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Shuttle Plasmid Vectors for *Lactobacillus casei* and *Escherichia coli* with a Minus Origin

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Recombinant plasmids which can be used as shuttle vectors between *Escherichia coli* and the industrially used strains of *Lactobacillus casei* were constructed. They have replication regions closely related to those of pUB110 and are likely to replicate by a rolling-circle mechanism via a plus-strand-specific DNA intermediate in *L. casei*. Both orientations of *palA* from the staphylococcal plasmid pC194 and those of the intergenic region from coliphage M13 are identified as active minus origins in *L. casei*, in contrast to the pAMα1Δ1-derived BA3 minus origin which does not function in *L. casei*. Stability of the plasmids increased in *L. casei* when one of these two active minus origins was inserted. All the DNA sequences of the constructed vectors were known.

Lactobacillus casei S-1 and its derivatives are industrially useful strains. Lactic acid beverages and fermented milk have been produced from skim milk culture of the strains for several decades (34). In addition, heat-killed cells of the derivative strain exhibit antitumor and immunostimulating activities (29, 35), and development of a drug that makes use of these activities is now in progress. Recently, a blood pressure-lowering effect was also observed in hot water extracts from autologous lysates of the derivative strain (14). Therefore, development of a host-vector system is needed for improvement of these strains by genetic manipulation.

Many attempts have been made to transform or transfect *L. casei* strains by using protoplasts and liposomes (8, 45); however, these methods are neither efficient nor reproducible. Electroporation is a technique by which cells are exposed to electrical fields for permeation of nucleic acids and is used for transformation and transfection of eucaryotic and procaryotic cells (10). In 1987, Chassy and Flickinger first showed that this technique is applicable to some *L. casei* strains (9). Therefore, we have used this technique to obtain plasmid vectors that can transform *L. casei* S-1 derivatives. However, transformation of our strains was unsuccessful when available plasmids were tested. Therefore, it was necessary to construct vectors useful for transformation of the *L. casei* strains.

A staphylococcal plasmid, pUB110, replicates by a rolling-circle mechanism via a single-stranded (ss) DNA intermediate in *Bacillus subtilis* and *Staphylococcus aureus* (2, 56), and this mechanism is widespread among gram-positive bacteria (16, 39). When pUB110 replicates in cells of *B. subtilis* or *S. aureus*, the plasmid regions, *rep*, *ori*, and BA3, function as a structural gene that encodes a replication protein, an initiation site of replication for the plus strand of the plasmid DNA (plus origin), and a signal for efficient conversion from the plus-stranded plasmid DNA to the double-stranded DNA (minus origin) by synthesis of the minus strand of the plasmid DNA, respectively (2, 7, 33, 56).

pHY320PLK is a shuttle plasmid vector which was constructed for use in *Escherichia coli* and *B. subtilis* (25) (see Materials and Methods and Fig. 1). Transformants of pHY320PLK were not obtained in *L. casei* S-1 derivatives. However, this plasmid was expected to replicate in the *L. casei* S-1 derivatives for the following reasons. (i) pHY320PLK has *rep* (*repα1*), *ori* (*oriα1*), and BA3 from pAMα1Δ1 (40), and their DNA sequences are identical to those of equivalent regions of pUB110 except for one base in the dispensable region between *rep* and *ori* (23, 36, 37). (ii) Replication via a rolling-circle mechanism is likely among some plasmids derived from *Lactobacillus* spp. because of sequence homology to plasmids known to replicate by this mechanism or accumulation of ssDNA (1, 3, 6, 28, 48).

This report describes the construction of pHY320PLK-based shuttle vectors able to replicate both in *E. coli* and in the *L. casei* S-1 derivatives. In contrast to the case with *B. subtilis*, BA3 did not function as a minus origin in *L. casei*. Subsequently, two DNA segments that did serve as active minus origins in *L. casei* were identified: (i) *palA* from the staphylococcal plasmid, pC194, which was known to function as a minus origin in *S. aureus* (17) but inefficiently in *B. subtilis* and *Streptococcus pneumoniae* (12, 56); and (ii) the intergenic (IG) region from ssDNA phage M13, which was reported to be similar to the *palA*-type minus origin (12). When a fragment including one of these regions was inserted in either orientation, the stabilities of the vectors in *L. casei* were improved.

MATERIALS AND METHODS

Bacterial strains. *E. coli* HB101 (r_B^- m_B^-) and JM109 (r_K^- m_K^+) were obtained from Takara shuzo (Kyoto, Japan). *L. casei* C257, which is a φFSW prophage-cured derivative of S-1 (46), and strain MSK248, which is a plasmid-free derivative of C257, were described previously (43).

Plasmids. pHY300PLK (22, 24, 26) and pHY163PLK (23) were described previously. pHY300PLK consists of *oriα1*, *repα1*, and the tetracycline resistance gene from pAMα1Δ1 (40), the RNA primer region for initiation of DNA replication in *E. coli* from plasmid p15A, and the ampicillin resistance gene from Tn*3* via pACYC177. pHY320PLK was constructed as a shuttle plasmid vector between *E. coli* and *B. subtilis* by the insertion of the BA3 minus origin sequence downstream of the tetracycline resistance gene of pAMα1Δ1 into the *Eco*RI site of pHY300PLK in the natural orientation (25). The BA3 region between the *Eco*47III and *Bbe*I sites in pHY320PLK includes a DNA sequence identical to that of pUB110, extending from nucleotide 1136 to 1553 (36, 37) (Fig. 1). pLY201 is a deletion derivative of pAMβ1 and is

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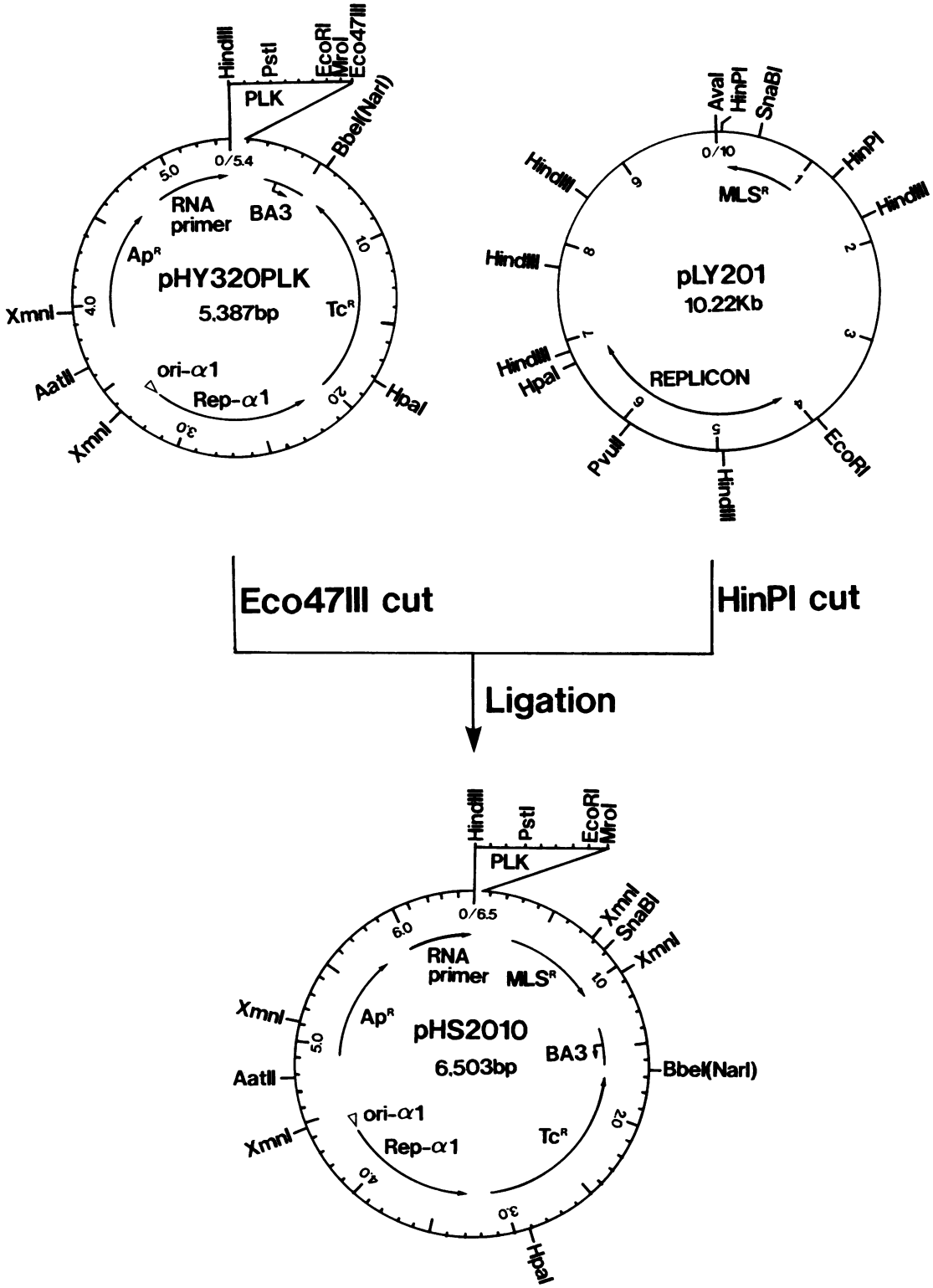

FIG. 1. Construction of pHS2010. The termini of the 1.1-kb *Hin*PI fragment of pLY201 (27) containing the MLS resistance gene was filled in with T4 DNA polymerase and ligated with the dephosphorylated *Eco*47III fragment of pHY320PLK (25). Only *Hin*PI sites of interest are indicated in pLY201. The replicon region in pLY201 was deduced from Swinfield et al. (50).

able to replicate in an *L. casei* S-1 derivative (27). pSA3 (11), pGKV11 (52), pUC119 (54), and pC194 (21) were obtained from J. Ferretti of the University of Oklahoma; J. Kok of the University of Groningen, the Netherlands; Takara shuzo; and T. Tanaka of Mitsubishi-kasei Life Science Institute, Tokyo, Japan, respectively.

Transformation and DNA manipulations. *E. coli* cells were transformed by the $CaCl_2$-RbCl method (18). *L. casei* cells were electrotransformed with an exponential decay pulse generated by a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) with a pulse controller as follows. Cells of the *L. casei* recipient strain were grown to mid-exponential phase at 37°C in 20 ml of Rogosa's medium (13) and were harvested by centrifugation. The cells were washed twice with 20 ml of 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.0, and once with 1 ml of 10% glycerol at 0°C and suspended in 200 μl of 10% glycerol. Forty microliters of the cell suspension were transferred to a chilled Gene Pulser cuvette (electrode gap, 2 mm) onto which DNA solution (<80 μl) had already been added. After a pulse (peak voltage, 1.5 kV; capacitance, 25 μF; resistance, 200 Ω) was applied to the cells, 1 ml of Rogosa's medium was added to the cell suspension as soon as possible. The mixture was incubated at 37°C for 1 h for gene expression and then plated on a Rogosa's agar plate containing 20 μg of erythromycin per ml. Under these conditions, the transformation frequency was approximately 2×10^5/μg of DNA.

All the DNA manipulations for construction of shuttle vectors were performed in *E. coli* by the method of Sambrook et al. (42).

DNA sequencing analysis. DNA sequences of all sources consisting of the constructed vectors, pHY300PLK (24), pHY163PLK (23), pHY320PLK (the accession number of pHY320PLK is D00946 in DDBJ, EMBL, and GenBank), the 1.1-kbp *Hin*PI macrolides-lincosamides-streptogram B (MLS) fragment from pAMβ1 (5), the IG region of M13 (53), and pC194 (21) are available. DNA sequences of the constructed vectors were known by first connecting the above sequences by using the SDC Genetyx program (Software Development Co. Ltd., Tokyo, Japan) and by then confirming them by sequencing the junction regions by using the dideoxynucleotide chain-termination method with double-stranded DNA (20) and primers synthesized as described previously (41).

Construction of strand-specific probes. A segment of each DNA strand of plasmid pHS2010 that included *repα1* was cloned into M13mp18 (57), and strand-specifically ^{32}P-labeled DNAs were prepared with the universal primer. Replicative form of M13mp18 was purchased from Takara shuzo. The 1.6-kb *Hpa*I-*Xmn*I fragment of pHS2010 including *repα1* was inserted into the *Hin*cII site of M13mp18 replicative form. Among the ss virion DNAs of the clones obtained, clone 5 had the template strand of Repα1 in the insert, and clone 17 had the reverse strand of Repα1 in it. After ss virion DNAs were extracted from clones 5 and 17, [α-^{32}P]dCTP (2,000 to 3,000 Ci/mmol; Amersham, Buckinghamshire, England) was incorporated with Sequenase version 2.0 (obtained from Toyobo, Osaka, Japan) from the universal primer preannealed with the ssDNA under the conditions suggested by the supplier. The reaction product was separated from the unincorporated nucleotides by gel filtration with Sephadex G-50.

ssDNA accumulated in *L. casei* cells. ssDNA in the cell lysate was detected by Southern hybridization (49) with the strand-specific probes. Lysates of *L. casei* MSK248 cells carrying the plasmids were prepared as follows. Cells were grown to late exponential phase in 40 ml of Rogosa's medium containing 20 μg of erythromycin per ml at 37°C and harvested. The cells were washed twice by centrifugation, resuspended in 8 ml of lysis mixture containing 1 M sucrose, 25 mM sodium bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane, pH 6.8, 5 μg of *N*-acetylmuramidase SG (Seikagaku Kogyo, Tokyo, Japan) per ml, and 100 μg of egg white lysozyme (Sigma, St. Louis, Mo.) per ml and incubated at 37°C for 25 min. Cells were then collected by centrifugation, suspended in 4.5 ml of Tris-EDTA (TE) buffer containing 50 mM Tris-hydrochloride, pH 8.0, and 20 mM sodium EDTA and frozen at −20°C. Cell lysis was accomplished by thawing at 65°C. The lysate was centrifuged at 30,000 rpm at 15°C for 10 min in a Hitachi RP55T rotor, and the supernatant was extracted twice with phenol-chloroform (1:1 [vol/vol] of water-saturated phenol and chloroform-isoamyl alcohol [24:1 {vol/vol}]), once with chloroform-isoamyl alcohol (24:1 [vol/vol]), and twice with ether. Finally, the sample was treated with 40 μg of RNase A (Sigma) per ml at 37°C for 30 min. If necessary, S1 nuclease (Takara shuzo) was added at 37°C for 30 min at a final concentration of 250 U/ml. After electrophoresis, the DNA in an agarose gel slab was transferred to a nitrocellulose filter as described previously (46, 49), except that the alkali treatment was omitted, and hybridized with the strand-specific probes described above. Procedures for electrophoresis and hybridization were described previously (46). Density of the hybridization bands was measured with an LKB 2202 Ultroscan laser densitometer.

Stability of the plasmids in *L. casei*. Plasmid-containing *L. casei* MSK248 cells grown overnight at 37°C in Rogosa's medium containing 20 μg of erythromycin per ml were suspended in the same volume of Rogosa's medium without the drug (generation 0). A 10^{-5} dilution was inoculated into Rogosa's medium without the drug, and then this culture was grown at 37°C to early stationary phase. The resultant culture was successively transferred several times without the drug as described above. In the serial cultures, the numbers of CFU were estimated on Rogosa's agar plates with or without the drug. The number of generations during each transfer was calculated as $\log_2$(total CFU at each stage/initial CFU).

Nucleotide sequence accession number. The accession number of the DNA sequence of pH2515 is D90381 in the DDBJ, EMBL, and GenBank nucleotide sequence data bases.

RESULTS

In order to develop a host-vector system in *L. casei* S-1 derivatives, attempts were made to introduce by electroporation the shuttle vectors pSA3, pGKV11, and pHY320PLK into *L. casei* MSK248, a plasmid-free derivative of S-1. However, no transformants that expressed the MLS resistance of pSA3 and pGKV11, the chloramphenicol resistance of pGKV11, or the tetracycline resistance of pHY320PLK were obtained. Since pHY320PLK was expected to replicate in *L. casei* S-1 derivatives and the MLS resistance gene of pAMβ1 was reported to be expressed in an S-1 derivative (27), the 1.1-kb *Hin*PI fragment including the MLS gene was isolated from pLY201, a deletion derivative of pAMβ1, and inserted into the *Eco*47III site of pHY320PLK after filling in the termini of the fragment (Fig. 1). The resulting plasmid was designated pHS2010 and is 6,503 bp long. pHS2010 provided MLS-resistant transformants of *L. casei* C257 and MSK248 following electroporation. MICs of tetracycline and

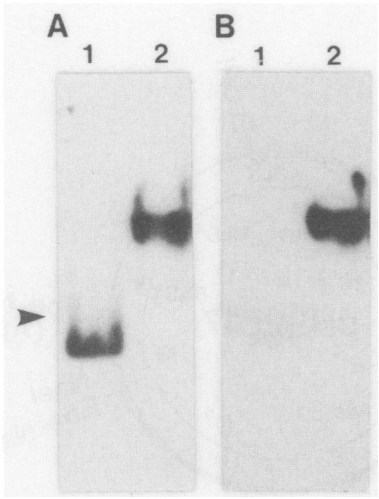

FIG. 2. Accumulation of the plus-strand-specific ssDNA in *L. casei* MSK248(pHS2010). Lysate of *L. casei* MSK248(pHS2010) (lane 1) and M13mp18 virion DNA (lane 2) were electrophoresed in an agarose gel and transferred to a nitrocellulose filter without an alkali-denaturing step and hybridized with the strand-specific probe 5 (A) or 17 (B) as described in Materials and Methods. The arrowhead indicates the position of covalently closed circular form of pHS2010 DNA which was detected in the hybridization with the alkali-denaturing step.

ampicillin for the transformants were indistinguishable from those without pHS2010 (data not shown). Therefore, it is unlikely that the tetracycline resistance gene from pAMα1 or the ampicillin resistance gene from Tn*3* on pHS2010 is expressed in *L. casei* S-1 and its derivatives. Transformation frequencies in *L. casei* MSK248 obtained by using pHS2010 DNA isolated from this strain were similar to that from *E. coli* HB101.

pHS2010 contains *repα1*, *oriα1*, and BA3 (derived as illustrated in Fig. 1), and their DNA sequences are almost identical to those of the equivalent regions of pUB110, which replicates by a rolling-circle mechanism via an ssDNA intermediate in *B. subtilis* and *S. aureus* (2, 56). Therefore, in order to elucidate whether pHS2010 replicates by a rolling-circle mechanism in *L. casei*, accumulation of plus-strand-specific ssDNA in the plasmid-carrying cells was examined by Southern hybridization using blots obtained without an alkali-denaturing step. The strand-specific fragments, 5 and 17, were used as probes in these experiments. As shown in Fig. 2, probe 5 detected a DNA species in MSK248(pHS2010) cells but probe 17 did so only scarcely. The intensity of the band detected by probe 5 was 74 times stronger than that detected by probe 17 when they were standardized with the band intensity of M13mp18 ssDNA, and both of the DNAs were sensitive to S1 nuclease (see Fig. 5, lane 1, for probe 5 and data not shown for probe 17). The probe 5-detected DNA migrated faster than the double-stranded DNA with covalently closed circular form which was detected by using Southern hybridization with the alkali-denatured gel (data not shown). These results indicate that MSK248(pHS2010) cells contained ssDNA consisting of the same strand known to be accumulated in *B. subtilis* cells carrying BA3-deleted pUB110 derivatives (plus strand) (2, 56). Therefore, it is likely that pHS2010 also replicates by a rolling-circle mechanism in *L. casei* S-1 derivatives and that the plus strand of the plasmid DNA is generated from *oriα1* by using *repα1* functions. However, the BA3 minus origin did not convert the plus strand of the plasmid DNA efficiently to the double-stranded form in *L. casei* by replication of the minus strand of the plasmid DNA compared with that in *B. subtilis*.

Since the activities of minus origins are known to vary among genera in which they reside (12, 17, 51), the BA3 minus origin in pHS2010 was replaced with a different minus origin or with a minus origin-like sequence or was deleted to examine the activity of the BA3 minus origin in *L. casei*. For the replacement, the *palA* minus origin from the staphylococcal plasmid pC194 and the IG region from coliphage M13 which included a *palA*-like sequence (12) were used.

A derivative carrying the IG region, pH2515, was constructed by recombination of two precursor plasmids as shown in Fig. 3. One precursor plasmid, designated pHS2517, was constructed by insertion of the *Apa*LI-*Bbe*I fragment of pUC119 including the IG region into the *Bbe*I site of pHS2010 after attaching the synthetic *Bbe*I/*Nar*I linker(s), 5′-pAGGCGCCT-3′, at the *Apa*LI prefilled terminus. The orientation of the IG region is shown in Fig. 3. The other precursor plasmid, designated pMSK721, was constructed by insertion of the 1.1-kb *Hin*PI MLS fragment of pLY201 into the *Sma*I site (between the unique *Bam*HI and *Eco*RI sites within the polylinker) of pHY163PLK after filling in the termini of the inserted fragment. Then, the *Eco*RI-*Nar*I fragment of the pHS2517 including the MLS resistance gene and the BA3 minus origin was replaced with the 1.1-kb *Eco*RI-*Bam*HI MLS fragment of pMSK721 after filling in the *Nar*I and *Bam*HI termini. Another derivative of pHS2010, pH2516, was isolated from pH2515 by inversion of the *Bbe*I fragment including the IG region. A BA3-deleted derivative of pHS2010, pH2012, was constructed from pH2515 by deletion of the *Bbe*I IG fragment. As shown in Fig. 4, derivatives carrying the *palA* minus origin, pH2311 and pH2312, were constructed by insertion of the 0.38-kb *Sau*3AI-*Ban*III fragment of pC194 including the *palA* region into the *Aat*II site of pH2012 in each orientation after blunting the termini of the fragments. In pH2312, the terminal one base of the *Sau*3AI site was lost during the manipulation.

pH2311 has the *palA* minus origin in the orientation to function efficiently in *B. subtilis*, and pH2312 has it in the reverse orientation. In pH2515, the direction of the IG region is the same as that of the similar sequence, *palA*, in pH2311.

Figure 5 shows accumulation of ssDNA in *L. casei* cells carrying the plasmids constructed by Southern hybridization without an alkali-denaturing step by using probe 5. Cells carrying pHS2010 and pH2012 accumulated S1 nuclease-sensitive DNA, that is, ssDNA, at a similar level, while those of pH2311, pH2312, pH2515, and pH2516 did not. The detected ssDNAs were plus-strand-specific because probe 17 scarcely detected them (data not shown). These results suggest that both orientations of *palA* of pC194 and those of the IG region of M13 exert minus origin activities in *L. casei*.

Since the BA3 minus origin has the ability to increase stability of the plasmid in its active host (7, 33), effects of *palA* and the IG region on plasmid stability were examined in *L. casei* MSK248. Figure 6 shows that the proportions of plasmid-carrying cells in cultures of MSK248(pH2311), MSK248(pH2312), MSK248(pH2515), and MSK248(pH2516) were similar to each other but larger than that of MSK248(pHS2010) when these strains were cultured for approximately 50 generations in Rogosa's medium without selective pressure. These results indicate that *palA* of pC194 and the IG region of M13 provide increased stability to the plasmids in which they have been incorporated in *L. casei*.

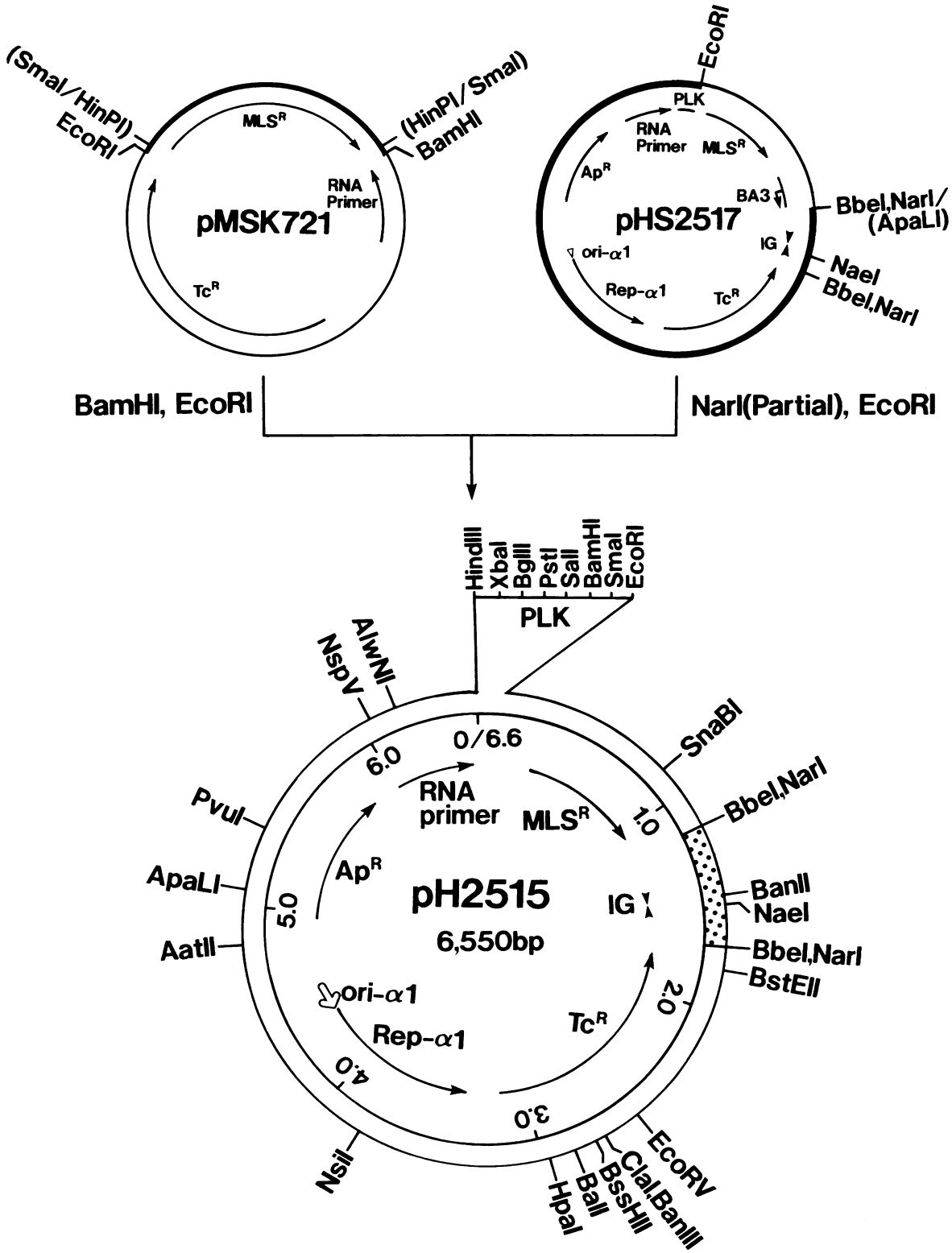

FIG. 3. Construction of pH2515 from two precursor plasmids, pHS2517 and pMSK721. Detailed procedures are described in the text. Bold lines in the precursor plasmids represent the regions which were used for construction of pH2515. Only restriction sites of interest are indicated in pHS2517 and pMSK721, and unique restriction sites plus two *Bbe*I sites in pH2515 are indicated. The IG region from coliphage M13 is indicated by dots in pH2515.

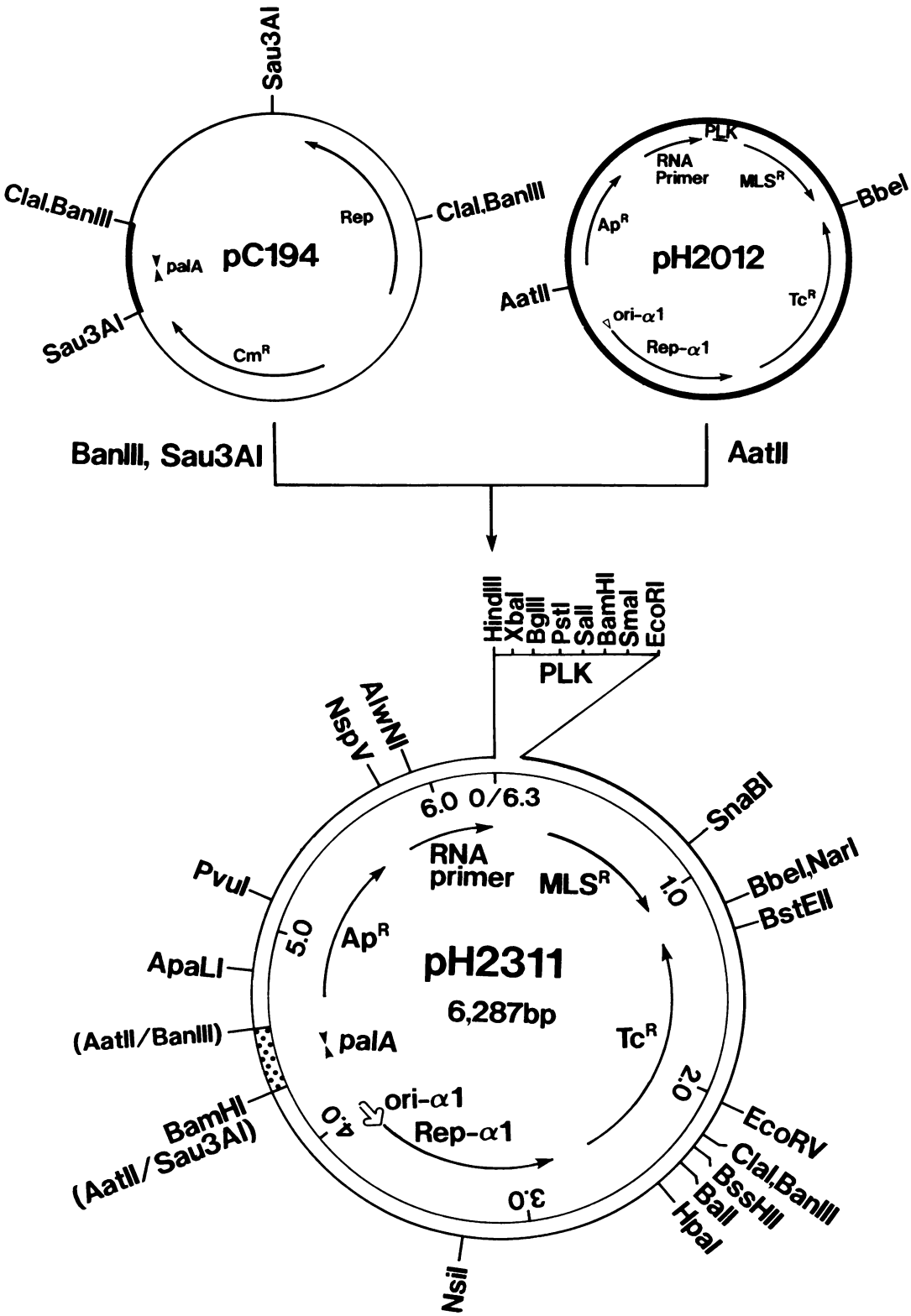

FIG. 4. Construction of pH2311 from pC194 and pH2012. Detailed procedures are described in the text. Bold lines in pC194 and pH2012 represent the regions which were used for construction of pH2311. Only restriction sites of interest are indicated in pC194 and pH2012, and unique restriction sites plus two *Bam*HI sites in pH2311 are indicated. One of the *Bam*HI sites was generated by insertion of the *palA* segment from staphylococcal plasmid pC194, which is indicated by dots in pH2311.

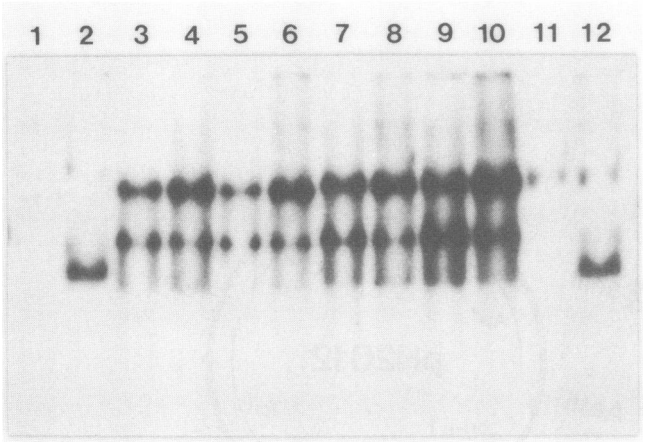

FIG. 5. Effects of the *palA* segment from staphylococcal plasmid pC194 and the IG region from coliphage M13 on accumulation of ssDNA in the plasmid-carrying *L. casei* MSK248 strains. Lysates of MSK248 cells having pHS2010 (lanes 1 and 2), pH2311 (lanes 3 and 4), pH2312 (lanes 5 and 6), pH2515 (lanes 7 and 8), pH2516 (lanes 9 and 10), and pH2012 (lanes 11 and 12) with the treatment of S1 nuclease (lanes 1, 3, 5, 7, 9, and 11) or without the treatment (lanes 2, 4, 6, 8, 10, and 12) were electrophoresed, transferred to a nitrocellulose filter without an alkali-denaturing step, and hybridized with probe 5 as described in Materials and Methods.

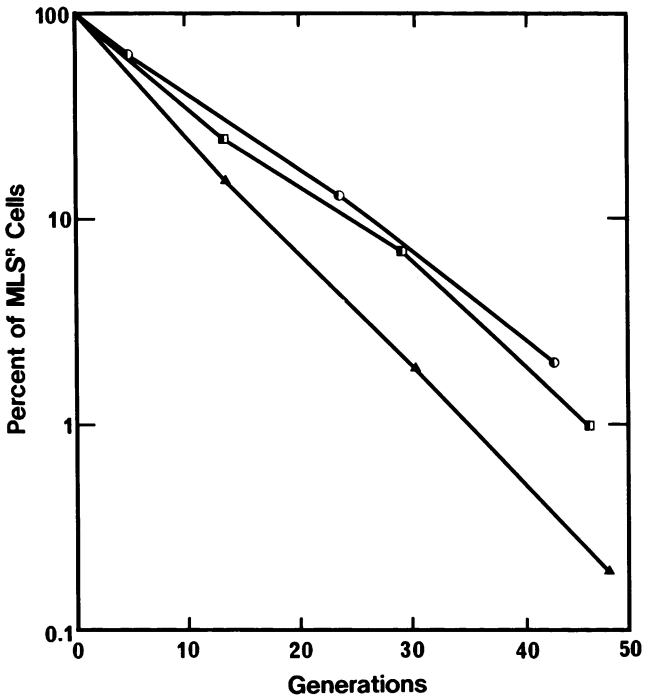

FIG. 6. Stabilities of the constructed plasmids in the cells of *L. casei* MSK248. MSK248 carrying pHS2010 (▲), pH2311 (■), pH2312 (□), pH2515 (●), and pH2516 (○) were successively transferred under the nonselective culture conditions, and plasmid-carrying cells were counted as MLS-resistant CFU as described in Materials and Methods.

DISCUSSION

Efficient transformation of some *L. casei* strains with electroporation was first reported by Chassy and Flickinger (9). We have used this technique to develop a host-vector system in *L. casei* S-1 derivatives used industrially. We also have constructed recombinant plasmids having replication regions for *L. casei* from the enterococcal plasmid pAMα1Δ1, which is closely related to those of staphylococcal plasmid pUB110. These recombinant plasmids can be used as shuttle vectors between *E. coli* and *L. casei*, since the β-galactosidase gene of *E. coli* was cloned into them, and they are stably maintained in both hosts (47). They are useful for restriction mapping of inserts after cloning, since DNA sequences of the vector plasmids are known.

Since pUB110 is known to replicate via rolling-circle mode in *B. subtilis* and *S. aureus* (2, 56), the constructed vectors were expected to replicate in the same fashion in *L. casei* S-1 derivatives. This was confirmed by Southern hybridization with the strand-specific probes to detect plus-strand-specific ss plasmid DNA accumulated in the cells carrying our primitive vector pHS2010. This also suggested that pHS2010 does not have a sequence able to function as an efficient minus origin in *L. casei* S-1 derivatives, although there is a sequence of the pAMα1Δ1-derived BA3 minus origin active in *B. subtilis* and *S. aureus* in the plasmid.

Lack of a minus origin was reported to result in plasmid instability, probably because of inefficient synthesis of the minus strand of the plasmid DNA (7, 12, 33). This prompted us to search for a minus origin active in *L. casei* S-1 derivatives and to introduce it into the plasmid vector. The *palA* segment from the staphylococcal plasmid pC194 and the IG region from coliphage M13 were identified as minus origins functioning in *L. casei*, since accumulation of ssDNA was not detected in the cells carrying the plasmids with inserts of these regions. In the life cycle of M13, the IG region functions as initiation sites for both plus- and minus-strand DNA syntheses by a rolling-circle replication mechanism and as a packaging site in virion morphogenesis (58). The sequence similarity between the IG region and *palA*-type minus origins was evident from an analogy to their functional similarity in DNA replication (12). In this study, it was confirmed that the IG region was able to function as a minus origin in *L. casei*. It is also noteworthy that both orientations of the *palA* segment and those of the IG region are active in *L. casei*. To our knowledge, minus origins active in other bacteria are orientation specific (2, 12, 17). It has been reported that pLAB1000, a plasmid derived from *Lactobacillus hilgardii*, contains a sequence that reduces accumulation of ss plasmid DNA. However, the active segment was not specified, and the plasmid does not include a sequence homologous to *palA*-type minus origins (28).

Among the constructed plasmids having active minus origins, a rolling-circle type of DNA replication was apparent in *L. casei*, although the combination of the plus (*oriα1*) and minus (*palA* or IG region) origins had been artificially constructed. When these plasmids were introduced into *L. casei*, their stabilities were increased relative to those of the corresponding plasmids carrying the BA3 minus origin.

As shown in Fig. 5, S1 nuclease-resistant DNAs were detected in cells of *L. casei* carrying the plasmids with the active minus origins. Since the covalently closed circular form of the plasmid DNA was not detected under our hybridization conditions, the detected S1 nuclease-resistant DNAs could be similar to high-molecular-weight plasmid multimers which were reported to exist in *B. subtilis* cells carrying pUB110 derivatives containing inserts of foreign DNA or in the cells of an ATP-dependent DNase-deficient mutant (*addA5*) of *B. subtilis* carrying pUB110 (15, 55).

Transformation frequencies of *L. casei* MSK248 with

pHS2010 DNA isolated from this strain were similar to those observed with the same plasmid isolated from a modification enzyme-deficient *E. coli*. It was reported that DNA introduced into *Lactobacillus helveticus* subsp. *jugurti* by electroporation was likely to be restricted by host-controlled mechanism (19). In contrast, Langella and Chopin (31) showed that host-controlled restriction systems did not affect transformation frequencies when some *Lactococcus lactis* strains were transformed with plasmid DNAs by electroporation. A restriction-modification system was detected in phage ϕFSW-infected *L. casei* MSK248 (44). Therefore, whether this system affects electrotransformation in *L. casei* S-1 derivatives is yet to be elucidated. The possibility that pHS2010 does not have recognition sites for the putative restriction enzyme(s) of MSK248 remains.

Although pSA3 and pGK12 could be introduced to *L. casei* 393 by electroporation (9, 32), MLS-resistant transformants were not obtained from our *L. casei* MSK248 by using pSA3 and pGKV11, which has the same replication region and MLS gene as pGK12 (30). The replication region of pSA3 is derived from pIP501 and is closely related to that of pAMβ1 (4, 50), which is able to replicate in a derivative of MSK248 (27). In addition, the tetracycline gene from pAMα1Δ1, which was not expressed in MSK248, was expressed in other *L. casei* strains, IAM1045 and JCM1053 (38). Gene expression systems may be different among *L. casei* strains.

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