Surface-Displayed Expression of a Neutralizing Epitope of ApxIIA Exotoxin in *Saccharomyces cerevisiae* and Oral Administration of It for Protective Immune Responses against Challenge by *Actinobacillus pleuropneumoniae*

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A neutralizing epitope fragment of ApxIIA toxin (ApxIIA#5) of the Korean *Actinobacillus pleuropneumoniae* serotype 2 strain was expressed and immobilized on the cell surface of *Saccharomyces cerevisiae* for efficient vaccine development. Expression of ApxIIA#5 was confirmed by Western blot analysis using cell-wall proteins, and the surface display of ApxIIA#5 was further visualized under confocal microscopy. Quantitative ELISA revealed that the recombinant ApxIIA#5 directed to the cell surface consisted of approximately 16% cell-wall proteins, estimated to be 35 mg of ApxIIA#5 protein per liter of cultured cells. An immunoblot assay revealed that antigen-specific antibodies against ApxIIA#5 were present in the sera of mice fed recombinant ApxIIA#5-expressing yeast, but not in mice fed the wild-type nor the vector-only transformant. Moreover, the mice fed the recombinant epitope-expressing yeast were protected from injection of a lethal dose of *A. pleuropneumoniae*.

Key words: surface display; APX toxin; *Actinobacillus pleuropneumoniae*; *Saccharomyces cerevisiae*; oral vaccination

*Actinobacillus pleuropneumoniae*, the causal agent of porcine pleuropneumonia, results in severe and fatal hemorrhagic necrotizing pneumonia and fibrinous pleuritis in pigs, and it is currently spreading throughout the pig industry. Gram-negative *A. pleuropneumoniae* has been classified as having two biotypes, based on the requirement for nicotinamide adenine dinucleotide, and 15 serotypes, based on surface polysaccharide antigens.1,2 Although multiple factors such as capsular polysaccharides, outer membrane proteins, Apx exotoxins, lipopolysaccharides, permeability factors, and iron-regulated proteins are believed to be involved in the virulence of *A. pleuropneumoniae*,2,3,5,10 Apx toxins are thought to be of particular importance in the induction of protective immunity, as previously demonstrated using several mutants.3,5–7)

Apx toxins, which belong to a family of repeats in ToXin (RTX) toxins secreted by several Gram-negative bacteria, are capable of creating pores in the host cell membrane. Genes for active RTX toxins comprise rtxC, A, B, and D,8 which encodes the post-translational activator, the structural toxin, and proteins required for secretion of the activated toxin respectively.2,3,9 RtxB and RtxD are required for the secretion of a functional toxin complex composed of RtxA and RtxC. The structural toxin RtxA is initially synthesized in an inactive form and is then processed to become a functional toxin through acylation and complex formation with RtxC. The ApxIIA toxin is produced in a similar way, except that it is secreted by ApxIB and ApxID. To synthesize active Apx toxins, the activity of four genes, apxC, apxA, apxB, and apxD, is required.2,3,9 Most *A. pleuropneumoniae* serotypes produce one or two exotoxins among the Apx, II, and III proteins. Both ApxI and ApxII of *A. pleuropneumoniae* are essential for full virulence and the development of clinical signs and typical lung lesions.2,3,5,10,11 Among the Apx toxins, ApxII is expressed in all but serotype 10, while the other two major toxins, ApxI and ApxIII, are expressed in fewer serotypes. In Korea, more than half of all *A. pleuropneumoniae* isolates obtained from infected pigs have been classified as serotype 2 and have been found to secrete ApxII and ApxIII.8 Therefore, a vaccination strategy against ApxII might be an effective approach to reduce porcine pleuropneumonia caused by a broad range of serotypes of *A. pleuropneumoniae*.

*A. pleuropneumoniae* is a good model system for the development of a vaccination strategy leading to a mucosal immune response for the following reasons: First, it gains access to its host through the mucosal surfaces of the respiratory tract. Secondly, we have reported that oral administration of *Saccharomyces cerevisiae* expressing full-length ApxIIA and a transgenic plant expressing the full-length ApxIIA toxin of the Korean strain are capable of inducing an antigen-specific immune response, and have suggested that orally administered ApxIIA can induce an effective immune response against *A. pleuropneumoniae* infection.12–15 However, improved strategies for efficient
antigen presentation and immune responses are still needed for enhanced protection against pathogen infection.

Recently, we characterized the antigenic determinants within ApxIIA required to induce a protective immune response against this bacterial infection (Seo et al., manuscript in preparation). The most effective neutralizing epitope of ApxIIA (ApxIIA#5), rather than the full-length protein, was considered as a vaccine candidate for *A. pleuropneumoniae* infection, since this smaller fragment is easy to handle and can be produced in large quantities by a heterologous expression system.

As an antigen delivery system, cell-surface display in *S. cerevisiae* is desirable in vaccine development due to the stable maintenance of surface-displayed epitopes because of a rigid, thick cell wall architecture and a high-density protein display (approximately 5 × 10⁶ molecules/cell). Several genes of antigenic determinants, including hepatitis B virus surface antigen, have been expressed on the yeast cell surface in the development of possible oral vaccines. Taken together, these data suggest that *S. cerevisiae* expressing antigenic determinants on its surface is a good candidate for a live oral vaccine carrier.

In this study, we expressed the neutralizing epitope of the ApxIIA toxin from the Korean strain in *S. cerevisiae*, directed the recombinant ApxIIA#5 onto the cell surface for efficient vaccine delivery against *A. pleuropneumoniae* infection, confirmed antigen specific systemic immune responses by oral administration of ApxIIA#5 expressing *S. cerevisiae*, and then demonstrated protection against the challenge using *A. pleuropneumoniae*.

### Materials and Methods

**Chemicals and enzymes.** Unless otherwise specified, all the chemicals, media, and enzymes used in this study were purchased from Sigma Chemical (St. Louis, MO), Difco Laboratories (Detroit, MI), and Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were purchased from Genotech (Daejeon, Korea).

**Strains and culture conditions.** The plasmids were maintained and propagated in *E. coli* Top10 or DH5α following Sambrook et al. The pApxIIA5 plasmid harboring the neutralizing epitope of ApxIIA (ApxIIA#5) corresponding to aa residues 439-801 of Korean *A. pleuropneumoniae* was used, and *S. cerevisiae* 2805 (MATα pep4::HIS3 prb1-α can1 GAL2 his3 ura3-52) was used as the recipient cell for surface display of ApxIIA5. *S. cerevisiae* was maintained in 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 acids, 2% dextrose, and 2% agar) was used to screen the transformants without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acids, 2% dextrose, and 2% agar. The expression cultures were grown at 30°C with agitation (200 rpm), after which cells were harvested and neutralizing epitope, as described previously, were fused by RicAmy1A (Rice Amylase1A) signal peptide (ASP) and the ApxIIA#5 gene encoding ApxIIA#5, as described previously, were fused by RicAmy1A (Rice Amylase1A) signal peptide (ASP) and the ApxIIA#5 gene encoding ApxIIA#5, as described previously.

**Construction of the vector and transformation of yeast.** The amylase 1A (RicAmy1A) signal peptide (ASP) and the ApxIIA#5 gene encoding aa residues 439-801 (GenBank accession no. AF363362) for the neutralizing epitope, as described previously, were fused by overlap extension PCR to create BamHI and EcoRI restriction sites at the 5’ and 3’ ends respectively, using primers forward 5’-GGATCCGATGCAGTTGC-3’ and reverse 5’-GGGAAATTCTG-GAATTC-3’ and overlap-forward 5’-TCTAACC-GACAGCCCGGCAAGGTTATGATTCTCTCGGT-3’ and overlap-reverse 5’-ACGGAATCTATAACCTTGCCCGGCTGTCAAGTTAGA-3’.

The amplification was cloned in pGEM-T Easy Vector (Promega, Madison, WI), analyzed by restriction enzyme digestion, and confirmed by DNA sequencing. To anchor ApxIIA#5 to the yeast surface, an anchor DNA fragment containing the 3’ half of the α-agglutinin gene (AGA1-C320) encoding the C-terminal 320 amino acids was prepared by PCR using primers 5’-GGGAAATTCTGCC-GAAGACTCCTTTTATC-3’ and 5’-GGTGCAGATTAGAATGCG-TTGAACA-3’, as described previously. The resulting amplified PCR fragment of AGA1-C320 was cloned in EcoRI/Sall of pBluescript II SK (Stratagene, La Jolla, CA), analyzed by restriction enzyme digestion, and confirmed by DNA sequencing. To construct a surface-displaying yeast vector, the ASP::ApxIIA#5 fusion fragment and the anchoring partner of AGA1-C320 were excised from the cloning vectors by digestion with BamHI/EcoRI and EcoRI/Sall respectively, and then ligated simultaneously to the BamHI/Sall sites in pYECPD vector having the same restriction enzyme sites between the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and the galactose-1-P uridyl transferase (GAL7) terminator. The direction of the recombinant yeast vector was confirmed by restriction enzyme digestion and DNA sequencing. The resulting plasmid was denoted pYECA5 (Fig. 1).

The constructed recombinant vector, pYECA5, was introduced into *S. cerevisiae* 2805 by the lithium acetate procedure. The stability of the introduced plasmids in the yeast was measured by counting colony-forming units (CFUs) on ura⁻ selective and non-selective plates, as described previously.

**Northern blot analysis.** Total RNA was extracted by a procedure described previously. The amount of RNA was measured by UV-spectrophotometry, and total RNA (30 μg/lane) was separated on a 1.2% formaldehyde-agarose gel. Before blotting, the gel was stained with ethidium bromide to confirm that similar amounts of RNA were loaded on each lane.
had been loaded for the samples. The RNA was transferred to a Hybond membrane as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was performed in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 250 mM NaPO₄, pH 7.2) at 65°C. The probe was labeled with α-[³²P]-dCTP using a random labeling kit (Amersham).

**Immunostaining and microscopy.** Immunofluorescent labeling of cells was carried out as previously described.²⁻⁷ Cultivated yeast cells (48 h) were washed twice with PBS/BSA solution after centrifugation at 3,000 x g for 5 min at room temperature. They were suspended in the same solution, and 100 µl of cell suspension (10⁶ cells/ml) was placed on a glass slide and air-dried for immuno-
lected and observed by confocal laser scanning microscopy (Carl Zeiss, Zena, Germany).²⁻⁷

Western blot analysis. Preparation of the cell-wall fraction (CWF) was performed as described by Pitarch et al.,²⁹ with some modifications. In brief, the cells were grown for 3 d, harvested, washed twice with lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride), and homogenized 3 times in a beaker ( Biospec Products, Bartsville, OK) for 1 min, with 3-min cooling intervals on ice.²⁷ The homogenate was observed by microscopy to verify that the cells were broken, and then centrifuged (10,000 x g, 10 min). The resulting supernatant was filtered through a cellulose filter, (0.4 µm) to prepare cell-free extract (CFE). The CWF, recovered as a precipitate after centrifugation, was washed 3 times with ice-cold water and rinsed 3 more times with each of the following ice-cold solutions: 5% NaCl, 2% NaCl, 1% NaCl, and water. Isolated cell walls were extracted by boiling with extraction buffer (50 mM Tris–HCl, pH 8.0, 2% SDS, 10 mM DTT, 0.1 mM EDTA) for 10 min. The concentration of isolated cell-wall proteins (CWP) was determined by Bradford assay (Bio-Rad, Hercules, CA) and the CWP was separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE), followed by blotting onto a nitrocellulose filter. After blocking, the filter was incubated with anti-ApxIIA#5 antiserum, followed by binding to an anti-mouse IgG conjugated to alkaline phosphotase as secondary antibody (Sigma). Color was developed using BCIP/NBT (USB, Cleveland, OH) in TMN buffer (100 mM Tris, pH 9.5, 5 mM MgCl₂, and 100 mM NaCl), by methods described previously.¹²,¹³

**ELISA quantification of the recombinant ApxIIA#5 protein.** The quantity of ApxIIA#5 expressed in CWP was determined by quantitative ELISA, as described previously.¹³ In brief, CWP at a 10-fold dilution were utilized to coat a 96-well microtitration plate at a concentration of 100 µl well in bicarbonate buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃) and incubated overnight at 4°C. The wells were washed 3 times in PBS containing 0.05% Tween-20 (PBST), blocked for 5 h at 25°C with the addition of 300 µl 1% BSA in PBS, and washed again 3 times in PBST. The plates were incubated with a 1/5,000 dilution of anti-ApxIIA antiserum, as in our previous study,³¹ and in 0.01% PBS containing 0.5% BSA for 2 h at 25°C, and then washed 3 times with PBST. The plates were then incubated with a 1/1,000 dilution of anti-mouse IgG conjugated with horseradish peroxidase (Promega) in 0.01% PBS containing 0.1% BSA, and then washed 3 times with PBST buffer. The plate was developed with the addition of 100 µl TMB substrate kit for peroxidase (PharMingen, San Jose, CA) at room temperature in darkness for 30 min. The plate was read at 405 nm in an ELISA reader (MRA-006; Packard Instruments, Meriden, CT) and quantified by comparison with a known quantity of purified bacterial ApxIIA#5. Purified bacterial ApxIIA#5, obtained from our previous study,¹³ was serially diluted with a concentration range of 8–30 ng.

**Experimental animals, immunization, and sample collection.** Oral immunization and sample collections were conducted as described previously.¹³,¹⁴ In brief, 5-week-old BALB/c female mice (Breeding and Research Center, Daejeon, Korea) were used throughout the study following the Policy and Regulations for the Care and Use of Laboratory Animals of the Laboratory Animal Center (Jeonju, Korea). All animals were provided with standard mouse chow and water ad libitum.

Oral immunization was preceded by overnight fasting of the mice (water was provided ad libitum). Freshly harvested 2.5 x 10⁷ or 2.5 x 10⁸ cells were dissolved into 1 ml of 0.9% saline and orally administered at 200 µl/mouse through an oral gavage at 10-d intervals, 4 times. Serum samples were collected 3 d after each immunization.

**Measurement of ApxIIA#5 specific antibody immune responses.** The level of antigen-specific antibody in the mouse serum samples was determined using ELISA after the plate was coated with ApxIIA#5.¹⁴ Each well in the ELISA plate was coated with 50 µl of 10 µg/ml of recombinant ApxIIA#5 in bicarbonate buffer by incubation overnight at 4°C and then blocked with 10% fetal bovine serum (PBS) (HyClone, Logan, UT) in PBST for 2 h at 37°C. After washing of the wells with PBS, 50 µl of the individual samples, which had been serially diluted in PBS, was added to each well and this was incubated overnight at 4°C. Goat antibodies were specific to mouse IgG (Promega) used as a secondary antibody to determine the total amount of antigen-specific antibody, and the substrate was added to develop color. The plate was read at 405 nm in an ELISA reader. In order to quantify the level of antigen-specific antibody with specific isotypes, control IgG was used as standard, as described previously.¹³

**Challenge assay.** Control and immunized mice were challenged with A. pleuropneumoniae serotype 2 prepared as described previously.¹³,¹⁴ The immunized mice were injected intraperitoneally with 200 µl of an A. pleuropneumoniae preparation (about 1 x 10⁸ CFU) or buffer (control) after 10 d of final immunization. Five mice in each experimental group were challenged, and each mouse was then monitored every 6 h for 3 d. The number of mice alive at 3 d after challenge was recorded. These mice were considered to have been protected from the challenge.⁵,²⁹

**Results**

**Transformation of S. cerevisiae using the cell-surface expression plasmid.** For surface expression of ApxIIA#5, plasmid pYEGAXA5 was constructed as described in “Materials and Methods” (Fig. 1). This plasmid was a multi-copy episomal plasmid for expression of the ApxIIA#5::AGA1-C320 fusion gene containing the secretion signal sequence of amylase 1A (RamyA1) under the control of the GPD promoter. After transformation of S. cerevisiae using plasmid pYEGAXA5, more than 20 transformants were randomly selected on ura medium, and then used to confirm the presence of pYEGAXA5 by plasmid extraction, followed by back transformation into Escherichia coli. Southern blot analysis using undigested genomic DNA from the transformants yielded hybridizing signals only in the recombinant vector-sized-DNA region, not in the chromosomal-sized DNA region, suggesting no integration of the vector DNA into the host chromosomal DNA (data not shown). The plasmid stability of the selected transformants was good, and more than 83% of the plated cells harbored plasmids after 72 h of cultivation in nonselective liquid media. The growth rate of the selected transformants was similar to that of the control strain, and appeared to be similar to those of other previous studies.²⁰,²⁷ In addition, no morphological abnormalities were observed in the selected transform-
intrinsically high enough for a good immune response.

**Western blot analysis of the surface-displayed ApxIIA-epitope**

Cells at the early stationary growth phase in a rich medium (YEPD) were collected by centrifugation, followed by isolation of the CWF and subsequent extraction of CWPs. Western blot analysis using antisera against ApxIIA#5 showed that the CWPs of cells harboring pYEGAXA5 had a band corresponding to the fusion protein with the expected size, of 73 kDa, but no band was found in the cell-free extract of the transformant cells or the corresponding cell fractions of the control cells (data not shown). The antigen-antibody reaction to CWPs, but not to a soluble cytosolic fraction, confirmed anchored expression of ApxIIA#5, and was in good agreement with results of microscopy. Compared to other proteins displayed on the yeast cell surface using the same expression cassette,

**ELISA quantification of the recombinant ApxIIA-epitope protein**

Quantitative ELISA was conducted to estimate the amount of recombinant ApxIIA#5 protein. The quantity of yeast-derived recombinant ApxIIA#5 was estimated by comparing relative light units (RLUs) from a known quantity of the bacterial ApxIIA#5 protein-antibody complex with that emitted by a known amount of the yeast CWP. In addition, values for the recombinant ApxIIA#5 were normalized by deducting the values for the control cells due to the possible cross-reactivity of the antibody. When the recombinant protein levels were plotted against dilutions of the CWP, a correlative increase in ApxIIA#5 protein levels was observed within the optimal concentration range of the total protein (30–250 μg). However, when the concentration of CWPs deviated from this range, the quantity of detected ApxIIA#5 protein did not correlate with the increase in concentration of total protein. This can be ascribed to the binding characteristics of the ApxIIA#5 protein in a mixture of CWPs on the microtiter plates. In addition, compared to previous studies, the optimal concentration range for ELISA was quite narrow. This might be explained as steric hindrance of the antigen-antibody interaction due to the presence of the C-terminal region of the AGA1 protein in the fusion construct. Quantitative ELISA revealed that the surface-displayed ApxIIA#5 comprised 16% CWPs, or an estimated 35 mg/l of cultured cells, which is comparable to other protein products in previous studies. No rapid decline in the protein products was observed after the heat-kill procedure, indicating again that the surface-displayed ApxIIA#5 appeared to be well-maintained and did not affect the cell-wall architecture.
In order to evaluate the efficacy of oral administration of the ApxIIA#5 expressing recombinant yeast in inducing immune responses, the levels of ApxIIA#5-specific antibodies were measured in the sera of the mice fed the recombinant yeast. As shown in Fig. 3, ELISA revealed that the level of ApxIIA#5-specific antibodies present in the sera of those mice was higher than that obtained from mice fed vector-only transformants. These results indicate that a partial fragment of ApxIIA corresponding to aa 439–801 was effective for induction of the immune response in mice after oral administration. In addition, the antigen-specific antibodies time-dependently increased under oral immunization with the recombinant yeast. For example, after 40 d of feeding, antigen-specific antibody levels were obviously higher in the sera fed the recombinant yeast than those at 30 d of feeding. Dose-dependent immunization, although further treatments with various concentrations of yeast cells must be tested, was also observed based on the fact that 5 × 10⁷ cells/feeding resulted in an earlier, stronger immune response as compared to that for 5 × 10⁶ cells/feeding.

**Induction of protective immune responses against A. pleuropneumoniae challenge**

Because oral administration of the recombinant yeast induced systemic immune responses, the survival of the immunized mice after *A. pleuropneumoniae* challenge was recorded. As shown in Fig. 4, the survival rates of the vaccinated groups were higher than those of the control groups. All the mice in the control groups died 36 h after challenge while the survival rates were 60% and 40% in the 5 × 10⁷ and 5 × 10⁶ cell oral-vaccinated groups at the same time point, respectively. The final survival rates of the 5 × 10⁷ and 5 × 10⁶ cell oral-vaccinated groups were 40% and 20% respectively 72 h after challenge, while none of the mice in the control groups survived. These results indicate that the surface-displayed ApxIIA#5 antigen can induce *A. pleuropneumoniae*-specific antibodies in mice, resulting in protection against infection by *A. pleuropneumoniae*. In addition, survival in these mice appeared to be dose-dependent, in that only a 20% survival rate in the 5 × 10⁷ cell oral-vaccinated group was observed 72 h after challenge, which is consistent with the results of ELISA, measuring the serum IgG specific to the ApxIIA#5 antigen.

**Discussion**

Currently, vaccine development is strongly focused on the development of live oral vaccines due to their convenience and effectiveness. In the case of a toxin-based vaccine strategy, oral administration is safer than injection because it avoids the risk of intramuscular injection of toxins and takes advantage of the specialized protective and detoxifying properties of the digestive system. *Saccharomyces* is a unique eukaryotic expression system for the development of edible vaccines, because it is safe in oral use and can be run cheaply. Moreover, derivatives of *S. cerevisiae* have adjuvant properties enhancing the immune response. Our previous studies using *S. cerevisiae* as an expression host for ApxIIA indicate that the recombinant yeast expressing full-length ApxIIA can induce an immune response in mice by injection. Furthermore, we have also found that the recombinant yeast can induce both local and systemic immune responses through a common mucosal immune system after oral administration. However, further studies on efficient antigen expression and delivery are required to develop an efficient edible vaccine.

Antigens expressed on the cell surface have the advantage of easy access to antibodies and should therefore be easily detected by the immune system; low
amounts of very small peptides can be immunogenic when presented on a large surface. 18) Saccharomyces is an even better anchoring system because it has a rigid cell-wall architecture maintaining the structure of the displayed antigen as well as high-density protein display. Recently, we identified the internal region of ApxIIA corresponding to aa 439–801 of Korean A. pleuropneumoniae as a neutralizing epitope (Seo et al., manuscript in preparation), which comprises only 38% of the full-length ApxIIA, consisting of 956 aa residues. Therefore, together with the above advantages, surface display of ApxIIA#5 is relevant to develop a live oral subunit vaccine against Korean porcine pleuropneumonia.

In summary, a neutralizing epitope of ApxIIA exotoxin of Korean A. pleuropneumoniae was successfully displayed on the cell surface of *S. cerevisiae*, as confirmed by microscopic observation and Western blot analysis. In addition, the amount of surface-displayed ApxIIA#5 was comparable to other protein products in previous studies. Moreover, immune response and protection assay indicated that the surface-displayed epitope is effective in inducing an immune response after oral administration and that this small epitope itself is sufficient to protect against pathogen infection. Thus this study, which demonstrated anchoring of the recently-identified neutralizing epitope on the surface of *S. cerevisiae* and protection against pathogen infection through an immune response after oral administration, perhaps furthers the development of a live oral vaccine against Korean porcine pleuropneumonia.

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