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A novel plasmid for delivering genes into mammalian cells with noninvasive food and commensal lactic acid bacteria

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Abstract

Using food and commensal lactic acid bacteria (LAB) as vehicles for DNA delivery into epithelial cells is a new strategy for vaccine delivery or gene therapy. However, present methods for DNA delivery with LAB have suffered low efficiency. Our goal was to develop a new system to deliver DNA into epithelial cells with high efficiency using food and commensal LAB. An *Escherichia coli*-LAB shuttle plasmid, pLKV1, for DNA delivery into eukaryotic cells was constructed. Two reporter plasmids with green and red fluorescent protein genes were also constructed to monitor the uptake of protein and DNA, respectively. Bacteria delivering these reporter plasmids into Caco-2 cells were monitored by fluorescence microscopy. Several methods that weaken the bacterial cell wall prior to co-culture with Caco-2 cells were evaluated for their role in the improvement of gene transfer efficiency. Treating *Streptococcus gordonii* with penicillin and lysozyme greatly increased its rate of gene delivery to mammalian cells compared to untreated control bacteria, while glycine pretreatment promoted the highest gene transfer rate for *Lactococcus lactis*. Uptake of green fluorescent bacteria by Caco-2 cells showed that the cell wall-weakening treatment promoted the internalization of the noninvasive bacteria into Caco-2 cells. In conclusion, we have developed a noninvasive system using LAB as a vehicle for vaccine delivery or gene therapy, and tested this system *in vitro* with Caco-2 cells.

Keywords

DNA vaccine; gene therapy; gene delivery; reporter plasmid; *Lactococcus*; *Streptococcus*

1. Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating bacteria that includes species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, all of which produce a common metabolic end product, lactic acid, from the fermentation of carbohydrates. Food LAB refers to those species and strains that are used in food- and feed-fermentation processes. Commensal LAB refers to those that inhabit in

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various anatomic sites of vertebrate animals, including the oral cavity, gastrointestinal and genital tracts.

Recently, there has been increasing interest in the use of bacteria to deliver DNA molecules as vaccines or gene therapies to mammalian cells. Attenuated pathogenic bacteria such as *Shigella*, *Listeria* and *Salmonella* have been used as live vectors to deliver DNA into mammalian cells (Detmer and Glenting, 2006; Vassaux et al., 2006). These bacteria can invade mammalian cells and deliver eukaryotic expression vectors resulting in cellular expression of the gene of interest and subsequent antigen presentation. However, their safety is of concern because a possible reversion to a virulent phenotype may occur in these attenuated bacteria.

Due to safety reasons, noninvasive bacteria, such as food and commensal LABs, have been used as DNA delivery vehicles to develop DNA vaccines (Wells and Mercenier, 2008). However, the DNA delivery efficiency with these bacteria has been low (Guimaraes et al., 2006). To improve DNA delivery into mammalian cells, invasive genes from pathogenic bacteria, such as the internalin gene *inlA* from *Listeria monocytogenes* (Guimaraes et al., 2005, 2009), have been cloned into the noninvasive food bacterium *Lactococcus lactis*. Although carrying an invasive gene significantly increased the efficiency of DNA delivery from *L. lactis* to mammalian cells, the success rate of DNA delivery is still low, only about 1% (Guimaraes et al., 2005, 2009). Moreover, by expressing an invasive protein of a pathogenic bacterial origin, the previous noninvasive bacterium becomes invasive, which could render the LAB strain unsafe for human use.

It has been reported (Granette et al., 2004) that bacterial cell wall mutants of LABs as a vaccine delivery vector could induce enhanced mucosal immune responses. This suggested that methods to weaken the cell wall of noninvasive bacteria might improve their DNA delivery into eukaryotic cells. Therefore, the objective of this study was to develop a safer and more effective DNA vaccine delivery system with LAB by weakening the bacterial cell wall.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture media

Bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* subsp. *cremoris* NZ3900 was grown in M17 medium containing 0.5% glucose (GM17) and *Streptococcus gordonii* V288 was grown in Todd Hewitt broth (Difco Laboratories). *Escherichia coli* strains were grown on Luria–Bertani medium and incubated at 37 °C with vigorous shaking. Antibiotics were added at the indicated concentrations as necessary: kanamycin, 600 µg/ml for *L. lactis*; 250 µg/ml for *S. gordonii*, 50 µg/ml for *E. coli*.

2.2. pLKV1 vector design and construction

pLKV1 contains three parts: a plasmid replication origin of pA13 derived from *Lactobacillus plantarum* (Kojic et al., 2010), which replicates in both Gram-negative and positive bacteria; a kanamycin resistance marker derived from Tn1545 of *Streptococcus pneumoniae* (Courvalin and Carlier, 1987) and can express in both Gram-negative and positive bacteria; and an eukaryotic gene expression cassette derived from pVAX1 (Invitrogen, Carlsbad, California). Due to safety records, kanamycin resistance marker is recommended for DNA vaccine delivery by the Food and Drug Administration (FDA). Therefore, the construction of the vaccine delivery vector included two steps. First, a small-sized, 3.7 kb kanamycin resistant *E. coli*-LAB shuttle plasmid pLK1 was constructed by combining a 2.1 kb DNA fragment containing the pA1 origin from the plasmid pA13 (Kojic et al., 2010) and the 1.6 kb DNA fragment containing the kanamycin resistance marker of

Tn1545 from pAK267. Second, an 860-bp fragment containing a DNA vaccine cloning cassette, a CMV promoter, a multiple cloning site, and a poly-A tail, was obtained from pVAX1. The DNA fragment was generated by polymerase chain reaction (PCR) with TaKaRa e2TAK™ DNA Polymerase (Fisher Scientific) and primers pVAX1-F (5' GGAGATCT GCGTTACATAACTTACGG 3') and pVAX1-R (5' TAGAAGCCATAGAGCCC 3') introducing respectively a *Bg*/II (underlined) and a blunt sites in the fragment. PCR was performed using the Techne thermalcycler (Techne, Princeton, NJ, USA). The reaction mixture (final volume of 50 µl) contained: 100 ng template DNA; 1 unit e2TAK™ DNA polymerase; 1 × reaction buffer; deoxynucleoside triphosphates 0.1 mmol l⁻¹ each; primer, 50 pmol each. The thermal cycling program used was as follows: initial denaturation at 94 °C for 3 min, 35 cycle of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min. Finally, there was an extension step at 72 °C for 10 min. The PCR DNA products were analyzed for correct size and purity on 1.2% agarose gel.

The amplified PCR product was cut with *Bg*/II and cloned into the shuttle vector pLK1 predigested with *Bam*HI and *Pvu*II (Fig. 1) resulting in pLKV1. Due to a 0.3-kb deletion in pLK1 after the double enzyme digestion, the size of pLKV1 was 4.4 kb. The plasmid was established by transformation in *E. coli* DH5α and then in *L. lactis* NZ3900 and *S. gordonii* strains (Table 1). The integrity of the pLKV1 sequence was confirmed by DNA sequencing.

2.3. Reporter plasmid construction

A promoterless 0.7-kb '*rfp*' (*rfp*) cassette encoding the red fluorescent protein DsRed2 was obtained from the plasmid pDsRed2 (Clontech) by digestion with restriction enzymes *Bam*HI and *Eco*RI. It was subcloned into pLKV1 multiple cloning site (MCS) downstream of the CMV promoter resulting in pLKV-Red2 (5.1 kb). The new plasmid carries a reporter DsRed2 gene that expresses only in eukaryotic cells in order to evaluate the functionality of the DNA vaccine plasmid pLKV1.

A promoterless 0.8-kb '*gfp*' (*gfp*) cassette encoding the GFPmut3* was amplified from pCM18 (Hansen et al., 2001) by PCR with the TaKaRa e2TAK™ DNA Polymerase. The primers were Gfpm3-F1 (5'-ATGCGTAAAGGAGAAGAACTT-3') and Gfpm3-Rx (5'-TAGCTCTAGATTATTATTTGTATAGTTCATCC-3') introducing one blunt end and one sticky end (*Xba*I, underlined). The PCR condition was the same as above. The DNA fragment was subcloned into the *Sca*I and *Xba*I site of pNZ8150 to yield pNZ8150-gfp. The '*gfp*' cassette encodes the mutant GFP protein GFPmut3*, which exhibits 100-fold-greater fluorescence intensity than the wild-type GFP protein when expressed in *E. coli* (Cormack et al., 1996). The '*gfp*' cassette in pNZ8150-gfp expresses the GFPmut3* protein only in bacteria containing the nisin-controlled gene expression system (NICE) (Mierau and Kleerebezem, 2005). The fluorescent protein expresses at such a high level that the bacteria show bright green color and can be visible after entering the eukaryotic cells. The expression of the green fluorescent protein in *L. lactis* NZ3900 cells was confirmed under an epifluorescent microscope (Olympus Model BX50F) after nisin induction suggested by the supplier (MoBiTec).

2.4. Genetic transformation

The plasmid was transformed into *S. gordonii* V288 by natural transformation as previously described (Kilic et al., 2004). Briefly, V288 was grown for 16 h in Todd-Hewitt broth supplemented with 5% heat-inactivated horse serum at 37 °C. The culture was diluted 1:40 in the same broth and continued to incubate for exactly 2 h. The plasmid pLKV-Red2 DNA at 0.1 µg was added to the culture. After 1 h, the bacterial cells were plated on Todd-Hewitt agar supplemented with kanamycin at 250 µg. Transformants were isolated after 24 h incubation in a candle jar. Transformation of *L. lactis* NZ3900 was carried out based on the

protocol described by Papagianni et al. (2007) with some modifications. Briefly, a lactococcal culture was grown in 5 ml GM17 broth overnight at 30 °C. Second morning, the culture was inoculated into 20 ml pre-warmed GM17 broth and continued to incubate at 30°C for 2–3 h to reach early exponential phase ($OD_{600} = 0.3–0.6$). Penicillin G was added to a final concentration of 100 µg/ml and the culture was continued to incubate for 1 h. The cells were harvested by centrifugation and resuspended in 1 ml lithium acetate solution (100 mM LiAc; 10 mM DTT; 0.6 M sucrose; 10 mM Tris-HCl, pH 7.5; filter-sterilized) and incubated for 30 min at room temperature. The cells were washed with sterile deionized water twice, 50 mM EDTA once, and sterile deionized water three times, and finally resuspended in 2 ml 0.3 M sucrose. At this point, the cells could be stored at –80 °C for future use or being used directly for electroporation.

The plasmid pLKV-Red2 DNA was added at 0.1 µg into 80 µl cells in a 1 mm pre-cooled electroporation cuvette. The Bio-Rad Gene Pulser II Electroporation system was used. The voltage was set at 1.8 kV, resistance at 400 Ω, and capacitance at 25 µF. After delivery of the electric pulse, 1 ml recovery medium (GM17 supplemented with 0.3 M sucrose) was added and the cells were retrieved into an eppendorf test tube and incubated at 30 °C for 2 h. The cells were plated on GM17 agar supplemented with kanamycin at 600 µg/ml or chloramphenicol at 10 µg/ml for selection of pLKV-Red2 or pNZ8149-gfp, and incubated in a candle jar for 24–48 h at 30 °C.

2.5. Transfection assays of pLKV-Red2 into Caco-2 cells

The pLKV-Red2 plasmid was assayed for DsRed2 expression in the human colon cancer cells (Caco-2 cells). Fifty to 80% confluent Caco-2 cells were cultured in Dulbecco modified Eagle medium, 10% fetal calf serum, 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium), 100 U penicillin and 100 g streptomycin.

Caco-2 cells were transfected with 1.0 µg of pLKV-Red2 or pLKV1 (negative control) with Lipofectamine TM reagent (Invitrogen). The red fluorescent protein-producing cells were visualized 48 h after transfection with an epifluorescent microscope (Zeiss, Axio Observer Z1 Motorized Inverted Research Microscope).

2.6. Gene Transfer from bacteria to Caco-2 cells

To demonstrate the efficacy of pLKV1 as a delivery vector with noninvasive food and commensal bacteria, *L. lactis* NZ3900 and *S. gordonii* V288 were transformed with pLKV-Red2, and *L. lactis* NZ3900 was transformed with pNZ8150-gfp. To facilitate the gene transfer, *L. lactis* NZ3900 and *S. gordonii* containing pLKV-Red2 and *L. lactis* NZ3900 containing pNZ8150-gfp were treated with several different methods to weaken the cell wall prior to adding to the mammalian cells.

The *L. lactis* strain was grown at 30 °C in GM17 supplemented with 600 µg/ml kanamycin for selecting pLKV-Red2 or 10 µg/ml chloramphenicol for selecting pNZ8150-gfp. The *S. gordonii* strain was grown at 37 °C in Todd-Hewitt broth supplemented with 200 µg/ml kanamycin.

For penicillin or glycine treatment, overnight cultures were diluted 1:5 (2 ml into 8 ml) with pre-warmed medium and grown for 2 h. Penicillin (100 µg/ml) or glycine (2.5% w/v) was added and the culture was continued for 1 h and harvested, washed 3 times with sterile deionized water, and resuspend in 0.5 ml 10% glycerol. At this stage, the cells were either electroporated or treated with lysozyme before electroporation.

For lysozyme treatment, the bacterial cells in 10% glycerol were added with lysozyme at 20 µg/ml and incubated for 1 h at 37 °C. The cells were then washed twice with 10% glycerol

to remove lysozyme and resuspend in 0.5 ml 10% glycerol. For electroporation treatment, the Bio-Rad Gene Pulser II Electroporation system and 4 mm cuvettes were used. The voltage was set at 2.5 kV, resistance at 400 Ω , and capacitance at 25 μ F. After the delivery of electric pulse, the cells were kept on ice and used within 2 h.

Assays of bacteria into human cells were performed using the human colon carcinoma cell line Caco-2. Briefly, eukaryotic cells were cultured in P6 wells plates containing 1×10^6 cells per dish in RPMI supplemented with 2 mM L-glutamine and 10% fetal calf serum. The multiplicity of infection (MOI) was 10^3 bacteria/cell. After 3 h of culture at 37 °C to allow internalization of bacteria, cells were washed 5 times to remove extracellular bacteria. The expression of fluorescent proteins in Caco-2 cells and in *L. lactis* bacteria were visualized with an epifluorescent microscope (Zeiss, Axio Observer Z1 Motorized Inverted Research Microscope). Both fluorescent and phase contrast photos of the same microscopic field were taken. Transfection efficiency was calculated by counting the number of red fluorescent cells among a total of 1,000 cells in the superimposed phase contrast microscopic photo.

3. Results

3.1. pLKV1 characteristics

To facilitate delivery of DNA vaccines into eukaryotic cells by lactic acid bacteria, we have constructed a new eukaryotic expression, *E. coli*-LAB shuttle plasmid vector pLKV1. As shown in Fig. 1, pLKV1 contains the eukaryotic region with the CytoMegaloVirus promoter (pCMV), a multiple cloning site (MCS), and the polyadenylation signal of bovine growth hormone (BGH polyA) needed for a gene expression by eukaryotic host cells. Its prokaryotic region contains the RepA replication origin for a broad host range expression in both *E. coli* and LAB strains. The MCS inserted between the eukaryotic promoter pCMV and the BGH polyA, provides five unique restriction enzyme recognition sites, *Bam*HI, *Spe*I, *Eco*RI, *Pst*I and *Not*I, which can be used to clone a gene of interest and the T7 primer binding site for its sequencing. The complete sequence of the plasmid has been determined. Its size is 4,448 bp. The sequence has been deposited in GenBank with an accession number of HM569775.

3.2. Transfection assay of pLKV1 into Caco-2 cells

The reporter red fluorescent protein (RFP) gene DsRed2 was subcloned into pLKV1 to form pLKV-Red2 (5.1 kb). pLKV-Red2 was directly transfected with LipofectamineTM reagent (Invitrogen) into Caco-2 cells. Forty-eight hours later, we observed red fluorescent protein (RFP) expression in these epithelial cells, but no RFP expression was observed after transfection with the blank shuttle DNA-delivery vector pLKV1 (data not shown). This result demonstrates that pLKV1 was functional for gene expression in mammalian cells and it could be used for further experiments.

3.3. Effect of cell wall treatments on bacteria entering Caco-2 cells

Both *L. lactis* NZ3900 and *S. gordonii* V288 containing pLKV-Red2 were used for gene delivery into Caco-2 cells. To facilitate the uptake of the noninvasive food and commensal bacteria, the bacteria were treated with various agents and/or procedures to weaken their cell walls. These included glycine, penicillin, lysozyme and/or electroporation prior to the transfection assay. As shown in Table 2, without treatment to weaken the bacterial cell walls, the gene transfer by both bacteria was near zero. When *L. lactis* NZ3900 was used to deliver the reporter gene into Caco-2 cells, treatment with glycine showed the highest transfection frequency. Additional treatment with lysozyme and electroporation did not improve or even reduced this effect. When the reporter gene was delivered by *S. gordonii* V288, the treatment with penicillin and lysozyme gave the best result with all Caco-2 cells

showing strong red fluorescence (Fig. 2A). Electroporation of the bacteria to physically damage the cell wall did not further improve the transfection result. To study whether the improved gene transfer is associated with increased internalization of the bacteria into the mammalian cells, the bacterium *L. lactis* NZ3900 containing pNZ8150-gfp, which turns the bacteria fluorescent green, was used with and without 2.5% glycine pretreatment. The glycine-treated bacteria showed significant increase in bacteria uptake by Caco-2 cells (Fig. 3).

4. Discussion

We have constructed a novel *E. coli*-LAB shuttle plasmid vector, pLKV1, for DNA-delivery into eukaryotic cells. This plasmid has several advantages over many existing plasmids for DNA vaccine delivery (Gram et al., 2007; Guimarães et al., 2005; 2006; 2009). First, it can express in both *E. coli* and many LAB species, including *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Weissella*. This feature allows relatively easy construction of DNA vaccines in *E. coli* and be transformed into a suitable LAB strain for vaccine delivery. Second, it has a small size (4.4 kb), so it can be used to clone genes or DNA vaccines of relatively larger sizes and be easily transformed into various bacterial species. Its parent plasmid pA13 has successfully cloned a 14.4-kb DNA fragment (Kojic et al., 2010). Third, it has a high copy number (about 65 per cell), so that it can deliver DNA vaccines with a higher dose. Fourth, it carries a kanamycin resistance marker that expresses in both Gram-negative and Gram-positive bacteria. This feature is desirable because kanamycin resistance marker is recommended by the FDA for use in DNA vaccine construction due to a better safety record than other antibiotic resistance markers (FDA, 1996). This new plasmid vector has been successfully tested *in vitro* by delivering a red fluorescent reporter gene into cultured Caco-2 cells (Fig. 2), the human tongue squamous carcinoma UM1 cells, the human oral keratinocytes and also *in vivo* into the mouse cells in the dermal and subdermal tissues of a skin wound (data not shown).

Previously, it was hypothesized that bacteria-mediated DNA delivery into eukaryotic cells requires invasion or infection by the bacterial carrier. As a result, invasive pathogenic bacteria such as *Listeria*, *Salmonella*, *Shigella*, and *Yersinia* have been used as DNA-delivery bacterial vectors (Detmer and Glenting, 2006). If a non-invasive bacterium is used, it must express a cloned invasion gene from a pathogen (Guimarães et al., 2005; Innocentin et al., 2009), which allows the bacterium to enter the host cells and to achieve an increased DNA delivery. Without carrying a cloned invasive gene, the native lactococcus showed very low efficiency in DNA vaccine delivery (Guimarães et al., 2006). Even with the cloned internalin gene, *inlA* from *Listeria monocytogenes*, the rate of the DNA delivery into Caco-2 cells was still low, only about 1% (Guimarães et al., 2005; 2009; Innocentin et al., 2009). Our DNA vaccine delivery system has achieved a transfection rate of about 100% using noninvasive, native lactococcal and streptococcal strains. This achievement indicates that invasion or infection is not required for DNA vaccine delivery with bacterial vectors. The human mucosal epithelial cells may serve as “non-professional” phagocytic cells that can take up bacteria (Blanchette et al., 2009), especially when the bacterial cell walls are disrupted by various chemical treatments.

The success of noninvasive DNA delivery into mammalian cells may include four stages: (i) DNA protection before entering the eukaryotic cells; (ii) DNA delivery across cell membranes as the bacteria entering the cells; (iii) DNA release in the phagosome; and (iv) DNA entering the nucleus. It is obvious that disrupting the integrity of bacterial cell wall can facilitate DNA release inside the eukaryotic cells. However, the critical stage affected by the bacterial cell wall disruption appears to be at the stage of internalization. The uptake of green fluorescent bacteria by Caco-2 cells was significantly enhanced by the treatment of the

bacterial cells with cell wall-disrupting agents (Fig. 3). The exact mechanism for the enhanced bacterial internalization is unknown. We propose several possibilities. One, bacteria with an intact cell wall may be more resistant against “non-professional” phagocytosis by the mammalian epithelial cells. Two, the surface of food or commensal bacteria may carry specific markers recognizable by the mammalian gut epithelial cells (Artis, 2008). Disrupting the bacterial cell wall may render these markers unrecognizable. Three, the bacteria may carry phagocytosis-associated receptors embedded in the cell wall or its associated structures (Melendez and Tay, 2008). Disrupting bacterial cell wall could expose such receptors and subsequently result in phagocytosis by the gut epithelium-derived Caco-2 cells.

To facilitate vaccine development, we constructed novel protein and DNA vaccine reporter plasmids with the green and red fluorescent protein genes, respectively. These reporter vaccine plasmids not only can be used for monitoring *in vitro* vaccine uptake by cultured cells, but also can be used for monitoring vaccine uptake *in vivo* in animals. The uptake and expression of these fluorescent reporter vaccines in a small animal can be monitored in real time with a noninvasive whole body imaging system, such as the UVP iBox® Scientia Small Animal Imaging System (www.uvp.com/ibox.html).

5. Conclusions

We have developed a novel system with noninvasive food and commensal bacteria as a vehicle to deliver genes into mammalian cells. In addition to gene therapy, this system may be useful for delivery of DNA vaccine, interference RNA and/or bioactive peptide. It has several advantages over existing methods. These include safety, high efficiency and capability to monitor the gene delivery with fluorescent reporter genes in real time.

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Fig. 1. Construction of pLKV1 and pLKV-Red2. The MCS in pLKV1 contains five unique restriction enzyme sites (from left to right): *Bam*HI, *Spe*I, *Eco*RI, *Pst*I, and *Not*I.

Fig. 2.
Streptococcus gordonii V288 delivers the red fluorescent reporter plasmid pLKV-Red2 into Caco-2 cells with (A) and without (B) the penicillin and lysozyme treatment.

Fig. 3.

Caco-2 cell uptake of GFPmut3*-expressing *Lactococcus lactis* NZ3900-pNZ8150-gfp with (A) and without (B) glycine treatment to weaken the bacterial cell wall. The phase contrast and fluorescent images were superimposed so that the unstained Caco-2 cells can be visualized.

Table 1

Bacterial strains and plasmids used in this work.

Strain/plasmid	Characteristics ^a	Source/Reference
<i>E. coli</i> DH5α	(F-80dlacZΔM15 Δ(lacZYA- argF)U169 endA1 recA1 hsdR17(rk- mk+) deoR thi-1 supE44 λ- gyrA96 relA1)	Invitrogen
<i>L. lactis</i> NZ3900	lacF-, pepN::nisRnisK; food-grade expression strain	MoBiTec
<i>S. gordonii</i> V288	Oral commensal strain	F. Macrina
NZ3900-pLKV-Red2	NZ3900 harboring pLKV-Red2/Km ^{±R}	This study
NZ3900-pNZ8150-gfp	NZ3900 harboring pNZ8150-gfp/Cm ^R	This study
V288- pLKV-Red2	V288 harboring pLKV-Red2/Km ^{±R}	This study
pA13	<i>E. coli</i> -LAB shuttle cloning plasmid/Em ^R	Kojic et al., 2010
pAK267	Plasmid carrying the Km ^{±R} cassette from Tn1545	This study
pVAX1	Expression vector containing pCMV, MCS and BGH polyA/Km ^{-R}	Invitrogen
pCM18	Plasmid carrying the <i>gfpmut3*</i> gene/Em ^R	Hansen et al., 2001
pDsRed2	Plasmid carrying the DsRed2 gene/Amp ^R	Clontech
pNZ8150	Nisin-induced expression vector/Cm ^R	MoBiTec
pNZ8150-gfp	pNZ8150 containing the <i>gfpmut3*</i> gene/Cm ^R	This study
pLK1	<i>E. coli</i> -LAB shuttle cloning plasmid/Km ^{±R}	This study
pLKV1	<i>E. coli</i> -LAB shuttle DNA vaccine plasmid/Km ^{±R}	This study
pLKV-Red2	Red fluorescent reporter DNA vaccine plasmid/Km ^{±R}	This study

^a Amp^R: ampicillin resistance; Cm^R: chloramphenicol resistance; Em^R: erythromycin resistance; Km^{-R}: kanamycin resistance only in Gram-negative bacteria; Km^{±R}: kanamycin resistance in both Gram-negative and positive bacteria.

Table 2

Various treatments of bacterial vehicles on the transfection efficiency of pLKV-Red2 into Caco-2 cells

Treatment	<i>L. lactis</i> NZ3900	<i>S. gordonii</i> V288
Untreated control	3%	1%
Penicillin	2%	65%
Glycine	100%	10%
Lysozyme	5%	1%
Penicillin + lysozyme	80%	100%
Glycine + lysozyme	10%	15%
Electroporation alone	0%	0%
Penicillin + electroporation	5%	0%
Glycine + electroporation	10%	2%
Lysozyme + electroporation	30%	80%
Penicillin + lysozyme + electroporation	20%	100%
Glycine, + lysozyme + electroporation	20%	30%