, and pHW800) were grown for 50 and 100 generations without selection. Cultures were then plated on nonselective media, and at least 200 single isolates were transferred to selective medium to confirm the retention of chloramphenicol resistance.

Restriction mapping determined the size of pMBB1 to be approximately 2.85 kb (Fig. 1). The region required for replication of pMBB1 was located by using two approaches. In the first, *Mbo*I-digested pMBB1 was ligated to the 1.4-kb *Mbo*I

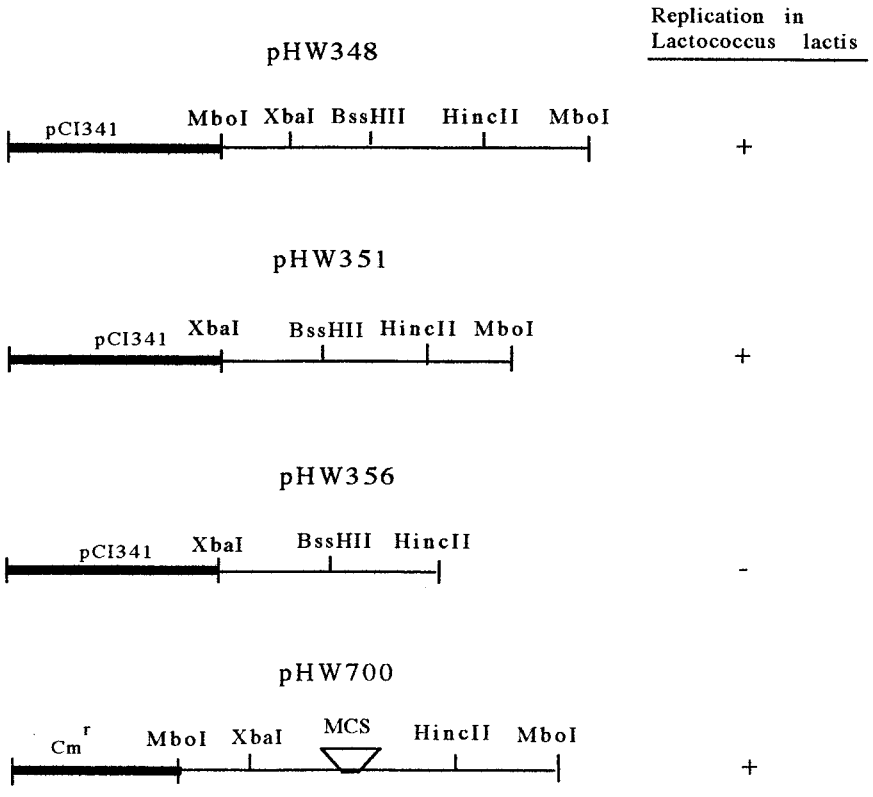

FIG. 2. Subclones of pMBB1 localizing the origin of replication. MCS, multiple cloning site.

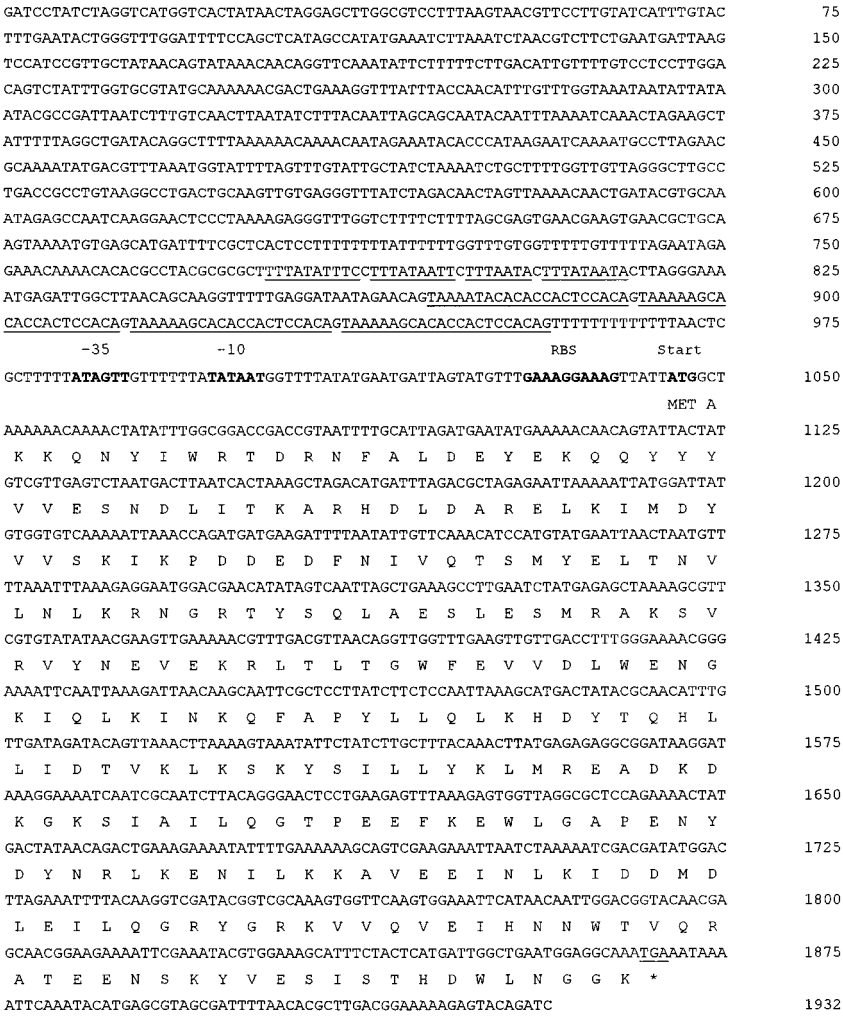

FIG. 3. Complete nucleotide sequence of the 1,932-bp *Mbo*I fragment of pMBB1. The putative amino acid sequence of the *repB* product is shown. The significant promoter sequences are boldfaced. The two series of direct repeats are underlined. RBS, ribosome binding site.

fragment of pGK12 which contains the Cm^r marker (16) and was transformed into *L. lactis* LM0230. Only constructions (pHW348) containing the larger *Mbo*I fragment of pMBB1 were isolated, indicating that the origin was located on this fragment. In the second approach, *Mbo*I-digested pMBB1 was ligated to the *Bam*HI-digested replication probe vector pCI341 and was transformed into *E. coli* MC1061. The resulting constructions were then used to transform *L. lactis* LM0230. Only the constructions containing the 1.9-kb *Mbo*I fragment were able to transform *L. lactis* to Cm^r, again suggesting that the replicon resided on this fragment.

A series of subclones of the 1.9-kb *Mbo*I fragment was constructed in pCI341 to further locate the minimal region which allowed replication (Fig. 2). The 1.3-kb *Xba*I-*Mbo*I region was able to allow replication of pHW351 in lactococci, indicating that the 600-bp *Mbo*I-*Xba*I fragment was not required for replication. However, the deletion of the 0.5-kb *Hin*cII fragment from the right side (pHW356) negated the ability of the plasmid to replicate in *L. lactis* LM0230. The insertion of a 173-bp multiple cloning site into the *Bss*HII (pHW700) site did not eliminate the ability of the origin to replicate in *L. lactis*; however, it did decrease the ability of the plasmid to be stably maintained in *L. lactis* (Table 2). Thus, the minimal replicon resides within the 1.3-kb *Xba*I-*Mbo*I fragment.

The sequence of the region shown to contain the origin of replication for pMBB1 is shown in Fig. 3. The G+C content of the fragment was 33.8%, slightly below the 38 to 39% G+C reported for the genome of *Enterococcus faecium* (20). The sequence had approximately 60% homology to the 1,661-bp region sequenced from the *L. lactis* plasmid pCI305 (11). The

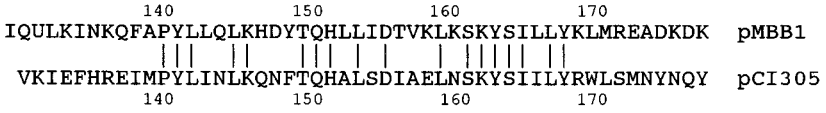

FIG. 4. Region of amino acid homology between the RepB proteins of pCI305 and pMBB1.

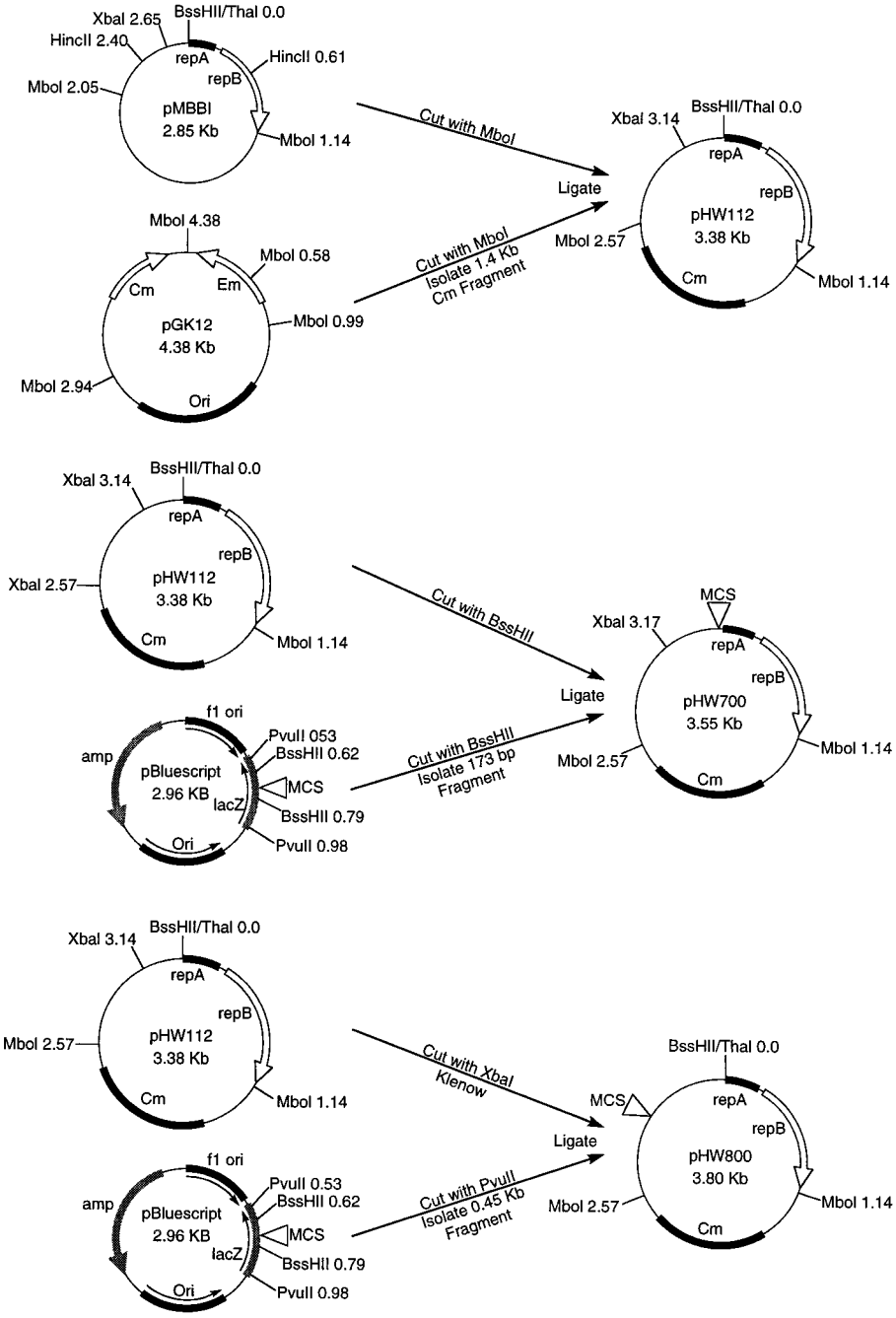

FIG. 5. Cloning strategy used in construction of plasmids pHW112, pHW700, and pHW800. MCS, multiple cloning site.

region of greatest homology spanned the first 150 nucleotides of the pCI305 sequence and nucleotides 660 to 810 of the pMBB1 sequence.

Analysis of the nucleotide sequence within the 1,932-bp region revealed the presence of one large open reading frame (ORF) (*repB*). This ORF extended from position 1045 to position 1867, spanning 822 nucleotides, and has the potential to encode a protein 274 amino acids in length with a molecular mass of 32,628 Da. The ORF is preceded by a consensus −10 region (10) (TATAAT), a putative −35 region (ATAGTTG), and an area which resembles the ribosomal binding site of some gram-positive promoters (7, 25) (GAAAGGAAAG). The predicted amino acid sequence of the ORF exhibited some similarity to the replication protein of pCI305 in the region spanning amino acids 140 to 170 (Fig. 4).

Analysis also revealed the presence of two series of direct repeats. The larger series consisted of four nearly perfect direct repeats 22 bp in length, and the smaller series contained four repeats that ranged from 7 to 10 bp in length (underlined in Fig. 3).

Two extremely AT-rich segments preceded the ORF. The first was approximately 35 bp in length and preceded the first series of direct repeats (nucleotides 708 to 742). The second AT-rich segment, which was approximately 55 bp in length,

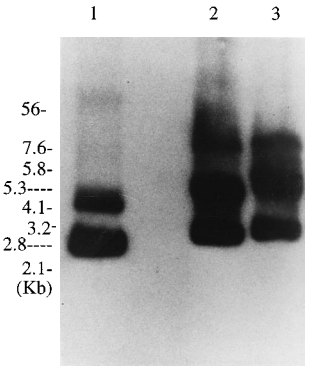

FIG. 6. Hybridization analysis of *Enterococcus faecium* 226 (lane 1), *L. lactis* LM0230 containing pHW112 (lane 2), and *L. lactis* LM0230 containing pHW800 (lane 3).

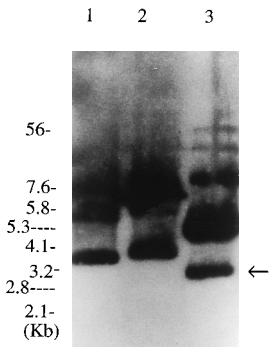

FIG. 7. Hybridization analysis of *L. lactis* LM0230 cultures containing pHW112 (lane 1), pHW800 (lane 2), and pGK13 (lane 3). The single-stranded form of pGK13 is indicated (arrow).

was directly in front of the start of the ORF and encompassed the −35 and −10 regions. The total AT-rich area (*repA*) spanned approximately 350 bp and included the two series of direct repeats. These characteristics (i.e., tandem repeats and runs of adenine residues) are analogous to many plasmid origins of replication (22).

The cloning strategies used in constructing plasmids used in this study are shown in Fig. 5. The 1,932-bp *Mbo*I fragment of pMBBI was ligated to the 1,434-bp *Mbo*I fragment of pGK12, which contains the chloramphenicol resistance gene of pC194 but lacks the ori sequence, to form pHW112. pHW700 was constructed by ligating the 173-bp *Bss*HII fragment containing the multiple cloning site of pBluescript II KS− into the single *Bss*HII site of pHW112. The vector pHW800 was constructed by ligating the 448-bp *Pvu*II fragment of pBluescript II KS− containing the multiple cloning site into the Klenow-treated *Xba*I site of pHW112.

The stability of pMBB1 became apparent during attempts to cure *Enterococcus faecium* 226 of the plasmid. In several attempts, derivatives of the culture lacking the plasmid could not be isolated (data not shown). In studies using pMMBB1 derivatives (Table 2), the ability of the replicon to be stably maintained was evident. Plasmids pHW112 and pHW800 were maintained by more than 95% of the population after 100 generations of growth.

Hybridizations of whole-cell lysates failed to detect the presence of any single-stranded intermediates of pMBB1 or its derivatives. In Fig. 6, lysates containing pMBB1, pHW112, and pHW800 were hybridized to a radiolabeled probe consisting of the 1.9-kb *Mbo*I fragment of pMBB1. Only bands corresponding to the molecular weights of the double-stranded forms were detected. In Fig. 7, a positive control (pGK13) which is known to produce single-stranded DNA is included with the pMBB1 derivatives pHW112 and pHW800. Hybridization with the labeled 1.4-kb *Mbo*I fragment of pGK12 which contains the chloramphenicol resistance gene showed the presence of a single-stranded intermediate in the pGK13 lysate, but no such forms were present with the pMBB1 derivatives.

Unlike many small gram-positive plasmids, pMBB1 lacks a consensus ori site of the type (27) (TACTACGA) which is found in many rolling-circle replicating plasmids of the pE194 class. Also, the lack of homology to any of the vectors of this type and the failure to detect any single-stranded forms of pMBB1 verify that this replicon does not belong to that class of plasmids and most likely replicated via a theta mechanism.

The replication region of pMBB1 resembles the motif of the lactococcal plasmid pCI305 and other gram-positive plasmids (11, 17) by containing an AT-rich region followed by an ORF which presumably encodes a replication protein. The lack of extended homology between pCI305 and pMBB1 in the sequence which is essential for replication of pMBB1 (i.e., *repB*) suggests only a moderate relationship between these plasmids. The amino acid homology between the predicted RepB protein of pMBB1 and the RepB protein of pCI305 is shown in Fig. 4. This region is conserved in the family of pCI305-like vectors (23) but is only one of several conserved regions identified in this vector family. Two more differences between the replication region of pMBB1 and the pCI305 family of plasmids (23) point to a limited relationship. No inverted repeats, centered around the translational start of the *repB* region of the pCI305-related vectors, were identified in pMBB1, and no ORF with amino acid homology to ORF X exists in pMBB1.

The origin of replication of pMBB1 has a somewhat broader range of functionality than does the replicon of pCI305 (12). pMBB1 derivatives have been successfully transformed into *Lactococcus*, *Leuconostoc*, and *Pediococcus* hosts (data not shown). However, like the pCI305 replicon, pMBB1 derivatives do not replicate in *E. coli*.

The stability of the pMBB1 replicon is demonstrated by plasmids pHW112 and pHW800. After growth for 100 generations without selection, less than 5% of the culture had lost the plasmid. In contrast, pHW700 was lost at a much higher rate. At 100 generations, more than 80% of the population had lost the plasmid. This indicates that the insertion of the 173-bp multiple cloning region next to the AT-rich region disrupted the replication function. The construction of a stable vector similar to pHW800 should be useful in future studies, especially for constructing lactococcal starter strains with improved fermentative and bacteriocin producing abilities.

Nucleotide sequence accession number. The nucleotide sequence of the 1,932-bp *Mbo*I fragment of pMBB1 has been submitted to GenBank under accession number U26268.

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