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ORIGINAL PAPER

Enhanced expression of PCV2 capsid protein in *Escherichia coli* and *Lactococcus lactis* by codon optimization

Wentao Kong · Jian Kong · Shumin Hu · Wenwei Lu · Ke Wang · Mingjie Ji

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Abstract Capsid protein (Cap) of porcine circovirus type 2 (PCV2) encoded by *orf2* is a main structural protein with strong immunoreactivity. However, capsid protein is expressed poorly in prokaryotic organisms because of differences in codon usage. In this study, we introduce 24 synonymous mutations into *orf2* by mutagenic primers and overlap extension polymerase chain reaction (OE-PCR) technique. Fourteen rare codons of *orf2* were replaced with preferable codons used in *Escherichia coli* cells. Moreover, the nuclear localization signal (NLS) region rich in rare codon clusters at the 5′ end was deleted. The codon-optimized genes demonstrated higher levels of expression compared with wild-type genes. The influence of rare codons on the gene expression was eliminated by mutation. Western blot analysis confirmed the immunoreactivity of the proteins expressed by mutated genes. Further testing demonstrated that the mutated genes were also expressed successfully in *Lactococcus lactis* NZ9000. The immunologically active Cap proteins produced by recombinant strains have the potential applications for serological diagnostic assays and vaccine development against PCV2-associated diseases.

Keywords *orf2* · Rare codons · *Escherichia coli* · *Lactococcus lactis* · Expression

Introduction

Porcine circovirus 2 (PCV2) is considered a pathogen causing post-weaning multisystemic wasting syndrome (PMWS) in swine (Allan and Ellis 2000; Chae 2005). This disease has resulted in significant economic losses in the swine industry. PCV2, a non-enveloped virus with single-stranded circular genomic DNA, has been identified as the genus circovirus of the Circoviridae family (Tischer et al. 1982). The genome of PCV2 contains three major open reading frames (ORF) (Mankertz et al. 1998a): ORF1 encodes a Rep protein related to viral replication (Mankertz et al. 1998b); ORF3 encodes a small protein involved in apoptosis (Liu et al. 2005); and ORF2 encodes the 28 kDa capsid protein, a unique structural protein that has been known to take strong immunoreactivity with serum from PCV2-infected swine (Nawagitgul et al. 2000). Capsid proteins are the preferred antigen in many serological tests and in vaccine development. In addition, capsid protein is expressed successfully in many eukaryotic cells, such as insect cells (Fan et al. 2007) and mammalian cells (Fan et al. 2008).

Escherichia coli (*E. coli*) is one of the most frequently used prokaryotic expression host for overproduction of heterologous proteins. However, codon usage in *E. coli* displays a bias. The coding sequence of *orf2* from PVC2 contains high proportion of rare codons that are rarely used by bacteria. Moreover, a nuclear localization signal (NLS) region with rare codon clusters, such as AGA/AGG, is located at the N-terminus of the gene (Liu et al. 2001a). To examine the effect of rare codons on heterologous expression, the *orf2* gene of PCV2 has been transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL strains; the expression has been performed successfully (Trundova and Celer 2007). The expression of the dCap protein by NLS deletion from *orf2* has been conducted in *E. coli* (Zhou et al. 2005). Co-expression of *orf2* gene with glutathione-S-transferase (GST) or maltose binding protein (MBP) allows for production at low levels (Liu et al. 2001b). *Lactococcus lactis*

W. Kong · J. Kong (✉) · S. Hu · W. Lu · K. Wang · M. Ji
State Key Laboratory of Microbial Technology, Shandong University, 250100 Jinan, Shandong, China
e-mail: kongjian@sdu.edu.cn

(*L. lactis*) are non-invasive and non-pathogenic bacteria that are generally recognized as safe (GRAS). They are used to manufacture a variety of fermented dairy products. Recently, *L. lactis* has received heightened attention after being recognized as a promising vaccine delivery vehicle candidate. The nisin-controlled gene expression (NICE) system is one of the most successfully and widely used tools for regulated gene expression in Gram-positive bacteria (Mierau and Kleerebezem 2005). Expression of immunogenic proteins in *L. lactis* for oral vaccines has been demonstrated with laboratory animals (Mercenier et al. 2000; Enouf et al. 2001; Lee et al. 2001).

To overproduce capsid protein of PCV2 in bacteria, specifically in *E. coli* or *L. lactis* for further studies or vaccine purpose, we attempted to replace the rare codons of PCV2 *orf2*. Multiple site-directed mutagenesis and deletion of NLS were adopted in this paper. The expressions of both the mutant fragments of *morf2* and *dmorf2* were performed successfully with relatively high levels in *E. coli* and *L. lactis*.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking. For *L. lactis* NZ9000, GM17 (M17/oxoid supplemented with 0.5% glucose) was used as culture medium. If necessary, antibiotics were added as follows: for *E. coli*, chloramphenicol (5 μg/ml) and ampicillin (100 μg/ml); for *L. lactis*, chloramphenicol (5 μg/ml).

DNA manipulation

Restriction enzymes, *pfu* DNA polymerase, and T4 DNA ligase were used according to manufacturer instructions (TaKaRa, Japan). *E. coli* strains were transformed by the standard $CaCl_2$ heat-shock protocol (Sambrook and Russell 2001). Plasmids isolation from *E. coli* was performed by the alkaline lysis method (Birnboim and Doly 1979). Unless otherwise indicated, plasmid constructions were first established in *E. coli*, and then prepared and transformed into electrocompetent *L. lactis* cells. The electrocompetent *L. lactis* cells were prepared as described by Holo and Nes (1989), and electroporation was performed with Bio-Rad Gene Pulser at 2.5 kV, 200 ohms, and 25 μF.

Construction of the *orf2* expression cassettes and codon optimization

With plasmid pGEM-TCap as the template, the *orf2* gene was amplified by polymerase chain reaction (PCR) using oligonucleotide primers: (forward NcoF: 5′-TGCCCATGG CGTATCCAAGGAG-3′, containing the *Nco* I cloning site (underlined); and reverse XhoR: 5′-GATCTCGAGGGGT TTAAGTGG-3′, containing the *Xho* I cloning site (underlined)). PCR amplification was conducted as follows:

Table 1 Bacterial strains and plasmids used in this study

Materials	Relevant features	Source or reference
Bacteria		
E. coli DH5α	Used for gene cloning	TaKaRa
E. coli BL21 (DE3)	Used for gene expression under T7 promoter	Novagen
E. coli Rosetta (DE3)	Cm^r, expresses six rare tRNAs and facilitates expression of genes that encode rare *E. coli* codons	Novagen
L. lactis NZ9000	*nisRK* genes integrated to the chromosome of *L. lactis* MG1363	(Kuipers et al. 1998)
Plasmids		
pET-22b(+)	Ap^r, T7 promoter	Novagen
pSec:leiss:Nuc	Cm^r, pWV01 replicon, expresses Nuc under PnisA control	(Le Loir et al. 1998)
pGEM-TCap	Ap^r, PCV2 *orf2* inserted into pGEM-T easy vector	Our lab
pET-*orf2*	pET-22b(+), contains the native *orf2* gene	This study
pET-*morf2*	pET-22b(+), contains the mutated *orf2* gene	This study
pET-*dorf2*	pET-22b(+), contains the *orf2* gene without the nuclear location signal	This study
pET-*dmorf2*	pET-22b(+), contains the mutated *orf2* gene without the nuclear location signal	This study
pSec:leiss:*orf2*	Cm^r, with the *orf2* gene expressed under PnisA control	This study
pSec:leiss:*morf2*	Cm^r, with the *morf2* gene expressed under PnisA control	This study
pSec:leiss:*dorf2*	Cm^r, with the *dorf2* gene expressed under PnisA control	This study
pSec:leiss:*dmorf2*	Cm^r, with the *dmorf2* gene expressed under PnisA control	This study

initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 55°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified PCR products were then ligated into *Nco* I and *Xho* I sites of the pET-22b (+) vector, generating the plasmid pET-*orf2*. The XhoR primer did not contain a stop codon; thus, the *orf2* gene could be fused into the His6 tag at the C-terminal of the pET-22b (+) vector.

NLS-deleted capsid protein gene (*dorf2*) was amplified by PCR using plasmid pGEM-TCap as the template with a pair of primers (forward: NLSF 5′-GAACCATGGATGGCATCTTCAACAC-3′, *Nco* I site is underlined; reverse primer was XhoR), PCR amplification was performed as described above. PCR products were digested, and then cloned onto the *Nco* I and *Xho* I sites of the pET-22b (+) vector, generating the plasmid pET-*dorf2*.

To replace the rare codons distributed in the *orf2* gene, five primers containing mutant bases were designed (Fig. 1). MF3 (5′-AACGTAATCAGCTGTGGCTGCGTTTAC-3′, synonymous mutations are underlined) and MR3 (5′-GTAAACGCAGCCACAGCTGATTACGTT-3′) are reverse complementary primers that were used to mutate the rare codons at the 3′ end by overlap extension PCR. With pET-*orf2* as the template, two overlapping fragments were amplified by PCR using the two pairs of primers: NcoF/MR3 and MF3/XhoR. Then, the two fragments obtained above were purified and fused by overlap extension PCR. PCR amplification was performed as follows: without primers and templates, equal amounts of the two overlapping fragments were added to PCR reaction mixtures. After heating at 94°C for 3 min, 5 cycles (94°C for 30 s, 55°C for 40 s, 72°C for 1 min) were employed. Then, the 5′ end NcoF and the 3′ end XhoR primers were added, and another 30 cycles were performed under the same conditions. PCR products were digested and ligated into the *Nco* I and *Xho* I sites of the pET-22b (+) vector, generating the plasmid pET-M3. To mutate the bases at the 5′ end and rare codons located in the middle region of the *orf2* gene, primers MF11 (5′-GG**CCATGG**CGTATCCACGTCGTCGCTACCGTCGTCGTCGTCACC-3′, containing the *Nco* I cloning site (bold), synonymous mutations are underlined), MF12 (5′-CGCTACCGTCGTCGTCGTCACCGCCCTCGCAGCCATCTTG-3′), and MF2 (5′-CA**GAATTC**AACCTTAACCTTACGAATACGGTAGTATTCAAATGG-3′, containing the *Eco*R I cloning site (bold)) were designed. Using pET-M3 as the template, PCR product M2 was amplified by the MF12 and MF2 primer pairs. Then, a second round of PCR was performed using primers M11 and M2 with M2 DNA as the template. The second-round PCR products were digested and ligated into the *Nco* I and *Eco*R I sites of pET-M3, generating the plasmid pET-*morf2*. The *dmorf2* products were amplified with NLSF and XhoR primers. Then, pET-*dmorf2* was constructed by cloning the *dmorf2* fragment onto the pET-22b (+) vector. The mutant genes in the pET-*morf2* and the pET-*dmorf2* were sequenced.

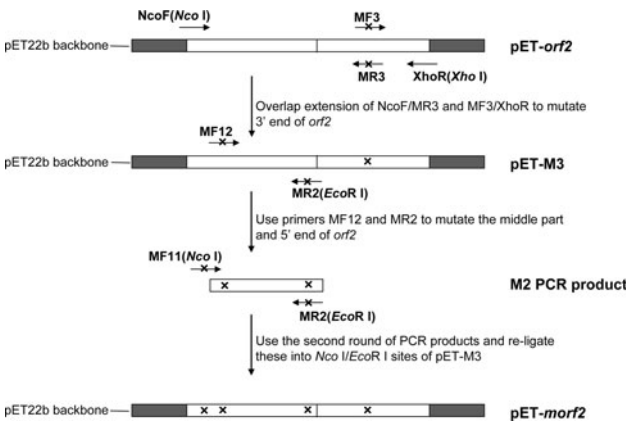

Fig. 1 Schedule of the mutagenesis and distributions of mutagenic primers in the *orf2*. Primers and sequences that contain synonymous mutations are indicated by the *symbol* (×)

Expression of the *orf2* gene and its mutants in *E. coli*

Plasmids pET-*orf2*, pET-*morf2*, pET-*dorf2,* and pET-*dmorf2* (constructed above) were transformed into *E. coli* BL21 (DE3) and Rosetta (DE3) competent cells. The transformants were selected on LB media plates containing 100 μg/ml ampicillin. For expression studies, a single clone from the plates was inoculated into 5 ml of LB broth medium with ampicillin, and incubated at 37°C with vigorous shaking at 200 rpm overnight. The cultures were inoculated into 10 ml of fresh LB media at a 1:100 dilution. When the cell density at 600 nm (OD_{600}) reached 0.8, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM. After induction for 3 h, the cultures were diluted to $OD_{600} = 1.2$ using fresh LB broth in order to achieve same levels of cell concentration. Cells from the 10 ml dilution were harvested by centrifugation at 12,000×*g* for 10 min. The pellets were resuspended in 1 ml of phosphate-buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and pH 7.4) and lysed by sonication. Then, 1 ml of 2× SDS loading buffer were added into the lysates, which were then heated at 100°C for 5 min. Next, 20 μl of the samples were analyzed by SDS-PAGE (8–15% gradient gel) and stained with Coomassie blue. Quantification of the Cap protein was determined by scanning and the analysis of SDS-PAGE gels with Image Quant 400 gel imaging system (GE Healthcare). Bovine Serum Albumin (BSA, Sigma) was used as standard protein. The amounts of protein were obtained by using Image Quant TL V2005 software, and by comparing signals to those of known amounts of BSA.

Western blot analysis

The proteins on the SDS-PAGE gel were electrotransferred onto a polyvinylidene fluoride membrane at 100 mA for 2 h. The membrane was blocked with 5% non-fat milk for 1 h and then incubated overnight with monoclonal antibody at a 1:10,000 dilution. The membrane was washed with PBS buffer twice for 10 min each time, and incubated with HRP-conjugated secondary antibody at a dilution of 1:1,000 for 1 h. Immunoblots were prepared by using Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the instruction manual.

Expression of the *orf2* gene and its mutant genes in *L. lactis* NZ9000

Fragments of *orf2* and *morf2* were amplified by PCR using a pair of primers orfF (5′-CCAGCCGGATGCATCTGG CGCGTATCCA-3′) and orfR (5′-TGGTGGATCGATT AGGGTTTAAGTGG-3′), and *dorf2* and *dmorf2* with primers dcapF (5′-GTTATGCATTCTTCAACACCCGCC TCTC-3′) and orfR. PCR products were digested and ligated onto *Nsi* I and *Cla* I sites of pSec:leiss:Nuc vector. The generating vectors pSec:leiss:*orf2*, pSec:leiss:*morf2*, pSec:leiss:*dorf2,* and pSec:leiss:*dmorf2* were electransformed into *L. lactis* NZ9000, and the recombinants were selected on GM17/Cm plates. A single clone from the plate was inoculated into 5 ml of GM17 broth supplemented with 5 μg/ml chloramphenicol. Overnight cultures were diluted at 1:50 in fresh medium. Bacteria were grown to an OD_{600} of 0.8 and induced with 10 ng/ml nisin. After 6 h, the cells in 5 ml cultures were harvested by centrifugation. The pellets were resuspended in 500 μl PBS buffer with lysozyme at a final concentration of 5 mg/ml, and then incubated at 37°C for 30 min. Proteins in the supernatant were concentrated to 50 μl by ultrafiltration using Amicon Ultra-4 (Millipore). After adding 2× SDS loading buffer and denaturation at 100°C for 5 min, proteins were analyzed by SDS-PAGE (8–15% gradient gel) and stained with Coomassie blue.

Results

Codon optimization of the *orf2* gene in *E. coli*

The *orf2* gene of PCV2 encoded 233 amino acids. There were 14 AGG/AGA, 1 CGA, 3 CUA, 3 AUA, 12 CCC, and 1 CGG, or a total of 34 rare codons, used in the *E. coli* at a frequency of <0.5%. In particular, an NLS region was observed with high GC content and rare codon clusters at N-terminus. The expression of a gene containing a triplet CGG (arginine) or AGA (arginine) gave rise to erroneous incorporation of amino acids in the growing peptide or frameshift during translation (McNulty et al. 2003; Forman et al. 1998; Calderone et al. 1996). In terms of the heterologous expression of the Cap protein of PVC2 in *E. coli*, two strategies were applied. First, multiple directed-site mutagenesis was conducted by mutagenic primers and overlap extension PCR amplification. Two pairs of primers, NcoF/MR3 and MF3/XhoR, were designed to mutate rare codons of 3′ end using overlap extension PCR. To mutate rare codons of 5′ end, and to avoid mismatch of long primers in the high GC content region, the two primers of MF11 and MF12 were designed. After three rounds of PCR amplification using the designed primers under different reaction conditions (Fig. 1), 24 bases in the wild-type gene, *orf2,* covering 14 rare codons (6% of the total amino acids) used in *E. coli* were substituted. The mutated sequence of *morf2* was confirmed by DNA sequencing, and aligned with the original gene *orf2* (Fig. 2). The DNA fragments *orf2* and *morf2* were cloned into vector pET-22b (+), generating the plasmids pET-*orf2* and pET-*morf2*, respectively. Next, a primer NLSF was used to delete the NLS at N-terminus from *orf2* and *morf2* to generate fragments *dorf2* and *dmorf2*. Then, *dorf2* and *dmorf2* were inserted into the vector pET-22b (+), resulting in the plasmids pET-*dorf2* and pET-*dmorf2*.

Expression of the *orf2* and its mutants in *E. coli*

The recombinant plasmids constructed above were transformed as two expression hosts, *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) that could express six rare tRNA genes. As shown in Fig. 3, the wild-type *orf2 gene* was expressed at the level similar to *morf2*, as well as *dorf2* and *dmorf2* in Rosetta (DE3). These reveal that the host cells have the ability to recognize rare *E. coli* codons. In contrast, the expression of original Cap protein encoded by *orf2* could not be detected in BL21 (DE3) (Fig. 3). The Cap protein (encoded by *morf2* gene) was the major protein produced with approximately 20% of total cellular proteins present in the cell lysates from BL21 (DE3). The expression level of *dmorf2* also increased to 22% of the total cellular proteins after removal of the NLS, indicating that the effects of the preferential codon usage in *E. coli* were minimized by multiple site-directed mutagenesis and NLS deletion. However, the expression level of *morf2* from BL21 (DE3) was equivalent to that of *morf2* and *orf2* in Rosetta (DE3) (Fig. 3), suggesting that the influence of rare codon clusters in *E. coli* was eliminated.

Expression in lactic acid bacteria

After transformation with recombinant plasmids, the transformants were cultured and induced by nisin for 6 h.

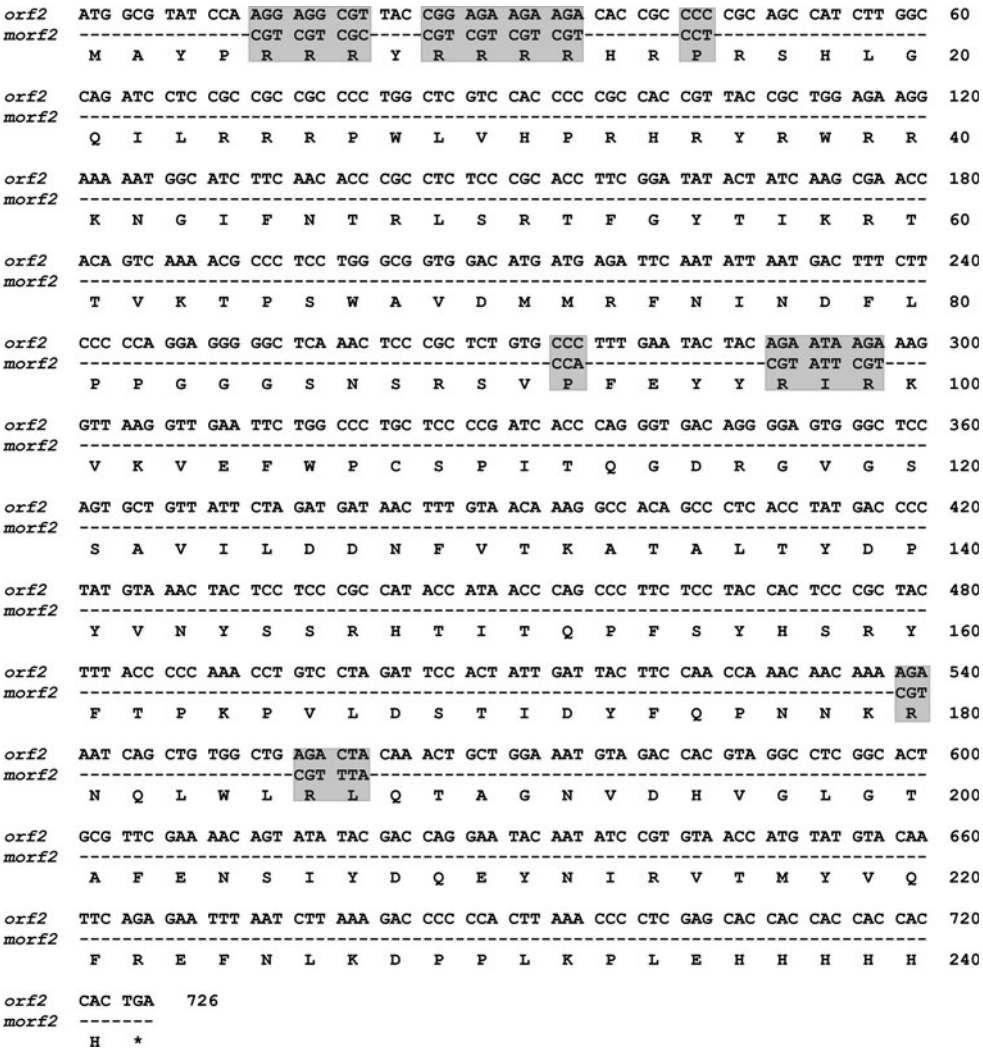

Fig. 2 Alignment of sequences of *orf2* and *morf2*. The *grey* shadows indicate the replaced codons; 41 amino acids at N-terminus are nuclear localization signal

The total cell proteins and concentrated cultural supernatants were analyzed by SDS-PAGE (Fig. 4). The *morf2* gene was expressed at approximately 10% of total cellular proteins, while *dorf2* or *dmorf2* was expressed at 12% of total cellular proteins; the wild-type *orf2* could not be expressed in *L. lactis* NZ9000 (Fig. 4).The content of *dorf2* and *dmorf2* in the supernatant was approximately 600 μg/l. These indicate that the expression level of Cap protein in *L. lactis* also increased.

Confirmation of the expressed Cap protein by Western blot

As for *E. coli,* several reports have claimed that rare codons may give rise to erroneous incorporation of amino acids. Genetic alteration of rare codons in the *orf2* gene without modifying the encoded protein has been attempted in this paper. To determine the immunoreactivity of the proteins produced by mutant genes, the whole cell lysates from *E. coli* BL21 (DE3) were analyzed by Western blot using anti-Cap monoclonal antibody. As shown in Fig. 5, obvious bands corresponding to Cap and dCap proteins were observed, suggesting that the codon-optimized *orf2* gene was produced successfully by the *E. coli* in the cytoplasm. The expressed proteins also displayed immunoreactivity. However, the band of original Cap protein was not visible because of poor expression in *E. coli* BL21 (DE3).

Discussion

Clusters of rare arginine codons AGG/AGA, leucine codon CUA, isoleucine codon AUA, and proline codon CCC can reduce the quality and quantity of synthesized proteins in *E. coli* (Kane 1995). To avoid the influence of rare codons on the expression of PCV2 *orf2* gene in *E. coli*, mutagenic primers and overlap extension PCR were used to introduce multiple-site mutagenesis into the *orf2* gene. PCR amplification-based multiple mutagenesis is an efficient tool to introduce mutations embedded in oligonucleotide primers

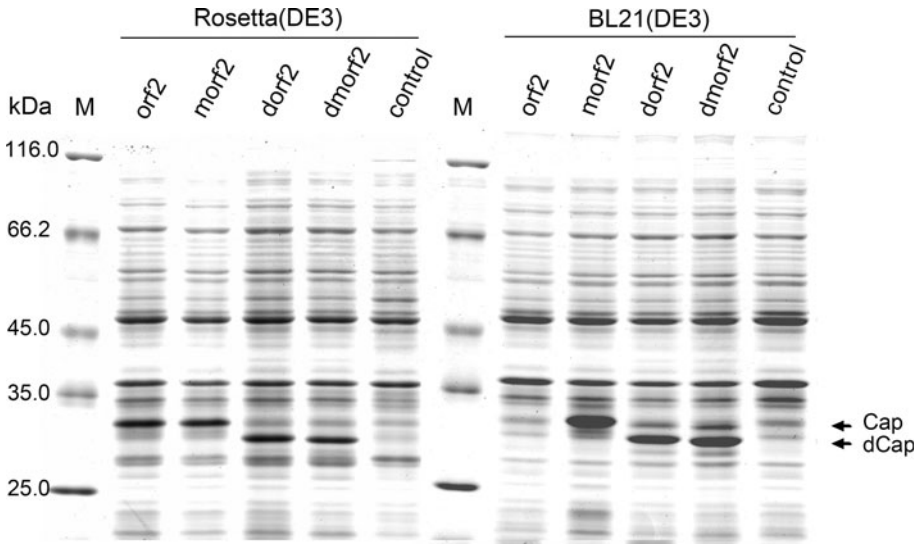

Fig. 3 Comparison of native *orf2* and its mutants for expression in *E. coli* cells. Equivalent amounts of total proteins (based on harvest OD_{600}) were analyzed by SDS-PAGE (8–15% gradient gel). Size of the molecular mass marker proteins are indicated on the *left*

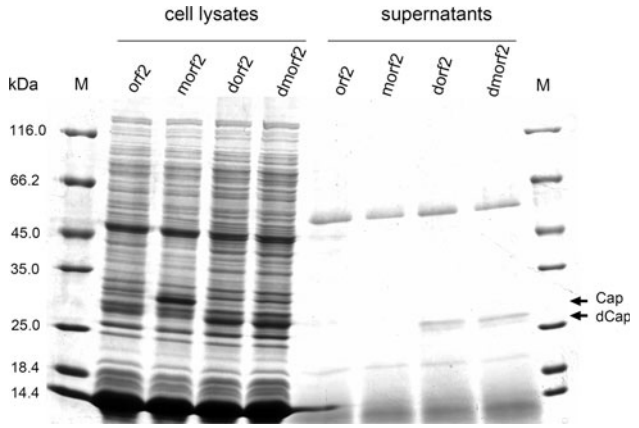

Fig. 4 Expression of *orf2* in *Lactococcus lactis* NZ9000. Equivalent amounts of total proteins (based on harvest OD_{600}) and same volume of supernatants were analyzed by SDS-PAGE (8–15% gradient gel). Sizes of the molecular mass marker proteins are indicated on the *left*; the bands of Cap and dCap protein are indicated by *arrows*

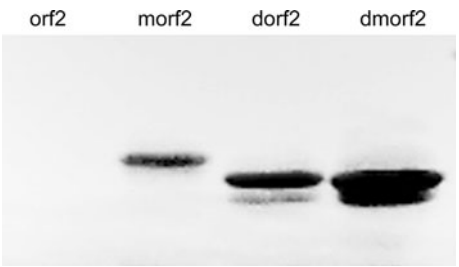

Fig. 5 Western blot analysis of the expressed Cap proteins. Total cell proteins of BL21 (DE3)/*orf2*, BL21 (DE3)/*morf2*, BL21 (DE3)/*dorf2* and BL21 (DE3)/*dmorf2* were analyzed by Western blot using monoclonal antibody of PCV2

(Ge and Rudolph 1997; Kumar and Rajagopal 2008). The negative influence of rare codons was eliminated after replacing the 14 rare codons with preferred ones in the *E. coli*. This is an effective way to change codons for genes containing consecutive rare codons. Codon optimization increased the expression of *orf2* gene in *E. coli* from being initially undetectable to comprising 20% of total bacterial proteins. No significant difference was observed in the expression level of *dorf2* and *dmorf2* in both hosts, indicating that the rare codons in the N-terminus play a key role in *orf2* expression. Confirmed by Western blot, the Cap proteins expressed by mutant genes have the similar immunoreactivity with the native Cap protein.

The use of lactococci as cell factories for the production of bacterial and viral antigens has been achieved by Villatoro-Hernandez et al. (2008) and Bahey-El-Din et al. (2008). For the development of oral vaccines against PCV2 disease, *L. lactis* NZ9000, one of the most widely used hosts, was used as a delivery vehicle in the production of Cap protein and its derivatives. The genome of lactic acid bacteria has low GC content (35–38%); the lactic acid bacteria also display a bias in codon usage (Fuglsang 2003). Some codons, such as AGG, AAG, CUG, CGG, CCC, and AUA, are generally used in *L. lactis* at a frequency of <0.4%. AGG, CGG, CCC, and AUA are also rare codons for *E. coli*; therefore, the replacement of these codons in *E. coli* is also applicable for *L. lactis*. In this study, the mutant gene derivatives were also expressed successfully in *L. lactis* NZ9000 in both the cytoplasm and the media.

In conclusion, this work provides an approach to produce Cap and dCap proteins in *E. coli* and *L. lactis* efficiently. The expressed Cap proteins and recombinant strains may be applied in serological assays and vaccines against PCV2 infection.

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Conflict of interest statement None.

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