Genetic and Antigenic Diversity of the Surface Protective Antigen Proteins of *Erysipelothrix rhusiopathiae* ▼

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The surface protective antigen (Spa) protein of *Erysipelothrix rhusiopathiae* has been shown to be highly immunogenic and is a potential candidate for a new vaccine against erysipelas. In this study, we cloned and sequenced spa genes from all *E. rhusiopathiae* serovar reference strains as well as from a serovar 18 strain which was not classified as any species in the genus *Erysipelothrix*. Sequence analysis revealed that the Spa proteins could be classified into three molecular species, including SpaA, which was previously found in serovars 1a and 2, and the newly designated SpaB and SpaC proteins. The SpaA protein is produced by *E. rhusiopathiae* serovars 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, and N, the SpaB protein is produced by *E. rhusiopathiae* serovars 4, 6, 11, 19, and 21, and the SpaC protein is produced only by serovar 18. The amino acid sequence similarity was high among members of each Spa type (96 to 99%) but low between different Spa types (~60%). The greatest diversity in Spa proteins was found in the N-terminal half of the molecule (50 to 57% similarity), which was shown to be involved in immunoprotection. Coinciding with this, immunoblot analysis revealed that rabbit antisera specific to each Spa reacted strongly with the homologous Spa protein but weakly with heterologous Spa proteins. A mouse cross-protection study showed that the three recombinant Spa (rSpa) proteins elicited complete protection against challenge with homologous strains but that the level of protection against challenge with heterologous strains varied depending on the rSpa protein used for immunization. Our study is the first to demonstrate sequence and antigenic diversity in Spa proteins and to indicate that rSpaM may be the most promising antigen for use as a vaccine component because of its broad cross-protectiveness.

*Erysipelothrix rhusiopathiae* is a small gram-positive rod bacterium that causes erysipelas in swine and a variety of diseases in other animals, as well as eryspeloid, a skin disease of humans (20). *E. rhusiopathiae* was once thought to be the only species in the genus *Erysipelothrix* and was classified into 25 serovars based on peptidoglycan antigens of the cell wall. At present, the genus contains at least the following two species: *E. rhusiopathiae*, including serovars 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, and 21 and type N; and *Erysipelothrix tonsillarum*, including serovars 3, 7, 10, 14, 20, 22, and 23. Serovars 13 and 18 are unclassified but are considered to be assigned to genetically distinct groups from the above two species (15).

*E. rhusiopathiae* serovars 1 and 2 are most frequently isolated from swine with clinical erysipelas (11, 16), but other serovars of *E. rhusiopathiae* are occasionally isolated from swine with septicaemia, urticaria, arthritis, lymphadenitis, and endocarditis (17). Because of their high frequency of isolation, serovar 1a (Koganei 65-0.15) and serovar 2 (Tama-96) strains have been used to prepare live and killed vaccines, respectively, in Japan. Both vaccines elicit a cross-protective immune response in immunized pigs against challenge with *E. rhusiopathiae* serovars 1 and 2 (6), but it is not known whether they confer cross-protection against other *E. rhusiopathiae* serovars.

In swine erysipelas, antibodies against a cell surface component(s) of *E. rhusiopathiae* have been known to play an important role in protection. A 64- to 66-kDa cell surface antigen in Triton X-100 extracts of bacterial cells has been reported to be a protective molecule (2). Recently, a gene encoding surface protective antigen A (SpaA) was cloned from serovar Tama-96 (serovar 2) (9) and Fujisawa (serovar 1a) (14), and its nucleotide sequence was determined. The genetic region responsible for protective immunity in the SpaA molecule has also been identified (5, 14). Southern and immunoblot analyses showed that *E. rhusiopathiae* serovars 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17 and type N possess the spaA gene and express the SpaA protein (9); however, whether the remaining *E. rhusiopathiae* serovars, i.e., serovars 4, 6, 11, 19, and 21, can produce Spa proteins or not is still unclear.

In this study, we analyzed spa-related genes of all *E. rhusiopathiae* serovars and of an unclassified serovar 18 strain in the genus *Erysipelothrix* and found that three spa-related molecules are present in the genus *Erysipelothrix*. We then analyzed the immunological properties of the three Spa proteins, mainly focusing on their cross-protectivity, using a mouse model.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains used in this study included 16 strains of *E. rhusiopathiae* (Fujisawa, serovar 1a; 422/1E1, serovar 1b; ATCC 19414T, serovar 2; Doggerscharbe, serovar 4; Pe’cs 67, serovar 5; Dolphin E-1, serovar 6; Goda, serovar 8; Kaparek, serovar 9; IV128, serovar 11; Pe’cs 9, serovar 12; Pe’cs 3597, serovar 15; Tanzania, serovar 16; 545, serovar 17; 2017, serovar 19; Bâno 36, serovar 21; and MEW 22, type N). 5 strains of *E. tonsillarum* (ATCC 43339T, serovar 7; ATCC 43338, serovar 7; Lengyel-P, serovar 10; 2553, serovar 20; and Bâno 107, serovar 22), and two unclassified strains in the genus *Erysipelothrix* (Pe’cs 56, serovar 13; and 715, serovar 18). *E. rhusiopathiae* strains Fujisawa (serovar 1a), ATCC 19414T (sero-
var 2), Dolphin E-1 (serovar 6), and 715 (serovar 18) were used to challenge mice. The properties of the strains are described elsewhere (15). The vector plasmid pGEM-T Easy (Promega, Madison, WI) was used to clone spa genes. Protein expression vectors pQE9 and pQE30 (QIAGEN, Santa Clarita, CA) were used for the construction and expression of histidine-tagged fusion proteins. Escherichia coli XL1-Blue was used as the host strain for replication of these plasmids.

Esrypelotrix strains were grown in tryptose phosphate broth supplemented with 1% proteose peptone no. 3 (Difco Laboratories, Detroit, MI) and 0.1% Tween 80 (pH 7.8). Escherichia coli strains were grown in Luria-Bertani medium. When appropriate, the medium was supplemented with ampicillin (100 μg/ml) or isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM).

PCR amplification. Chromosomal DNAs of Esrypelotrix strains were prepared as described previously (19). The following primers were custom synthesized (Sawady Technology Co., Ltd., Tokyo, Japan): primer 1, 5′-AGGATCCATGAAAAAGAAAAAACACC TTCCATCATGTT-3′; primer 2, 5′-AGGATCCATGAAAAACAGAAAAACACCTATTTTCCGAGAATA-3′; and primer 4, 5′-GAAGCTTCTATTTTAAACTTC-3′. Primers 1 and 3 correspond to the sense strand at positions 2 to 1 and 1 to 33, respectively, of the spa gene of E. rhusiopathiae strain Fujisawa (14), with the addition of a new HindIII restriction site at the 5′ end. Primers 2 and 4 correspond to the antisense strand at positions 1881 to 1860 and 1881 to 1848, respectively, of the spa gene of strain Fujisawa, with the addition of a new HindIII restriction site at the 3′ end. Primers 1 and 2 were used for amplification of spaA from serovars 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, and N, and primers 3 and 4 were used for amplification of spaB from serovars 4, 6, 11, 19, and 21 and for amplification of spaC from serovar 18. PCR was performed essentially as described previously (10). Briefly, amplification products were subjected to an initial denaturation step at 94°C for 3 min and then to 25 cycles of denaturation at 94°C for 45 s, annealing at 55°C (primers 1 and 2) or 60°C (primers 3 and 4) for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 5 min.

Cloning and sequencing of spa genes. PCR products were ligated to the plasmid pGEM-T Easy and then transformed into Escherichia coli XL1-Blue by electroporation with a Gene Pulser and pulse controller set at 2.5 kV, 25 μF, and 1 μF. E. rhusiopathiae XL1-Blue was used as the host strain. The predicted molecular masses of the Spa proteins ranged from 69.9 to 76.9 kDa.

PCR amplification, cloning, and sequence analysis of spa genes. We designed several primer pairs and successfully amplified genes encoding putative Spa proteins from 16 serovars of E. rhusiopathiae and a serovar 18 strain by PCR (data not shown). No products were amplified when DNAs from serovars 1, 2, 4, 6, 5, 8, 11, 12, and 16 were used. The PCR products were cloned into the plasmid pGEM-T Easy to determine their nucleotide sequences. Sequence analysis of the amplified DNA fragments revealed that the sizes of the Spa nucleotide sequences of 17 Erypelotrix strains have been submitted to the DDBJ/EMBL/GenBank data base under accession no. AB259652 (strain Fujisawa [serovar 1a]), AB259653 (442/E1 [serovar 1b]), AB259654 (ATCC 19414T [serovar 2]), AB259655 (Pe′cs 67 [serovar 5]), AB259656 (Goda [serovar 9]), AB259657 (Pe′cs 9 [serovar 12]), AB259658 (Pe′cs 397 [serovar 15]), AB259659 (Tanzania [serovar 16]), AB259660 (545 [serovar 17]), AB259661 (MEW22 [serovar N]), AB238211 (Doggerscharbe [serovar 4]), AB238212 (Dolphin E-1 [serovar 6]), AB238213 (IV128 [serovar 11]), AB238214 (2017 [serovar 19]), AB238215 (Bano 36 [serovar 21]), and AB238210 (715 [serovar 18]).

RESULTS

PCR amplification, cloning, and sequence analysis of spa genes. We designed several primer pairs and successfully amplified genes encoding putative Spa proteins from 16 serovars of E. rhusiopathiae and a serovar 18 strain by PCR (data not shown). No products were amplified when DNAs from serovars 1, 2, 4, 6, 11, 12, and 16 were used. The PCR products were cloned into the plasmid pGEM-T Easy to determine their nucleotide sequences. Sequence analysis of the amplified DNA fragments revealed that the sizes of the Spa open reading frames (ORFs) ranged from 1,818 to 1,992 nucleotides (encoding 606 to 664 amino acids). The Spa ORFs varied in length depending on the E. rhusiopathiae serovar strain. The predicted molecular masses of the Spa proteins ranged from 69.9 to 76.9 kDa.

Based on their deduced amino acid sequence similarities, Spa proteins could be divided into three species of molecules (Table 1). We named the first Spa protein, from E. rhusiopathiae serovars 1a and 1b, SpaA, and the second Spa protein, from serovar 6, SpaB, and the third Spa protein, from serovar 18 only, SpaC.” The amino acid sequence similarities within each Spa type for various serovar strains were 96 to 99% for SpaA and 96 to 99% for SpaB. In contrast, the similarities between different Spa types were 61 to 64% (between SpaA and SpaB), 63 to 65% (between SpaA and SpaC), and 66 to 67% (between SpaB and SpaC), indicating that SpaA, -B, and -C are apparently differ-
ent molecular species. SpaA proteins contain 626 amino acids, with a deduced molecular mass of 72.3 kDa, with the exception of the SpaA protein of serovar 9, which contains 606 amino acids, with a deduced molecular mass of 69.9 kDa. SpaB and SpaC proteins contain 630 and 664 amino acids, respectively, with deduced molecular masses of 72.9 and 76.9 kDa, respectively.

Figure 1 shows a schematic diagram of the domain structures of the three Spa proteins. The 29-amino-acid signal sequence, immunoprotective domain, proline-rich region, and 20-amino-acid repeat domain are indicated by solid (black), open, stippled, and numbered boxes, respectively.

A phylogenetic tree was constructed based on the deduced amino acid sequences of Spa proteins (Fig. 2). The branch lengths between molecules are proportional to the similarities

### Table 1. Deduced amino acid sequence similarities among Spas from 17 *Erysipelothrix* strains

| Serovar of Spa | 1a | 1b | 2 | 5 | 8 | 9 | 12 | 15 | 17 | N | 4 | 6 | 11 | 19 | 21 | 18 |
|---------------|----|----|---|---|---|---|----|----|----|---|---|---|---|---|---|---|
| 1a            | 100|    |   |   |   |   |     |     |     |   |    |     |    |     |     |     |
| 1b            | 99 | 100|   |   |   |   |     |     |     |   |    |     |    |     |     |     |
| 2             | 98 | 98 | 100|   |   |   |     |     |     |   |    |     |    |     |     |     |
| 5             | 97 | 98 | 99 | 100|   |   |     |     |     |   |    |     |    |     |     |     |
| 8             | 99 | 98 | 97 | 96 | 98 | 100|     |     |     |   |    |     |    |     |     |     |
| 9             | 98 | 98 | 97 | 96 | 98 | 100|     |     |     |   |    |     |    |     |     |     |
| 12            | 99 | 99 | 98 | 99 | 98 | 100|     |     |     |   |    |     |    |     |     |     |
| 15            | 97 | 98 | 99 | 100| 98 | 96 | 98 | 100|     |   |    |     |    |     |     |     |
| 16            | 99 | 99 | 98 | 98 | 99 | 98 | 99 | 98 | 98 | 100|     |     |     |     |     |     |
| 17            | 98 | 98 | 97 | 97 | 99 | 97 | 99 | 97 | 98 | 100|     |     |     |     |     |     |
| 4             | 63 | 63 | 63 | 63 | 64 | 61 | 63 | 63 | 63 | 64 | 64 | 64 | 64 | 64 | 64 | 100|
| 6             | 63 | 63 | 63 | 63 | 64 | 61 | 63 | 63 | 63 | 64 | 64 | 64 | 64 | 64 | 64 | 100|
| 11            | 64 | 64 | 64 | 64 | 64 | 64 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 100|
| 19            | 62 | 63 | 62 | 63 | 63 | 61 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 100|
| 21            | 62 | 63 | 62 | 63 | 63 | 61 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 63 | 100|
| 18            | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 100|

* Values showing ≥96% amino acid sequence similarity are highlighted in bold.
of the sequences. This tree confirms that Spa proteins from 16 *E. rhusiopathiae* serovars and serovar 18 could be divided into three molecular species, namely, SpaA, SpaB, and SpaC.

**Expression and purification of rSpas.** To produce rSpas, ORFs encoding SpaA, -B, and -C were cloned into the expression vector pQE9 or pQE30. We chose Spa ORFs of serovars 1a and 6 as representatives to produce rSpaA and rSpaB, respectively. A Spa ORF from serovar 18 was used to produce rSpaC. Whether Spa ORFs were correctly inserted downstream of the promoter sequence was confirmed by nucleotide sequence analysis. His-tagged rSpa fusion proteins were expressed in *Escherichia coli* and purified with column chromatography on Ni-nitrilotriacetic acid resin.

SDS-PAGE analysis of purified rSpaA, rSpaB, and rSpaC showed single bands of approximately 73, 70, and 77 kDa, respectively (Fig. 3A). rSpaA and rSpaC migrated at their expected molecular masses, whereas the rSpaB protein showed a lower molecular mass than predicted. Aberrant migration of rSpaB might result from posttranslational modifications.

SDS-PAGE profiles of alkaline extracts of *E. rhusiopathiae* strains, which contained native SpaA, SpaB, and SpaC proteins, are shown in Fig. 3B. Major bands detected in the extracts of *E. rhusiopathiae* serovars 1a and 6 were located at the same positions as those of rSpaA and rSpaB, respectively. However, in the extract of serovar 18, only a faint band was detected at the same position as that of rSpaC, and a major band was located at a lower-molecular-weight position than that of rSpaC. These results indicated that the alkaline extracts of *E. rhusiopathiae* strains contained mainly native Spa proteins and that SpaC is easily degraded.

**Reactivity of anti-rSpa sera to homologous and heterologous Spa proteins.** To examine whether all 16 *E. rhusiopathiae* serovars and the serovar 18 strain express Spas, rabbit antisera specific to rSpaA, rSpaB, and rSpaC were incubated with whole-cell lysates prepared from homologous serovar strains by immunoblotting. i.e., the rSpaA antiserum was incubated with lysates of serovars 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, and N; the rSpaB antiserum was incubated with those of serovars 4, 6, 11, 19, and 21; and the rSpaC antiserum was incubated with that of serovar 18. The results showed that all antisera specific to each Spa protein recognized an approximately 70-kDa protein antigen, which corresponds to the expected molecular mass of the Spas, in the lysates of 16 *E. rhusiopathiae* serovars and the serovar 18 strain (Fig. 4). This result demonstrates that all serovars of *E. rhusiopathiae* produce at least one of the Spa proteins.

Next, to analyze the cross-reactivity among the three Spa proteins, rabbit antisera specific to rSpaA, rSpaB, and rSpaC were incubated with homologous and heterologous Spas (both recombinant and native) by immunoblotting. When rSpases were used as antigens, anti-rSpa sera reacted strongly with homologous rSpases but weakly with heterologous rSpas (Fig. 5A). This lower reactivity to heterologous Spa proteins than to homologous Spa protein was more clearly demonstrated when native Spa proteins were used as antigens. Anti-rSpaA and anti-

**FIG. 2.** Phylogenetic tree constructed from the deduced amino acid sequences of Spa proteins from 16 *E. rhusiopathiae* serovars and one unclassified serovar in the genus *Erysipelothrix*. The designations shown to the right of the tree show serovars. A scale bar is shown at the bottom. The tree was constructed based on the neighbor-joining method. Bootstrap proportions were plotted at the main internal branches of the phylogram to show support values.

**FIG. 3.** SDS-PAGE profiles of purified rSpa proteins and alkaline extracts of *E. rhusiopathiae* strains containing native Spa proteins. The gels were stained for protein with Coomassie brilliant blue. (A) Purified recombinant proteins. Lane 1, rSpaA protein prepared from the spaA gene of strain Fujisawa (serovar 1a); lane 2, rSpaB protein prepared from the spaB gene of strain Dolphin E-1 (serovar 6); lane 3, rSpaC protein prepared from the spaC gene of strain 715 (serovar 18); lane M, molecular masses in kilodaltons. (B) Alkaline extracts of *E. rhusiopathiae* strains Fujisawa (serovar 1a: lane 1’) and Dolphin E-1 (serovar 6: lane 2’) and the unclassified *Erysipelothrix* strain 715 (serovar 18: lane 3’).
rSpaB sera reacted strongly with their homologous native SpaA and SpaB proteins (Fig. 5b, panel A, lane 1/H11032, and panel B, lane 2/H11032, respectively), in contrast to the weak reactivity observed with heterologous native Spas; however, anti-rSpaC antiserum reacted strongly with the native SpaC protein (Fig. 5b, panel C, lane 3/H11032) and even reacted with heterologous SpaA and SpaB proteins, with a moderate degree of intensity (Fig. 5b, panel C, lanes 1/H11032 and 2/H11032, respectively). In addition, the native SpaC fraction was found to contain several antigenic proteins, with molecular masses of 60, 45, 36, and 30 kDa, which appeared to be degradation products of the intact SpaC protein. An alignment of deduced amino acid sequences of SpaA (from serovar 1a strain Fujisawa), SpaB (from serovar 6 strain Dolphin E-1), and SpaC (from serovar 18 strain 715) is shown in Fig. 6. These results suggest that the heterogeneity in the primary sequences of the three representative Spas might contribute to differences in cross-reactivity.

**Cross-protection study using rSpas as immunogens.** To examine whether cross-protection could be induced by immunization with different rSpas as antigens, we injected each rSpa protein twice into mice and challenged the mice with three *E. rhusiopathiae* strains expressing homologous and heterologous Spas to the immunizing rSpa.

After challenge with the serovar 1a strain Fujisawa, which expresses SpaA, all nonimmunized control mice died within 4 days (Fig. 7A). In contrast, all mice immunized with rSpaA (homologous) survived until 14 days, when the experiment was terminated. The difference between the rSpaA-immunized group and the control group was significant (*P* = 0.0000). Only 4 of 10 (40%) mice in the rSpaB (heterologous)-immunized group survived (*P* = 0.0433 [significant]), indicating that the protection conferred by immunization with rSpaB was partial. In the rSpaC (heterologous)-immunized group, 9 of 10 (90%) mice survived, showing that rSpaC induced efficient protection against challenge with strain Fujisawa (*P* < 0.0001 [significant]). Similar results were observed when mice were challenged with the serovar 2 strain ATCC 19414T, which expresses...
SpA: 100%, 50%, and 90% of mice in the rSpA-, rSpB-, and rSpC-immunized groups, respectively, survived under the challenge conditions where all mice in the nonimmunized control group died (Fig. 7B).

When challenged with the serovar 6 strain Dolphin E-1, which expresses SpaB, all of the nonimmunized control mice died within 3 days (Fig. 7C), whereas all mice immunized with rSpaB (homologous) survived ($P = 0.0000$ [significant]). Only 5 of 10 mice (50%) in the SpaA (heterologous)-immunized group survived ($P = 0.0163$ [significant]); however, 9 of 10 mice in the SpaC (heterologous)-immunized group survived ($P < 0.0001$ [significant]).

After challenge with the serovar 18 strain 715, which expresses SpaC, all nonimmunized control mice died within 6 days (Fig. 7D), whereas all mice immunized with rSpaC (homologous) survived. Seven of 10 mice (70%) in the SpaA (heterologous)-immunized group survived ($P = 0.0163$ [significant]); however, 9 of 10 mice in the SpaC (heterologous)-immunized group survived ($P < 0.0001$ [significant]).

After challenge with the serovar 18 strain 715, which expresses SpaC, all nonimmunized control mice died within 6 days (Fig. 7D), whereas all mice immunized with rSpaC (homologous) survived. Seven of 10 mice (70%) in the SpaA (heterologous)-immunized group survived ($P = 0.0163$ [significant]); however, 9 of 10 mice in the SpaC (heterologous)-immunized group survived ($P < 0.0001$ [significant]).

These results indicate that all rSpas have potent protective immunogenicity against challenge with E. rhusiopathiae strains expressing homologous Spas; however, the levels of immunogenicity of the rSpas were variable against E. rhusiopathiae strains producing heterologous Spas. These data also demonstrate that the rSpaC protein is the most potent cross-protective antigen in this mouse challenge model.

**DISCUSSION**

The Spa protein of E. rhusiopathiae has been shown to be localized at the bacterial cell surface and to be a potent protective antigen against E. rhusiopathiae infection (5, 14). The present study is the first to describe genetic and antigenic diversity in the Spa proteins of 16 E. rhusiopathiae serovars and one unclassified serovar in the genus Erysipelothrix. Our results demonstrate that (i) Spa proteins can be classified into three molecular species, named SpaA, SpaB, and SpaC, based on their amino acid sequence similarities; (ii) the three Spa proteins are antigenically different, as antisera reacted strongly with homologous Spas but reacted moderately or weakly with heterologous Spas; and (iii) that the degree of cross-protection conferred in mouse immunoprotection tests by immunization with each Spa protein varies among Spas and that SpaC is the most broadly cross-protective antigen among the three Spa proteins.

SpA was previously identified and characterized by Makino et al. (9), using E. rhusiopathiae strain Tama 96 (serovar 2), and by Shimoji et al. (14), using strain Fujisawa (serovar 1a). Makino et al. found that SpaA is produced by only 11 (1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17, and N) of 16 E. rhusiopathiae serovars. However, when the remaining five serovars (4, 6, 11, 19, and 21) were used as samples, a weak positive reaction was shown by enzyme-linked immunosorbent assay with a monoclonal antibody specific to SpaA, but no positive signal was detected by immunoblotting with the monoclonal antibody and Southern blot analysis using the spaA gene as a probe; thus, they concluded that these five serovar strains do not produce SpaA. In the present study, we demonstrated that these five serovar strains produce SpaB, which shows approximately 60% homology to SpaA but is apparently a distinct and novel molecule. Consequently, all E. rhusiopathiae serovar strains produce at least one Spa protein, SpaA or SpaB, as we showed in this study (Fig. 4). In contrast, we could not detect any spa-related genes by PCR or any Spa-related proteins by immunoblot analysis in E. tonsillarum serovar strains (data not shown). This finding suggests that Spa proteins may be virulence factors specifically involved in the pathogenesis of E. rhusiopathiae, and from a practical point of view, the presence or absence of spa may be a suitable marker for the differentiation of E. rhusiopathiae and E. tonsillarum.

Regions or stretches of sequence conservation among Spas are likely to represent domains of protein constrained by a common function. In our alignment of Spas from 17 Erysipe-
lothrix strains, high sequence identity was found within the signal and repetitive amino acid regions. In particular, the amino acid sequences of the signal sequence regions, located at the most N-terminal part of the Spas, were completely identical in the Spa proteins. These data suggest that Spa proteins may cross bacterial cell membranes and eventually be secreted from the bacterial cell by using a secretion machinery common to E. rhusiopathiae strains. The regions containing 20-amino-acid tandem repeats are also structurally similar among the three Spas. The repeat region has been suggested to function as an anchor for binding Spa proteins tightly to the bacterial surface (8), as it was found in other surface proteins of gram-positive bacteria (1, 4, 22). This suggests that the anchoring mechanism of the bacterial cell surface protein may be common not only to E. rhusiopathiae strains but also to other gram-positive pathogenic bacteria.

Sequence diversity among Spa proteins was largely confined to the α-helical N-terminal half, which could be defined as a hypervariable domain (~50% identity). Importantly, the N-terminal half has been found to play a major role in immunoprotection against E. rhusiopathiae infection (5, 14). In fact, immunoblot analysis showed that antisera raised against rSpaA, rSpaB, or rSpaC reacted strongly with homologous Spas but moderately or weakly with heterologous Spas when native Spas in E. rhusiopathiae whole-cell extracts as well as rSpas purified from recombinant Escherichia coli were used as antigens. Various degrees of cross-protection among different E. rhusiopathiae serovars have been described (13). In our cross-protection study, mice immunized with rSpaA, rSpaB, or rSpaC were completely protected against homologous challenge, but the degree of cross-protection varied for heterologous challenge. However, it was noteworthy that rSpaC induced good protection against even heterologous strains expressing SpaA or SpaB. This high degree of cross-protection induced by immunization with SpaC was unexpected because of the high sequence diversity in the protection domain found between SpaC and other Spa proteins. One possible explanation is that short linear epitopes which are similar between SpaC and other Spas may exist in the protective domain. Another possible explanation is that conformational epitopes in SpaC which work as cross-protective epitopes may exist. Further studies concerning the identification and characterization of epitopes which may be present in Spa molecules should be performed to clarify why the SpaC protein can induce cross-protection in immunized animals.

Our results showed that serovar 18 is a unique serovar which produces SpaC. At present, the genus Erysipelothrix contains at least two species, E. rhusiopathiae and E. tassilum. Serovar 18 strains, however, have not been assigned to these two species and are considered to be classified in a genetically distinct group, based on the observation that they hybridize at low levels with the type strains of E. rhusiopathiae and E. tassilum (15). The serovar 18 reference strain 715, together with other serovar 18 strains, has been shown to be virulent for mice and swine (21), raising the possibility that SpaC plays some role in their virulence. The function of SpaC, as well as those of SpaA and SpaB, and its possible role in E. rhusiopathiae virulence should be elucidated by analyzing a Spa gene knockout mutant.

The present findings concerning the diversity of Spa proteins in the genus Erysipelothrix may shed new light on the pathogenesis of Erysipelothrix species and may help to classify various serovar strains in the genus Erysipelothrix. Currently, serovars 18 and 13 are not classified into species. We found that a serovar 13 strain did not have spa-related genes or produce any Spa proteins, as detected by PCR and immunoblot analysis (data not shown). This is an important characteristic for discriminating serovar 13 strains from serovar 18 or other E. rhusiopathiae serovar strains. The finding that the spa-related genes and their products are found only in E. rhusiopathiae species, not in E. tassilum species, may be applied to the development of a spa gene-targeted PCR assay and an rSpa antigen-based serodiagnostic test for differential diagnosis. Most importantly, Spa proteins have been shown to be potent immunogens and thus may become good candidates for a vaccine component. This study revealed that rSpaC can induce broad cross-protectiveness against challenge with E. rhusiopathiae serovar strains producing different Spas; therefore, a component vaccine containing rSpaC or a more advanced chimeric vector vaccine producing rSpaC may be useful for the eradication of erysipelas from swine herds.

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