Vaccination and Protection of Pigs against Pleuropneumonia with a Vaccine Strain of *Actinobacillus pleuropneumoniae* Produced by Site-Specific Mutagenesis of the ApxII Operon

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The production of toxin (Apx)-neutralizing antibodies during infection plays a major role in the induction of protective immunity to *Actinobacillus pleuropneumoniae* reinfection. In the present study, the gene encoding the ApxII-activating protein, apxIC, was insertionally inactivated on the chromosome of a serovar 7 strain, HS93. Expression of the structural toxin, ApxI, and of the two genes required for its secretion, apxIB and apxID, still occurs in this strain. The resulting mutant strain, HS93C Amp+, was found to secrete the unactivated toxin. Pigs vaccinated with live HS93C Amp+ via the intranasal route were protected against a cross-serovar challenge with a virulent serovar 1 strain of *A. pleuropneumoniae*. This is the first reported vaccine strain of *A. pleuropneumoniae* which can be delivered live to pigs and offers cross-serovar protection against porcine pleuropneumonia.

*Actinobacillus pleuropneumoniae* is a member of the family Pasteurellaceae and is the etiological agent of porcine pleuropneumonia, an acute or chronic infection affecting pigs of all ages. The disease, characterized by hemorrhagic, fibrinous, and necrotic lung lesions, is highly contagious and causes major losses to the swine industry (25). To date, 12 serovars have been identified worldwide (serovars 1 to 12). Within a geographical region a small number of serovars predominate; for example, in Australia serovars 1, 7, and 12 make up approximately 90% of isolates.

A number of potential virulence factors have been identified for *A. pleuropneumoniae*, including a family of secreted toxins (3, 5, 26, 29). These secreted toxins, or Apx toxins, are members of the RTX toxin family (11–13). The role of Apx toxins in *A. pleuropneumoniae* virulence was first demonstrated with spontaneous and chemically induced nonhemolytic mutants which were found to be completely or partially avirulent; this role was later confirmed by using transposon mutagenesis (1, 15, 17, 29, 30, 33, 34). At least three different Apx toxins are produced by *A. pleuropneumoniae*, designated ApxI, ApxII, and ApxIII. ApxI shows strong hemolytic activity, and ApxII is nonhemolytic but strongly cytotoxic, with a host range including porcine alveolar macrophages and neutrophils (19, 29). Currently, no identified serovar of *A. pleuropneumoniae* produces all three Apx toxins, with the majority producing only two, while a small number produce only one (8, 10–12, 19, 29).

Production and secretion of active RTX toxins requires the activity of at least four genes, apxC, apxC, -A, -B, and -D. The apxA gene encodes the structural toxin, and the apxB 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 (18). Activation of ApxA is required for target cell binding. The apxB and apxD genes encode proteins that are required for secretion of the activated toxin (7, 36). ApxI and ApxIII are encoded by operons that consist of the four contiguous genes (-C, -A, -B, -D) expressed from a single promoter located 5' of the apxC gene. The ApxII operon contains only the apxA and apxC genes expressed as a single RNA transcript. Secretion of ApxII is dependent on the activity of the apxB and apxD gene products (13).

Vaccination against porcine pleuropneumonia has utilized, to date, bacterins or subunit vaccines based on various components of *A. pleuropneumoniae*. Results obtained with bacterin vaccines have offered, at best, homologous protection against the serovar used to prepare the vaccine material. In contrast, natural infection of pigs with any one serovar serves to prevent natural reinfection with any serovar (24). Apx's are thought to be of particular importance for the induction of protective immunity; nonhemolytic mutants cannot induce protective immunity in animals (17), and commercial bacterin vaccines that lack Apx do not provide adequate protection (16). Previously we (26) demonstrated the ability of an *A. pleuropneumoniae* mutant deficient in chromosomal apxA and apxC genes to express and secrete an unactivated form of ApxI from a plasmid-encoded apxA gene. This engineered strain was found to be attenuated in a mouse model and, when administered as a live vaccine, offered protection against homologous and heterologous challenge.

The use of a plasmid-borne protective antigen in a live vaccine strain is limited due to the potential of the plasmid to be lost during in vivo replication of the vaccine. Here we describe the construction of an *A. pleuropneumoniae* vaccine strain by using site-specific mutagenesis of the apxIIC gene on the chromosome. The resulting strain produces and secretes an unactivated ApxIIA by using chromosomally encoded genes, thus ensuring that the protective antigen is maintained within the vaccine strain, unlike in previous experiments, in which ApxIIA was expressed from a plasmid and could therefore be lost from replicating bacteria. The potential of this modified strain to protect pigs from cross-serovar challenge with virulent *A. pleuropneumoniae* was investigated.**

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**SITE-SPECIFIC MUTAGENESIS OF A. PLEUROPNEUMONIAE**

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *A. pleuropneumoniae* bacterial strains used in this study (serovar 1, HS25; serovar 7, HS93) were isolated from pigs and kindly supplied by Pat Blackall (Animal Health Research Institute, Yeerongpilly, Queensland, Australia). Strains of *A. pleuropneumoniae* were grown in brain heart infusion broth (BHI), supplemented with antibiotics (ampicillin, 50 μg/ml; and ampicillin, 5 μg/ml, unless stated otherwise. *Escherichia coli* DH5α was used throughout this study, by standard techniques (31).

**Isolation of genomic DNA.** Isolation of *A. pleuropneumoniae* genomic DNA was performed as described by Prideaux et al. (26), using lysozyme and proteinase K digestion followed by phenol-chloroform-isoamyl alcohol extraction. Amplification of specific regions of the *A. pleuropneumoniae* genome was achieved by PCR, using the buffer and cycle conditions described previously (26) and a Perkin-Elmer Cetus DNA thermal cycler.

**Construction of recombination plasmids.** The plasmid pEP-Camp was constructed for use in site-specific mutagenesis of the *apxIIC* gene. A 3.4-kb fragment containing the *apxIIC* gene was isolated by PCR. Specific oligonucleotides for use in PCR were synthesized with a Pharmacia Gene Assembler Plus DNA synthesizer (5′, CCACCAACGTGTCGGGC; 3′, TTAACAGCTGAGTCCAG) based on the sequence described by the ADPribosylation factor A (23). The resulting plasmid, pEP-Camp, was electroporated into *A. pleuropneumoniae* HS25 (serovar 7, ApxI and ApxII). A toxin-deficient strain of HS93 resulting in size corresponded to the size of the Ampr gene. Products of the PCRs were further characterized by Southern blot hybridization with the isolated Ampr or apxC gene as a probe (Fig. 1). Hybridization of the apxIIC gene probe to the PCR products from HS93 and HS93C Ampr confirmed that the region of the chromosome containing the *apxIIC* gene had been amplified. The PCR product obtained from HS93C Ampr, confirming that this strain contained the gene associated with the apxC gene. The PCR product obtained when HS93 genomic DNA was used as template did not hybridize to the Ampr gene probe.

**Characterization of the apxIIC mutant.** Site-specific mutagenesis of the *apxIIC* gene utilized the recombination plasmid pEP-Camp. This plasmid contains the *apxIIC* open reading frame insertionally inactivated by the introduction of an Amp gene into the unique XbaI site. The inactivated *apxIIC* gene is flanked by 2.0 kb of genomic DNA upstream (5′) and 900 bp downstream (3′). pEP-Camp was linearized and electroporated into *A. pleuropneumoniae* HS93, and the products of homologous recombination were selected by plating on blood agar plates containing ampicillin.

Genomic DNA was extracted from the nonzoning, ampicillin-resistant mutants designated HS93C Ampr and the parent strain, HS93. PCR was used to examine the region of the *A. pleuropneumoniae* chromosome containing the *apxIIC* gene. The PCR product (Fig. 1) obtained by using HS93C Ampr genomic DNA (3.5 kb) was approximately 1.8 kb larger in size than that obtained with the parent strain, HS93. This increase in size corresponded to the size of the Ampr gene. Products of the PCRs were further characterized by Southern blot hybridization with the isolated Ampr or apxC gene as a probe (Fig. 1). Hybridization of the apxIIC gene probe to the PCR products from HS93 and HS93C Ampr confirmed that the region of the chromosome containing the *apxIIC* gene had been amplified. The *A. pleuropneumoniae* gene probe hybridized to the PCR product obtained from HS93C Ampr, confirming that this strain contained the Ampr gene associated with the apxC gene. The PCR product obtained when HS93 genomic DNA was used as template did not hybridize to the Ampr gene probe.

**Characterization of Apx expression by HS93C Ampr.** Logarithmic cultures of HS93 and HS93C Ampr were examined by Western blotting with antisera raised in rabbits against the secreted proteins of *A. pleuropneumoniae* HS25 (serovar 1, ApxI and ApxII). A toxin-deficient strain of HS93 resulting from deletion of the *apxIIC* and *apxIIA* genes (26) was used as a negative control. The Apx-deficient mutant (HS93 Tox−) did not react specifically with the anti-Apx sera in the region corresponding to the ApxII molecular weight. Supernatant and cellular material from both the HS93C Ampr mutant and the parent strain, HS93, produced a single polypeptide, corresponding in size to ApxII, that reacted with the anti-Apx rabbit sera (Fig. 2). A potential high-molecular-weight HS93 Tox− polypeptide may have reacted with the antisera. The preabsorption of the sera with HS93 Tox− prior to use and the position of the band suggest that it corresponds to nonspecific cross-reaction with material remaining in the loading well.

**Evaluation of HS93C Ampr virulence in mice.** To test the relative virulence of *A. pleuropneumoniae* HS93 and HS93C Ampr in mice, various dilutions of each bacteria (2 × 10⁸ to 1 × 10⁶ CFU/mouse) were prepared in bacterial growth me-
dium (BHI-NAD) and administered to mice i.p. The number of mice that had received a sublethal dose was determined 24 h postchallenge. Under our conditions, all mortalities occurred within the first 24 h postchallenge. A comparison of the deaths obtained with each isolate showed that 2310^8 CFU of the parent strain, HS93, was sufficient to kill 100% of mice, while an equivalent challenge with HS93C^2Ampr was sublethal (Table 1).

**Vaccination and challenge of pigs.** Two groups of nine 6-week-old pigs were vaccinated with either 1 ml of BHI containing 10^9 CFU of HS93C^2Ampr or 1 ml of sterile BHI (unvaccinated) via intranasal inoculation on days 0 and 14. Two weeks after secondary vaccination, six of the HS93C^-Ampr-vaccinated and six of the unvaccinated pigs were challenged intranasally with 2 ml of growth medium containing 2310^9 CFU of HS25. The number and severity of lung lesions present in pigs at autopsy 5 days postchallenge were recorded (Table 2). It is our experience that no additional lesions resulting from experimental challenge of pigs with this protocol develop beyond day 5 postchallenge and that at this time a number of lesions detected have commenced to resolve. The three pigs that were neither vaccinated nor challenged had no detectable lung lesions present at autopsy. Similarly, pigs (three) that were vaccinated and challenged with HS25, only one had a lesion at autopsy; this was in the form of a single adhesion between the lung and the rib cage. Upon closer examination, this adhesion appeared to be older than the 5 days since challenge. Bacteria isolated from this adhesion were not NAD dependent and are therefore unlikely to have been *A. pleuropneumoniae*. Lung samples that were taken from vaccinated and challenged pigs, homogenized, and plated on blood agar (BHI-NAD) did not yield bacteria.

**DISCUSSION**

Apx toxins are known to play a major role in both the virulence of and induction of protective immunity to *A. pleuropneumoniae*, the causative agent of porcine pleuropneumonia. The principal phagocytic cells of the lung and the first line of defense against bacterial invasion are the alveolar macrophages. It is possible that *A. pleuropneumoniae* colonizes the lung through the production of Apx toxins which lyse these cells and thus compromise the primary immune responses of the lung (37).

**TABLE 1. Virulence of *A. pleuropneumoniae* strains in mice**

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<tr>
<th>Challenge level (10^5)</th>
<th>% of mice dead 24 h postchallenge with indicated strain</th>
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<tr>
<td></td>
<td>HS93</td>
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<tr>
<td>200</td>
<td>100</td>
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<tr>
<td>20</td>
<td>15</td>
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* Levels below those previously found to give 0% death were not tested (NT).
Targets of the Apx toxins include erythrocytes, the lysis of which leads to an increase in the availability of free iron for bacterial growth. In addition, the Apx toxins contribute to lung damage through the lysis of leukocytes, which leads to localized inflammation. Binding of Apx to target cells requires posttranslational activation of the structural toxin by the ApxIIA protein (18). In this study we utilized site-specific mutagenesis to inactivate the apxIIIC gene on the A. pleuropneumoniae chromosome and examined the effect of this mutation on virulence, induction of protective immunity following infection, and potential to generate mutants for use as live vaccines.

Site-specific mutagenesis of the apxIIIC gene was achieved with suicide, or nonreplicating, plasmid vectors. This method has the advantage of rapid screening of products, as theoretically only those bacteria that have undergone recombination with the plasmid vector, resulting in the transfer of the marker gene onto the bacterial chromosome, will grow under selective conditions. A number of attempts within our laboratory have failed to transform A. pleuropneumoniae with pEP2, leading to the conclusion that A. pleuropneumoniae is a nonpermissive host for this plasmid vector. A serovar 7 strain of A. pleuropneumoniae producing ApxII alone was chosen for use in this study, as the genes required for ApxII secretion are not co-transcribed with the structural and activating genes. In addition, serovar 7 is relevant as a vaccine candidate in Australia, as serovar 7 isolates are responsible for large numbers of porcine pleuropneumonia outbreaks.

Homologous recombination leading to the insertion of the ApxIIA gene into the apxIIIC gene on the chromosome was confirmed by PCR and Southern blot hybridization. Insertion of the ampicillin resistance gene into the chromosomal copy of the apxIIIC gene did not prevent transcription or translation of the apxIIA gene, as evidenced by the ability to detect ApxII in Western blots (Fig. 2). It appears that transcription initiates at the apxII promoter and continues through the ampicillin resistance gene and into the apxIIA gene. Although translation of an active apxIIIC gene product is prevented by the presence of the ampicillin resistance gene (Fig. 1), translation of the apxIIA gene must recommence further downstream. Chang et al. (3) have described a potential ribosome binding site, located between the apxIIIC and apxIIA genes, which may serve to reinitiate translation of ApxII. The orientation of the ampicillin resistance gene is opposite that of the apxII operon, and therefore the ampicillin resistance gene promoter cannot contribute to ApxIIA expression. Insertion of the ampicillin resistance gene into the apxIIIC gene appears to have reduced the level of ApxII production, possibly due to polar effects of the ampicillin resistance gene promoter on the level of downstream apxIIA transcription. A potential solution to this possible limitation would be to clone the ampicillin resistance gene in the same orientation as the apxIIA gene, though licensing of the vaccine strain for commercial use would require the removal of any antibiotic resistance gene from the chromosome. The presence of ApxIIA in the culture supernatant would also indicate that activation of ApxIIA is not required for secretion. A similar observation has been made for both E. coli and P. haemolytica, where the RTX toxins produced by these bacteria have been shown to be secreted without activation (6, 35).

Inactivation of the apxIIIC gene on the chromosome resulted in reduced virulence, as observed in a mouse model in which $2 \times 10^8$ CFU of the apxIIIC-deficient mutant resulted in no mortalities compared to a mortality rate of 100% when mice were inoculated with the same level of the parent strain, HS93 (Table 1). This is in agreement with our previous observations (26) with a toxin-deficient strain of A. pleuropneumoniae expressing an unactivated form of ApxIA from a plasmid, where it was found that unless the toxin was activated, it did not contribute to bacterial pathogenesis.

To test the protective efficacy of the vaccine in the target species, we vaccinated pigs with HS93C<sup>−</sup> Amp<sup>+</sup>, a serovar 7 strain, via the intranasal route and challenged with HS25 (Table 2), which belongs to serovar 1 and produces both ApxI and ApxII. This combination of Apx production is associated with the most severe outbreaks of pleuropneumonia (13, 20). Prior to vaccination, pigs were determined to be free of both HS93- and ApxI-specific antibodies, therefore ensuring their naive status for both vaccination (HS93) and challenge (HS25: ApxI) strains. Vaccination and challenge were both via the intranasal route. This method of delivery was chosen because it best mimics the natural route of exposure of pigs to A. pleuropneumoniae. The three pigs that were vaccinated and not challenged had no lung lesions present at autopsy, indicating that the vaccine strain does not cause lesions in pigs that are evident at 3 weeks postvaccination. Previously we had administered the toxin-deficient strain HS93 Tox<sup>−</sup> to pigs at doses similar to that of the challenge used in this experiment and autopsied the pigs at day 5 but observed no lesions. ApxI-deficient mutants of APP produced by either chemical or transposon mutagenesis have previously been shown to have a reduced ability to induce lung lesions (1, 15, 17, 29, 30, 33, 34). The six unvaccinated pigs challenged with HS25 showed numerous lung lesions that were visible on autopsy, indicating that the level of challenge used was sufficient to induce lesions in unprotected animals. In contrast, only one of the six vaccinated pigs showed any sign of infection, in the form of a single lung adhesion, which was unlikely to be a result of the challenge. The ability to achieve cross-serovar protection following live vaccination, but not after vaccination with bacterin preparations, suggests that cross-serovar protection may be dependent on the presentation of in vivo-regulated proteins to the immune system. In addition, the route of vaccination may also play a role in the level of cross-protection obtained. Intranasal vaccination was chosen because it best mimics the natural route of A. pleuropneumoniae infection, which is known to induce an immune response that is cross-protective. In contrast, bacterin vaccines are delivered by subcutaneous or intra-
muscular injection. It has been demonstrated previously that the immune responses induced by a commercial vaccine are very different from those induced following aerosol exposure of pigs to *A. pleuropneumoniae* (14). Sera obtained from animals postvaccination and prior to challenge responded weakly to ApxIIA by enzyme-linked immunosorbent assay (results not shown). Additional work is ongoing to further characterize the immune responses obtained through vaccination with H593C’ Amp and to compare them with those obtained during natural infection.

The findings of this protection study demonstrate the potential of H593C’ Amp to be delivered via the nasal route as a vaccine to protect pigs against porcine pleuro pneumonia. Activation of ApxIIA was found to be necessary for virulence in a mouse model but not for secretion. The ability of H593C’ Amp to protect pigs from virulent *A. pleuropneumoniae* challenge, together with the central role of Apx immunity in protecting pigs from *A. pleuropneumoniae* infection, suggests that activation of the toxin is not required to induce protective immunity. This is the first report of a live vaccine strain of *A. pleuropneumoniae* that is suitable for use in pigs and offers cross-serovar protection.

**ACKNOWLEDGMENT**

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**REFERENCES**