Growth Ability and Immunological Properties of *Erysipelothrix rhusiopathiae* Serotype 2

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**ABSTRACT.** Five field strains of *Erysipelothrix rhusiopathiae* belonging to serotype 2 were compared for their growth ability, immunogenicity in mice, SDS-PAGE profile of cell surface proteins and their immunoblotting patterns. Strain Tama-96 showed the most stable growth in Feist medium and tryptose phosphate broth with Tween 80 (TPB), and its immunogenicity was highest in a mouse protection test using the inactivated vaccines prepared from 20-h TPB culture. The 50% mouse protective dose of the vaccine was only 12 μl. SDS-PAGE and immunoblotting patterns of the proteins were similar among the strains in general and indicated that 66 to 64 kDa protein antigens were dominant. — **KEY WORDS:** *Erysipelothrix rhusiopathiae*, immunogenicity, serotype 2.

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*Erysipelothrix rhusiopathiae* is a small gram-positive rod causing swine erysipelas, and the disease continues to be a major problem in swine producing areas of the world. Prevention of the disease has commonly been undertaken by vaccination using live-attenuated organism, essentially based on the vaccine developed more than 100 years ago by Pasteur and Thillier [9], and bacterin originally developed by Traub [18]. This bacterin depended upon the selection of specially immunogenic strain of serotype 2 [11].

In Japan, a live-organism vaccine has been used for prevention of the disease, however, its effectiveness depends on the susceptibility of swine to be vaccinated. The maternal antibody makes piglets unsusceptible to the vaccine, therefore, revaccination become necessary. This is however, a laborious task and uncertain in outcome, because the duration of the maternal antibody is irregular. Another problem is in SPF pig production, where contrasting the vaccine sometimes causes strong responses, like urticaria or stress, due to high susceptibility of the animals. Therefore, it is considered important to develop a more effective and safe, inactivated vaccine.

The objective of the present study was to characterize several strains of serotype 2 isolated in Japan for their growth abilities and immunological properties for the development of a more effective inactivated vaccine.

*E. rhusiopathiae* strains 82-510, 82-527, Shizuoka-63 and Tama-96 isolated from swine with chronic disease in several prefectural meat inspection offices or centers, and strain 44 isolated from an urticarial lesion [17], in Japan, were used as candidates for a vaccine. An acriflavin-fast attenuated strain Kg-2 [20], originally designated as Koganei 65-0.15 [11], and strain SE-9, which is one of the strains used for USDA standard vaccine [23], provided by Dr. R.L. Wood, National Animal Disease Center, Ames, IA, U.S.A., were used as references. All the above strains belonged to serotype 2. A virulent strain, Fujisawa (serotype 1a), isolated from swine with septicemia was used for challenge exposure as described previously [11].

A modified Feist medium [3], containing 6 g of glucose, 5 g of proteose peptone No. 2 (Difco Laboratories, Detroit, MI), 5 g of yeast extract (Difco), 0.5 g of arginine (Merck, West Point, PA), and 0.5 ml Tween 80 (Sigma, St. Louis, PA) dissolved in 1 liter of 0.2 M sodium phosphate buffer (pH 8.0), and tryptose phosphate broth (Difco) containing 0.1% Tween 80 (TPB) were used for the growth test. Colonies from 48-hr culture of each strain on tryptose phosphate agar (TPA) plate were suspended in sterilized 0.02 M phosphate-buffered saline (PBS, pH 7.2), and the optical density (OD) was adjusted to 0.5 at 420 nm for each strain. Five ml of the medium were inoculated with 50 μl of the bacterial suspension and incubated at 37°C. The growth pattern was recorded by plotting the value of OD against the corresponding time for 23 hr. The growth ability was determined by the viable bacterial number and OD.

Vaccine was prepared essentially as described previously [18]. Briefly, 20-hr cultures of each strain in each broth medium were inactivated with formalin (Wako Pure Chemical Industries, Co., Ltd, Tokyo) to 0.3% (v/v). The concentration of bacterial cells was adjusted to OD 0.5, then mixed with 1/5 volume of aluminium hydroxide gel (kindly provided by Dr. T. Yagihashi, Nippon Institute for Biological Science, Ohme, Tokyo) at room temperature for at least 2 hr. This was kept at 4°C, and cut to half of the volume by removing clear supernatant 24 hr later. Five 5-week-old female ddY mice (Japan SLC, Inc., Hamamatsu, Shizuoka), each of 3 groups, were inoculated subcutaneously with 0.5 ml of the vaccine diluted serially in 33% aluminium hydroxide gel suspension. Three weeks later the immunized mice were challenge-exposed with 0.1 ml of 24-hr TPB culture of strain Fujisawa, containing 6.5 x 10³ colony forming units (CFU) (approx. 1,000 LD₅₀). The mice were observed for 14 days and the responses were determined by the quantal (live-dead) method. The 50% protective dose (PD₅₀) was calculated by the method of Spearman and Kaerber [2].

Samples for SDS-PAGE and immunoblotting were
prepared from bacterial cell surfaces as follows. A colony of each strain produced on TPA plate, after overnight incubation, was inoculated into 200 ml of TPB and incubated at 37°C for 20 hr. Then the broth culture was centrifuged at 12,000 x g for 20 min. The bacterial cells were washed 3 times with PBS and suspended in a sample buffer of 62.5 mM Tris-hydrochloride (pH 6.8) containing 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.002% bromophenol blue. The proportions of bacterial pellet and sample buffer were 1:3 (w/v), and the total protein concentration of this preparation were 1,500 µg per ml. SDS-PAGE was performed by applying 10 µl of sample to 12% polyacrylamide slab gel prepared in Mini-PROTEAN II (Bio-Rad Laboratories, Richmond, CA) by the method of Laemmli [7]. The approximate molecular masses were determined by comparing the migration pattern with the patterns of proteins of known molecular masses (Pharmacia LKB Biotechnology, Uppsal, Sweden). After SDS-PAGE, solubilized cell surface proteins of bacteria were transferred to a nitrocellulose membrane by electrophoresis at 30 volts for 18 hr by transblot apparatus (Bio-Rad), according to the Western blot method of Towbin et al. [19]. The strip was blocked by 3% bovine serum albumin in a Tris-buffered saline (20 mM Tris, 150 mM sodium chloride; pH 7.2). It was washed in the same buffer and treated with antiserum of swine, which had survived from infection with strain Fujisawa, at room temperature for 2 hr. The antiserum showed a growth-agglutinating antibody titer of more than 1:1,024 and was diluted 100 times before use. After washing, the strip was incubated for 1 hr with horseradish peroxidase labeled anti-porcine immunoglobulin G (IgG) rabbit serum (Cappel, Durham, NC), and washed. The coloring of the strip was developed by 4-chloro-1-naphtol (Sigma).

The number of viable bacteria was more varied among the strains in the Feist medium than in TPB. The bacterial growth in both media was in the logarithmic phase as early as 4 hr after incubation except for strain 82-510. The OD decreased soon after peaking at 14 hr in the Feist medium, however, the growth of strain Tama-96 was more stable (Fig. 1). In TPB the growth patterns of each strain were similar, and at 23 hr the cultures were still in the stationary phase (Fig. 2).

The immunogenicity of the strains was expressed as a PD50 value as shown in Table 1. All mice in the control group died within 4 days after challenge exposure to strain Fujisawa. Strain Tama-96 was most immunogenic, PD50 of the vaccine prepared from TPB culture of the strain for mice being only 12 µl (approx. 5 x 107 bacterial cells per mouse). Then strain Tama-96 was used for comparing 3 media for antigen production: BHI containing 0.1% Tween 80, BHI supplemented with 10% horse serum (HS), and Feist medium. The vaccine prepared from the culture in BHI with HS was most immunogenic (Table 2). Western blot analysis revealed neither quantitative nor qualitative disparities among the strains (Fig. 3). Antigens of the

![Graph](image1)

**Fig. 1.** Growth pattern of *E. rhusiopathiae* strains in Feist medium. Strains 82-510 (○), 82-527 (●), Shizuoka-63 (□), 44 (■), Tama-96 (△), SE-9 (▲) and Kg-2 (×).

![Graph](image2)

**Fig. 2.** Growth pattern of *E. rhusiopathiae* strains in tryptose phosphate broth. Strains 82-510 (○), 82-527 (●), Shizuoka-63 (□), 44 (■), Tama-96 (△), SE-9 (▲) and Kg-2 (×).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dilution of vaccine</th>
<th>PD50 (µl)¹</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Original</td>
<td>1:5</td>
</tr>
<tr>
<td>Test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82-510</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>82-527</td>
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<tr>
<td>Shizuoka-63</td>
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<tr>
<td>44</td>
<td>5/5</td>
<td>5/5</td>
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<tr>
<td>Tama-96</td>
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<td>5/5</td>
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<tr>
<td>Reference</td>
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<td>SE-9</td>
<td>5/5</td>
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<tr>
<td>Kg-2</td>
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<tr>
<td>Control</td>
<td>0/5</td>
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</tbody>
</table>

¹ 50% of protective dose of vaccine.  
² No. of survived mice/No. tested. Mice were immunized subcutaneously with 0.5 ml of aluminium gel vaccine prepared from each strain cultured in tryptose phosphate broth. Challenge exposure was done subcutaneously with 1,000 LD50 of strain Fujisawa 3 weeks after immunization.
proteins 76, 74, 66–64, 56, 45 and 38 kDa were generally recognized, and 66 to 64 kDa protein antigens were dominant in comparison with the others. Strains Shizuoka-63 and reference strain SE-9 were void of 45 kDa protein.

The growth pattern of strains obtained by plotting OD of their cultures against their corresponding times showed that multiplication of the strains entered the logarithmic phase 4 hr after incubation in both TPB and Feist medium. The maximum growth was attained at approximately 10 hr in TPB and was in the stationary phase until 23 hr. In Feist medium the OD of the cultures showed more variety but generally reached a peak at approximately 14 hr and then decreased as described previously [5]. The decrease of OD in Feist medium may indicate autolysis due to exhaustion of the nutrients in the medium as a result of a very high rate of cell synthesis. Interestingly, strain Tama-96 was less susceptible to lysis. The stability of the growth of this strain appeared to be helpful for further immunological study.

The immunogenicity of the strains was tested using an aluminium gel adsorbed vaccine, prepared from whole broth culture, because protective antigens of *E. rhusiopathiae* were reportedly abundant also in the culture filtrate [10, 11, 13, 21]. The protective activity of the vaccine prepared from the strains conferred various degrees of protection as expressed by PD50 value in the present study. Amongst the Japanese isolates strain Tama-96 was most immunogenic, and this strain was therefore used for the selection of the best medium, from among the three tested, for antigen production. BHI was used as the basal medium in this experiment, because its components were considered to be similar to those of the medium for the preparation of the

Table 2. Immunogenicity of *E. rhusiopathiae* strain Tama-96 grown in various media in mice

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilution of vaccine</th>
<th>PD50 (µl)a)</th>
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<tbody>
<tr>
<td></td>
<td>Original</td>
<td>1:5</td>
</tr>
<tr>
<td>Feist medium</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td>BHI+0.1% Tween 80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BHI+10% horse serum</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Control</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

a) 50% protective dose of vaccine.

b) No. of survived mice/No. tested. Mice were immunized subcutaneously with 0.5 ml of aluminium gel vaccine prepared from culture of each medium. Challenge exposure was done subcutaneously with 1,000 LD50 of strain Fujisawa 3 weeks after immunization.

c) Brain heart infusion.

original inactivated vaccine [18]. Our present results showed that the vaccine prepared using BHI supplemented with HS was more immunogenic than those of BHI containing 0.1% Tween 80 or Feist medium. Lipoprotein in horse serum was considered to be protective-antigen production-enhancing factor [22], whereas Feist medium, compared with BHI supplemented with 10% HS, was reportedly superior for antigen production [4]. This discrepancy may be due to differences in the properties of the bacterial strains used for vaccine preparation and challenge exposure in the respective experiments.

The SDS-PAGE patterns of cell surface protein from the strains were uniform, and Western blot analysis of the protein antigens against swine antisera showed no qualitative difference. These results were similar to antigens driven by immunoabsorbent affinity chromatography [5]. Anti-culture filtrate sera against serotype 1a strain recognized these protein antigens which were extracted from the cells with NaOH in Western blotting [6]. This, coupled with our present results obtained with serotype 2 strains, suggests no difference in protein patterns between serotypes 1a and 2. Antigens of 60 to 64 kDa protein shown in our present study were apparently similar to those involved in protection as reported previously [5]. The protective antigen was reportedly rich in culture supernatant [10, 11, 13, 21], and its antisera [12, 14, 15] was highly protective, however, a study in passively immunized mice suggested that opsonization played an important role in protection [15]. Thus, antibodies directed to surface antigens of the bacteria are dominant in protection. These results confirmed that protective antigens in culture filtrates were released from the cell surface of the bacteria [12]. Although the strain sensitive to autolysis was immunogenic [24] but stability of the protective antigens may be degraded by bacterial protease released from the cytoplasm [16], since the protective antigens are protein [10, 20, 21]. Therefore, autolysis may shorten stability of vaccines prepared from such strain. Strain Tama-96, which is more resistant to autolysis, may conserve its immunogenic cell surface antigens.

Finally, from the view of immunogenicity and autolysis resistance, strain Tama-96 was considered to be a suitable E. rhusiopathiae strain for use in the preparation of an inactivated vaccine, and for further study of the protective antigen.

REFERENCES