Expression of the Apx Toxins of *Actinobacillus pleuropneumoniae* in *Saccharomyces cerevisiae* and Its Induction of Immune Response in Mice

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Abstract *Actinobacillus pleuropneumoniae* is an important pig pathogen, which is responsible for swine pleuropneumonia, a highly contagious respiratory infection. To develop subunit vaccines for *A. pleuropneumoniae* infection, the Apx toxin genes, *apxI* and *apxII*, which are thought to be important for protective immunity, were expressed in *Saccharomyces cerevisiae*, and the induction of immune responses in mice was examined. The *apxI* and *apxII* genes were placed under the control of a yeast hybrid *ADH2-GPD* promoter (AG), consisting of alcohol dehydrogenase II (*ADH2*) and the *GPD* promoter. Western blot analysis confirmed that both toxins were successfully expressed in the yeast. The ApxIA and ApxIIA-specific IgG antibody response assays showed dose dependent increases in the antigen-specific IgG antibody titers. The challenge test revealed that ninety percent of the mice immunized with ApxIIA or a mixture of ApxIA and ApxIIA, and sixty percent of mice immunized with ApxIA survived, while none of those in the control groups survived longer than 36 h. These results suggest that vaccination of the yeast expressing the ApxI and ApxII antigens is effective for the induction of protective immune responses against *A. pleuropneumoniae* infections in mice.

Keywords: Actinobacillus pleuropneumoniae, Saccharomyces cerevisiae, ApxIA, ApxIIA, vaccines

INTRODUCTION

Actinobacillus pleuropneumoniae (APP), an etiological agent of infection, causes severe and fatal fibrinous hemorrhagic necrotizing pneumonia, especially in growing pigs [1]. Virulence factors described for APP include: capsular polysaccharides, outer membrane proteins, Apx toxins, lipopolysaccharides, permeability factors and iron-regulated proteins [2-4]. Of these, Apx toxins are thought to be of particular importance for the induction of protective immunity, as previously demonstrated with several different mutants [5-9]. To date, 15 serotypes, which secrete different combinations of four cytotoxins, ApxI, ApxII, ApxIII and ApxIV, have been reported [10-12], and the serovars producing ApxI and ApxII are known to be the most virulent [3,7,8]. For the synthesis of active toxins, the activity of four genes, *apxC*, *apxA*, *apxB*, and

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apxD, which encode the posttranslational activator, structural toxin and two proteins required for the secretion of activated toxin, respectively, are required [3,9,10].

The primary requirement for the control of APP infection is vaccination, which works on pigs in a potent, safe and cost effective manner. The current APP vaccines may reduce losses caused by disease, but none provide absolute protection or prevent development of the carrier state of an APP infection [11-13]. Considering the other successful reports on the use of yeast expressed antigens as oral vaccines [14,15], to develop a new subunit vaccine against APP infection, the yeast, *Saccharomyces cerevisiae*, was employed as an oral delivery system, in which we attempted to express the APP antigens. The yeast based expression system is unique compared to other expression systems, as it combines the advantages of both a prokaryotic system, such as high expression levels, easy scale-up and inexpensive growth media, and a eukaryotic system in carrying out most of the post-translational modification. Moreover, yeast is a GRAS (Generally Recognized as Safe) organism, with a long history of applications

[16-18]. Consequently, it has been used as a tracer for the oral application of vaccines and drugs because it is relatively stable, nonpathogenic and noninvasive in the gut compared to other biodegradable vehicles [19,20].

In this study, we expressed the *apx*IA and *apx*IIA toxin genes in *S. cerevisiae*, and examined the immunogenicity of the yeast-derived Apx toxins by injecting mice, before their application as an oral vaccine candidate, to protect against an APP infection.

MATERIALS AND METHODS

Strains, Vectors and Culture Conditions

A. pleuropneumoniae serotype 5, isolated from the lungs of Korean pigs with pleuropneumonia, was used to clone the *apx*IA gene, as previously described [21]. *E. coli* Top10 and M15, and *S.cerevisiae* 2805 (*MAT*α *pep*4:: *HIS*3 *prb*1-δ *Can*1 *GAL*2 *his*3 *ura*3-52), were used as hosts for the transformation and expression of the ApxIA and ApxIIA. The TOPO, pBluescript IIKS(+), for *E. coli*, and YEpAG, containing a yeast hybrid (AG) promoter [22], for *S. cerevisiae*, were used as vectors for cloning and expression.

The *S. cerevisiae* was maintained in YEPD medium (yeast extract, 10 g/L; peptone, 20 g/L; dextrose, 20 g/L), with a uracil deficient selective medium (yeast nitrogen base without amino acids, 6.7 g/L; adenine and tryptophan, 0.03 g/L; casamino acid, 5 g/L; dextrose, 20 g/L; agar, 20 g/L) used to screen the transformants at 30°C. To produce the ApxIA and ApxIIA in *S. cerevisiae*, the primary culture was prepared by inoculating a well-grown single colony on ura⁻ plate into a test tube, containing either ura⁻ or YEPD broth, and incubating for 12 h at 30°C, while agitating vigorously (300 rpm). A total of 2×10^8 cells from the primary culture were then transferred to a 300-mL Erlenmeyer flask containing 40 mL of an appropriate expression medium. Since the upstream activating sequence (UAS) of the yeast hybrid (AG) promoter is known to cause repression as well as depression in the presence of excess glucose and ethanol, respectively, and 1.5% (v/v) ethanol supplementation gave the best results for the production of foreign protein in our previous study [22], YEPD-EtOH (YEPD containing 1.5% ethanol) medium was used as an expression medium for the AG promoter. Expression cultures, grown at 30°C, were then harvested, and the cellular proteins extracted by vortexing with an extraction buffer (50 mM Tris-HCl, 10% glycerol, 10 mM EDTA) and glass beads. The extracted protein was collected by centrifugation at 7,000 rpm for 5 min at 4°C, and analyzed by Western blot using a mono-specific polyclonal antibody.

Cloning of *A. pleuropneumoniae apx*IA and *apx*IIA Gene

The *apx*IA and *apx*IIA genes were amplified by PCR, using primers designed based on the sequence from GeneBank (Accession no. D16582 and M30602, respec-

tively), and cloned into the TOPO cloning vector kit (Invitrogen). The primers used for the *apx*IA and *apx*IIA gene amplifications were forward 5'-GGATCCATGGCT AACTCTCAGCTCGAT-3' and reverse 5'-GGATCCTTAA GCAGATTGTGTTAAATA-3', and forward 5'-GGATCC ATGTCAAAAATCACTTTGTCA-3' and reverse 5'-GGA TCCTTAAGCGGCTCTAGCTAATTG-3', respectively. The PCR included 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, polymerization at 72°C for 3 min and final polymerization at 72°C for 7 min. The cloned genes were analyzed using the restriction enzymes, *Eco*RI, *Hind*III and *Kpn*I (Gibco/BRL), and the correct clones sequenced using an automatic sequencer (ABI PRISM 377XL).

To perform cloning into the *S. cerevisiae* using the YEpAG vector, appropriate enzyme sites were generated by subcloning the *apx*IA and *apx*IIA genes into the pBluescript IIKS vector. Briefly, the 5 and 3 ends of the *apx*IA and *apx*IIA genes were blunted with a Klenow fragment (Gibco/BRL) and cloned with *Eco*RV-digested pBluescript IIKS. The orientation of the inserted fragment was confirmed by digestion with restriction endonuclease. Subsequently, the *apx*IA and *apx*IIA genes were excised from the pBluescript IIKS through digestion with *Bam*HI and *Sal*I, and ligated with the yeast expression vector, YEpAG, which had been digested with same restriction enzymes. After ligation, the yeast expression vectors were transformed into the *S. cerevisiae* 2805, using the LiAc method [23].

Western Blot

Proteins expressed in the *E. coli* or yeast *S. cerevisiae* 2805 were analyzed by Western blot [24,25], using the mono-specific polyclonal antibodies produced from mice injected with *E. coli*-expressing either the ApxIA or ApxIIA. For SDS-PAGE, the total proteins (10 μg) from the *S. cerevisiae* 2805 harboring vector, with the *apx*IA and *apx*IIA genes, or the vector only, were treated with the sample buffer (60 mM Tris-HCl, pH 6.8, 25% Glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) and subjected to electrophoresis in a 10% polyacrylamide gel, at 20 mA, for 2 h. For immunological analysis of the ApxIA and ApxIIA proteins expressed in *S. cerevisiae*, the proteins separated by SDS-PAGE, as described above, were electrophoretically transferred on to nitrocellulose membranes (Bio-Rad), which were then incubated in 5% skim milk (Sigma Co.) in Tris buffered saline (TBS, pH 7.5) for 2 h at 37°C. After washing three times with TBS, the membranes were incubated at room temperature with 1:500 diluted mono-specific mouse anti ApxIA or ApxIIA antiserum for 2 h. After the immunoreaction, the membranes were washed again, as described above, and then reacted with 1:7,000 diluted alkaline phosphate conjugated goat anti-mouse IgG antibody (Sigma Co.). After removal of the unreacted antibodies, by washing the sample with TBS, the immunoreactive bands were visualized, in the dark, with an enhanced AP conjugate substrate kit (Bio-Rad).

Experimental Animals, Immunization and Sample Collections

Five-week-old BALB/c female mice (Breeding and Research Center, Seoul National University, Korea) were used throughout this study, in accordance with the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University, Korea). All animals were provided with standard mouse chow and water ad libitum.

Immunization of the mice with the yeast-derived antigens was performed by injection. Briefly, five mice per group were subcutaneously injected with 1 mg of protein extract prepared from *S. cerevisiae*, following emulsification with complete Freund's adjuvant (Sigma). This was followed 10 days later by a booster immunization with the same amount of antigens after their emulsification with Incomplete Freund's adjuvant (Sigma). The final immunization was performed in the same way 10 days after the booster immunization. Blood for the collection of serum was drawn 5 days after the final booster immunization. Finally, the challenge test and IgG antibody response assays were conducted to confirm the immunogenicity of the yeast-derived antigens.

Measurement of ApxIA and ApxIIA-specific Antibody Immune Responses

The level of antigen-specific antibodies (IgG) in the serum samples was determined using an enzyme-linked immunosorbent assay (ELISA). One hundred µg of the recombinant ApxIA or ApxIIA, suspended in 100 µg of coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.1 mM NaN_3, pH 9.6), was added to a microplate for the ELISA (Greiner, Alphen aan den Rijn, the Netherlands), and incubated overnight at 4°C. The plate was washed three times with PBST (0.05% Tween 20 in PBS) and blocked with PBST containing 1% bovine serum albumin (BSA) for 1 h at 37°C. For the first antibody, the sera collected from immunized mice and 5 mg of the total protein from each homogenized sample, described above, were used in the IgG analysis. A 1:10 diluted primary antibody was then added to the plate, which was incubated for 1 h at 37°C. After washing with PBST, 100 µg of goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad) was added to the plate, and incubated for 1 h at 37°C. The color was developed by the addition of 100 µL of ABTS substrate solution (Bio-Rad) to the plate. After incubation for 20 min at room temperature, the OD value was measured at 405 nm, using an ELISA reader (Molecular Device, Sunnyvale, CA, USA).

Challenge Test

Five mice per each experimental group were challenged with a subcutaneous injection of a minimal lethal dose (MLD, 4.5×10^7 CFU/mL) of an *A. pleuropneumoniae* serotype 2 field isolate after 10 days of the final immunization. Each mouse was then monitored every 6 h for 72 h.
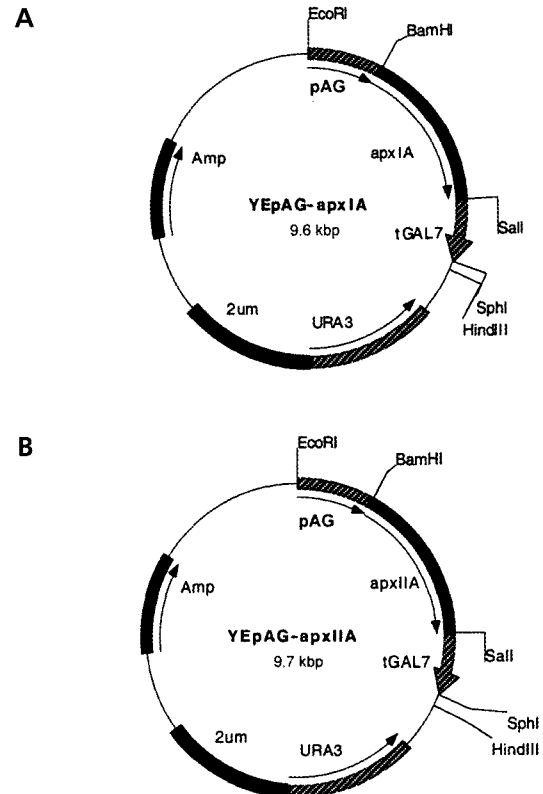
Fig. 1. Schematic diagrams of YEpAG-ApxIA (A) and YEpAG-ApxIIA (B) for expression of the *apx*IA and *apx*IIA genes in *Saccharomyces cerevisiae*.

Statistical Analysis

Statistical analyses of the results were performed using Excel and the GraphPad Prism software package version 2.0 (North Andover, MA, USA). All results were expressed as the mean ± standard deviation (SD). Differences between the groups were analyzed with the Student's t-test and were considered significant when probability values of $p<0.05$ were obtained.

RESULTS AND DISCUSSION

Cloning and Expression of *A. pleuropneumoniae apx*IA Gene and *apx*IIA Gene in *S. cerevisiae*

In the first step of developing a new subunit vaccine using a yeast expression system, the *apx*IA and *apx*IIA genes were successfully amplified as 2.8 and 3.1 kb PCR products, respectively, and cloned using the TOPO cloning vector. The cloned genes were sequenced, was and found to be identical to those reported previously in the GenBank (Accession no. D16582 and M30602, respectively). After sequencing of the cloned genes, the *apx*IA and *apx*IIA were subcloned into the pQE31 vector, and expressed in *E. coli* M15.
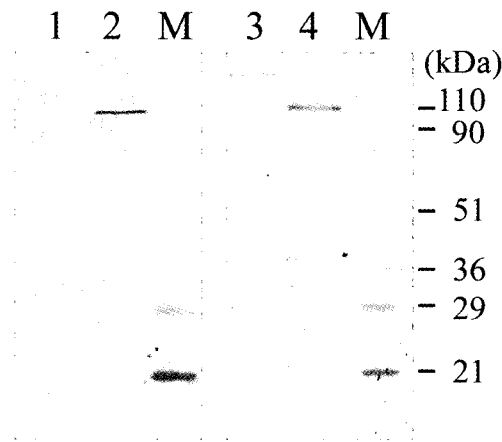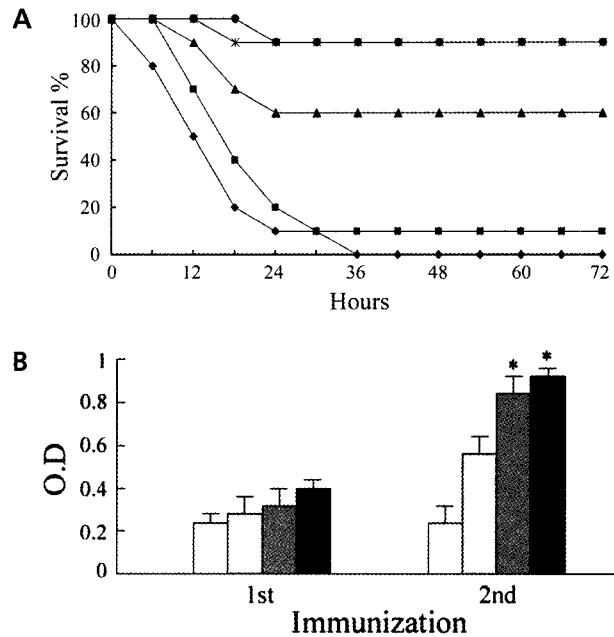
Fig. 2. Western blot analyses of ApxIA and ApxIIA expressed in *S. cerevisiae*. Lane 1, *S. cerevisiae* containing the YEpAG vector; lane 2, *S. cerevisiae* containing YEpAG-ApxIA; lane 3, vector control; lane 4, *S. cerevisiae* containing YEpAG-ApxIIA; M, molecular weight marker.

Fig. 3. Protective efficacy (A) and IgG levels (B) in mice immunized with the yeast-derived ApxIA and ApxIIA. The protective efficacy was determined by a subcutaneous injection of 1 mg of total protein extract prepared from the *S. cerevisiae* expressing Apx toxins (*, ApxIIA; ▲, ApxIA; ●, mixture of ApxIA and ApxIIA; ■, vector control; ◆, non-treated control). The level of serum IgG antibody was determined by ELISA after a subcutaneous injection of 1 mg of the total protein extract prepared from the *S. cerevisiae* expressing Apx toxins (white bar, vector control; light gray bar, ApxIA; dark gray bar, ApxIIA; black bar, mixture of ApxIA and ApxIIA). The asterisks in panel (B) indicate p values less than 0.05.

The cloned *apx*IA and *apx*IIA genes were also subcloned into the YEpAG vector, and expressed in *S. cerevisiae* 2805, as described in Materials and Methods. Several transformants of *S. cerevisiae*, representing each recombinant plasmid, were selected on ura⁻ medium. The plasmid DNAs from these transformations were isolated, and reintroduced into the *E. coli* to confirm the presence of the recombinant plasmid in the yeast. The expression levels of the *apx*IA and *apx*IIA were measured by Northern blot analysis, which revealed variations in the transcriptional level of the *apx*IA and *apx*IIA genes between strains transformed with the same expression construct (data not shown). A wide variation in the heterologous gene expression level in *S. cerevisiae* is not unusual when episomal 2 μ ori-based plasmids are used, possibly due to variations in the plasmid copy number between the different transformants [22]. Thus, transformants showing the highest expression levels were selected, and used to confirm the production of the recombinant proteins.

The expressed ApxIA and ApxIIA proteins were shown to be 105 kDa and 110 kDa, respectively, by Western blot (Fig. 2).

Induction of the Immunogenicity of the Yeast Derived Antigens and Challenge Test

The immunogenicity of the yeast-derived antigens, ApxIA and ApxIIA, or a mixture of both, was examined by performing a challenge test and IgG antibody response assays. Ninety percent of the mice immunized with the protein prepared from the ApxIIA-expressing yeast, and the protein mixture from the ApxIA and ApxIIA-expressing yeasts, survived to 72 h after the challenge with the minimal lethal dose (MLD, 4.5×10^7 CFU/mL), whereas sixty percent of the mice immunized with the protein prepared from the ApxIA-expressing yeast survived for 72 h with the same MLD challenge. None of the mice in the

control groups survived longer than 36 h (Fig. 3). Also, the levels of IgG in mice immunized with protein prepared from the ApxIA-, ApxIIA- and protein mixture from the ApxIA and ApxIIA-expressing yeasts, were significantly higher than those of the control groups ($p<0.05$) (Fig 3). These results suggest that the antigens, ApxIA and ApxIIA, expressed in the yeast retain the immunogenicity of the authentic bacterial antigen and; moreover, that the ApxIIA or mixture of ApxIA and ApxIIA has a higher immune response than that of ApxIA alone.

Until recently, the yeast *S. cerevisiae* has been largely applied legally in food, feed additives, or oral vaccines and drugs, as it is a good system for the expression of many kinds of recombinant protein, such as pharmaceutical proteins, enzymes and antigens [22,26-28], and has a generally regarded as a safe (GRAS) status. In this study, for the development of an oral yeast vaccine, the ApxIA and ApxIIA proteins were produced in *S. cerevisiae*, and their immunogenicity was examined in mice. The yeast strains expressing Apx toxins, as used in this study, might be a useful oral delivery system for the prevention of porcine pleuropneumonia, and will be investigated further in future studies.

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