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 1 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.

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*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, 26, 32, 34). Species other than *E. rhusiopathiae* are of low virulence in swine (25). Because of the importance of swine erysipelas, killed and attenuated live vaccines having been used extensively. However, despite widely practiced vaccination, the importance of this disease has not decreased (34). In Japan, annually about 2,000 pigs are affected with acute and subacute septicemia and about 2,000 pigs are condemned by meat inspection authorities because of arthritis.

There are many reports on the characterization of protective antigens of *E. rhusiopathiae*. A mouse protective antigen was identified in culture supernatant (30, 31) and in 10 mM NaOH extracts of bacterial cells (18, 19). Neuraminidase was also considered a protective antigen, because mice were protected by passive immunization with rabbit antiserum against *E. rhusiopathiae* neuraminidase (16). In 1 mM EDTA extract of T28 (serotype 2), one major polysaccharide capsular antigen of 14.4 to 22 kDa and two main protein antigens of 64 and 48 kDa were revealed by immunoblotting with rabbit antiserum (10). However, the protective activity of these antigens was not examined in the work reported. Groschup et al. (7) showed that protective antisera from pigs recognized prominent bands of 64 to 66 and 39 to 40 kDa in 1 mM EDTA and 10 mM NaOH extracts of T28. Both antigens were trypsin sensitive and contained no detectable polysaccharide. Mice immunized with preparations of the 64- 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 E1-6P (serotype 1a) (5). Guinea pig antiserum 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-acids repeat 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* for enzyme-linked immunosorbent assay (ELISA), and challenge of mice and pigs. For challenge of pigs, which is avirulent in mice, could confer complete protective immunity to mice (21).

**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* R2-875 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.

**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>
</tr>
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<tbody>
<tr>
<td><em>E. rhusiopathiae</em> (serotype)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fujisawa (1a)</td>
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<td>Koganei (1a)</td>
<td>Japanese official live vaccine strain</td>
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<td>SE-9 (2a)</td>
<td>U.S. official bacterin strain</td>
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<td>ATCC 19414T (2b)</td>
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<td>Marienfelde (1a)</td>
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<tr>
<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>
<td>Reference strains for serotyping</td>
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<td><strong>E. coli</strong> XL1-Blue</td>
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<td>plasmids</td>
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<td>Recombinant plasmids</td>
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<td>This study</td>
</tr>
<tr>
<td>pA1.0</td>
<td>Recombinant pQE32 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>
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**E. coli XL1-Blue**

<table>
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<th>Plasmids</th>
<th>Relevant characteristic</th>
<th>Reference or source</th>
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<td>This study</td>
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<tr>
<td>pA1.0</td>
<td>Recombinant pQE32 of <em>spaA/Fujisawa</em></td>
<td>This study</td>
</tr>
<tr>
<td>Recombinant <em>E. coli</em> XL1-Blue (pA1.0)</td>
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<td>This study</td>
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**Expression and purification of fusion protein.** A KpnI 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 KpnI site was located in spaA, and another 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 specified 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 (A600 = 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 buffer (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 filtered...
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 300 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 Fujiwasa subcutaneously 3 weeks after immunization. The infections were monitored for 12 days, and the cause of death was confirmed by isolation of the organism.

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 × 10³ Fujiwasa (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 × 10³ 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 titers were evaluated by double-antibody sandwich ELISA with intact SpaA, indirect ELISA with HisSpa1.0, and growth agglutination test.

Preparation of intact Spa in alkaline extract of E. rhusiopathiae. E. rhusiopathiae Fujiwasa 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 0.1 g (wet weight)/ml, and incubated at 20°C overnight with gentle shaking. After neutralization, the suspension was centrifuged at 10,000 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 0.1% Tween 80, 0.3% Tris, kanamycin, 5% Fetal calf serum and 25 mM HEPES (pH 7.2) (RPMI-FCS). Monocytes and neutrophils were harvested and washed with distilled water, then resuspended in 10 mM phosphate-buffered saline, pH 7.2 (PBS).

FIG. 2. SDS-PAGE and immunoblot analysis of affinity purification of HisSpa1.0 on an Ni-NTA-agarose column. Lane 1: extract; 2, dialyzed through fraction; 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.

RESULTS

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 comple-
TABLE 2. Protection of pigs immunized with HisSpa1.0 against challenge with Fujisawa (serotype 1a) and 82-875 (serotype 2b)

<table>
<thead>
<tr>
<th>Challenge strain</th>
<th>Immunization dose (µg, given twice)</th>
<th>Result for pig no. 1/pig no. 2</th>
<th>Symptom after challenge</th>
<th>Isolation of E. rhusiopathiae at necropsy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujisawa</td>
<td>0</td>
<td>Death/death</td>
<td>++++/++++</td>
<td>NT/NT (erythema)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-/-</td>
<td>+/ (+) (tonsils)</td>
<td>NT/(+) (erythema)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-/-</td>
<td>(+)/- (heart, lung, lymph nodes)</td>
<td></td>
</tr>
<tr>
<td>82-875</td>
<td>0</td>
<td>Septicemia/generalized erythema</td>
<td>++++/++++</td>
<td>NT/NT (erythema)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-/-</td>
<td>(+)/- (heart, lung, lymph nodes)</td>
<td></td>
</tr>
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</table>

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

completely against intradermal challenge with virulent strain Fujisawa (serotype 1a) and strain 82-875 (serotype 2b) (Table 2). They showed no clinical symptoms and no urticarial lesions at the injection site. Bacterial isolation 1 week after challenge was also negative by both direct culture and enrichment culture.

All two control pigs challenged with Fujisawa died from septicemia 3 to 4 days after challenge, and numerous E. rhusiopathiae bacteria were isolated from all body organs examined. In contrast, both of the control pigs challenged with 82-875 survived, although they showed systemic symptoms such as severe septicemia or generalized skin erythema. Many E. rhusiopathiae bacteria were isolated from the tonsils of these pigs by direct culture 1 week after challenge. By enrichment culture, E. rhusiopathiae was isolated from the spleen, heart, lungs, and lymph nodes of one pig which showed septicemia and from spleen and skin erythema lesions of another pig which showed generalized skin erythema.

The antibody response of all pigs immunized with HisSpa1.0 was sensitively detected by double-antibody sandwich ELISA using intact SpaA and by indirect ELISA using HisSpa1.0 from 2 weeks after the first immunization. The specificity of the antibody response was confirmed by immunoblotting. On the other hand, a conventional growth agglutination test could not detect the antibody response in pigs immunized with HisSpa1.0. The results are shown in Fig. 3.

**Effect of pig serum immunized with HisSpa1.0 on in vitro phagocytosis.** In vitro phagocytosis activity of swine neutrophils observed by Giemsa staining was significantly enhanced by opsonizing Fujisawa cells with pig antisera immunized with HisSpa1.0. Nearly 50% of neutrophils showed phagocytosis when the cells were opsonized, but only 20% showed phagocytosis when the cells were not opsonized. On the other hand, phagocytosis of monocytes was not affected by opsonization of the bacteria; 18 and 29% of monocytes showed phagocytosis.

In contrast, the number of live bacteria in neutrophils was much less than that in monocytes. Furthermore, upon opsonization of the cells, the number of live bacteria in neutrophils decreased significantly, from $10^5$ to less than $10^4$ CFU/ml. On the other hand, the viable bacterial count in monocytes was not affected by opsonization, being about $10^6$ CFU/ml regardless of opsonization. From these results (Fig. 4), we attributed the major protection mechanism of pigs immunized with HisSpa1.0 to the enhancement of bacterial killing activity of neutrophils by opsonizing the bacteria with antibody.

**DISCUSSION**

In this study, we showed that purified fusions of truncated SpaA/Fujisawa, constructed with the N-terminal 342 amino

**DNA sequence analysis 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 19414<sup>7</sup>, 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 spaAs agreed with the immunoblotting results for intact SpaAs (data not shown).
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 (SpaA) (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 strains 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.

**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 seemed to 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.

ACKNOWLEDGMENT

We thank T. Takahashi for providing *E. rhusiopathiae* 82-875.

REFERENCES


FIG. 6. RFLP analysis of PCR products of spaA (bp 16 to 1,871) of Fujisawa (serotype 1a), Koganei (1a), SE-9 (2a), ATCC 19414T (2b), and reference strains of *E. rhusiopathiae*, representing all 12 serotypes harboring the gene. Products of all strains but two showed the same restriction fragment size as Fujisawa with all enzymes used; products of R32E11 (2a) and Kaparek (9) gave about 300-bp longer and about 100-bp shorter C-terminal fragments, respectively, with all enzymes used. Restriction fragments showing size variation are indicated as checked boxes.


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