Hyaluronidase Is Not Essential for the Lethality of *Erysipelothrix rhusiopathiae* Infection in Mice

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**ABSTRACT.** To investigate the role of hyaluronidase in the pathogenicity of *Erysipelothrix rhusiopathiae*, transposon Tn916 was transferred from *Enterococcus faecalis* CG110 to a virulent strain of *E. rhusiopathiae*, and hyaluronidase-deficient mutants were isolated. A virulence assay in the mice showed that of the seven hyaluronidase-deficient mutants tested, six mutants were avirulent, but that one mutant, designated AST121, was as virulent as its parental strain. Western immunoblotting with a monoclonal antibody specific to the capsule, revealed that all of the avirulent mutants had lost the capsular antigen, whereas the mutant AST121 did not. These results suggest that the lack of virulence of the six hyaluronidase-negative mutants could be due to a loss of the capsule and that hyaluronidase does not contribute to the lethality of *E. rhusiopathiae* infection in mice.

**KEY WORDS:** *Erysipelothrix rhusiopathiae*, hyaluronidase, virulence.

*Erysipelothrix rhusiopathiae* is a gram-positive bacterium that causes a wide spectrum of disease in animals, birds, and humans [29]. Erysipelas in swine can occur as an acute septicemia or chronic disease, which is characterized by arthritis and endocarditis [29]. In mice, the organism usually causes acute septicemia and chronic arthritis following the acute disease, and the mouse sepsis model has been alternatively used for bacterial virulence and vaccine efficacy determination assays in pigs [18, 19, 26].

Studies of virulence mechanisms of the bacterium have shown (i) the importance of the capsule in pathogenicity [20–23], (ii) a strong association between neuraminidase production and the pathogenicity of strains [12], and (iii) a better adherence of virulent strains to porcine kidney cells in vitro than avirulent strains [25]. In addition, the organism produces hyaluronidase, an enzyme capable of breaking down hyaluronate. Hyaluronidase is produced by a number of pathogens including bacteria, helminths, and arthropods, and it has been considered to be associated with their virulence [1–5, 7, 9–11, 13, 14, 27, 28]. The possible role of hyaluronidase as a virulence factor is postulated to be a spreading factor facilitating the dissemination of pathogens into tissues [3, 7, 9, 10]. The importance of the enzyme in the pathogenesis of *E. rhusiopathiae* infection has been suggested by Mann [15]. However, Nørrung [16] examined the virulence and hyaluronidase production of sixty-two isolates from joints or regional lymph nodes of pigs with arthritis, and concluded that there was no association between the organisms' hyaluronidase production and their virulence. Thus, the role of the enzyme in the pathogenesis of the infection remains controversial; however, genetic analysis regarding this point has not been conducted. In the present study, we constructed transposon mutants deficient in hyaluronidase production and investigated the role of the enzyme in the pathogenesis of the infection.

Mutants defective in hyaluronidase production were constructed by transposon mutagenesis with Tn916. Transposon Tn916, a 16.4-kilobase transposon carrying a tetracycline resistance gene, is located on the chromosome of *Enterococcus faecalis* CG110 and is capable of conjugative transfer in the absence of plasmid DNA [6, 8]. The recipient strain, Fujisawa-SmR [23], is a streptomycin-resistant mutant of a highly virulent *E. rhusiopathiae* strain, Fujisawa (serovar 1a), that was isolated from a septicemic pig. *E. rhusiopathiae* strain Fujisawa-SmR and *E. faecalis* CG110 were grown in a brain heart infusion (BHI; Difco Laboratories, Detroit, MI) containing 0.1% Tween 80 (pH7.6) (BHI-T80). Tn916 was transferred from *E. faecalis* to *E. rhusiopathiae* by filter-mating, as previously described [23]. Briefly, bacterial cells of donor and recipient strains were mixed at a 1:10 ratio and then deposited on membrane filters. The filters were incubated for approximately 14 hr, and the bacteria were removed and plated on medium containing tetracycline (12.5 µg/mL) and streptomycin (200 µg/mL). Tetracycline-resistant transconjugants were screened for hyaluronidase activity on hyaluronidase-test-agar [24]. BHI (Difco) containing 0.1% Tween 80 (pH 6.8) and 1% Noble Agar (Difco) were autoclaved at 121°C for 15 min and cooled to 46°C. Hyaluronic acid sodium salt (Nacalai Tesque Inc., Kyoto, Japan) and bovine albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) were sterilized by filtration and added to cooled media to give final concentrations of 400 µg/mL and 1%, respectively. After incubation of the transconjugants at 37°C for 24–30 hr, the plates were flooded with 2 N acetic acid for 10 min for visualization of hyaluronidase activity. This method permits the screening of a large number of samples for enzyme activity and indicated in the present study that hyaluronidase-positive mutants produce clear zones around their colonies (Fig. 1). Approximately 10,000 transconjugants were generated from the virulent strain Fujisawa-SmR and screened for a loss of hyaluronidase production. The results showed that
The virulent Fujisawa-SmR strain generated hyaluronidase-negative mutants at a high frequency, with this generation correlating with a change in colonial morphology, and a total of forty-nine hyaluronidase-negative mutants were isolated. With the exception of one mutant, designated AST121, colonies of these hyaluronidase-negative mutants displayed circular flat edges in contrast to those of the parent strain, which were convex and irregular in shape (data not shown). Of the hyaluronidase-negative mutants isolated, seven mutants were chosen for further experiments.

Southern hybridization was performed as previously described [23], and the presence of a Tn\textsuperscript{916} transposon insertion in the chromosome of the seven hyaluronidase-negative mutants was confirmed. It was found that the mutants contained one to four transposon insertions within the chromosome (Fig. 2), indicating that at least one copy of Tn\textsuperscript{916} is inserted within a structural gene or regulatory gene(s) of hyaluronidase, resulting in a loss of hyaluronidase production.

The virulence of parent and mutant strains was assayed in mice. The bacterial strains were grown overnight at 37°C in BHI-T80 broth and then diluted with BHI-T80. Seven to nine-week old female BALB/C mice (Japan SLC Inc., Hamamatsu, Japan) were inoculated subcutaneously with 0.1 m\textsuperscript{l} of the dilutions. The number of mice still alive 14 days after challenge was recorded, and the LD\textsubscript{50}s were determined by the Reed and Muench method [17]. It was found that of the seven mutants tested, six hyaluronidase-negative mutants were avirulent for mice, whereas the mutant AST121 which had the same colonial morphology was as virulent as its parental strain (Table 1).

Table 1. Virulence of \textit{E. rhusiopathiae} strains in mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hyaluronidase production</th>
<th>Capsule antigen expression</th>
<th>LD\textsubscript{50}\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>+</td>
<td>+</td>
<td>10\textsuperscript{1.2}</td>
</tr>
<tr>
<td>Mutant</td>
<td>28G12</td>
<td>–</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>28G5</td>
<td>–</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>11A2</td>
<td>–</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>AST121</td>
<td>–</td>
<td>10\textsuperscript{1.2}</td>
</tr>
<tr>
<td></td>
<td>566</td>
<td>–</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>736</td>
<td>–</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>IRN</td>
<td>–</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>33H6</td>
<td>+</td>
<td>&gt;10\textsuperscript{8}</td>
</tr>
</tbody>
</table>

\textsuperscript{a) LD\textsubscript{50}s are given as CFU per mouse.}

The mutant AST121 was confirmed by culturing homogenates of spleens and livers removed from dead animals on hyaluronidase-test-agar. No hyaluronidase-producing revertant colonies from the organs of animals were observed, suggesting that the deaths of mice were due to factor(s) other than hyaluronidase.

We have previously observed that the change in colonial morphology of \textit{E. rhusiopathiae} bacteria correlates with their virulence [23]. To examine the discrepancy in the virulence of the hyaluronidase-negative mutants, we examined expression of the capsule, a major virulence factor of the organism, in these mutants. The bacterial surface antigens were prepared, and Western immunoblotting was carried out as previously described [21] by using a monoclonal antibody (MAb) ER21 (isotype IgM) that is specific for capsular antigen [20]. The MAb ER21-secreting hybridoma was
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is that both hyaluronidase and capsule production may be regulated by a common genetic locus or loci, and that in these hyaluronidase-negative mutants, insertion of Tn916 occurred in regulatory region(s), resulting in a loss of both hyaluronidase and capsule production.

Our results, however, do not exclude the possibility that the enzyme contributes to virulence of the organism. It is possible that the enzyme plays a role in helping the organism escape from local host defenses that could not be tested by this model. This hypothesis is strengthened by the finding that in Streptococcus pneumoniae, mutagenesis of either hyaluronidase or pneumolysin genes has no effect on virulence, whereas a double mutation in these genes has a significant impact [3]. Because E. rhusiopathiae causes a variety of diseases other than septicemia [29], it would be of interest to test the hyaluronidase-deficient mutant (AST121) obtained in this study in other disease models.

Fig. 3. Detection of a capsular antigen by Western immunoblotting. The bacterial surface antigens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was treated with MAb ER21 specific to capsular antigen. Lane 1, Fujisawa-SmR; 2, 33H6; 3, 28G12; 4, 28G5; 5, 11A2; 6, AST121; 7, 566; 8, 736, 9, IRN.

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