Role of Surface Protective Antigen A in the Pathogenesis of Erysipelothrix rhusiopathiae Strain C43065

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Introduction

Erysipelothrix rhusiopathiae is a facultative, non-spore-forming, small gram-positive bacillus that causes erysipelas in a variety of wild and domestic animals, including swine, sheep, birds, and fish, as well as erysipeloid in humans [27]. In current taxonomy, the genus Erysipelothrix contains two main species, E. rhusiopathiae and E. tonsillarum. Each species consists of various serotypes, including serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 10, 11, 12, 15, 16, 17, 19, 21, and N in E. rhusiopathiae, and serotypes 3, 7, 10, 14, 20, 22, and 23 in E. tonsillarum [25, 27]. Serotypes 13 and 18 are two unclassified groups considered genetically distinct from both E. rhusiopathiae and E. tonsillarum [23]. Based on DNA-DNA hybridization analysis of numerous isolates from different organisms, E. rhusiopathiae was considered to be the pathogenic species of the genus [24]. In particular, serotypes 1 and 2 are most frequently isolated from pigs with clinical erysipelas [16] and are commonly used to develop live or killed vaccines against infection.

Surface protective antigens of gram-positive bacteria have been implicated in both virulence and protection during bacterial infection [10, 27]. Among several cell surface proteins identified in E. rhusiopathiae, SpaA has been shown to be a 64 kDa surface protective antigen associated with the induction of highly protective antibodies against E. rhusiopathiae after infection in both mice and pigs [5, 14, 17, 28]. Structural and sequence analyses have revealed that the C-terminal region of SpaA is very similar to the choline-binding proteins of Streptococcus pneumoniae, which can bind to choline residues of teichoic acid [8]. SpaA has been shown to form a complex by binding to

To clarify the role of surface protective antigen A (SpaA) in the pathogenesis of Erysipelothrix rhusiopathiae C43065 (serotype 2), the spaA deletion mutant of E. rhusiopathiae ΔspaA was constructed by homologous recombination. The virulence of the ΔspaA mutant decreased more than 76-fold compared with that of the wild-type strain C43065 in mice. The mutant strain was sensitive to the bactericidal action of swine serum, whereas the wild-type strain was resistant. The adhesion of wild-type strain to MEF cells was inhibited significantly by treatment with rabbit antiserum against recombinant SpaA (rSpaA) as compared with the treatment with normal rabbit serum, but the mutant strain was not affected. The mutant strain was readily taken up by mouse peritoneal macrophages in the normal rabbit serum, whereas the wild-type strain was resistant. Whereas the rabbit antiserum against rSpaA promoted the phagocytosis of wild-type strain by macrophages, the mutant strain was not affected. In addition, mice vaccinated with the formalin-killed mutant strain were provided 40% protection against challenge by the homologous virulent strain as compared with those with wild-type strain, NaOH-extracted antigen, or rSpaA, which provided more than 80% protection against the same infection. These suggested that SpaA has an important role in the pathogenesis of E. rhusiopathiae infection and could be a target for vaccination against swine erysipelas.

Keywords: Erysipelothrix rhusiopathiae, surface protective antigen A, homologous recombination, virulence, protection
lipoteichoic acid (LTA) in Bacillus subtilis and Staphylococcus aureus, suggesting an important role in the bacterial infection process [15]. Recently, the SpaA-mediated adhesion of E. rhusiopathiae to porcine endothelial cells was demonstrated [6]. These studies suggested that SpaA is involved in E. rhusiopathiae infection and virulence. However, the role of SpaA in bacterial virulence is entirely unknown. In this study, we generated a spaA-deleted mutant from a highly virulent strain, C43065 (serotype 2), which is responsible for the majority of swine erysipelas in China, to examine its particular virulence and protective capacities.

Materials and Methods

Bacterial Strains and Plasmids

E. rhusiopathiae strain C43065 (serotype 2) was originally isolated from an infected pig in Shanxi Province, China and was purchased from the Chinese Veterinary Culture Collection Center. E. rhusiopathiae strains were cultured in brain heart infusion (BHI) broth or BHI agar (Difco Laboratories, Detroit, MI, USA) plate supplemented with 0.1% Tween 80 (BHI-T). E. coli DH5α and M15 containing recombinant plasmid were cultured in Luria broth (LB) or LB agar (Difco) plates at 37°C. When required, both broth and agar were supplemented with ampicillin (100 µg/ml) or tetracycline (5 µg/ml for E. coli and 2.5 µg/ml for E. rhusiopathiae). Plasmids pBR322 and pUC18 were purchased from TaKaRa Biotechnology Co., Ltd., Dalian, China. The prokaryotic expression plasmid pQE-30 and its host M15 [pREP4] were purchased from Qiagen, Valencia, CA, USA.

Cloning, Expression, and Purification of Recombinant SpaA

Genomic DNA of E. rhusiopathiae C43065 was isolated by using a bacterial genomic DNA extraction kit (TaKaRa). The spaA gene, in the absence of the putative signal peptide coding sequence, was amplified using PCR primers P-1 and P-2 from the genomic DNA of C43065 (Table 1). The PCR product was inserted into the pQE-30 vector between the NdeI and HindIII restriction sites. The pQE-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>Restriction site</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>CGCGGATCCGATCCGACGATATTTTC</td>
<td>BamHI</td>
<td>88-104&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P2</td>
<td>CGCAAGCTTCTATTTTTAAGCTCCATC</td>
<td>HindIII</td>
<td>1881-1864&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>P3</td>
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<td>SamI</td>
<td>1-19&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>P4</td>
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<td>HindIII</td>
<td>1881-1861&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td>1293-1273&lt;sup&gt;d&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Position relative to the sequence deposited in GenBank (EF688317); <sup>b</sup>Position relative to the sequence deposited in GenBank (J01749); <sup>c</sup>Underlined sequence refers to the restriction sites.

Preparation of Rabbit Antiserum Against Recombinant SpaA

Antiserum against purified rSpaA was prepared by immunization of adult male New Zealand white rabbits (SLAC Laboratory Animal Co., Ltd., Hunan, China). Rabbits were subcutaneously injected with 200 µg of protein emulsified in an equal volume of complete Freund’s adjuvant (Sigma, St. Louis, MO, USA). The injection was repeated three times at 30-day intervals with the antigen emulsified in incomplete Freund’s adjuvant (Sigma). Blood was collected 7 days after the final injection. Antiserum was absorbed with whole-cell lysates of E. coli M15 [pREP4] to remove nonspecific antibodies, as described previously [13]. The NaOH-extracted antigen of C43065 was prepared according to a method described previously [11]. The purified rSpaA and NaOH-extracted antigens were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. The gel for separated proteins was transferred to a nitrocellulose membrane (Amersham Biosciences KK, UK) by a semi-dry system. The SpaA protein was probed with the primary antibody from rabbit antiserum against rSpaA, followed by incubation with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) (Sigma). Protein bands were visualized by incubation with 3, 3’-diaminobenzidine solution (Sigma).

Construction of Targeting Vector pUC18ΔspaA

PCR primers for spaA were designed based on the gene sequence of E. rhusiopathiae C43065, whereas the PCR primers Tet-1 and Tet-2 were designed based on the sequence of the tetracycline resistance cassette (Tet'f) in pBR322. These primers were custom synthesized with an oligonucleotide synthesizer (TaKaRa). The primer sequences are listed in Table 1. The 1,881 bp DNA fragment

spA was transformed into E. coli DH5α, and then transferred into E. coli M15 [pREP4] for expression. The recombinant protein was purified by nickel-implunodiacetic acid (Ni-IDA) affinity chromatography (Sangon Biotechnology Co., Ltd., Shanghai, China) following induction with isopropyl-β-D-1-thiogalactopyranoside (TaKaRa). The concentration of recombinant SpaA protein was determined by Bio-Rad assay.

Table 1. Primer sequences used for construction and expression plasmids and targeting vector.

of spaA was amplified from the genomic DNA of C43065 by PCR using primers P-3 and P-4 (Table 1), which was inserted between the Smal and HindIII restriction sites of pUC18 to generate pUC18-spaA. The 1,191 bp DNA fragment of the tetracycline-resistant cassette (Tet') was amplified from pBR322 by PCR using primers Tet-1 and Tet-2 (Table 1). After digestion with PstI and Fhal, the Tet gene was inserted into pUC18-spaA to generate the targeting vector pUC18ΔspaA (Fig. 2A). The targeting vector pUC18ΔspaA was analyzed by restriction enzymes and DNA sequencing.

**Construction and Analysis of spaA Deletion Mutant**

*E. rhusiopathiae* competent cells were prepared for electroporation according to the method described previously [21]. Briefly, 10 µg of DNA from the targeting vector pUC18ΔspaA containing the mutated spaA gene was transformed into the wild-type strain C43065 by electroporation using the Gene Pulser Xcell Electroporation System (Bio-Rad, Laboratories, Inc., Hercules, USA). After electroporation, the bacteria were incubated at 37°C for 2 h without antibiotic selection and then plated onto BHI-T plates containing 2.5 µg/ml of tetracycline. After incubation at 37°C for 48 h, the tetracycline-resistant transformants were screened for the spaA mutant by colony PCR using the spaA-specific primers IN-1 and IN-2 (Table 1).

The spaA deletion mutant was derived by replacing a 691 bp DNA fragment with the Tet' cassette in strain C43065. The mutant locus in the genomic DNA of the spaA deletion mutant was identified by PCR using primer pairs P-3/P-4, Tet-1/Tet-2, and IN-1/IN-2 (Table 1). The PCR products were cloned into the pMD18-T vector using *E. coli* DH5α. The sequences of the inserts in pMD18-T were determined to confirm the incorporation of the mutant locus.

**Confirmation of spaA Deletion Mutant by RT-PCR and Western Blotting**

The *E. rhusiopathiae* strains were grown to an optical density value of 0.5 at 600 nm, and the total RNA was isolated using the RNAPrep pure cell/bacteria kit (Tiangen Biotechnology Co., Ltd., Beijing, China) per the manufacturer’s instructions. The extracted RNA was treated with DNase to eliminate any contaminating DNA. cDNA was then synthesized using the random octamers provided in the quantscript first-strand synthesis kit (Tiangen) for RT-PCR per the manufacturer’s instructions. The primers used for RT-PCR analysis were IN-1 and IN-2 (Table 1). PCR products on the RT-PCR analysis were IN-1 and IN-2 (Table 1). The RT-PCR per the manufacturer’s instructions. The primers used for RT-PCR analysis were IN-1 and IN-2 (Table 1). The RT-PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The RT-PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

**Virulence of spaA Deletion Mutant in Mice**

The spaA deletion mutant and wild-type strain C43065 were separately inoculated in BHI-T broth and cultured at 37°C until reaching log phase growth. Serial 10-fold dilutions were prepared in sterile PBS (pH 7.4) and plated on agar plates for quantification. For virulence testing, 0.1 ml of the serially diluted cultures was injected intramuscularly into 7-week-old female BALB/c mice (SLAC) to obtain the 50% lethal dosage (LD50). The infected mice were observed daily, and their mortality rate was recorded.

**Serum Resistance Assay**

Normal porcine serum consisted of a poll of sera collected from a healthy piglet (3–4 weeks old) from a farm free of swine erysipelas. The serum was filter sterilized (0.22 µm) and aliquots were stored at −80°C. Some aliquots of serum were treated at 96°C for 30 min to inactivate complement. Bacterial testing was performed as described previously [4]. Briefly, bacterial suspension (10^6 CFUs/ml) were incubated with either fresh porcine serum or with heat-inactivated porcine serum at a ratio of 1:9 for 3 h, and then appropriate dilutions of the bacteria were spotted onto BHI-T plates or LB plates. After incubation for 24 h, the numbers of bacteria on each plate were counted. Tests were performed in triplicate for *E. rhusiopathiae* strains and *E. coli* DH5α.

**Adhesion Assay**

Mouse embryonic fibroblast (MEF) cells were obtained according to standard procedures [9] from 12- day-old embryos of BALB/c mice (SLAC). The adhesion assay was performed as described by Boorathybay et al. [2]. Briefly, MEF cells (3 × 10^5 cells/ml) were seeded onto 24-well tissue culture plates in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 µg/ml of streptomycin, and 100 µg/ml of penicillin. Cells were cultured at 37°C in a humidified incubator with 5% CO2 for 24 h, washed three times with PBS, and then infected with approximately 10^5 CFUs/ml of *E. rhusiopathiae* strains. After incubation at 37°C for 1 h, one set of tissue-cultured cells was washed, fixed, and stained with Giemsa solution for microscopic evaluation, and the other three sets of cells were washed, lysed with 0.1% Triton X-100 in PBS (pH 7.4), and plated onto BHI-T plates for quantification of the number of bacterial cells. Adherence data were expressed as CFUs/ml of the bacterial inoculums.
Adhesion Inhibition Assay
To determine whether SpaA was involved in the adherence of *E. rhusiopathiae* to MEF cells, bacterial cells (10^8 CFUs/ml) were sensitized with rabbit antiserum against rSpaA at 37°C for 1 h. Sensitization with normal rabbit serum was done as the control. After sensitization by serum, the bacterial cells were washed in the same manner as described above and used for the adhesion assay. Adherence data were expressed as CFU/ml of the bacterial inoculums.

Phagocytosis Assay
Peritoneal macrophage cells of BALB/c mice were isolated using the method of Kumagai et al. [12]. A phagocytosis assay was performed as described previously [22]. Briefly, bacterial cells (10^7 CFUs) were sensitized with rabbit antiserum against recombinant SpaA or with normal rabbit serum at 37°C for 10 min, washed with RPMI1640 medium three times, and then resuspended in RPMI1640 medium containing 10% fetal calf serum. Subsequently, the bacterial suspensions were incubated with rotation at 37°C for 1 h with mouse peritoneal macrophages (3 × 10^6 cells). Phagocytosis was stopped by shaking the tubes in ice, and the uningested bacterial were removed by washing with cold PBS containing 5% fetal calf serum. Cytospin smears were prepared, stained with Giemsa’s solution, and then observed by light microscopy under oil immersion. The experiment was repeated three times, and the results are expressed as the phagocytosis index, which is defined as the percentage of bacterium-ingesting macrophages times the average number of bacteria in a macrophage times 100.

Protection of spaA Deletion Mutant in Mice
The *E. rhusiopathiae* strains were cultured in BHI-T broth containing 10% horse serum at 37°C for 24 h, yielding approximately 2 × 10^9 CFUs total bacteria per 10 ml. Bacteria were killed by adding formaldehyde at a final concentration of 0.5% (v/v). This bacterial suspension was incubated at room temperature on a rotator for 48 h and then centrifuged. The collected bacterial cells were adjusted to a concentration of 3 × 10^8 CFUs/ml in sterile PBS (pH 7.2). The immunogens were prepared at appropriate concentrations and completely emulsified with an equal volume of Freund’s incomplete adjuvant (Sigma) and kept at 4°C before usage. The dosages of each immunogen are shown in Table 3. For protection tests, the 7-week-old BALB/c mice of each group were subcutaneously inoculated with 0.1 ml of each immunogen twice per dose at 2-week intervals. Two weeks after the second vaccination, all five groups of mice were challenged with 0.1 ml of bacterial suspension containing 9 × 10^7 CFUs of the wild-type C43065 strain. The mice were observed for 10 days post-challenge. Seven days after the second vaccination, five mice from each group were bled from the eye, and their antibody titers were determined by indirect ELISA. All animal procedures were performed under the ethical guidelines of Jishou University.

Statistical Analysis
A two-tailed Student’s *t*-test was used to determine the significant differences between the control and exposure groups. Statistical analysis was performed using SPSS 18.0 software, and *p* < 0.05 was considered to be statistically significant.

Results

Expression and Purification of Recombinant SpaA
The recombinant SpaA (rSpaA) protein was expressed by the plasmid pQE-spaA, containing the *spaA*-coding sequence from *E. rhusiopathiae* C43065 tagged with 6-histidine (at N-terminal), in *E. coli* M15. As shown in Fig. 1, native SpaA appeared in NaOH-extracted antigen of C43065, whole-cell proteins of rSpaA-expressing bacteria, and nickel-purified samples at approximately 64 kDa by SDS-PAGE and Coomassie staining. Consistently, both native SpaA and rSpaA were detected at their corresponding positions by western blot analysis using antiserum against the rSpaA protein (Fig. 1). These results indicated that the rSpaA protein was expressed with high immunogenicity in *E. coli* M15.

Construction and Analysis of spaA Deletion Mutant
To make the targeting vector pUC18ΔspaA, the *spaA* gene of C43065, derived by the deletion of a 691 bp fragment replaced with a tetracycline-resistant cassette

![Fig. 1. Expression, purification, and examination of rSpA in E. coli M15.](image-url)
(Tet') flanked by the homologous arms of endogenous spaA, was cloned into pUC18 for homologous recombination (Fig. 2A). The pattern of restriction analysis confirmed the presence of the 1,191 bp tetracycline-resistant cassette, the 616 bp upstream homologous arm, and the 574 bp downstream homologous arm in the targeting vector (Fig. 2B). Furthermore, sequencing results confirmed that each part of the targeting vector was correct (data not shown). These results validate the pUC18ΔspaA targeting vector.

The targeting vector pUC18ΔspaA was transformed into the wild-type strain C43065 by electroporation. Through homologous recombination, a 691 bp fragment of the endogenous spaA gene was replaced by the 1,191 bp Tet’ gene, which also served as a positive selection marker for the spaA deletion mutant. Through selection, two tetracycline-resistant transformants were obtained, followed by colony PCR confirmation using spaA-specific primers (IN-1 and IN-2). As shown in Fig. 2C, an expected 668 bp fragment was generated by PCR amplification from the wild-type strain, but not spaA deletion mutants, suggesting a lack of endogenous spaA in the knockout bacteria. One of these tetracycline-resistant transformants was designated ΔspaA.

To further identify the mutant strain, the genomic DNA of the ΔspaA mutant strain was examined by multiplex PCR. As shown in Fig. 2D, the expected 2,381 bp fragment...
1,881 bp fragments were detected by PCR using primers P3 and P4 from the ∆spaA mutant and wild-type strain C43065, respectively. Consistent with the presence of the Tet' gene, the 1,191 bp PCR products, representing the tetracycline-resistant gene, were detected in the mutant strain, but not the associated wild-type strain (Fig. 2D). In good agreement with the deletion of the 691 bp fragment from the spaA gene, the 668 bp PCR product was detected in the wild-type strain, but not the mutant strain, using the spaA-specific primers (Fig. 2D). Finally, PCR products amplified using P3 and P4 primers were determined by sequence analysis, which confirmed the presence of the tetracycline-resistant cassette (1,191 bp), the upstream homologous arm (616 bp), and the downstream homologous arm (574 bp) of the spaA gene. These results demonstrate that the 691 bp fragment of the spaA gene was successfully replaced by the Tet' cassette utilizing homologous recombination in the spaA deletion mutant.

Confirmation of spaA Deletion Mutant by RT-PCR and Western Blotting

In order to determine the level of mRNA expression of spaA in the mutant strain, we utilized RT-PCR using the spaA-specific primers IN-1 and IN-2. As shown in Fig. 3A, total RNA from the ∆spaA mutant and wild-type strain C43065 was successfully isolated using the RNAprep pure cell/bacteria kit (Tiangen), respectively. The results of RT-PCR showed that a single band of the expected length (668 bp) was detected only in the wild-type strain C43065, but not in the ∆spaA mutant (Fig. 3B). These results demonstrated the absence of spaA mRNA expression in the mutant strain.

To determine SpaA protein expression, we carried out protein analysis by western blotting. Briefly, whole-cell proteins from either the ∆spaA mutant or the wild-type strain C43065 were compared by SDS-PAGE, followed by Coomassie staining and western blotting. Although several nonspecific proteins (14, 20, 28, and 66 kDa protein bands) were evident in both samples by Coomassie staining, the 64 kDa protein only appeared in samples of the wild-type strain, but not in samples of the mutant strain (Fig. 3C). Consistently, the same size protein was only detected in whole-cell proteins of the wild-type strain, and not in the whole-cell proteins of the mutant strain, when utilizing polyclonal antibodies against the rSpaA (Fig. 3C), thus confirming loss of SpaA expression in the mutant strain.

Virulence of spaA Deletion Mutant in Mice

Although it has been postulated that SpaA proteins are virulence factors in E. rhusiopathiae, mechanistic data are sorely lacking. To address this issue, we performed an assay for virulence examination and evaluation by intramuscular injections of the wild-type strain C43065 or the ∆spaA mutant into mice. As shown in Table 2, all five mice were killed by the wild-type strain at a minimum dosage of 82 CFUs. In contrast, the minimum dosage for 100% mortality of experimental mice by the mutant strain was 5,900 CFUs, suggesting that loss of SpaA led to

![Fig. 3. Confirmation of the ∆spaA mutant by RT-PCR and western blotting.](image-url)
significant attenuation of virulence in *E. rhusiopathiae*. These data indicated that SpaA is an important virulence factor in *E. rhusiopathiae*.

**Resistance of spaA Deletion Mutant to Complement-Mediated Serum Killing**

To determine if the SpaA of *E. rhusiopathiae* was involved in serum resistance, wild-type and mutant strains were incubated with swine serum. A serum-sensitivity control of *E. coli* DH5α was also examined to confirm the presence of complement activity in the serum. As shown in Table 3, the ∆spaA mutant exhibited a significant reduction in serum resistance as compared with the wild-type strain C43065 (*p* < 0.01). Heat-inactivated serum permitted growth of the wild-type strain and mutant strain as well as *E. coli* DH5α. No statistically significant differences were observed between the bacteria number of wild-type strain and mutant strain, after incubation in heat-inactivated serum.

**Adhesion of spaA Deletion Mutant to MEF Cells**

The light microscopy images of infected, Giemsa-stained MEF cells showed obvious impairment of adherence of the ∆spaA mutant as compared with the wild-type strain C43065 (Fig. 4A). The level of impairment in adherence of ∆spaA mutant was determined by counting bacteria adhering to MEF cells after extensive washing and cell lysis. The mutant strain showed a significant reduction in adherence to MEF cells of 2 orders of magnitude (*p* < 0.01) compared with the wild-type strain (Fig. 4B). These data suggest that SpaA is involved in the ability of *E. rhusiopathiae* to adhere to MEF cells.

**Adhesion Inhibition Assay by Treatment with Rabbit Antiserum Against rSpaA**

The influence of treatment of wild-type strain C43065 and ∆spaA mutant with rabbit antiserum against rSpaA on their ability to MEF cells is shown in Fig. 5. The treatment with anti-rSpaA rabbit serum caused significant reduction (48.2%) of bacterial numbers of wild-type strain adherent to MEF cells (*p* < 0.01) as compared to normal rabbit serum treatment, but mutant strain was not affected. These demonstrated that the SpaA protein on the surface of *E. rhusiopathiae* serotype 2 strains mediated their adhesion to MEF cells.

**Phagocytosis of spaA Deletion Mutant by Mouse Peritoneal Macrophages**

The light microscopic examination showed that the ∆spaA mutant was readily taken up by mouse peritoneal macrophages in the normal rabbit serum, whereas the wild-type strain C43065 was resistant (Fig. 6A).

### Table 2. Virulence of wild-type strain C43065 and ∆spaA mutant in mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Challenge dose (CFUs)</th>
<th>Dead/total</th>
<th>Mortality (%)</th>
<th>LD₅₀</th>
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<tbody>
<tr>
<td>C43065</td>
<td>8.2 × 10⁴</td>
<td>5/5</td>
<td>100</td>
<td>9CFU</td>
</tr>
<tr>
<td></td>
<td>8.2 × 10³</td>
<td>5/5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.2 × 10²</td>
<td>5/5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.2 × 10¹</td>
<td>5/5</td>
<td>100</td>
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<tr>
<td></td>
<td>8.2 × 10⁰</td>
<td>3/5</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>∆spaA</td>
<td>5.9 × 10⁶</td>
<td>5/5</td>
<td>100</td>
<td>698CFU</td>
</tr>
<tr>
<td></td>
<td>5.9 × 10⁵</td>
<td>5/5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.9 × 10⁴</td>
<td>2/5</td>
<td>40</td>
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<td></td>
<td>5.9 × 10³</td>
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<td>0</td>
<td></td>
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<tr>
<td></td>
<td>5.9 × 10²</td>
<td>0/5</td>
<td>0</td>
<td></td>
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<tr>
<td>Control</td>
<td>BHI broth</td>
<td>0/5</td>
<td>0</td>
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### Table 3. Resistance of wild-type strain C43065 and ∆spaA mutant to porcine serum.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum heat treatment</th>
<th>CFUs/before treatment</th>
<th>CFUs/after treatment</th>
<th>Survival ratio*</th>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>-</td>
<td>3.4 × 10⁷</td>
<td>1.2 × 10⁷</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.4 × 10⁷</td>
<td>4.5 × 10⁶</td>
<td>132 ± 52b</td>
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<tr>
<td><em>E. rhusiopathiae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C43065</td>
<td>-</td>
<td>2.5 × 10⁶</td>
<td>3.6 × 10⁶</td>
<td>144 ± 59</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.5 × 10⁶</td>
<td>4.2 × 10⁵</td>
<td>168 ± 67</td>
</tr>
<tr>
<td>∆spaA</td>
<td>-</td>
<td>2.8 × 10⁷</td>
<td>5.4 × 10⁷</td>
<td>0.03 ± 0.04b,c</td>
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<tr>
<td></td>
<td>+</td>
<td>2.8 × 10⁶</td>
<td>3.9 × 10⁶</td>
<td>139 ± 55b</td>
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*Survival rate = (CFUs/ml at t = 3 h)/(CFUs/ml at t = 0 h).

bSurvival ratios for serum and heated serum were significantly different (*p* < 0.01).

cSurvival ratios for wild-type C43065 strain and ∆spaA mutant in the swine serum were significantly different (*p* < 0.01).
antiserum against rSpaA promoted the phagocytosis of wild-type strain by macrophages, but the mutant strain was not affected (Fig. 6B). This result strongly suggested that the anti-SpaA antibodies act as opsonins.

Protective Effects of spaA Deletion Mutant in Mice

To test whether spaA is a potential target for vaccine development for the treatment of *E. rhusiopathiae* infection, mice were immunized by formalin-killed C43065 and ΔspaA mutant, as well as by NaOH-extracted antigen and recombinant rSpaA, followed by challenge with the wild-type strain C43065. Although the formalin-killed ΔspaA mutant demonstrated significant protection compared with a saline control (Table 4), its protective rate of 40% was much less than that of the formalin-killed wild-type strain C43065 (80%), NaOH-extracted antigen (100%), and recombinant SpaA (100%). These data suggest that SpaA is a major protective antigen for developing a subunit vaccine to control swine erysipelas. The level of antibody titer induced by the whole-cell proteins of ΔspaA was significantly (*p < 0.05*) lower than those by the whole-cell
proteins of C43065, NaOH-extracted antigen, and rSpaA (data not shown).

Discussion

In the present study, based on homologous recombination technology, we first demonstrated that SpaA is a critical virulence factor in strain C43065, a highly virulent strain of *E. rhusiopathiae* responsible for major epidemics of swine erysipelas in China. Inspired by a recent study concerning the *szp* gene being deleted in *Streptococcus equi* ssp. *zoopneumoniae* through homologous recombination [7], we mutated the endogenous spaA gene in strain C43065 by the replacement of a fragment of endogenous spaA with a tetracycline-resistant cassette. Employing a proof-of-principle approach, we confirmed that spaA was effectively deleted at both the mRNA and protein levels in the C43065 strain by taking advantage of a pUC18-based selection with tetracycline.

SpaA, as a major surface protective antigen, has been suggested to be associated with virulence in *E. rhusiopathiae* isolates as well as disease in mice and swine [15, 18]. It has been proposed that SpaA may play an important role in the *E. rhusiopathiae* infection process and virulence owing to the structure and sequence similarities of its C-terminal region to the choline-binding proteins of *Streptococcus pneumoniae* [8, 15]. However, the role of SpaA in bacterial virulence is not fully understood. Utilizing the virulence test, we demonstrated that SpaA is one of several critical virulence factors in strain C43065, by showing that the LD50 associated with the ΔspaA mutant was 76-fold lower than the LD50 associated with its parental strain when mice were challenged by the wild-type strain C43065. Moreover, the adhesion of wild-type strain to MEF cells was inhibited significantly by treatment with rabbit antiserum against SpaA as compared with the treatment with normal rabbit serum, but the ΔspaA mutant was not affected. Recently, Harada *et al.* [6] also demonstrated that SpaA was responsible for the adhesion of *E. rhusiopathiae* to porcine endothelial cells. These studies suggested that SpaA is an adherence factor of *E. rhusiopathiae* to host cells. In this study, we also found that serum obtained from the rSpaA-immunized rabbit promoted the phagocytosis of the wild-type strain C43065 by mouse peritoneal macrophages, but the ΔspaA mutant was not affected. This result strongly suggested that the anti-SpaA antibodies act as opsonins, similar to the antibody against choline-binding protein B (CbpB), which is a major protective antigen of *E. rhusiopathiae* [18].

Live-attenuated vaccines are known to be an indispensable tool in the control of swine erysipelas, in part due to their ability to mimic the natural course of infection, thereby conferring cross-protection [20]. By using the self-conjugative transposon Tn916, Shimoji *et al.* [21] revealed that the live-attenuated vaccine strain from a defined acapsular mutant of *E. rhusiopathiae* was completely protected against the

### Table 4. Protection in BALB/c mice by various immunized vaccines.

<table>
<thead>
<tr>
<th>Type of vaccine</th>
<th>Dose/mouse</th>
<th>Survived/total</th>
<th>Protective rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin-killed ΔspaA</td>
<td>1.5 × 10⁶ CFUs</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>Formalin-killed C43065</td>
<td>1.3 × 10⁶ CFUs</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>NaOH-extracted antigen</td>
<td>100 µg</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>Recombinant SpaA</td>
<td>100 µg</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>Saline control</td>
<td>100 ml</td>
<td>0/5</td>
<td>0</td>
</tr>
</tbody>
</table>

Taken together, this study demonstrates that SpaA is one of many crucial virulence factors in the C43065 strain. Further studies are warranted to elucidate the underlying mechanism by which SpaA promotes virulence in this strain. The result of serum resistance assay was consistent with the role for SpaA in survival in vivo. Wild-type strain C43065 was resistant to the bactericidal action of swine serum, whereas the ΔspaA mutant was sensitive. Although the mechanism of inhibition of complement-mediated killing is not known, our results are consistent with other studies [1, 3, 4]. A common trait of microbial pathogens is the expression of factors that bind to molecules on host tissue cells. In this study, we found that the adherence rates of the ΔspaA mutant was approximately 31.6% lower than those of the wild-type strain C43065. Moreover, the adhesion of wild-type strain to MEF cells was inhibited significantly by treatment with rabbit antiserum against SpaA as compared with the treatment with normal rabbit serum, but the ΔspaA mutant was not affected. Recently, Harada *et al.* [6] also demonstrated that SpaA was responsible for the adhesion of *E. rhusiopathiae* to porcine endothelial cells. These studies suggested that SpaA is an adherence factor of *E. rhusiopathiae* to host cells. In this study, we also found that serum obtained from the rSpaA-immunized rabbit promoted the phagocytosis of the wild-type strain C43065 by mouse peritoneal macrophages, but the ΔspaA mutant was not affected. This result strongly suggested that the anti-SpaA antibodies act as opsonins, similar to the antibody against choline-binding protein B (CbpB), which is a major protective antigen of *E. rhusiopathiae* [18].
challenge of a homologous virulent strain. In this study, the formalin-killed ΔspaA mutant strain was shown to have a lower protective rate (40%) as an immunogen against the homologous virulent strain (C43065) challenge, suggesting that SpaA could be used as a vaccine antigen to control swine erysipelas. In agreement with previous studies [5, 14], NaOH-extracted antigen and recombinant SpaA, which provided a 100% protective rate in the virulence test, may be more effective reagents against *E. rhusiopathiae* infection in swine.

In summary, the effect of the *spaA* deletion mutant of *E. rhusiopathiae* strain C43065 on virulence, serum resistance, adherence, and antiphagocytosis were investigated. Our results showed that the virulence of the *spaA* deletion mutant in mice via an intramuscular route of infection was significantly attenuated as compared with the wild-type strain. The number of adherent organisms of wild-type strain was resistant. Moreover, antiserum against SpaA promoted the phagocytosis of wild-type strain by macrophages, but the mutant strain was not affected. The mutant strain was sensitive to the bactericidal action of swine serum, but the wild-type strain was resistant. The mutant strain was readily taken up by mouse peritoneal macrophages in the normal rabbit serum, whereas the wild-type strain was resistant. Moreover, antiserum against SpaA inhibited significantly by treatment with antisera against rSpaA, but the mutant strain not affected. The adhesion of wild-type strain to MEF cells was approximately two times as much as the mutant strain. The number of adherent organisms of wild-type strain was significantly attenuated as compared with the wild-type strain. The adhesion of wild-type strain to MEF cells was approximately two times as much as the mutant strain. The adhesion of wild-type strain was not affected. Our results demonstrate that SpaA is a major virulence factor of *E. rhusiopathiae*.

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**References**


