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Full Length Research Paper

Construction of an expression vector for *Lactococcus lactis* based on an indigenous cryptic plasmid

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To construct an expression vector for *Lactococcus lactis*, the EmPMT fragment which contained the erythromycin resistance gene, P_{32} promoter, multiple cloning site (MCS) and terminator (T) was subcloned into the small cryptic plasmid pAR141. The resulting vector, designated as pAR1411, was found to be stably maintained in *L. lactis* MG1363 after transformation for at least 100 generations under non-selective conditions. The vector was also demonstrated to be able to express the gene coding for chloramphenicol acetyltransferase (*cat*) in *L. lactis*.

Key words: Plasmid vector, transformation, expression.

INTRODUCTION

Lactococcus lactis is one of the best characterized lactic acid bacteria (LAB) and is widely used as a dairy starter culture traditionally. The extensive knowledge and the advancement of molecular biology techniques for this bacterium have not only enhanced its performance in dairy production, but also expanded its potential application in other areas (Konings et al., 2000). Due to its generally regarded as safe (GRAS) stature, this Gram-positive food-grade bacterium has been used as cell factories for the production of various chemicals for food industry (Luoma et al., 2001), pharmaceutical (Bermúdez-Humarán et al., 2003) and neutraceutical (Hugenholtz and Smid, 2002), in addition to being developed for vaccine delivery (Ramasamy et al., 2006; van Roosmalen et al., 2006; Mercenier et al., 2000). Recombinant *L. lactis* has also been used for the expression of several viral and eukaryotic proteins (Kunji et al., 2003; Madsen et al., 1999). Recently, the potential of *L. lactis* in nanobiotechnology has been exploited (Novotny et al., 2005).

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Most of these applications of *L. lactis* require the use of cloning and expression vectors for the introduction of foreign genes or genetic manipulations of the hosts. These vectors were generally derived from cryptic plasmids (Shareck et al., 2004; de Vos and Simons, 1994). Cryptic plasmids are extrachromosomal DNA elements that encode no recognizable phenotype besides their replication functions. Selectable markers are then incorporated into these plasmids to enable recombinant bacteria carrying them to be scored easily. The present study reported the construction of a constitutive expression vector for *L. lactis* based on an indigenous small cryptic plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* and *Escherichia coli* strains were cultured in GM17 (Terzaghi and Sandine, 1975) and LB (Sambrook and Russell, 2001) at 30 and 37°C, respectively. For *L. lactis*, erythromycin and chloramphenicol were used at a final concentration of 5 and 7.5 µg ml^{-1}. For *E. coli*, erythromycin at a concentration of 150 µg ml^{-1} was used.

Plasmids were isolated from *Lactococcal* strains using alkaline

Table 1. Bacterial strains and plasmids used in this study.

Strains/Plasmids	Relevant characteristics	Source/Reference
Strains		
L. lactis subsp. *cremoris* MG1363	Plasmid-free strain	Gasson, 1983
L. lactis subsp. *lactis* M14	Milk isolate, natural host of pAR141	Raha et al., 2006
E. coli TOP10	Cloning host	Invitrogen
E. coli XL1Blue	Cloning host	Stratagene
Plasmids		
pMG36e	3.6 kbp, Erm^r	van de Guchte et al., 1989
pNZ8048	3.3 kbp, Chl^r	Kuipers et al., 1998
pAR141	1.6 kbp, cryptic plasmid	Raha et al., 2006
pAR1411	3.2 kbp, Erm^r, pAR141 derivative containing EmPMT fragment	This study
pAR1411-cat	3.9 kbp, Erm^r, Chl^r, pAR1411 derivative containing *cat* as reporter gene	This study

analysis method (Sambrook and Russell, 2001). The only difference was that the growth medium for strains containing plasmid with anti-biotic resistance gene was supplemented with the appropriate antibiotic for the selection of plasmid-bearing cells. Analyses were carried out by agarose gel electrophoresis.

PCR Amplification of EmPMT Fragment from pMG36e and transformation into *L. lactis*

PCR was used to amplify a fragment (EmPMT) containing an erythromycin resistance gene (*ermC*), constitutive promoter P_{32}, multiple cloning site (MCS) and terminator T from pMG36e (van de Guchte et al., 1989) using EM1 (5′-CGA CAT ACT GTT CTT CCC-3′) and EM2 (5′-AAC CGT TTC TAC TCA ATG-3′) primers. The 50 µl PCR reaction mix comprised of 1× PCR Buffer with $MgSO_4$ (Fermentas), 0.2 mM of dNTP Mix (Fermentas), 0.08 mM of each EM1 and EM2 primers, 200 ng of pMG36e and 1.25 U of Pfu DNA polymerase (Fermentas). The PCR conditions were: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 3 min, and ended by final extension at 72°C for 10 min. The PCR product was then analysed with agarose gel electrophoresis.

The EmPMT fragment was cloned into the unique FspI site of pAR141 (Raha et al., 2006) with T4 DNA ligase (Fermentas). The ligation mix was transformed into competent *L. lactis* MG1363 by electroporation as described by Holo and Nes (1989) with minor modification. The electroporation was carried out with GenePulser (Bio-Rad; 2.3 kV, 25 µF and 200 Ω) and the transformants were screened on SGM17 (GM17 supplemented with 0.5 M sucrose) containing erythromycin after incubation at 30°C for 24 to 48 h. The recombinant plasmid was then isolated and verified by restriction enzyme digestion analysis. The stability of the recombinant plasmid was tested as described previously (Raha et al. 2006).

Transformation into *E. coli*

The newly constructed plasmid was then transformed into *E. coli* TOP10 and XL1-Blue competent cells using standard heat-shock method as described by Sambrook and Russell (2001). A widely used *lactococcal* shuttle vector pMG36e was used as the positive control for transformation. Transformants were selected from LB agar plates containing 150 µg/ml of erythromycin after overnight incubation at 37°C. Erythromycin resistant transformants were subjected to plasmid extraction followed by RE digestion analysis and PCR screening.

Cloning and expression of heterologous gene in pAR1411

Primers were designed to isolate the chloramphenicol acetyltransferase cat gene from a *lactococcal* vector pNZ8048. The open reading frame (ORF) of chloramphenicol acetyltransferase (cat) gene was PCR amplified from pNZ8048 using CmF (5′-GGC TCT AGA TAT GAA CTT TAA TAA AAT TGA TTT AG-3′) and CmR (5′-AAT CTG CAG TTA TAA AAG CCA GTC ATT AGG-3′) primers that contain Xba I and Pst I site at their respective 5′-end. The PCR conditions used were: initial denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR product was analysed by agarose gel electrophoresis, cloned into pAR1411 and transformed into *L. lactis* MG1363 as described above. The transformants were selected on SGM17 agar containing erythromycin, chloramphenicol or both. The recombinant plasmids were then isolated from the transformants and verified.

Verification of insertion of cat gene in pAR1411

Single colonies of transformants were subcultured in SGM17 broth standing culture containing erythromycin and chloramphenicol, or only chloramphenicol at 30°C. Plasmid DNA was then extracted from the culture. The insertion of cat gene in pAR1411 in the putative recombinant plasmid was verified through RE digestion analysis with PstI and XbaI. The RE digests were then subjected to agarose gel electrophoresis.

RESULTS

Each of the erythromycin resistant transformants selected

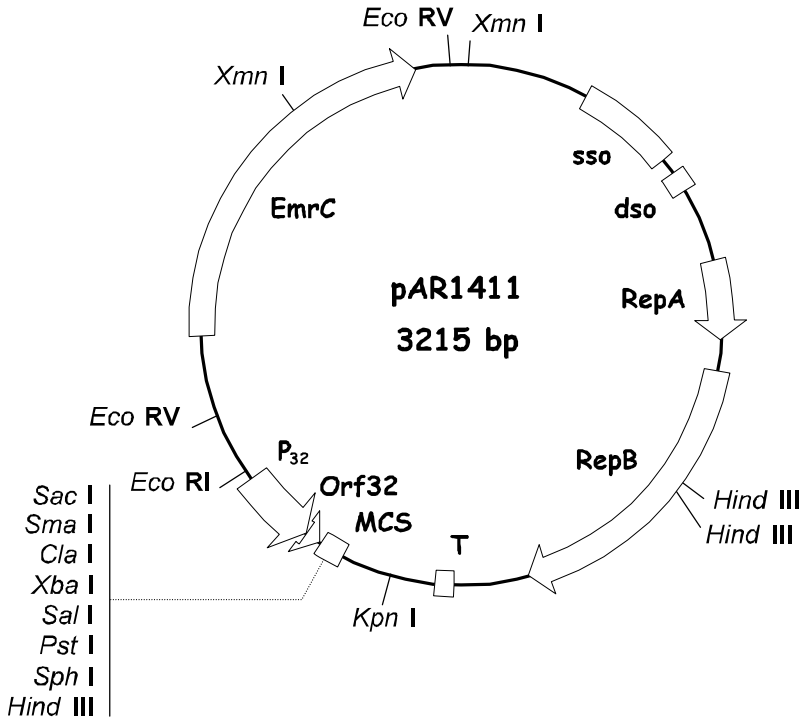

Figure 1. Schematic diagram of pAR1411.

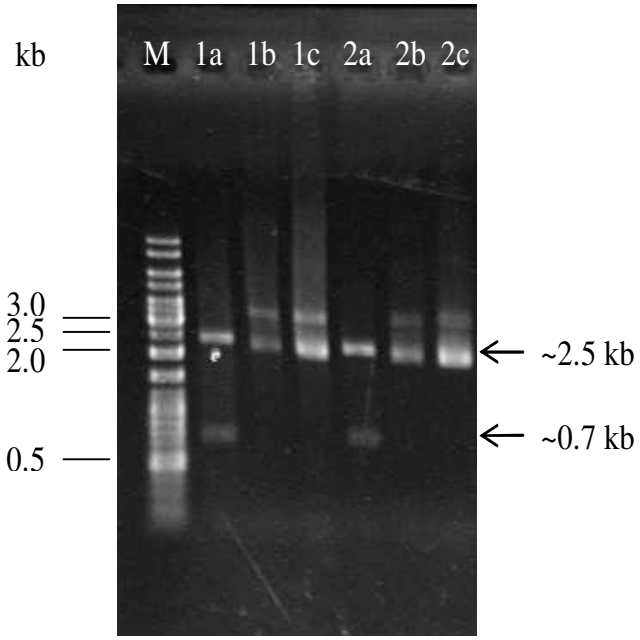

Figure 2. Analysis of putative recombinant plasmids digested with HindIII and Eco321. Lane M: GeneRuler™ DNA Ladder Mix (Fermentas); Lanes 1 and 2: putative pAR1411 from *L. lactis*; Lane a: HindIII-digested samples; Lane b: Eco321-digested samples; Lane c: undigested samples. The arrows showed the expected bands of HindIII digest. The yield of two visible bands of ~0.7 and ~2.5 kbp reveals that the ermC was in the same orientation with the repA and repB genes.

was found to contain a single plasmid that was larger than pAR141. The calculated size of the recombinant plasmid was about 3.2 kbp. Restriction enzyme analysis of these plasmids recovered from erythromycin resistant *L. lactis* transformants showed that the 1.6 kbp blunt-ended EmPMT fragment has been successfully cloned into pAR141. Digestion with HindIII yielded two visible fragments of ~0.7 and ~2.5 kb, which indicated that the ermC gene was in the same orientation with the repA and repB genes of pAR141 (Figures 1 and 2). Constructs with resistance and replication genes in opposite direction, which would have given rise to two detectable fragments of ~1.4 and ~1.8 kb, were not obtained. This newly constructed plasmid vector was designated as pAR1411.

Plasmid stability test showed that pAR1411 was highly stable (~100%) in *L. lactis* for at least 100 generations under non-selective conditions. However, after several attempts, pAR1411 could not be recovered from the *E. coli* erythromycin resistant transformants.

In order to examine the usefulness of pAR1411 as a cloning and expression vector, a gene coding for chloramphenicol was selected as the reporter marker. The cat gene was isolated from pNZ8048 by PCR amplification, devoid of its transcriptional and translational signals. Both the amplicon and pAR1411 were digested with *Pst*I and *Xba*I. The open reading frame of the cat gene (~0.7 kb) was then cloned in-frame with the P_{32} promoter in pAR1411, producing the contstruct pAR1411-

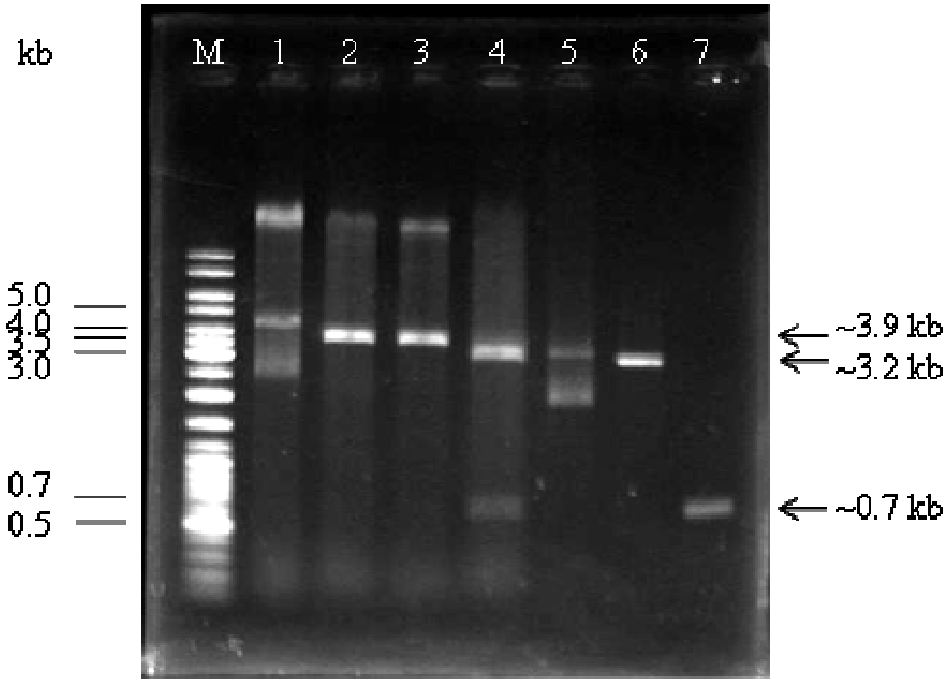

Figure 2. Restriction enzyme analysis of putative recombinant plasmid pAR1411-cat for verification. Lane M: GeneRuler™ DNA Ladder Mix (Fermentas); Lane 1: Undigested pAR1411-cat; Lane 2: pAR1411-cat digested with Pst I; Lane 3: pAR1411-cat digested with Xba I; Lane 4: pAR1411-cat digested with PstI and XbaI; Lane 5: pAR1411; Lane 6: pAR1411 digested with PstI and XbaI; Lane 7: amplicon of cat gene digested with Pst I and Xba I.

cat, and transformed into *L. lactis* MG1363. Initially, a total of 28 transformants were recovered from SGM17 agar plate containing erythromycin (5 µg/ml), but not on the SGM17 with erythromycin (5 µg/ml) and chloramphenicol (7.5 µg/ml). Interestingly, after two subsequent transfers, one colony grew in the broth medium containing both antibiotics. Plasmid extraction revealed that this erythromycin and chloramphenicol-double resistant strain contained a plasmid bearing the expected molecular size. The putative recombinant plasmid pAR1411-cat was further verified through RE digestion analysis Digestion analyses indicated that the plasmid extracted from the transformant harbored the expected fragment (Figure 3).

DISCUSSION

A lactococcal cloning and expression vector was constructed based on the indigenous plasmid pAR141. pAR 141 is a small plasmid isolated from *L. lactis* subsp. *lactis* M14 (Raha et al., 2006). Detailed examination of the plasmid sequence revealed that this cryptic plasmid only carried genes essential for its own replication. There are no selection markers which would enable the strains harboring it to be scored. In addition, pAR141 also contained limited unique RE sites for use in cloning. These features of pAR141 restricted its use as a cloning vector. Therefore, in this study a fragment from the *L. lactis* expression vector pMG36e was introduced into pAR141 to produce a cloning and expression vector, pAR 1411. Based on the sequence of pMG36e, primers were carefully designed to amplify the whole fragment of complete erythromycin resistance gene (ermC) including its expression signals, and the multiple cloning sites together with the promoter P_{32} and terminator (T) sequences. The other nonessential sequence was minimized to keep the newly constructed plasmid as small as possible.

Antibiotic resistance is generally used as a selection marker due to its easy selection. However, antibiotic resistance genes are not commonly present in *lactococcal* plasmids. Although several plasmids in *L. lactis* had been studied, many of these plasmids turned out to be cryptic (McKay, 1983), including pWV01 (Leenhouts et al., 1991), pSH1 (Gasson, 1983) and pAR141 (Raha et al., 2006). Only a limited number of the identified plasmids such as pAJ01 (Raha et al., 2002) encoded antibiotic resistances. Therefore, the erythromycin resistance marker, ermC which originated from *Staphylococcus aureus* plasmid, pE194, was selected because it

has been shown to express in *L. lactis*, *Bacillus. subtilis* and *E. coli* efficiently and utilized in various constructions of *lactococcal* plasmid vectors (de Vos and Simons, 1994).

Since pAR141 possess limited number of unique RE sites, a fragment constituting several restriction enzyme recognition sites was added to enhance cloning. With the addition of the multiple cloning sites, cloning of genes into the vector would be made easier as there are more choices of RE sites. Directional cloning could also be performed where two different REs are used to orientate the insertion and the problem of inappropriate insertions can also be avoided.

In addition to the selection marker and MCS, the constitutive P_{32} promoter and transcriptional terminator of pMG36e (van de Guchte et al., 1989) were also incurporated into pAR1411. These two gene expression signals flanked the MCS of pMG36e. Although useful, the constitutive promoter P_{32} which originated from the chromosome of *L. lactis* subsp. *cremoris* Wg2 is not a very strong promoter (van der Vossen et al., 1987). This may be caused by the difference in two nucleotides in the sequence of P_{32} from the consensus in the -35 region (van der Vossen et al., 1987). The fragment also encompassed the RBS sequence (GGAGG) and the 5′-end of the orf32 gene.

The transcriptional terminator used in pAR1411 originated from the proteinase gene of plasmid pWV05 of *L. lactis* Wg2 (Kok et al., 1988). The terminator consisted of two complementary IRs which could form a stem of 15 bp followed by a stretch of T's, which are the features of a rho-independent terminator. It is beneficial to have a transcriptional terminator downstream of the site in which to clone foreign genes (Stueber and Bujard, 1982). This could prevent read-through of the promoter to the replication region and affect the plasmid replication and its stability.

During the construction of the recombinant vector from the cryptic plasmid pAR141, care was taken that the replication function of the plasmid was not disturbed. The repA, repB genes and the sequence encoding dso are essential features for plasmid replication and maintenance. The successful transformation and recovery of pAR1411 in *L. lactis* indicated that the unique FspI site used for the insertion of pMG36e fragment is not located within the region important for replication of the plasmid. This observation also appeared as evidence that plasmids from *L. lactis* subsp. *lactis* could replicate in subsp. *cremoris*.

The replication of the recombinant plasmid pAR1411 in *E. coli* strains was tested. However, pAR1411 failed to be retrieved from the transformants. In some instances, plasmids isolated from the transformants showed differences in sizes and RE patterns as compared to the pAR1411 obtained from *L. lactis*. Most of the time, no plasmid could be extracted from the cells. This was unexpected because based on sequence similarity to pWV01 (Leenhouts et al., 1991), pAR141 and its derivative should be functional in a wide range of bacteria. These may indicate the structural and segregational instability of the construct in *E. coli*. The functionality of the replication system of this plasmid might be another point for these results. The possibility of contamination of the transformants was low as the control reaction with pMG36e was successful.

To test the functionality of the construct pAR1411 as cloning and expression vector in *L. lactis*, the cat gene conferring chloramphenicol resistantance was used. The use of antibiotic resistance gene as the reporter provided an easy selection of recombinants expressing the inframe inserted DNA. The cat gene has been used as a reporter gene in *B. subtilis* and *L. lactis* for the screening of promoters and transcriptional terminators (van der Vossen et al., 1985). The problems of low yield of transformants with double resistance encountered during the attempts of expression might be caused by several factors. The higher yield on the agar plates with erythromycin selection alone indicated that the electrotransformation processes were successful. However, a majority of these transformants were erythromycin sensitive indicating that the plasmid might have undergone self-ligation. This might be improved by using higher ratio of insert to vector in the ligation, or by dephosphorylation of the vector with calf/shrimp alkaline phosphatase to reduce self-ligation. At the same time, the failure to recover transformants on the agar plates supplemented with both antibiotics might be due to the high concentrations of the antibiotics. Kok et al. (1984) used only 5 and 1 µg/ml of chloramphenicol and erythromycin, respectively, while in this study, the concentrations were 7.5 and 5 µg/ml. These researchers also showed that reduced concentrations of antibiotics were required during selection in *E. coli* when plasmid with these two resistant markers was used.

A recombinant plasmid pAR1411-cat containing cat gene was successfully recovered from the *L. lactis* transformants. Although SDS-polyacrimide gel electrophoresis was not carried out to demonstrate the production of the CAT protein, the ability of the transformants to grow on the chloramphenicol-containing agar plate was a strong evidence that a functional substance conferring resistance to the antibiotic was present and expressed in the strain.

In conclusion, a recombinant vector pAR1411 was constructed by subcloning the EmPMT fragment from pMG36e into pAR141. This construct could replicate in *L. lactis*, but was found to be unable to transform the *E. coli* strains tested. pAR1411 could be used to clone and express catalase acetyltransferase, a heterologous reporter protein, in *L. lactis*. The expression vector could be further developed and improved to serve different purpose.

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