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A broad host range food-grade cloning vector for lactic acid bacteria

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Abstract: The genetic modification of lactic acid bacteria being used in medicine and food industries has been limited due to the scarcity of food-grade cloning vectors for the bacteria. The 4.46-kb food-grade cloning vector pUBU constructed in this study consisted of 3 major components from food-approved organisms, the theta-type replicon from pUCL287 of *Tetragenococcus halophilus*, the lactococcal cadmium resistance (Cd^r) determinant from pND918 and the promoter of L-lactate dehydrogenase (*ldh*L) gene from *Lactobacillus plantarum.* The Cd^r determinant was used as a dominant selectable marker and the *ldh*L promoter, a strong constitutive promoter, was used to drive the expression of inserted genes. The newly constructed vector was able to transform several genera of lactic acid bacteria and stable in the bacteria under non-selective pressure for at least 100 generations. In addition, it allowed inserted genes to express in lactic acid bacteria under the control of *ldh*L promoter. The host range of pUBU extended to *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Tetragenococcus.* These results suggest that pUBU is a potential food-grade cloning vector for genetic modification of a wide range of lactic acid bacteria.

Key words: cadmium resistance gene; food-grade cloning vector; lactic acid bacteria.

Abbreviations: Cd^r, lactococcal cadmium resistance; *gfp*uv, gene for a green fluorescence protein variant; GFPuv, a green fluorescence protein variant; GRAS, generally regarded as safe; *ldh*L, L-lactate dehydrogenase gene; MCS, multiple cloning site; MRS, deMan Rogosa Sharpe.

Introduction

Lactic acid bacteria are a heterogeneous group of gram positive bacteria that share a common metabolic property of producing lactic acid from the fermentation of carbohydrates (Carr et al. 2002; He et al. 2012). They belong to more than 10 genera under the family of Lactobacillaceae including *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Sporolactobacillus*, *Tetrgenococcus*, *Vagococcus* and *Weissella* (Mayo et al. 2008). They are ubiquitous, being found in food and in gastrointestinal tract of healthy persons. Therefore, they earn generally regarded as safe (GRAS) status and have been widely applied in medicine and food industries (Mercenier et al. 2000; Giraffa et al. 2010; Widyastuti et al. 2014; Saez-Lara et al. 2015).

Lactic acid bacteria for medicinal and industrial uses are generally genetically modified to gain the most suitable traits. Such genetic modifications require cloning vectors to deliver desired genes into the bacteria. However traditional cloning vectors for lactic acid bacteria usually contain one or more antibiotic resistance selectable markers. These vectors are not acceptable for medicinal and food uses because of concern over the transfer of antibiotic resistance genes to intestinal microflora in humans, thereby generating new antibiotic resistant microorganisms. Due to the restricted use of cloning vectors having antibiotic resistance genes as selectable markers, food-grade cloning vectors, consisting exclusively of DNA from GRAS microorganisms or food-approved organisms, are developed for use in genetic modifications of lactic acid bacteria being used in medicine and food industries.

For the construction of food-grade cloning vectors for lactic acid bacteria, two major types of selectable markers are used: dominant selectable markers and complementary selectable markers (De Vos 1999). Genes commonly used as dominant selectable markers are the nisin resistance gene (Froseth & McKay 1991; Hughes & McKay 1992; von Wright & Raty 1993), the cadmium resistance gene (Liu et al. 1997; Wong et al. 2003; Rattanachaikunsopon & Phumkhachorn 2012) and the copper resistance gene (Liu et al. 2002, 2005). The selection of bacteria carrying such markers is straightforward depending on the presence of the markers in the bacteria. The use of complementary selectable markers requires the initial specific mutations in host bacterial chromosomes and the subsequent complementation of the mutations by cloning vectors containing complementary selectable markers. Examples of complementary selectable markers are the alanine

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Table 1. Transformation frequencies obtained from electroporation of lactic acid bacteria with pUBU and stability of pUBU in transformants after 100 generations of growing under non-selective pressure.

Lactic acid bacteria	Transformation frequency (per μg of pUBU)[a]	Stability of pUBU (%)[a]
Enterococcus faecium SF9	$1.62 \pm 0.40 \times 10^3$	97.4 ± 1.14
Lactobacillus gasseri ATCC33323	$2.56 \pm 0.42 \times 10^3$	96.8 ± 1.64
Lactobacillus plantarum N014	$2.24 \pm 0.27 \times 10^3$	97.2 ± 1.30
Lactobacillus rhamnosus ATCC53103	$2.92 \pm 0.19 \times 10^3$	96.4 ± 1.52
Lactobacillus ruminis ATCC17782	$2.26 \pm 0.24 \times 10^3$	97.6 ± 1.14
Lactococcus garvieae ATCC49156	0	nd
Lactococcus lactis TFF221	0	nd
Leuconostoc mesenteroides TFF5	$3.86 \pm 0.21 \times 10^3$	95.6 ± 1.95
Pediococcus pentosaceus ATCC25745	$1.14 \pm 0.11 \times 10^3$	96.2 ± 2.28
Tetragenococcus halophilus PF	$5.40 \pm 0.21 \times 10^3$	98.2 ± 1.30

[a] Mean ± SD values of 5 replicates; nd = not done.

racemase gene used with alanine racemase deletion mutants (Bron et al. 2002, 2004; Nguyen et al. 2011) and the thymidylate synthase gene used with thymidine requiring mutants (Fu & Xu 2000; Sasaki et al. 2004). For medicinal and industrial uses, dominant selectable markers are more favourable because there is no need to generate mutants of lactic acid bacteria that are normally not readily available.

Most of food-grade cloning vectors for lactic acid bacteria that have been developed can be used with specific hosts, thereby limiting the application of the vectors. Examples of such vectors are pTRK418, a *Lactobacillus* vector (Allison & Klaenhammer 1996), pSL102, a *Propionibacterium* vector (Brede et al. 2007) and *Lactococcus* vectors pVS40 (von Wright & Raty 1993), pND919 (Wong et al. 2003) and pND632 (Liu et al. 2005). This drawback leads us to the development of a broad host range cloning vector with a dominant selectable marker that can be used in many genera of lactic acid bacteria. The newly constructed food-grade cloning vector may have a beneficial impact on genetic modification of lactic acid bacteria, thereby broadening their application in medicine and food industries.

Material and methods

Bacterial strains and growth conditions

Bacterial strains used in this study were *Escherichia coli* DH5α and 12 strains of lactic acid bacteria. *E. coli* DH5α was used for propagation of pGFPuv and pND918. It was grown in Luria-Bertani medium at 37 °C with shaking at 300 rpm. All strains of lactic acid bacteria were cultured in deMan Rogosa Sharpe (MRS) medium at 37 °C without shaking. Of 12 strains of lactic acid bacteria, *Tetragenococcus halophilus* ATCC33315 and *Lactobacillus plantarum* ATCC8014 were used as sources of pUCL287 and the promoter of L-lactate dehydrogenase gene (*ldh*L promoter), respectively. The other lactic acid bacteria listed in Table 1 are plasmid-free. They were used as bacterial hosts for the determination of host range of the newly constructed food-grade cloning vector. *T. halophilus* PF is the plasmid-cured derivative of *T. halophilus* ATCC33315. Plasmid curing was done by serial subculturing *T. halophilus* ATCC33315 in MRS broth at 42 °C for 10 rounds (18 h each round). Of the bacteria listed in Table 1, 4 strains are our laboratory strains including *Enterococcus faecium* (Phupaboon et al. 2016), *Lactococcus lactis* TFF221 (Rattanachaikunsopon et al. 2008), *Lactobacillus plantarum* N014 (Rattanachaikunsopon et al. 2006) and *Leuconostoc mesenteroides* (Rattanachaikunsopon et al. 2003). Bacterial stock cultures were stored as frozen cultures at –80 °C in appropriate culture media containing 20% glycerol (v/v).

Plasmids

Plasmids used in this study were pGFPuv, pND918, pUCL287, pUBU-GFP and pUBU. The 3.3-kb pGFPuv was purchased from Clontech Laboratories (Mountainview, CA, USA). The 9.3-kb pND918 is an *E. coli*/*Lactococcus* shuttle vector carrying the lactococcal cadmium resistance (Cd^r) determinant (Wong et al. 2003). This plasmid was obtained from DSM Food Specialties, New South Wales, Australia. The 8.7-kb pUCL287 is a cryptic plasmid of *T. halophilus* ATCC33315 carrying a theta-type replicon. The plasmid pUBU-GFP was constructed as an intermediate vector containing *gfp*uv gene. The plasmid pUBU, the food-grade cloning vector carrying the Cd^r determinant, was constructed in this study.

DNA manipulations

Chromosomal DNA of *L. plantarum* ATCC8014 was purified according to the protocol of Bor et al. (1992). Plasmid DNA was extracted from *E. coli* by the alkaline lysis method (Birnboim & Doly 1979) and from lactic acid bacteria by the method of Anderson & McKay (1983). Standard molecular cloning techniques were performed as described by Green & Sambrook (2012). Restriction endonucleases, *Taq* polymerase, and T4 DNA ligase were purchased from Promega (Madison, WI, USA) and used according to the recommendations of the manufacturer.

Electrotransformation

The lactic acid bacteria listed in Table 1 were electotransformed with pUBU by using our protocol described previously (Rattanachaikunsopon & Phumkhachorn 2009). Transformants were isolated on MRS agar plates containing $CdCl_2$ (0.3 mM). For each electrotransformation, 20 randomly selected transformants were tested for the presence of pUBU by plasmid isolation, restriction digestion analysis and Southern hybridization with the labelled Cd^r determinant as a probe. Transformation frequency was calculated as the number of transformants generated per μg of the vector.

The electrotransformation of lactic acid bacteria with pUBU-GFP was used to test the functionality of *ldh*L promoter in promoting gene expression. The expression of the *gfp*uv gene in the cadmium resistant transformants was determined by observing fluorescence ability of the transformants under fluorescence microscope according to the method of Phumkhachorn et al. (2007).

Table 2. Templates and primers used in this study.

Amplicons	Templates	Primers[b]	Recognition sites[c]
*ldh*L promoter	*L. plantarum*[a]	F: 5' CAAGAATTCGCGTTTATTAAGTGCA 3'	*Eco*RI
		R: 5' TTGGCAAGCTTGACAAAATAACT 3'	*Hin*dIII
repA region	pUCL287	F: 5' TAAGGGCCCTCTCGAACAGCGGTGC 3'	*Apa*I
		R: 5' GAACCTAGGTTCTTTGCTTTGAAA 3'	*Bam*HI
cadA-cadC region	pND918	F: 5' CAGGATCCAAAAAACAGCTCA3'	*Bam*HI
		R: 5' TAGGAATTCTTTTACTAGCAATCATGTTC 3'	*Eco*RI

[a] *L. plantarum* ATCC8014 chromosome.
[b] F and R, forward and reverse primers, respectively.
[c] Recognition sites within primers of restriction enzymes are underlined sequences in primers.

Southern hybridization
DNA was transferred from an agarose gel onto an Hybond-N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ, USA) and fixed to the membrane by UV cross-linking. The amplified Cd^r determinant (the *cadA-cadC* region) used as a probe was labelled with the DIG High-Prime DNA labelling kit (Roche Diagnostics, Palo Alto, CA, USA). Prehybridization, hybridization, and post-hybridization washes as well as detection were performed according to the manufacturer's instructions.

PCR amplification
PCR was used for the amplification of the 200-bp promoter of L-lactate dehydrogenase (*ldh*L promoter), the 1.24-kb *repA* region (replicon) of pUCL287 and the 2.97-kb *cadA-cadC* region (the Cd^r determinant) of pND918. Templates and primers used to produce the amplicons are shown in Table 2. PCR was carried out in a Thermocycler 480 (Perkin-Elmer, Norwalk, CT) using the amplification procedure described by Liu et al. (1997).

Plasmid stability
For testing the stability of pUBU, transformants (approximately 10^3 colony-forming units) were grown in 100 mL of MRS broth without $CdCl_2$ at 37°C and subcultured (0.1% inoculum) into the same medium every 10 generations. At the end of subculturing for 100 generations, the transformants were plated on nonselective MRS agar (without $CdCl_2$) and incubated at 37°C for 18 h. One hundred bacterial colonies were randomly selected from the nonselective agar to test for cadmium resistance on the selective MRS agar (with $CdCl_2$) by patching the colonies on the selective agar and to test for the presence of pUBU by plasmid isolation, restriction digestion analysis and Southern hybridization with the labelled Cd^r determinant as a probe. The percent of plasmid stability was calculated as 100× (number of cadmium resistant transformants carrying pUBU/number of tested transformants).

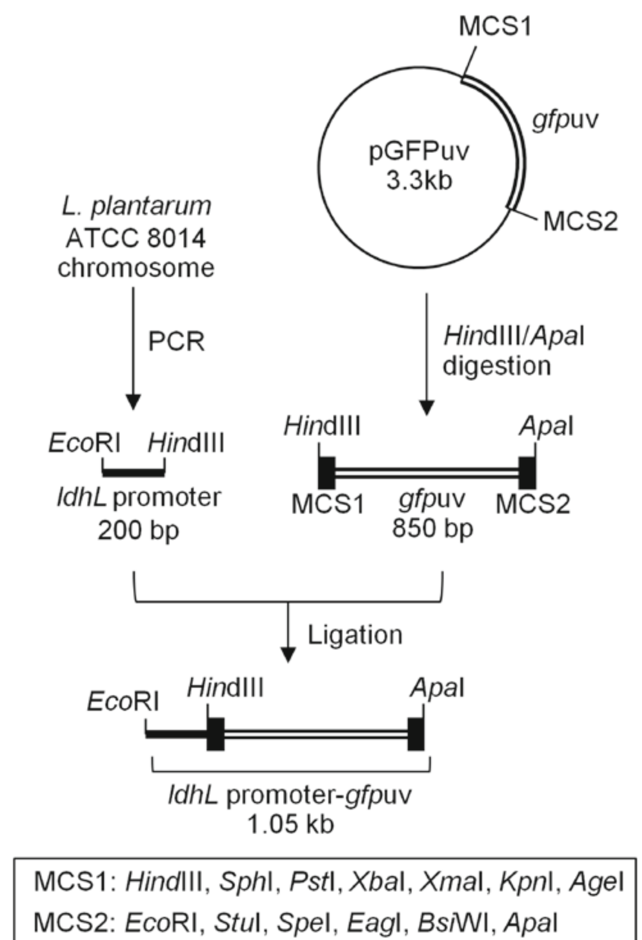

Fig. 1. A diagram showing the construction of *ldh*L promoter-*gfp*uv fragment. MCS1 and MCS2, 5' multiple cloning site and 3' multiple cloning site of pGFPuv, respectively.

Results and discussion

Construction of food-grade cloning vector, pUBU
The approach for construction of the food-grade cloning vector, pUBU, consisted of 3 main steps: (1) the formation of the 1.05-kb *ldh*L promoter-*gfp*uv fragment; (2) the formation of the 4.25-kb *repA-cadC-cadA* fragment; and (3) the ligation of both fragments to form pUBU.

The 1.05-kb *ldh*L promoter-*gfp*uv fragment took part in the construction of pUBU by providing a multiple cloning site and driving the expression of inserted genes. The fragment was constructed as shown in Fig. 1. The 850-bp *gfp*uv gene was derived from pGFPuv as a *Hin*dIII/*Apa*I fragment. Because *Hin*dIII cut at 5' end of 5' multiple cloning site (MCS1) of pGFPuv and *Apa*I cut at 3' end of 3' multiple cloning site (MCS2) of pGFPuv, the *Hin*dIII/*Apa*I digested fragment was flanked by MCS1 and MCS2. The MCS1 contains restriction sites for *Hin*dIII, *Sph*I, *Pst*I, *Xba*I, *Xma*I, *Kpn*I and *Age*I, whereas the MCS2 contains restriction sites for *Eco*RI, *Stu*I, *Spe*I, *Eag*I, *Bsi*WI and *Apa*I. The *ldh*L promoter, known to be a strong constitutive promoter, was generated by PCR as a 200-bp DNA fragment with

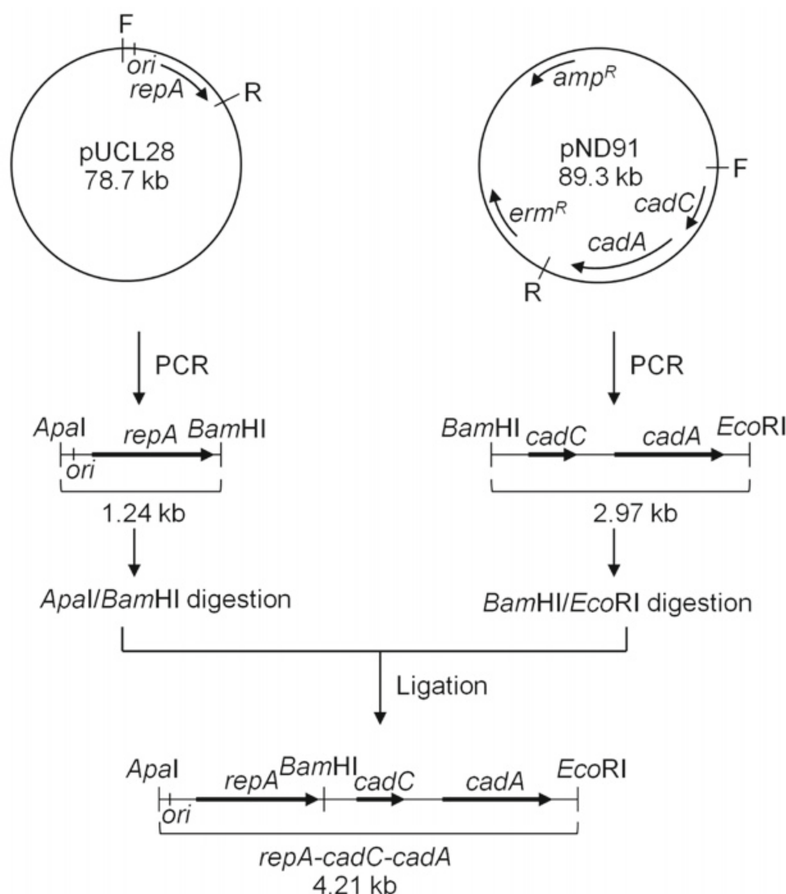

Fig. 2. A diagram showing the construction of *repA-cadC-cadA* fragment. amp^R, ampicillin resistance gene; erm^R, erythromycin resistance gene; *repA*, replication gene in pUCL287; *ori*, origin of replication; F and R, positions of forward and backward primers, respectively; *cadA* and *cadC*, cadmium resistance determinant.

an *Eco*RI site at one end and a *Hin*dIII site at the other end. The ligation of of 850-bp *gfp*uv gene and 200-bp *ldh*L promoter fragment at the *Hin*dIII restricted ends generated the 1.05-kb *ldh*L promoter-*gfp*uv fragment, which placed the *gfp*uv gene under the control of *ldh*L promoter.

In this study, we decided to use the *ldh*L promoter of *Lactobacillus plantarum* to drive the expression of cloned genes in the vector to be constructed because it has known to be a strong constitutive promoter. Besides this promoter, other strong promoters can also be used for the same purpose, such as the constitutive promoter P32 of *Lactococcus lactis* subsp. *cremoris* (Scott et al. 2000) and the nisin inducible promoter of *Lactococcus lactis* (Geoffroy et al. 2000). However, the use of inducible promoters to drive the expression of cloned genes in vectors is more complicated than that of constitutive promoters.

In general, food-grade cloning vectors require a replicon that supports the replication of the vectors in host bacteria and a food-grade selectable marker. The plasmids carrying theta-type replicons are expected to be more stable in lactic acid bacteria and are thus preferred candidates for the development of stable cloning vectors (Seegers et al. 1994). In this study, we chose the replicon of pUCL287 for constructing our food-grade cloning vector because it is a theta-type replicon and can function in many genera of lactic acid bacteria including *Enterococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* (Benachour et al. 1995, 1997). The replicon of pUCL287 consists of a replication origin (*ori*) and a replication gene (*repA*) encoding a 311 amino-acid protein.

The lactococcal Cd^r determinant, a dominant selectable maker, was chosen for use as the selectable marker in this study based on its simplicity of use. It consists of 2 adjacent genes, *cadA* and *cadC*. The *cadA* gene encodes a P-type ATPase which allows efflux of cadmium resulting in reduced accumulation of the toxic cation (Nucifora et al. 1989). The *cadC* gene encodes a small protein which acts as a transcriptional regulatory repressor. Its DNA binding capacity is diminished in the presence of cadmium (Endo et al. 1995). Recently, the lactococcal Cd^r determinant has been used as a selectable marker in the construction of several food-grade cloning vectors for lactic acid bacteria (Trotter et al. 2001; Wong et al. 2003; Liu et al. 2005; Rattanachaikunsopon & Phumkhachorn 2012).

The 4.21-kb *repA-cadC-cadA* fragment was used for the construction of pUBU by providing the theta-type replicon and a dominant selectable marker. The fragment was constructed as shown in Fig. 2. The 1.24-kb *repA* fragment was generated by PCR as a DNA fragment with an *Apa*I site at one end and a *Bam*HI site

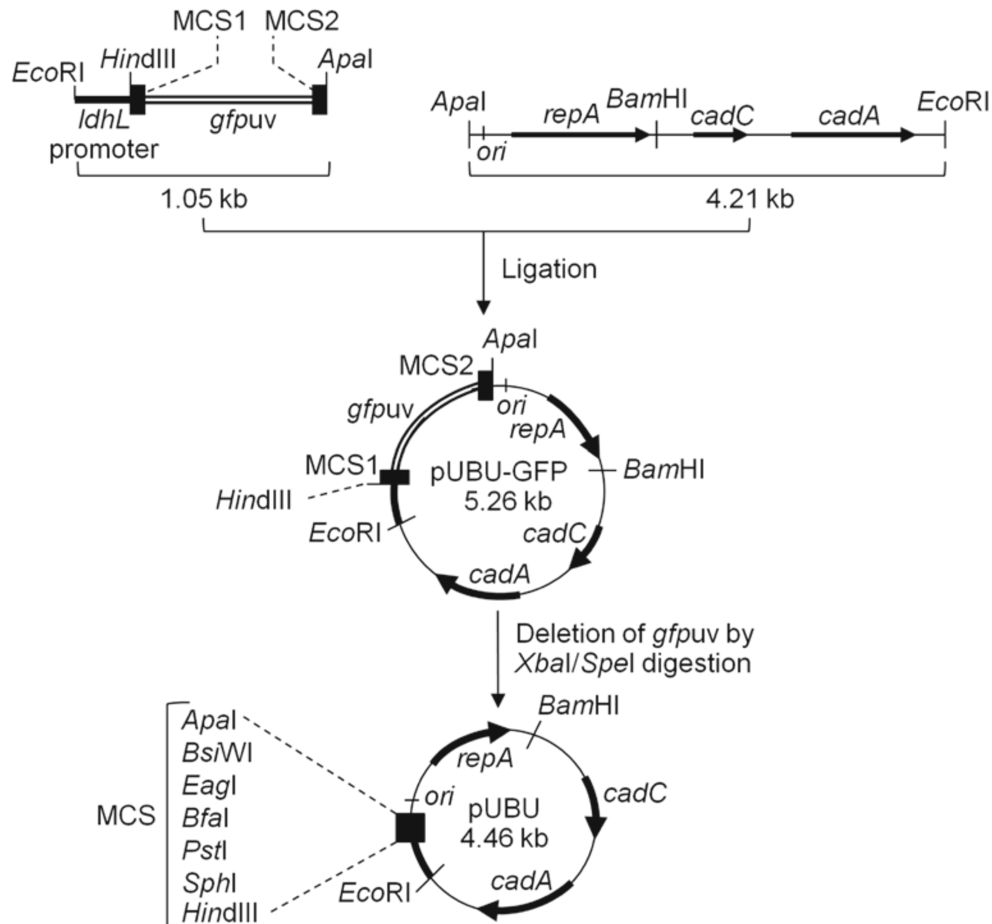

Fig. 3. A diagram showing the ligation of *ldh*L promoter-*gfp*uv fragment and *repA*-*cadC*-*cadA* fragment to form pUBU-GFP and pUBU. MCS, multiple cloning site in pUBU.

at the other end. Similarly, the 2.97-kb *cadC*-*cadA* fragment, the cadmium resistance (Cd^r) determinant fragment, was generated by PCR as a DNA fragment with a *Bam*HI site at the end near *cadC* and an *Eco*RI site at the end near *cadA*. The ligation of *Bam*HI restricted ends of 1.24-kb *repA* fragment and 2.97-kb *cadC*-*cadA* fragment generated the 4.21-kb *repA*-*cadC*-*cadA* fragment.

The ligation of 1.05-kb *ldh*L promoter-*gfp*uv fragment and 4.21-kb *repA*-*cadC*-*cadA* fragment led to an intermediate cloning vector, pUBU-GFP (Fig. 3). The deletion of the *gfp*uv gene from pUBU-GFP by *Xba*I/*Spe*I digestion created a new multiple cloning site (MCS) consisting of *Apa*I, *Bsi*WI, *Eag*I, *Bfa*I, *Pst*I, *Pst*I and *Hind*III. The resulting plasmid is a food-grade cloning vector, pUBU, with the size of 4.46-kb.

Electrotransformation of lactic acid bacteria with pUBU
The newly constructed cloning vector, pUBU, was introduced into *T. halophilus* PF by electroporation, and transformants were selected on MRS agar containing $CdCl_2$. The transformation efficiency was found to be 5.2×10^3 transformants per μg of the plasmid. Twenty transformant clones were randomly selected and subjected to plasmid isolation. The 4.46-kb isolated plasmid DNA was confirmed to be pUBU by restriction

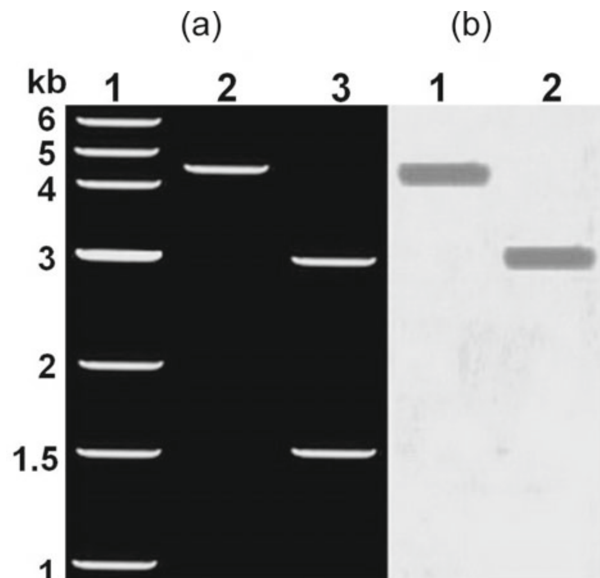

Fig. 4. Restriction digestion analysis and Southern hybridization of plasmid DNA isolated from pUBU transformed *T. halophilus* PF. (a) An agarose gel stained with ethidium bromide. Lane 1, 1 kb DNA ladder; lane 2, the uncut plasmid; lane 3, the plasmid digested with *Eco*RI/*Bam*HI. (b) Southern hybridization using the labelled Cd^r determinant as a probe. Lane 1, the uncut plasmid hybridized to the probe; lane 2, the 2.97-kb *Eco*RI/*Bam*HI fragment of the plasmid hybridized to the probe.

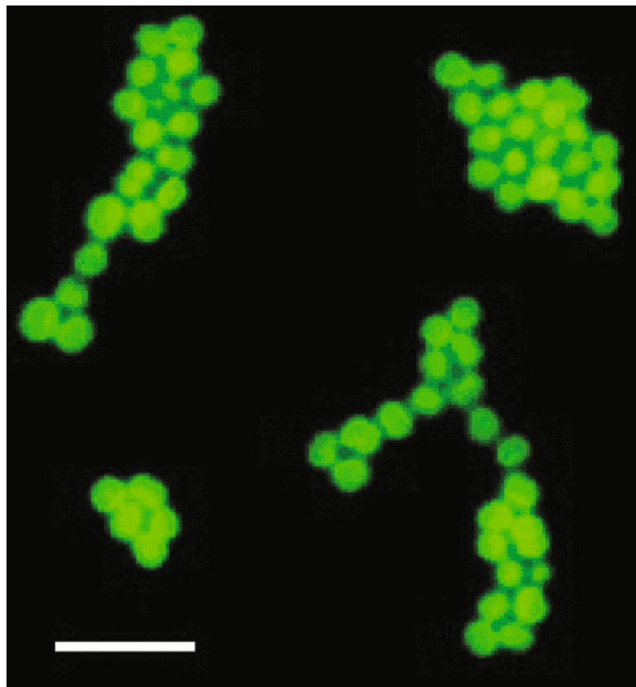

Fig. 5. A micrograph showing fluorescence ability of pUBU-GFP transformed *T. halophilus* PF. (Bar = 5 μm).

digestion analysis and Southern hybridization with the labelled Cd^r determinant as a probe. The digestion of 4.46-kb isolated plasmid DNA with *Bam*HI and *Eco*RI gave 2 DNA fragments with expected sizes of 1.49 and 2.97 kb (Fig. 4). Southern hybridization detected the 2.97-kb fragment as the fragment carrying the Cd^r determinant (Fig. 4). These results suggest that pUBU could replicate in *T. halophilus* and the lactococcal Cd^r determinant within pUBU could be expressed in *T. halophilus*. The electrotransformation with pUBU was also performed with several genera of lactic acid bacteria listed in Table 1. It was successful with all genera of lactic acid bacteria except *Lactococcus* with different transformation frequencies. These results suggest that pUBU is a broad host range vector that can replicate in several genera of lactic acid bacteria including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Tetragenococcus*. This feature of pUBU makes it an interesting cloning vector to be used for genetic modification of lactic acid bacteria.

Stability of pUBU

The stability of pUBU was studied only in lactic acid bacteria transformable by the vector. The pUBU transformed cells were cultivated in MRS broth without selective pressure for 100 generations, and isolated colonies were checked for cadmium resistance on MRS agar with $CdCl_2$ and for the presence of pUBU by plasmid isolation, restriction digestion analysis and Southern hybridization with the labelled Cd^r determinant as a probe. The number of cadmium resistant transformants carrying pUBU was used to calculate the percent of plasmid stability. In all tested strains of lactic acid bacteria, the percentages of plasmid stability were more than 95% after 100 generations of growth without selective pressure (Table 1). These results suggest that pUBU was very stable in all of the transformable lactic acid bacteria.

Expression of an inserted gene in pUBU

The ability of *ldh*L promoter to drive the expression of inserted genes at MCS was tested by electrotransformation of *T. halophilus* PF with pUGU-GFPuv, the plasmid containing the *gfp*uv gene under the control of *ldh*L promoter. The expression of the *gfp*uv gene in the transformants was checked by observing fluorescence ability of the transformants under a fluorescence microscope. Transformants from all of the randomly selected cadmium resistant colonies (20 colonies) were found to be brightly fluorescent (Fig. 5). This experiment was also performed with all lactic acid bacteria listed in Table 1 except bacteria in genus *Lactococcus*. The same result was obtained in all tested bacteria. The results suggest that pUBU is an expression vector allowing genes inserted in MCS to express.

In conclusion, this study presents the construction of pUBU, a broad host range food-grade cloning vector for lactic acid bacteria using the Cd^r determinant as a selectable marker. Its hosts include *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Tetragenococcus*. It can allow the expression of cloned genes in host bacteria under the control of strong constitutive *ldh*L promoter. This feature together with its high stability in host bacteria under non-selective pressure for at least 100 generations make pUBU to be a potential food-grade cloning vector for genetic modification of lactic acid bacteria being used in medicine and food industries.

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