Truncated Surface Protective Antigen (SpaA) of *Erysipelothrix rhusiopathiae* Serotype 1a Elicits Protection against Challenge with Serotypes 1a and 2b in Pigs

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*Erysipelothrix rhusiopathiae* is a causal agent of swine erysipelas, which is of economic importance in the swine industry by virtue of causing acute septicemia, chronic arthritis, and endocarditis. However, little is known about the genetic properties of its protective antigens. Recently, a surface protective antigen (SpaA) gene was identified from serotype 2 in a mouse model. We cloned spaA from virulent strain Fujisawa (serotype 1a) and determined that the N-terminal 342 amino acids without C-terminal repeats of 20 amino acids have the ability to elicit protection in mice. Fusions of 342 amino acids of Fujisawa SpaA and histidine hexamer (HisSpa1.0) protected pigs against challenge with both serotype 1 and serotype 2, the most important serotypes in the swine industry. Pigs immunized with HisSpa1.0 reacted well with both HisSpa1.0 and intact SpaA by enzyme-linked immunosorbent assay and immunoblotting. Serum collected at the time of challenge from a pig immunized with HisSpa1.0 markedly enhanced the in vitro phagocytic and killing activity of pig neutrophils against the bacteria. DNA sequences of protective regions of spaA genes from five strains of serotypes 1 and 2 were almost identical. The full DNA sequences also seemed to be conserved among strains of all 12 serotype reference strains harboring the spaA gene by restriction fragment length polymorphism analysis of PCR products. These results indicate that SpaA is a common protective antigen of serotypes 1a and 2 of *E. rhusiopathiae* in swine and will be a useful tool for development of new types of vaccines and diagnostic tools for effective control of the disease.

*Erysipelothrix rhusiopathiae* (formerly *E. insidiosa*) is a small gram-positive rod that causes erysipelas mainly in swine (34) and turkeys (2) and less frequently in other animals and humans. *E. rhusiopathiae* was once thought to be the only member of genus *Erysipelothrix* and was classified into 23 serotypes and type N based on peptidoglycan antigens of the cell wall (9, 34). The genus now contains two species, *E. rhusiopathiae* and *E. tonsillarum*, and other (two) genetically distinct unclassified groups (24, 25). Among 15 serotypes of *E. rhusiopathiae* (25), serotypes 1 (subdivided into 1a and 1b) and 2 (subdivided into 2a and 2b) are the most important in the pig industry (3, 4, 17, 25), and contained no detectable polysaccharide. Mice immunized with preparations of the 66- to 66-kDa band were protected against challenge with Frankfurt XI (serotype N). They also described the enhanced production of these protective antigens in serum-free modified Feist broth (6). However, some questions remain as to whether the band of 64 to 66 kDa is the only protective antigen of the bacteria, this band contains only one kind of protective antigen, and this antigen can elicit protection in swine.

Galan and Timoney first identified a mouse protective antigen gene in a 5.4-kb EcoRI fragment of chromosomal DNA of virulent strain El-6P (serotype 1a) (5). Guinea pig antisera against the recombinant clone of this gene reacted with *E. rhusiopathiae* protein antigens of 66, 64, and 43 kDa. These proteins are of the same size as the protective proteins mentioned above. However, the DNA sequences of the gene were not described, and it is also not known whether the clone contained only one gene. Recently, a novel surface protective antigen (SpaA) of *E. rhusiopathiae* was identified from serotype 2 in a mouse model using a monoclonal antibody recognizing 64-kDa proteins of most serotypes of *E. rhusiopathiae* (13). Mice immunized with live recombinant *Escherichia coli* intraperitoneally survived after challenge with the same strain of *E. rhusiopathiae*. In this study, the presence of 20-amino-acid repeats units at the C terminus was shown to be essential for protection.

In contrast to protein antigens, 14.4- to 22-kDa capsular
antigen appears to be not necessary for protection. A live acapsular mutant created by insertion and excision of Tn916, which is avirulent in mice, could confer complete protective immunity to mice (21).

The existence of a common protective antigen among serotypes of *E. rhusiopathiae* was identified experimentally and practically. Although killed vaccines are prepared from serotype 1a and live vaccines are from serotype 2, both vaccines can cross-protect pigs against challenge with strains of serotypes 1 and 2 (1, 23, 33). In this study, we cloned *spaA* from a Sau3AI library of virulent strain Fujisawa (serotype 1a), determined that the N-terminal 342 amino acids are necessary to elicit protection in mice, and evaluated the ability of SpaA of strain Fujisawa (SpaA/Fujisawa) to elicit cross-protection in pigs against challenge with serotypes 1 and 2 by the use of fusion of truncated SpaA/Fujisawa with a histidine hexamer (HisSpa1.0).

**MATERIALS AND METHODS**

Bacterial strains, vectors, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. rhusiopathiae* Fujisawa was used for cloning of *spaA*, preparation of intact SpaA for enzyme-linked immunosorbent assay (ELISA), and challenge of mice and pigs. For challenge of pigs, *E. rhusiopathiae* 82-875 was also used. Vector plasmid pBluescript II SK+ (Stratagene) was used for cloning of *spaA*, and expression vector pQE32 (Qiagen) was used to construct HisSpa1.0, in which the histidine hexamer tag was placed at the N terminus of the protein. *E. coli* XL1-Blue was used as the host strain for these plasmids.

*E. rhusiopathiae* was grown on modified Feist broth at 37°C. Recombinant *E. coli* carrying these plasmids could elicit protection in mice. The protective region of the gene was determined by analyzing the Exo-Mung deletion mutants of these plasmids created as instructed by the manufacturer (Stratagene) by immunoblotting and confirmed by a mouse protection test.

**Expression and purification of fusion protein.** A *Kpn*I fragment of recombinant plasmid pA containing bp 266 to 1,294 of *spaA* was ligated into the compatible site of expression vector pQE32 to construct pA1.0 as shown in Fig. 1. One *Kpn*I site was located in *spaA*, and another one was located in the multicloning site of pBluescript II. Recombinant fusion protein HisSpa1.0 was expressed in *E. coli* XL1-Blue (pA1.0) and purified as described by the manufacturer (Qiagen) under denaturing conditions. The culture was inoculated with a 1:50 dilution of overnight culture of recombinant *E. coli*, grown at 37°C to mid-exponential phase (*A*<sub>600</sub> = 0.8), and then induced with 2 mM isopropyl-β-D-thiogalactopyranoside for 5 h with vigorous shaking. Cells were harvested and resuspended in 6 M guanidine hydrochloride (pH 8.0) at 0.2 g (wet weight)/ml and stirred for 1 h at room temperature to solubilize the fusion protein. The slurry was centrifuged at 10,000 × g for 15 min; then the fusion protein in the supernatant was filter

**Determination of protective region of *spaA*.** Two *spaA* recombinant plasmids, pA and pB, obtained from a Sau3AI library of Fujisawa were used. Lysates of recombinant *E. coli* carrying these plasmids could elicit protection in mice. The protective region of the gene was determined by analyzing the Exo-Mung deletion mutants of these plasmids created as instructed by the manufacturer (Stratagene) by immunoblotting and confirmed by a mouse protection test.

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**DNA was prepared by a modified alkaline lysis-polyethylene glycol precipitation procedure in a dye terminator cycle sequencing protocol (Perkin-Elmer).**

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant characteristic</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>E. rhusiopathiae</strong> (serotype)</td>
<td></td>
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<tr>
<td>Fujisawa (1a)</td>
<td>Japanese official challenge strain</td>
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</tr>
<tr>
<td>Koganei (1a)</td>
<td>Japanese official live vaccine strain</td>
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</tr>
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<td>SE-9 (2a)</td>
<td>U.S. official bacterin strain</td>
<td>6</td>
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<tr>
<td>ATCC 19414 (2b)</td>
<td>Type strain</td>
<td>American Type Culture Collection</td>
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<td>Marienfelde (1a)</td>
<td>Growth agglutination strain</td>
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<td>ME-7 (1a), 422/E1 (1b), R32E11 (2a), NF4E1 (2b), Pécs 67 (5), Goda (8), Kaparek (9), Pécs 9 (12), Pécs 3597 (15), Tanzania (16), 545 (17), MEW 22 (N)</td>
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<td></td>
<td>Recombinant pOE32 of <em>spaA/Fujisawa</em></td>
<td>This study</td>
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<td>Recombinant <em>E. coli</em> XL1-Blue (pA1.0)</td>
<td></td>
<td>This study</td>
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**FIG. 1.** Map of *spaA/Fujisawa* in recombinant plasmids pA, pB, and pA1.0 and map of HisSpa1.0 in intact SpaA. Protective regions are indicated as dotted boxes.
sterilized and adsorbed on Ni-nitrilotriacetic acid (NTA)-Sepharose column. The Ni-NTA column was washed with guanidine buffer (pH 8.0), 8 M urea buffer (pH 8.0), and 8 M urea buffer (pH 6.3); then the fusion protein was eluted with urea buffer (pH 4.5) and dialyzed against 10 mM phosphate-buffered saline, pH 7.2 (PBS).

Mouse immunization and challenge. Four-week-old ddY female mice were immunized subcutaneously with 500 μg of sonicated extract of recombinant E. coli in Freund’s incomplete adjuvant or with 50 μg of purified HisSpa1.0 in complete adjuvant twice and challenged with 100% lethal doses of E. rhusiopathiae Fujisawa subcutaneously 3 weeks after immunization. The infections were monitored for 12 days, and the cause of death was confirmed by isolation of the organisms.

Pig immunization and challenge. Four-week-old specific-pathogen-free (SPF) pigs obtained from a farm free from swine erysipelas where no pigs were vaccinated against the disease were used. Six pigs were divided into three groups and immunized intramuscularly with 0, 100, and 500 μg of purified HisSpa1.0 in Freund’s complete adjuvant twice at 3-week intervals and 2 weeks later challenged intradermally with 4 × 107 Fujisawa (serotype 1a) organisms. Another four pigs were divided into two groups and immunized with 0 and 100 μg of purified HisSpa1.0 twice at 4-week intervals and 2 weeks later challenged with 8 × 107 82-875 (serotype 2b) bacteria. Dead pigs were autopsied on the day of death, and pigs that survived were euthanized and autopsied 1 week after challenge. Organs (heart, lung, liver, spleen, kidney, lymph nodes, and tonsils) and skin erythema lesions of all pigs were examined by bacterial isolation. Sera were collected from all pigs every week through the experiment, and antibody was titrated by double-antibody sandwich ELISA with intact SpaA, indirect ELISA with HisSpa1.0, and growth agglutination test.

Preparation of intact SpaA in alkaline extract of E. rhusiopathiae. E. rhusiopathiae Fujisawa was cultured in modified Feist broth at 37°C overnight (6). Cells were harvested and washed with distilled water, then resuspended in 10 mM NaOH at 61 g (wt wet)/ml and incubated at 4°C overnight with gentle shaking (7). After neutralization, the suspension was centrifuged at 110,000 rpm for 30 min; then the supernatant was sterilized by filtration and kept at −20°C. The extract was used in double-antibody sandwich ELISA.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (11) on 10% gels. After semidry electrophoretic transfer of the antigens to a nitrocellulose membrane, the membrane was blocked with 3% skim milk in PBS supplemented with 0.05% Tween 20 (PBS-Tw) for 30 min, incubated with pig antiserum diluted 1:100 with PBS containing 0.1% skim milk in PBS-Tw, washed with PBS-Tw for 3 times for 15 min, and then incubated with anti-pig immunoglobulin G (IgG) peroxidase conjugate (Rockland) diluted 1:1,000 with 1% skim milk in PBS-Tw for 1 h. Membrane was washed as described above and then developed with 1:12,000 (all dilutions were with 1% skim milk in PBS-Tw). Finally substrate solution (0.02% tetramethyl benzidine and 0.01% hydrogen peroxide in 0.1 M sodium phosphate–0.05 M citric acid buffer [pH 4.5]) was added, and the wells were developed for 60 min with 100 μl of substrate solution and 100 μl of 2 M H2O2. The absorbance was read at 450 nm.

RESULTS

In vitro phagocytosis assay. Peripheral blood was collected from an SPF pig not immunized against swine erysipelas. Mononuclear cells and neutrophils were isolated then and standard density gradient centrifugation on Ficoll-Paque (Pharmacia). The mononuclear cell fraction was washed and resuspended at 5 × 106/ml (monocytes = 105/ml) in 5 ml of RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum and 25 mM HEPES (pH 7.2) (RPMI-FCS). Monocytes were cultured as cells, and the mononuclear cells and were not separated from lymphocytes. Neutrophils were purified by ammonium chloride lysis of erythrocytes and after washing resuspended in 5 ml of RPMI-FCS at 106/ml. An over-night culture of Fujisawa was centrifuged and resuspended at 105/ml in 0.5 ml of a 1:1 mixture of inactivated pig serum, collected from an immunized pig or nonimmunized control pig at the time of challenge, and fresh germfree pig serum. The suspension was incubated at 37°C for 1 h with gentle agitation to opsonize the bacteria, then added to the each phagocyte suspension at a final concentration of 106 CFU/ml and incubated at 37°C with gentle shaking. After 30 min, the phagocytes were washed with medium twice, resuspended in 10 ml of RPMI-FCS containing penicillin (10 U/ml) to inhibit the growth of E. rhusiopathiae in the medium, and reincubated at 37°C. Each 1 ml of the suspension was collected after 0, 2, 4, and 18 h; 1 ml was used for Giemsa staining, and another 1 ml was used for counting the bacteria surviving in phagocytes.

PCR cloning of protective region of spaA. Each DNA fragment flanking the 1216-bp protective region of spaA was amplified by PCR from chromosomal DNA of Koganei (serotype 1a, Japanese official live vaccine strain), SE-9 (serotype 2a), Fujisawa (serotype 1a), and ATCC 19414T (serotype 2b) and cloned into pBlueScript II. DNA sequences of these fragments were determined by cycle sequencing on a model 3733S automated DNA sequencer (Applied Biosystems).

PCR-RFLP of spaAs of diverse serotypes. spaA sequence of almost full size (bp 16 to 1871) were amplified by PCR with a primer set designed from sequences of spaA/Fujisawa from chromosomal DNA of Fujisawa (serotype 1a), Koganei (serotype 1a), Fujisawa, and ATCC 19414T (serotype 2b) and E. rhusiopathiae reference strains of all 12 serotypes harboring this gene. Each 1 μg of purified PCR products was digested with restriction enzymes EcoRI, HpaII, KpnI, PstI, and SalI, respectively, and restriction fragment length polymorphism (RFLP) was analyzed by 1% agarose gel electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence of spaA/Fujisawa will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession no. AB019124. Nucleotide sequences of protective regions of spaA of Koganei, spaA/ATCC 19414T, and spaA/SE-9 will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession no. AB024082, AB024083, and AB024084, respectively.

FIG. 2. SDS-PAGE and immunoblot analysis of affinity purification of HisSpa1.0 on an Ni-NTA-agarose column. Lane 1, 1:1 mixture of inactivated pig serum; lane 2: empty extract; 3, purified protein eluted at pH 4.5. (A) Coomassie blue staining of polyacrylamide gel; (B) immunoblot detection with pig serum immunized with live E. rhusiopathiae.

Determination of protective region of spaA. The spaA/Fujisawa recombinant plasmids pA and pB had bp 1 to 1,294 of spaA in a 1.7-kb insert and bp 1 to 1,881 of full-length spaA in a 3.8-kb insert, as shown in Fig. 1. Analysis of Eco-Mung deletion mutants of them showed that an approximately 1.0 kb C-terminal region of the insert of pA was necessary to elicit protection in mice. This was confirmed by immunizing mice with 50 μg of purified HisSpa1.0 in complete adjuvant twice and successive challenge. After challenge, four of five mice survived.

Purification of truncated SpaA by affinity chromatography. The elution profile of HisSpa1.0 at each stage of the purification procedure analyzed by SDS-PAGE and immunoblotting (Fig. 2). Purified HisSpa1.0 showed the predicted molecular size of 45.5 kDa and was reactive with pig antisera immunized with E. rhusiopathiae. Both mouse and pig antisera against HisSpa1.0 reacted well with the 69.0-kDa intact SpaA in the alkaline extract and with a 43-kDa SpaA fragment in the culture supernatant of Fujisawa (data not shown).

Pig protection assay. All pigs immunized with purified HisSpa1.0 in Freund’s complete adjuvant were protected com-
isolation; (erythema)

Results for all body organs examined, otherwise indicated. –, no isolates by direct and enrichment culture; +, +++, few and numerous of colonies isolated; (+), isolation positive only by enrichment culture; NT, not tested (no erythema lesion).

**DISCUSSION**

In this study, we showed that purified fusions of truncated SpaA/Fujisawa, constructed with the N-terminal 342 amino acid sequences of diverse SpaAs. The DNA sequences of the protective regions of SpaAs of four strains of serotypes 1 and 2, Fujisawa, Koganei, SE-9, ATCC 19414T, and Tama, were almost identical, as shown by alignment of amino acid sequences (Fig. 5).

By PCR, all spaA genes of 16 strains of *E. rhusiopathiae* representing 12 serotypes were amplified well, and the sizes of all PCR products and restriction fragments were the same as for spaA/Fujisawa except in two strains, R32E11 and Kaparek. From these results, the nucleic acid sequences of diverse SpaAs seemed to be highly conserved among different serotypes (Fig. 6).

In R32E11 the PCR products and all C-terminal restriction fragments were about 300 bp longer, and in Kaparek they were about 100 bp shorter, compared to spaA/Fujisawa. Because these size variations were observed in the EcoRI C-terminal fragments of PCR products, encoding all C-terminal amino acid repeating units and four additional nucleic acids, we concluded that they reflect the number of amino acid repeating units as observed in Tama. These size variations of spaA agreed with the immunoblotting results for intact SpaAs (data not shown).

FIG. 3. Antibody response of pigs immunized with two injections of various doses of HisSpa1.0 at weeks 0 and 3 of the experiment. Sera were collected at 0, 1, 2, 3, and 5 weeks, and pigs were challenged at 5 weeks of the experiment. (A) Sandwich ELISA with intact SpaA; (B) indirect ELISA with HisSpa1.0; (C) growth agglutination test. Pigs 1 and 2, 0 µg; pigs 3 and 4, 100 µg; pigs 5 and 6, 500 µg.
acids (90 to 431) and histidine hexamer, could elicit complete protection in swine against challenge with virulent strains of *E. rhusiopathiae* of serotypes 1 and 2, and the C-terminal amino acid repeats of SpaA were not necessary for protection. In contrast, Makino et al. (13) emphasized the importance of the C-terminal amino acid repeats for protection, because in their study only recombinant *E. coli* expressing complete SpaA could elicit protection in mice. Although they created many Exo-Mung deletion derivatives, including two clones harboring complete *spaA*, they could not show protection with any of them (13). The contradiction in results can be attributed to the experimental method used by Makino et al. They immunized mice by intraperitoneal injection of a large dose of viable recombinant *E. coli*, and most mice died from endotoxin shock. In the paper they mentioned that their protection assay was very difficult to perform. It appears difficult to obtain reproducible results by their method.

The C-terminal 20-amino-acid repeat region of *E. rhusiopathiae* SpaA was shown by Makino et al. (13) to be necessary for SpaA to bind tightly to the bacterial surface like other gram-positive bacteria. This region has high sequence homology with the C-terminal amino acid repeats of pneumococcal surface protein A (PspA) (44.9% over 225 amino acids) and *Streptococcus pneumoniae* secretory IgA binding protein (SpA) (40.1% over 227 amino acids) (8, 35). On the other hand, the protective region of SpaA is located at the N-terminal region, like that of PspA. In PspA, epitopes eliciting protection in mice were present in the 43-kDa α-helical N-terminal half of the native 84-kDa molecule (27) and in amino acids 192 to 260 and 192 to 588 (14, 28). Despite the high diversity of the α-helical protective region of PspA (15, 22), a recombinant PspA of one strain can elicit cross-protection against pneumococci of different capsular types and PspA serological types (15, 28). In contrast to PspA, the DNA sequences of protective region of SpaA of *E. rhusiopathiae* of five strains of serotypes 1 and 2, the most important serotypes in pigs, were almost identical and highly conserved. Also, nucleic acid sequences of *spaA* of all serotypes of *E. rhusiopathiae* harboring this gene seemed to be well conserved when examined by PCR-RFLP. Although Makino et al. (13) showed strain differences of EcoRI fragment size in the *spaA* gene, such as 0.7 or 2 kb, by Southern hybridization, in this study all of these stains gave the same 0.7-kb fragment upon EcoRI digestion by PCR-RFLP. These results indicate that *spaA* genes are highly conserved among different serotypes of *E. rhusiopathiae* and this characteristic provides a major mechanism of the cross-protection activity of SpaA against challenge with different serotypes. Although the number of C-terminal amino acid repeats appears to vary among strains, this repeat has no role in protection.

Pigs immunized with recombinant truncated SpaA were

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FIG. 4. Effect of pig serum immunized with HisSpa1.0 on in vitro phagocytosis of monocytes (columns a and b) and neutrophils (columns c and d) of a nonimmunized SPF pig. (A) Percentage of cells showing the indicated phagocytosis score; (B) number of *E. rhusiopathiae* bacteria surviving in phagocytes. Bacteria were treated with control serum (columns a and c) or immunized serum (columns b and d) collected at the time of challenge.

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FIG. 5. Alignment of the 342-amino-acid sequences of protective regions (amino acids 76 to 438) of SpaA proteins of *E. rhusiopathiae* Fujisawa (serotype 1a), Koganet (1a), SE-9 (2a), ATCC 19414T (2a), and Tama-96 (2). Identical and different amino acids are marked with asterisks and dots, respectively.
completely protected against challenge with virulent strains of *E. rhusiopathiae*. By in vitro phagocytosis assay, we determined that the major protection mechanism in these pigs seemed to be the enhancement of the activity of neutrophils to phagocytose and kill the bacteria by effective opsonization with antibody produced against SpaA. On the other hand, the bacteria phagocytosed by monocytes of nonimmunized pigs tended to resist killing. This result suggested that activation of monocytes and macrophages may be also necessary for ready clearance of the bacteria. In immunized pigs, macrophages could be also activated readily after challenge.

The antibody response of all pigs immunized with HisSpa1.0 was sensitively detected by ELISA with intact SpaA/Fujisawa and HisSpa1.0 from 2 weeks after the first immunization. In contrast, the conventional growth agglutination test widely used in Japan did not detect the antibody response to HisSpa1.0, although the test is considered useful to assay the protective antibody in pigs (20). These results indicate that the growth agglutination test cannot directly detect the antibody response against SpaA, the protective antigen of *E. rhusiopathiae*, and can detect antibody responses against other antigens.

By immunoblotting analysis with pig antiserum against HisSpa1.0, it was shown that SpaA, like the 64- to 66-kDa protective protein described by Groschup and Timoney (6), was produced in larger amounts in modified Feist broth than in brain heart infusion, and SpaA was produced consistently in larger amounts by SE-9 than by Fujisawa.

In this study, we found that purified recombinant SpaA/Fujisawa can elicit protective immunity in pigs, the N-terminal 342 amino acids are necessary for protection in pigs, the nucleic acid sequences of this region are highly conserved among strains of serotypes 1 and 2, which are the most important serotypes in pigs, truncated SpaA of serotype 1 can elicit cross-protective immunity against challenge with serotype 2, and the sequences of full-size spaA also seemed to be highly conserved among all serotypes of *E. rhusiopathiae* harboring this gene. From these results, we conclude that this truncated SpaA may be useful for development of new types of vaccines such as component, vector, and DNA vaccines and of new diagnostic techniques such as ELISA to assay protective antibody of vaccinated pigs and maternal protective antibody of piglets.

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**REFERENCES**


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