S4-2

Characterization of pFMBL1, a Small Cryptic Plasmid Isolated from *Leuconostoc mesenteroides* SY2

Jeong Hwan Kim

Division of Applied Life Science, Graduate School, and Institute of Agriculture & Life Science
Gyeongsang National Universit

L. mesenteroides SY2 isolated from Kimchi harbors three plasmids (15, 8, and 4.6 kb). The smallest, pFMBL1, was cloned into pUC19 by using the unique *Sac*I site to yield pSJ46, and the construct was used for sequence determination. The complete nucleotide sequence (4,661bp) was determined. Sequence analysis revealed two open reading frames (ORFs), *orf1* and *orf2,* and six palindromic sequences. ORF1 (261 amino acids) had no significant homology to any other protein in the databases. ORF2 (150 amino acids) had 58% identity with a protein (81 amino acids in size) suspected to be involved in the replication of pTXL1, a small cryptic plasmid from *L. mesenteroides* ssp. *mesenteroides* Y110 (Biet et al., 2002). The homologous region was confined to the C-terminal half of ORF2. To determine the minimum region required for the replication of pFMBL1, different regions in pFMBL1 were PCR amplified and used for transformation experiments. Fig. 1 shows the location of each fragment in pFMBL1 and the recombinant plasmids were introduced into competent *L. mesenteroides* SY1 cells by electroporation. The only construct, which was able to replicate in *L. mesenteroides* SY1 and thus allowed the transformants to form colonies

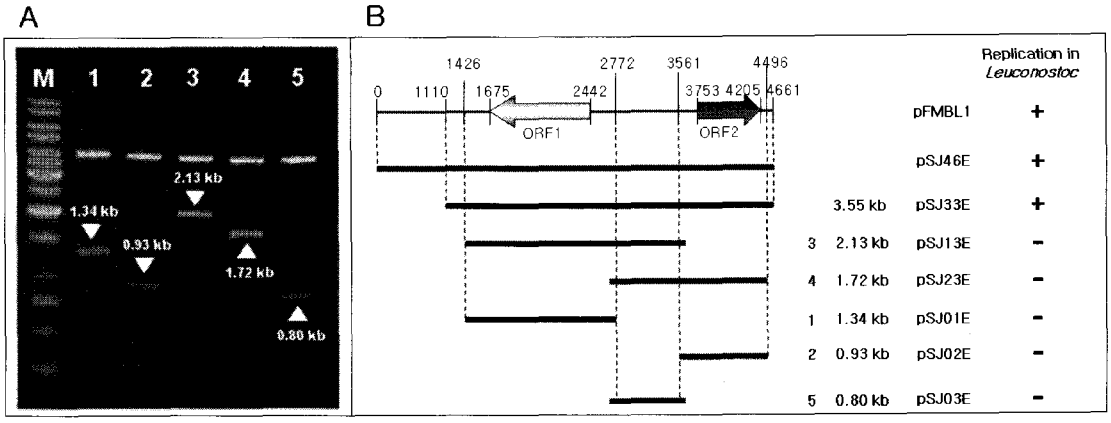

Fig. 1. Identification of the minimal replicon from pFMBL1. (A) Agarose gel electrophoresis of plasmids containing different regions from pFMBL1 for determining the minimal replicon. M, 1 kb ladder (MBI); lane 1-5, *Eco*RI digested pSJ01E (lane 1), pSJ02E (lane 2), pSJ13E (lane 3), pSJ23E (lane 4), and pSJ03E (lane 5). (B) Location of each fragment examined for the minimal replicon.

on MRS agar plates containing Em (5 μg/ml), was pSJ33E. pSJ33E was used for transformation of *L. mesenteroides* SY1, another Kimchi isolate (Kim et al., 2005), and it was successfully employed for the overexpression of *ccpA* in *L. mesenteroides* SY1. The transformation efficiency was quite low, 10^1 TFs/μg DNA. However, pSJ33E transformed other *Leuconostoc* species and *Lactobacillus brevis* 2.14 at higher efficiencies (see Table 1). Above all, *Leuconostoc lactis* showed the best efficiency (4×10^5). pSJ33E seems to be unable to replicate in lactococci. Northern blot experiment (Fig. 2) showed that only *orf2* transcript was detected and no transcripts from *orf1* or intergenic region were detected. The size of the detected *orf2* transcript, 580 nc, was in good agreement with the expected size for *orf2* transcript calculated from the DNA sequence data. To determine the replication mechanism of pFMBL1, the presence of ssDNA intermediate was examined. From whole-cell DNA preparation from *L. mesenteroides* SY1 [pSJ33E], no ssDNA band was observed regardless of S1 nuclease treatments (Fig. 3, lane b). In contrast, a fast migrating ssDNA band was detected when preparation from *L. lactis* ssp. *cremoris* MG1363 harboring pGK12, a RCR plasmid, was probed with radiolabelled pGK12. The results strongly suggested that pFMBL1 replicated via a theta-type replication mechanism. This indirect proof, together with the absence of any *orf* encoding a replicase as well as the lack of single-strand and double-strand origin sequences, is consistent with a mode of replication differing from the rolling-circle mechanism. Combined together,

Table 1. Electrotransformation efficiencies of pSJE with different hosts.

host	TFs/μg DNA
Leuconostoc mesenteroides SY1	1.1×10^1
Leuconostoc citrium	1.8×10^4
Leuconostoc gelidum	2×10^5
Leuconostoc lactis	4×10^5
Lactobacillus brevis 2.14	1.5×10^3

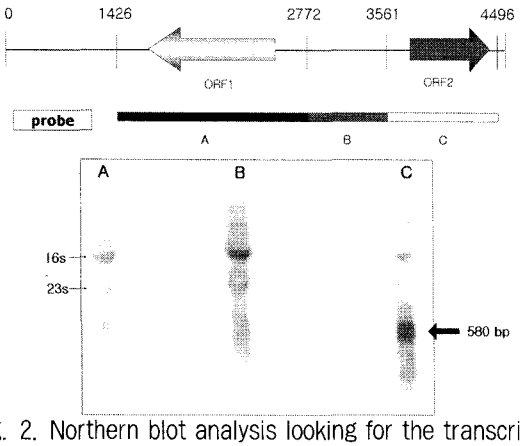

Fig. 2. Northern blot analysis looking for the transcripts from *orf1*, *orf2*, or intergenic region from pFMBL1 in *L. mesenteroides* SY1. The location and direction of transcription of *orf1* and *orf2* are shown. The size of the transcript is indicated by arrows. *orf1* (A), intergenic region (B), or *orf2* (C)- specific PCR product was used as a probe, separately.

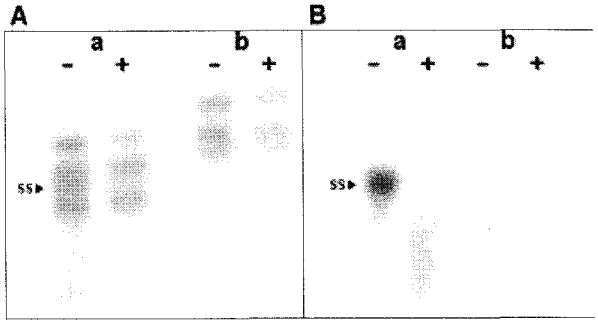

Fig. 3. Detection of ssDNA intermediate from (a) *Lactococcus lactis* ssp. *cremoris* MG 1363 [pGK12] and (b) *L. mesenteroides* SY1 [pSJ33E]. A, Alkaline Southern blot hybridization. B, Nonalkaline Southern blot hybridization. Whole-cell DNA preparations were prepared from cultures in MRS containing rifampicin (100 μg/ml). DNA prepararions treated with endonuclease S1 are indicated by +, and untreated by -. SS stands for ssDNA.

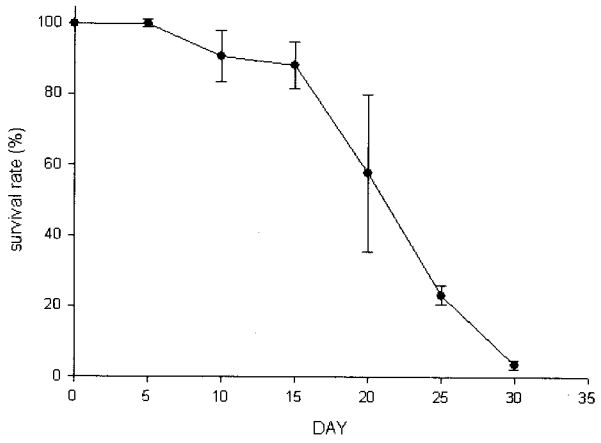

Fig. 4. Stability of pSJ33E in *L. mesenteroides* SY1

all the results indicated that *orf2* was essential for the replication of pFMBL1. It is unclear for the role of *orf1*. As shown in Fig. 4, pSJ33E was quite stable without antibiotic selection up to two weeks, i.e. more than 90% cells retained plasmid. After two weeks, the portion of cells which lost pSJ33E increased rapidly. Even after one month of daily subculturing in MRS broth without antibiotic, three percent of cells still retained plasmid. This number is much higher than those of many other small RCR-type plamids (Biet et al., 1999). The high stability of pSJ33E in *L. mesenteroides* SY1 is another indirect evidence that pFMBL1 replicates via theta replication mechanism. The high stability of pFMBL1 is an advantage when a food-grade vector is to be constructed from pFMBL1 since no antibiotic marker genes are allowed for food-grade vectors. In this respect, pFMBL1 might be useful for construction of a food-grade vector (Kiewiet et al., 1993).

Acknowledgments

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References

1. Biet, F., Cenatiempo, Y., Fremaux, C., 1999. Characterization of pFR18, a small cryptic plasmid from *Leuconostoc mesenteroides* ssp. *mesenteroides* FR52, and its use as a food grade vector. FEMS Microbiol. Lett. 179, 375-383.
2. Biet, F., Cenatiempo, Y., Fremaux, C., 2002. Identification of a replicon from pTXL1, a small cryptic plasmid from *Leuconostoc mesenteroides* subsp. *mesenteroides* Y110, and development of a food-grade vector. Appl. Environ. Microbiol. 68, 6451-6456.
3. Kiewiet, R., Bron, S., De Jonge, K., Venema, G., Seegers, J. F., 1993. Theta replication of the lactococcal plasmid pWV2. Mol. Microbiol. 10, 319–327.
4. Kim, J. H., Park, J. Y., Jeong, S. J., Chun, J. Y., Lee, J. H., Chung, D. K., Kim, J. H., 2005. Characterization of the α-galactosidase gene from *Leuconostoc mesenteroides* SY1. J. Microbiol. Biotechnol. 15, 800-808.