Surface Displayed Expression of a Neutralizing Epitope of Spike Protein from a Korean Strain of Porcine Epidemic Diarrhea Virus

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Abstract The neutralizing epitope (K-COE) of the spike protein from a Korean strain of porcine epidemic diarrhea virus (PEDV) has been shown to prevent and foster an immune response to PED, when orally adjusted. The cell surface of the budding yeast, *Saccharomyces cerevisiae*, was engineered to anchor the K-COE on the outer layer of the cell, and consequently, the altered yeast was applied as a dietary complement for animal feed, with immunogenic functions. In this study, the K-COE gene (*K-COE*) of the Korean strain of PEDV with the signal peptide of rice amylase 1A (*Ramy1A*), was fused with the gene encoding the carboxyterminal half (320 amino acid residues from the C terminus) of yeast α-agglutinin, a mating associated protein that is anchored covalently to the cell wall. The glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter was selected in order to direct the expression of the fusion construct, and the resulting recombinant plasmid was then introduced into *S. cerevisiae*. The surface display of K-COE was visualized via confocal microscopy using a polyclonal antibody against K-COE as the primary antibody, and FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse IgG as the secondary antibody. The display of the K-COE on the cell surface was further verified via Western blot analysis using the cell wall fraction after the administration of α-1,3-glucanase/PNGase F/β-mannosidase treatment. © KSBB

Keywords: surface display, porcine epidemic diarrhea virus, Saccharomyces cerevisiae

INTRODUCTION

Porcine epidemic diarrhea (PED) is characterized by severe enteritis and watery diarrhea, and is often fatal, with a mortality rate of up to 90%. Consequently, PED has been implicated as one of the production limiting factors in pig nurseries [1,2]. Since the initial identification of PED in Belgium and the United Kingdom in 1978, outbreaks of the disease have been reported in Korea and Europe [2-5]. Immunity is important for both the prevention and control of PED. Moreover, maternal antibodies derived from immu-

nized sows are currently the sole source of immunity to PED in highly susceptible neonates in the first few weeks following birth [6]. The porcine epidemic diarrhea virus (PEDV), a causal agent of acute digestive track infections in 1~2 week-old suckling pigs, is a member of coronavirus group I, and is an enveloped, single-stranded RNA virus [7]. In addition, its subgenomic mRNAs, which are transcribed from the viral genome, generate viral protein products, including the spike (S, 180~220 kDa), membrane (M, 27~32 kDa), and nucleocapsid (N, 55~58 kDa) proteins [8]. Among the viral proteins, S, a glycoprotein peplomer (surface antigen) on the viral surface, performs a crucial function in the attachment of the viral particles to the receptors of host cells, with subsequent penetration into the cells via membrane fusion. The S glycoprotein has also been identi-

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fied as a stimulating factor for the production of neutralizing antibodies within the host [9]. In our previous studies, we identified the neutralizing epitope of the spike gene from the Korean strain of PEDV, and demonstrated that oral feeding with a transgenic plant elicited efficient systemic and mucosal immune responses, thereby resulting in the inhibition of the virus [10-12].

Currently, the field of vaccine development is strongly focused on the development of live oral vaccines [13,14]. *Saccharomyces* has a long history of live oral use for the treatment of disease. Also, *S. boulardii* has been used for over 40 years to treat antibiotic-associated diarrhoea, and was proven safe for treatment in humans [15]. Orally administered *S. boulardii* in mice offered protection against *Clostridium difficile*-induced disease, and inhibited the translocation of gastrointestinal *Candida albicans* [16,17]. In addition, *S. cerevisiae* has been used both live and orally to treat *C. difficile* diarrhoea occurring in humans, and was shown to protect mice against mild influenza virus infections [18,19].

The yeast-based expression system is unique among expression systems, most notably because it combines the advantages of a prokaryotic system, such as high expression levels, ease of scale-up, and inexpensive growth media, as well as the inherent advantages of a eukaryotic system, for the execution of the majority of post-translational modifications. Moreover, yeast is a GRAS (Generally Recognized as Safe) organism, with a long history of application in the production of rich biomass yields of high-quality proteins and vitamins. This allows its use in pharmaceutical, livestock feed, and food industry applications. Recently, a number of variably-sized heterologous proteins have been displayed on the surfaces of yeast cells via a genetic engineering technique referred to as yeast cell-surface display, a method which has a variety of applications in biotechnology, including bioconversion, edible vaccines, bioremediation, and bioseparation [20-26].

Taking these data together, it has been suggested that *S. cerevisiae* expressing antigenic determinants on its surface, would be a good candidate for use as a live oral vaccine carrier. Several genes encoding for antigenic determinants, including the hepatitis B virus surface antigen, have been expressed on the surfaces of yeast cells in an effort to develop new oral vaccines [22]. Thus, in this study, we constructed an engineered yeast strain, on the surface of which the antigenic determinant of the Korean strain of PEDV was immobilized.

MATERIALS AND METHODS

Chemicals and Enzymes

Unless otherwise specified, all chemicals, media, and enzymes used in this study were purchased from the Sigma Chemical Co. (St. Louis, MO, USA), Difco Laboratories (Detroit, MI, USA), and Boehringer Mannheim (Mannheim, Germany), respectively.

Strains and Culture Conditions

The plasmids were maintained and propagated in *E. coli* HB101 or DH5 in accordance with the methods of Sambrook *et al.* [27]. The pMYV52 plasmid harboring the neutralizing epitope of Korean PEDV (K-COE) was used, and *S. cerevisiae* 2805 (*MAT. pep4: HIS3 prb1-Can1 GAL2 his3 ura3-52*) was used as the recipient cell for the surface display of K-COE [11,28].

The *S. cerevisiae* culture was maintained in a YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose), and a uracil-deficient selective medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acid, 2% dextrose, and 2% agar) was employed for the screening of the transformants at 30°C. The primary inoculum was prepared from 5 mL of the uracil selective medium, cultured for 24 h, and a total of 1×10^{7} cells were inoculated into a 300-mL Erlenmeyer flask containing 40 mL of the YEPD medium. The expression cultures were grown at 30°C with continuous agitation (200 rpm), after which, the culture itself was assayed for the presence of surface-displayed K-COE.

Construction of the Plasmid and Transformation of Yeast

The amylase 1A (*Ramy1A*) signal peptide (ASP) and the K-COE gene (*K-COE*) for the neutralizing epitope, which were described in several previous studies, were fused via overlap extension PCR, creating *Bam*HI and *Xba*I restriction sites at the 5′ and 3′ ends, respectively, using the following primers: forward 5′-<u>GGATCC</u>GCATCCAGGTGCTGAAC-3′ and reverse 5′-<u>TCTAGA</u>CTTGTACAGCTCGTCCAT-3′, and overlap-forward 5′-AACTTGACAGCCGGGCTGGC-AGTCCCCGCC-3′ and overlap-reverse 5′-GGCGGGGGA-CTGCCAGCCCGGCTGTCAAGTT-3′ [29-33]. The amplified gene was cloned in pBluescript IIKS (Stratagene, La Jolla, CA, USA), analyzed via restriction enzyme digestion, and confirmed by DNA sequencing. To construct a surface-displaying yeast vector, the ASP/K-COE fusion fragment excised from the pBluescript IIKS by digestion with *Bam*HI and *Xba*I, was inserted into the pYEGPD vector, which harbors the same restriction enzyme sites between the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and the galactose-1-P uridyl transferase (*GAL7*) terminator [34,35]. To anchor the K-COE on the surface of the yeast, a DNA fragment harboring the 3′ half of the α-agglutinin gene (*AGA*1-C320), encoding a C-terminal of 320 amino acids, was prepared via PCR using the following primers: 5′-<u>TCTAGA</u>GCCAAAAGCTCTTTTATCTCAA-3′ and 5′-<u>GT-CGAC</u>TTAGAATAGCAGGTAGGACA-3′, as previously described, and then digested with *Xba*I and *Sal*I. The resultant fragment was subsequently cloned into the *Xba*I/*Sal*I site between the ASP/K-COE and *GAL7* terminator [35]. The direction of the fusion construct was verified via *Xba*I/*Sal*I digestion and DNA sequencing. The resultant plasmid was designated as pYEAGPVAG (Fig. 1).

The constructed recombinant vector was then introduced

A

B
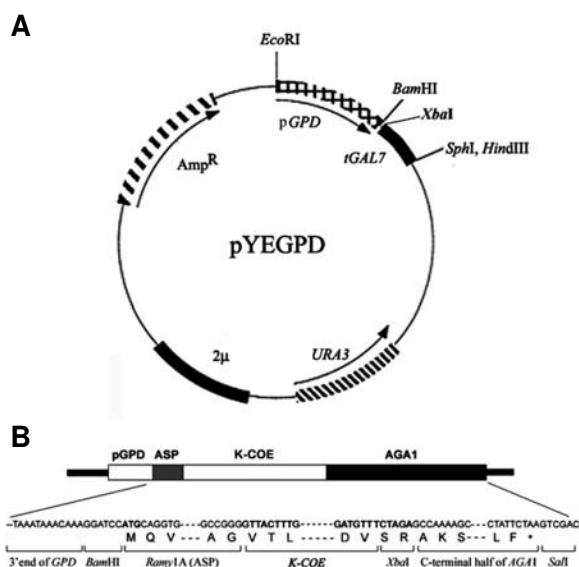
Fig. 1. (A) Schematic diagram of the pYEAGPVAG yeast expression vector. The boxes represent genes or their corresponding functional domains. (B) Schematic diagram of the fusion construct cloned in the pYEGPD plasmid and the sequence covering the links of the *GPD* promoter-Rice *Amy1A* signal peptides-*K-COE-AGA*1. The translation start codon and the first codon of K-COE are shown in bold letters. p*GPD*, promoter of glyceraldehyde-3-phosphate dehydrogenase; ASP, Rice *Amy1A* signal peptides; *K-COE*, neutralizing epitope of the spike protein from a Korean strain of PEDV; *AGA*1, C-terminal half (320 amino acid residues from the C terminus) of yeast α-agglutinin anchoring protein; t*GAL7*, terminator of galactose-1-P uridyl transferase.

into *S. cerevisiae* 2805, via the lithium acetate procedure [36]. The stability of the plasmids introduced into the yeast was measured as follows: samples grown in the non-selective YEPD medium were serially diluted with sterile H$_2$O to an expected level of 50 colony-forming units (CFUs) per plate, they were then plated on ura$^-$ selective and ura$^-$ non-selective plates, and the relative number of CFUs was determined.

Northern Blot Analysis

The transformed yeast cells were lysed using glass beads, and the total RNA was extracted via a previously described procedure [29]. The RNA was quantified via UV-spectrophotometry, and the total RNA (30 g per lane) was separated on 1% agarose gel (in 2.2 M formaldehyde). Prior to blotting, the gel was stained with ethidium bromide, thereby confirming that similar RNA quantities had been loaded for each of the samples. The RNA was then transferred onto Hybond membranes, in accordance with the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was conducted in Church buffer [7% (w/v) SDS, 1% BSA, 1 mM EDTA, 250 mM NaPO$_4$, pH

7.2] at 65°C [29]. The probe was labeled with α-[^{32}P]-dCTP, using a random labeling kit (Amersham Pharmacia Biotech).

Immunostaining and Microscopy

The cells were immunofluorescently labeled via a previously described procedure. In brief, the yeast cells, after 48 h of cultivation in 40 mL of YEPD, were washed twice with PBS/BSA solution by centrifugation at 3,000 × g for 5 min at room temperature [37]. The cells were then suspended and diluted to 10^5 cells/mL in the same solution. Anti-K-COE mouse polyclonal antibody, which was obtained in a previous study, was used as the primary antibody at a dilution rate of 1:10 [11]. One milliliter of the diluted cells and the primary antibody were then incubated over night at 4°C. After the cells were washed, the secondary antibody, FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse IgG (Sigma), diluted to a factor of 1:50, was reacted with the cells for 1 h at room temperature. After washing, the labeled cells were resuspended in 50 μL of PBS solution and observed via a C1si confocal laser scanning microscope (Nikon, USA) [37].
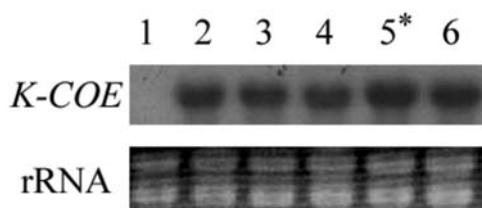
Western Blot Analysis

The preparation of cell-free extracts and the isolation of the cell wall fraction were conducted as previously described [38,39]. In order to prepare the cell-free extract, the cells were grown for three days, harvested, washed twice in extraction buffer (50 mM Tris-HCl, 2 mM EDTA), and homogenized three times in a bead beater (Biospec Products Inc., Bartlesville, OK, USA) for 1 min each, with 3 min intervals of ice-cooling. The homogenates were observed under a microscope to verify that the cells had been broken, and then centrifuged (10 min at 10,000 × g). The resultant supernatant was filtered through a cellulose filter (diameter 0.4 mm) in order to prepare the cell-free extract. The cell wall fraction recovered as precipitate after centrifugation was then washed three times in extraction buffer, and separated from the glass beads via standing. The fraction was then treated with α-1,3-glucanase in sodium acetate buffer (pH 5.2) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), in order to extract the K-COE from the cell wall [39]. A 150 mU amount of α-1,3-glucanase per gram of wet cell wall was used, and the reaction was conducted for 4 h at 37°C. PNGase F (New England Biolabs, Beverly, MA, USA) and jack bean β-mannosidase (Sigma) were used to deglycosylate the K-COE fusion construct, as described previously [40].

Sample aliquots of the cell-free extract and cell wall fraction were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently blotted onto nitrocellulose filters.

RESULTS AND DISCUSSION

Construction of the Cell Surface Expression Plasmid

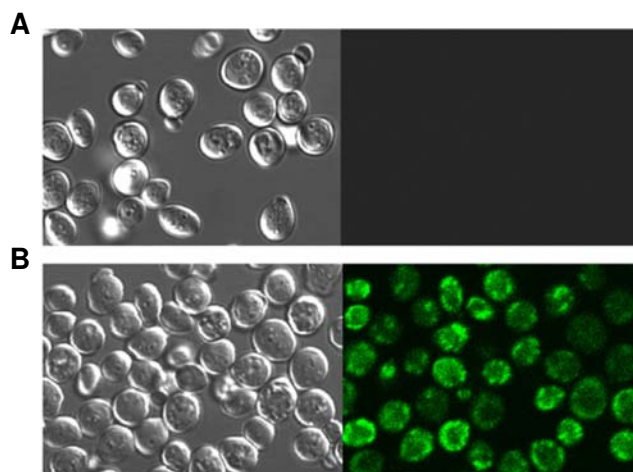The pYEAGPVAG plasmid was constructed as described

Fig. 2. Northern blot analysis of the accumulation of the *K-COE* transcript in transformed yeasts. The yeasts transformed with pYEAGPVAG were probed with labeled *K-COE*. Lane 1 contains the RNA of the recipient strain, and lanes 2~6 show those of five different transformants. The asterisk indicates the selected transformant of TYEAG-PVAG-4. Before blotting, the gel used for the blot in the upper panel was stained with ethidium bromide, to confirm that similar quantities of RNA had been loaded for each sample.


Fig. 3. Confocal laser scanning microscopy of recombinant yeast cells. Light (left) and FITC immunostained (right) micrographs are shown. No immunofluorescence was observed from the nontransformed control cells (panel A), and a clear immunofluorescence was observed from the transformed cells (panel B) with FITC-conjugated goat anti-mouse IgG. A C1si confocal system (Nikon, USA) was used for the analysis, and a 488-nm laser line of an Ar laser was used for excitation.

in the Materials and Methods (Fig. 1). The pYEGPAG plasmid was a multicopy plasmid for the expression of the K-COE/α-agglutinin fusion gene, which harbors the secretion signal sequence of amylase 1A (*Ramy1A*) under the control of the *GPD* promoter. Over 20 transformants of *S. cerevisiae* were randomly selected on ura$^-$ medium, and then used to confirm the presence of pYEAGPVAG via plasmid extraction, followed by back transformation into *E. coli*. The plasmid stability of the selected transformants was sufficiently good for more than 80% of the plated cells, harbouring the plasmids up to 72 h after cultivation in the non-selective liquid media.

The level of accumulation of the recombinant K-COE transcripts was assessed via Northern blot analysis, where the results revealed variations in the transcriptional levels of the recombinant K-COE genes among the strains transformed with the same expression construct (Fig. 2). Wide variation in the heterologous gene expression levels of *S. cerevisiae* is not unusual when using episomal 2-μm ori-based plasmids, possibly owing to variations in the plasmid copy number between different transformants [29]. Thus, the transformant showing the highest expression level, TYE-AGPVAG-4, was selected, and then employed in confirming the surface display of recombinant K-COE.

Detection of K-COE on the Cell Surface

With the cells at the exponential growth phase in the YEPD medium, the FITC fluorescence on the cell surface was visualized with a confocal microscope (Fig. 3). Immunofluorescence was observed in the transformant cells, but not in the control cells, which had been grown under identical conditions. The mouse and goat antibodies were not able to access the interior of the yeast cells under the labeling conditions employed; this indicates that the K-COE is expressed in such a manner that it can be detected in the outer layer of an intact cell by using an anti-K-COE antibody that does not cross-react with the yeast epitopes. Thus, we were

able to verify that the K-COE was actually anchored to the cell wall. Even after the administration of a heat-kill procedure via 1 h of incubation at 60°C, the immunofluorescence microscopy revealed that the cells had remained intact, and that the surface display of the K-COE had been unaffected (data not shown). However, we were not able to quantitatively calculate the number of K-COE molecules displayed on the surface of the yeast cells, because the standard amount of immunofluorescence of cells with FITC-anti-mouse IgG was not available for this study.

The immunofluorescence of the cell surfaces showed that the intensity in which the cells expressed the K-COE/α-agglutinin fusion gene varied slightly from cell to cell. This suggests differences in the expression levels among the individual cells. The number of foreign protein molecules expressed on the cell surface of yeast is known to be dependent on the copy number of the 2-μm plasmid maintained in the cell, as well as the size of the target protein. In addition, a spatial limitation is imposed by the intrinsic number of anchoring molecules, which has been estimated at 10^4~10^5 molecules per cell for the number of α-agglutinin-fused molecules on the cell surface [41].

Western Blot Analysis of Surface-displayed K-COE

The cell wall proteins were purified from the transformant cells harboring pYEAGPVAG as described in the Materials and Methods. Western blot analysis, using an antisera against K-COE, confirmed that the resultant α-1,3-glucanase and PNGase F/β-mannosidase extracts of the transformant
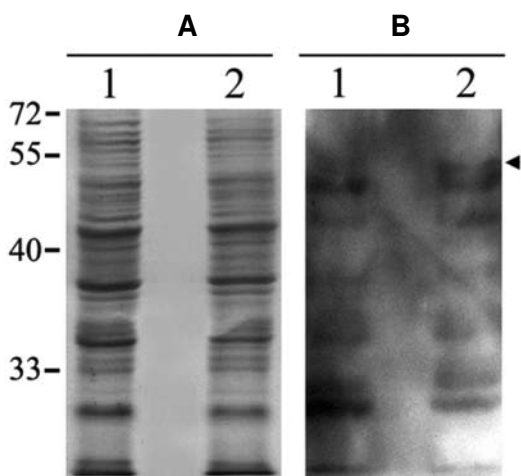
Fig. 4. Western blot analysis of the K-COE fusion construct. (A) Coommasie blue-stained SDS-PAGE of cell wall extracts treated with α-1,3-glucanase/PNGase F/β-mannosidase. (B) Antigen-antibody reaction of the corresponding gel of the cell wall extracts treated with α-1,3-glucanase/ PNGase F/β-mannosidase. Lane 1, 20 μL of the α-1,3-glucanase/PNGase F/β-mannosidase-treated cell wall extract of the recipient strain; lane 2, 20 μL of the α-1,3-glucanase/PNGase F/β-mannosidase -treated cell wall extract of the transformed strain. Numbers on the left refer to the estimated sizes in kDa, and the arrowhead at the right indicates the K-COE-AGA1-C320 fusion protein.

cells contained a band corresponding to the fusion protein at the expected size of 50 kDa (Fig. 4), but such a band was detected neither in the cell-free extract of the transformant cells, nor the corresponding cell fractions of the control cells (data not shown), indicating the K-COE was displayed on the cell surfaces of the yeast cells.

Yeast-based systems are known to be successful in terms of displaying large protein molecules, and not only of single-subunit proteins, but also of hetero-oligomeric multisubunits. Antigens expressed on the cell surface carry the advantage of easy access to antibodies, and should, therefore, be detectable by the immune system; even very small peptides, as well as vanishingly small quantities of peptides, can be immunogenic when presented on a larger surface [22,23]. Because *Saccharomyces* is such an excellent eukaryotic expression system, is cheap to produce, and is safe for oral use, it appears to be a superior candidate for the development of a live oral vaccine against mucosal diseases, in this case Korean PED.

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