

Expression of *apxIA* of *Actinobacillus pleuropneumoniae* in *Saccharomyces cerevisiae*

Sung Jae Shin, Jong Lye Bae¹, Young Wook Cho, Moon Sik Yang¹, Dae Hyuk Kim¹,
Yong Suk Jang¹ and Han Sang Yoo*

Department of Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology,
Seoul National University, Seoul, 151-742, Korea

¹Division of Biological Sciences and the Institute for Molecular Biology and Genetics, Chonbuk National University,
Chonju, 561-756, Korea

Actinobacillus pleuropneumoniae is an important primary pathogen in pigs, in which it causes a highly contagious pleuropneumoniae. In our previous study, *apxIA* gene amplified from *A. pleuropneumoniae* Korean isolate by PCR with primer designed based on the N- and C-terminal of the toxin was cloned in TA cloning vector and sequenced. The nucleotide sequences of *apxIA* gene was reported to GeneBank with the accession numbers of AF363361. Identity of the Apx IA from the cloned gene in *E. coli* was proved by SDS-PAGE and Western blot. Yeast has been demonstrated to be an excellent host for the expression of recombinant proteins with uses in diagnostics, therapeutics and vaccine productions. Therefore, to use the yeast as a delivery system in new oral subunit vaccine, *apxIA* gene was subcloned into *Saccharomyces cerevisiae*, and identified the expression of Apx IA protein. First, *apxIA* gene was amplified by PCR with the primers containing *Bam*HI and *Sal*I site at each end. Second, the DNA digested with *Bam*HI and *Sal*I was ligated into YEpGPD-TER vector, and transformed into *S. cerevisiae* 2805. Third, after identification of the correctly oriented clone, the 120-kDa of Apx IA protein expressed in *S. cerevisiae* 2805 was identified by SDS-PAGE and Western blot.

Key words: *Actinobacillus pleuropneumoniae*, *apxIA*, expression, *Saccharomyces cerevisiae*

Introduction

Porcine pleuropneumoniae caused by *Actinobacillus pleuropneumoniae* is a contagious, fibrinous, hemorrhagic,

and necrotizing disease that results in high mortality in acutely infected pigs, or localized small lung lesions in chronically infected ones [5,6,13]. A number of potential virulence factors have been identified in *A. pleuropneumoniae*, including a family of secreted toxins, or Apx toxins, which are members of the RTX (Repeat in ToXins) toxin family. Importance of Apx toxins in *A. pleuropneumoniae* virulence was demonstrated with several different mutants such as spontaneous, chemically induced, and transposon mutagenesis [1,7,8,14,16,23]. To date, 15 serotypes, which secrete different combinations of four cytotoxins belonging to the RTX toxin family, Apx I, Apx II, Apx III and Apx IV have been described [4,10,17]. Regional difference in the prevalence of serotypes and toxinotypes were reported [12,13]. The most prevalent serotypes in Korea are in the order of 2, 5 and 6 [12]. Although the virulence of *A. pleuropneumoniae* is multifactorial, studies indicate that virulence is strongly correlated with the production of Apx exotoxins, with serovars producing Apx I and Apx II being the most virulent [7,14,16,23]. At present, no identified serovars of *A. pleuropneumoniae* were found to produce all four Apx toxins, with the majority producing only two. Apx IA named hemolysin I (Hly I) or cytotoxin I (Cly I) is produced by serotypes 1, 5, 9, 10 and 11. This protein is strongly hemolytic and shows strong cytotoxic activity toward porcine macrophages and neutrophils [9].

Production and secretion of active RTX toxins require the activity of four genes, *apxC*, *-A*, *-B*, and *-D* [5,7,20]. The *apxA* gene encodes the structural toxin, whereas the *apxC* gene encodes a posttranslational activator, which is involved in the transfer of a fatty acyl group from an acyl carrier protein to the structural toxin. Activation of ApxA is required for target cell-binding. The *apxB* and *apxD* genes encode proteins that are required for the secretion of activated toxin. Apx I and Apx III are encoded by operons that consist of four contiguous genes (*-C*, *-A*, *-B*, *-D*)

*Corresponding author
Phone: +82-2-880-1263; Fax: +82-2-874-2738
E-mail : yoohs@plaza.snu.ac.kr

expressed from a single promoter located 5' of the *apxC* gene [7].

The effective controls of diseases are depending on vaccinations and antibiotic therapies which are based on injectable forms so far. However, these methods still pose problems such as induction and spreading of antibiotic resistance, presence of antibiotic residues in slaughter pigs, vaccination side effects, labor-intensive vaccination procedures, development of the carrier state [25,26]. Therefore, recent vaccine development has been strongly focused more on the development of oral vaccines. *Saccharomyces cerevisiae* has been part of our diet for centuries without adverse effects and is also considered to be superior to bacterial systems in respect to production of recombinant proteins in a conformation that more closely resembles that of native proteins [2,18].

Therefore, we attempted to develop an oral vaccine as a new trial to control porcine pleuropneumonia and, at the same time, minimize the problems following injection as low as possible.

As the first step of development of a new subunit vaccine, *apxIA* gene was amplified from *A. pleuropneumoniae* serotype 5 isolated from Korea by PCR with primer designed based on the N- and C-terminal of the toxin [21]. Also, Apx IA protein was expressed using *E. coli* system and yeast *Saccharomyces cerevisiae* then, the expressed proteins was identified by using SDS-PAGE and Western blot.

Materials and Methods

Bacterial strains and vectors

A. pleuropneumoniae serotypes 5 isolated from lungs of Korean pigs with pleuropneumonia was used for the cloning of *apxIA* gene as previously described [21]. *E. coli* Top 10 and M15 and *S. cerevisiae* 2805 were used as hosts for transformation and expression of the recombinant Apx IA. TOPO, pBluescript IKS (+), and pQE31 for *E. coli*, and YEpGPD for *S. cerevisiae* were used as vectors for cloning and expression.

Cloning, subcloning of *A. pleuropneumoniae apxIA* gene

apxIA gene was amplified by PCR with primers designed based on the sequence from GenBank (Accession no. D16582), and cloned with TOPO cloning vector kit (Invitrogen) after purification of the amplified PCR products from agarose gel using Gel extraction-QIA quick Gel extraction Kit (Qiagen). The primers used for *apxIA* gene amplification were forward 5'-GGATCCATGGCTA ACTCTCAGCTCGAT-3' and reverse 5'-GGATCCTTAAG CAGATTGTGTAAATA-3'. 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 gene was analyzed using restriction enzymes, *EcoRI*, *HindIII*, and *KpnI* (Gibco/BRL) and the correct clones were sequenced using an automatic sequencer (ABI PRISM 377XL).

To perform cloning in *S. cerevisiae* with YEpGPD, appropriate enzyme sites were generated by subcloning *apxIA* gene with pBluescript II KS cloning vector into *E. coli* Top 10. Briefly, 5 and 3 ends of *apxIA* gene were blunted with Klenow fragment (Gibco/BRL) and cloned with *EcoRV*-digested pBluescript II KS. Orientation of inserted fragment was confirmed by digestion with restriction endonucleases. Subsequently, *apxIA* of pBluescript II KS-*apxIA* was excised out through digestion with restriction endonucleases, *BamHI* and *SalI*, and ligated with the yeast expression vector, YEpGPD, digested with same restriction enzymes. After ligation, the yeast expression vector was transformed into the expression host *S. cerevisiae* 2805 using LiAc method [15].

Expression of *apxIA* gene in *Saccharomyces cerevisiae*

Transformed colonies were cultured onto selective medium (URA⁻; yeast nitrogen base 6.7 g, casamino acid 5 g, glucose 20 g, adenine 0.03 g, tryptophan 0.03 g, and bactoagar 20 g in 1000 ml of D.W.) for 12 hr, transferred into basic medium (YEPD; yeast extract 10 g, bactopectone 20 g, and glucose 20 g in 1000 ml of D.W.) and cultured until 0.6-0.7 at O.D.₆₀₀ for 2-3 days at 30°C. The cells were then harvested, and cellular proteins were extracted with an extraction buffer (Tris-HCl 50 mM, glycerol 10%, EDTA 10 mM) and glass beads by vortexing five times for 1 min. Extracted protein was collected by centrifugation at 7,000 rpm for 5 min at 4°C and analyzed by SDS-PAGE and Western blot using mono-specific polyclonal antibody against rApx IA.

SDS-PAGE and Western blot

Proteins expressed in *E. coli* or extracted from yeast *S. cerevisiae* 2805 were analyzed by SDS-PAGE [11] and Western blot [24] using mono-specific polyclonal antibody against rApx IA. For SDS-PAGE, total proteins (10 µg) from *S. cerevisiae* 2805 harboring vector with *apxIA* gene or only vector 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 electrophoresed into 10% polyacrylamide gel at 20 mA for 2 hr. The gels were then stained with Coomassie brilliant blue R-250.

For immunological analysis of expressed rApx IA protein in *S. cerevisiae*, the proteins separated by SDS-PAGE as described above were electrophoretically transferred on to nitrocellulose membranes (Bio-Rad). The NC membranes were incubated in 5% skim milk (Sigma Co.) in Tris buffered saline (TBS, pH 7.5) for 2 hrs at 37°C.

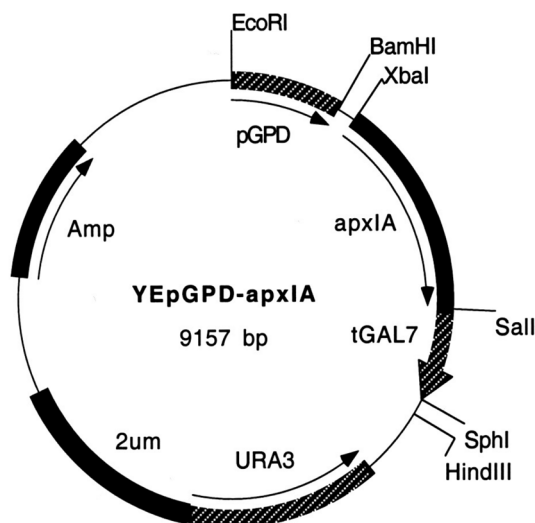


Fig. 1. Diagram of YE pGPD-*apxIA* for the expression of *apxIA* gene in *Saccharomyces cerevisiae*.

After washing three times with TBS, the membranes were incubated with 1 : 500 diluted mono-specific mouse anti-Apx IA antiserum for 2 hrs at room temperature. 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). After removal of unreacted antibodies by washing with TBS then immunoreactive bands were visualized with an enhanced AP conjugate substrate kit (Bio-Rad) in the dark.

Results

As the first step of development of a new subunit vaccine using yeast expression system, *apxIA* gene was amplified as a 3,069 bps PCR product from *A. pleuropneumoniae* isolated from Korea by PCR with primer designed based on the N- and C-terminal of the toxin and the cloned gene was sequenced, and the sequence was reported to GenBank with Accession no. AF363361. Identity of the cloned gene with reference strain was proved by comparison of the nucleotide sequence and phylogenetic analysis [21]. Identification of the expressed and purified protein was also confirmed by SDS-PAGE and Western blot analysis as previously described [21].

The cloned *apxIA* gene was successfully subcloned into YE pGPD vector through pBluescript II KS (f) to generate appropriate restriction enzyme sites (Fig. 1) and was expressed in *S. cerevisiae* 2805. To confirm the expression of Apx IA protein in *S. cerevisiae* 2805, SDS-PAGE and Western blot were performed. The 120-kDa size of expressed rApx IA protein in *S. cerevisiae* was detected as same as size of Apx IA in SDS-PAGE analysis and

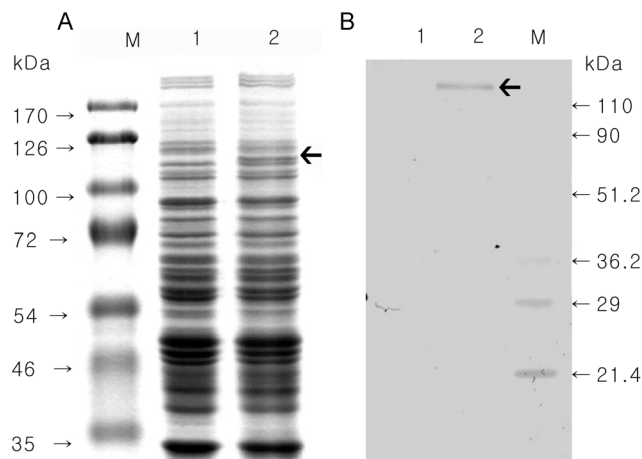


Fig. 2. Analysis of expressed Apx IA in *Saccharomyces cerevisiae* by 10% SDS-PAGE (A) and Western blot analysis (B). Lane 1, *S. cerevisiae* containing YE pGPD-TER; lane 2, *S. cerevisiae* harboring YE pGPD-TER-*apxIA*; and lane M, molecular weight marker. Arrows (←) indicate the expressed rApx IA in *S. cerevisiae*.

Western blot using mono-specific polyclonal antibody against rApx IA expressed in *E. coli* (Fig. 2).

Discussion

Recombinant DNA technology, and in particular yeast expression systems, have been successfully used to produce antigens such as malaria antigens, hepatitis B virus surface antigens [2,18]. Also, recombinant proteins can be produced from yeast in large quantities and at low cost with the possibility of widespread immunization compared with bacterial expression systems [2,19].

S. cerevisiae has been considered to be safe as diet in human without any side effects. It has a generally regarded as safe (GRAS) status and is generally a good expression system for heterologous proteins. Therefore, it has been legally used in food and pharmaceutical productions [18]. In addition, it has been used as tracer for the oral application of vaccines and drugs because it is relatively stable, nonpathogenic and noninvasive in gut compared to other biodegradable vehicles [3]. Also, cellular components of yeast such as β -glucan have immunostimulatory effects that might be beneficial when it works as adjuvant for the induction of broad-based cellular immune responses [2,22].

Therefore, with the development of yeast expressing Apx IA exotoxin, *S. cerevisiae* might be a useful delivery system for the prevention of porcine pleuropneumonia and the results obtained in this study could be used for the future study to develop a new oral vaccine to porcine pleuropneumonia.

Acknowledgments

This study was supported by Biogreen 21 Programs, Brain Korea 21 Project, and the Research Institute for Veterinary Science, Seoul National University, Korea.

References

- Anderson, C., Potter, A. A. and Gerlach, G. F. Isolation and molecular characterization of spontaneously occurring cytolysin-negative mutants of *Actinobacillus pleuropneumoniae* serotype 7. *Infect. Immun.* 1991, **59**, 4110-4116.
- Bathurst, I. C. Protein expression in yeast as an approach to production of recombinant malaria antigens. *Am. J. Trop. Med. Hyg.* 1994, **50**, 11-19.
- Beier, R. and Gebert, A. Kinetics of particle uptake in the domes of Peyer's patches. *Am. J. Physiol.* 1998, **275**, 130-137.
- Blackall, P. J., Klaasen, H. L. B. M., Van Den Bosch, H., Kuhnert, P. and Frey, J. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. *Vet. Microbiol.* 2002, **84**, 47-52.
- Bosse, J. T., Janson, H., Sheehan, B. J., Beddek, A. J., Rycroft, A. N., Kroll, J. S. and Langford, P. R. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Micro. Infect.* 2002, **4**, 225-235.
- Chiers, K., Donne, E., Van Overbeke, I., Ducatelle, R. and Haesebrouck, F. *Actinobacillus pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. *Vet. Microbiol.* 2002, **85**, 343-352.
- Frey, J. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. *Trends Microbiol.* 1995, **3**, 257-261.
- Fuller, T. E., Martin, S., Teel, J. F., Alaniz, G. R., Kennedy, M. J. and Lowery, D. E. Identification of *Actinobacillus pleuropneumoniae* virulence genes using signature-tagged mutagenesis in a swine infection model. *Microb. Pathog.* 2000, **29**, 39-51.
- Kamp, E. M., Poma, J. K., Anakotta, J. and Smits, M. A. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. *Infect. Immun.* 1991, **59**, 3079-3085.
- Komal, J. P. and Mittal, K. R. Grouping of *Actinobacillus pleuropneumoniae* strains of serotypes 1 through 12 on the basis of their virulence in mice. *Vet. Microbiol.* 1990, **25**, 229-240.
- Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970, **227**, 680-685.
- Min, K. and Chae, C. Serotype and *apx* genotype profiles of *Actinobacillus pleuropneumoniae* field isolates in Korea. *Vet. Rec.* 1999, **145**, 251-254.
- Nielson, R. Seroepidemiology of *Actinobacillus pleuropneumoniae*. *Can. J. Vet. Res.* 1998, **29**, 580-582.
- Prideaux, C. T., Lenghaus, C., Krywult, J. and Hodgson, A. L. M. Vaccination and protection of pigs against pleuropneumonia with a vaccine strain of *Actinobacillus pleuropneumoniae* produced by site-specific mutagenesis of the *Apx II* operon. *Infect. Immun.* 1999, **67**, 1962-1966.
- Prideaux, C. T., Pierce, L., Krywult, J. and Hodgson, A. L. Protection of mice against challenge with homologous and heterologous serovars of *Actinobacillus pleuropneumoniae* after live vaccination. *Curr. Microbiol.* 1998, **37**, 324-332.
- Reimer, D., Fery, J., Jansen, R., Veit, H. P. and Inzana, T. J. Molecular investigation of the role of *ApxI* and *ApxII* in the virulence of *Actinobacillus pleuropneumoniae* serotype 5. *Microb. Pathog.* 1995, **18**, 197-209.
- Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anbderson, T. J., MacInnes, J. I., Segers, R. P. and Frey, J. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology.* 1999, **145**, 2105-2116.
- Schreuder, M. P., Deen, C., Boersma, W. J. A., Pouwels, P. H. and Klis, F. M. Yeast expressing hepatitis B virus antigen determinants on its surface: implications for a possible oral vaccine. *Vaccine.* 1996, **14**, 383-388.
- Schreuder, M. P., Mooren, A. T. A., Toschka, H. Y., Verrips, T. C. and Klis, F. M. Immobilizing proteins on the surface of yeast cells. *Trends Biotechnol.* 1996, **14**, 115-120.
- Seah, J. N., Frey, J. and Kwang, J. The N-terminal domain of RTX toxin *ApxI* of *Actinobacillus pleuropneumoniae* elicits protective immunity in mice. *Infect. Immun.* 2002, **70**, 6464-6467.
- Shin, S. J., Cho, Y. W. and Yoo, H. S. Cloning, sequencing and expression of *apxIA*, *IIA*, *IIIA* of *Actinobacillus pleuropneumoniae* isolated in Korea. *Korean J. Vet. Res.* 2003, **43**, 247-253.
- Stubbs A. C., Martin, K. S., Coeshott, C., Skaates, S. V., Kuritzkes, D. R., Bellgrau, D., Franzusoff, A., Duke R. C. and Wilson, C. C. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. *Nat. Med.* 2001, **7**, 625-629.
- Tascon, R. I., Vanquez-Boland, J. A., Gutierrez-Martin, C. B., Rodriguez-Barbosa, I. and Rodriguez-Ferri, E. F. The RTX haemolysin *ApxI* and *ApxII* are major virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*: evidence from mutational analysis. *Mol. Microbiol.* 1994, **14**, 207-216.
- Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* 1979, **76**, 4350-4354.
- Vaillancourt, J. P., Higgins, R., Martineau, G. P., Mittal, K. R. and Lariviere, S. Changes in the susceptibility of *Actinobacillus pleuropneumoniae* to antimicrobial agents in Quebec (1981-1986). *J. Am. Vet. Med. Assoc.* 1998, **193**, 470-473.
- Van Overbeke, I., Chiers, K., Ducatelle, R. and Haesebrouck, F. Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing *Apx* toxins and transferrin-binding proteins. *J. Vet. Med. B.* 2001, **48**, 15-20.