Erysipelothrix tonsillarum sp. nov. Isolated from Tonsils of Apparently Healthy Pigs
TOSHI TAKAHASHI,1* TOMOHIO FUJISAWA,2 YOSHIHI BENNO,3 YUTAKA TAMURA,1 TAKUO SAWADA,1 SHOKO SUZUKI,1 MASATAKE MURAMATSU,1 AND TOMOTARI MITSUOKA2,3

National Veterinary Assay Laboratory, Kokubunji, Tokyo 185; 2Department of Biomedical Science, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113; and 3The Institute of Physical and Chemical Research, Wako, Saitama 351, Japan

A new species, Erysipelothrix tonsillarum, is proposed for avirulent Erysipelothrix strains of serotype 7, which is frequently isolated from tonsils of apparently healthy pigs. This species is morphologically and biochemically indistinguishable from the Erysipelothrix rhusiopathiae strains characterized to date. Seven strains of E. tonsillarum that belonged to serotype 7 were avirulent for swine, except for strain T-334, which induced a local urticarial lesion at the site of inoculation. The 50% lethal dose values in mice ranged from 1.0 CFU to 6.9 X 10^6 CFU. The E. rhusiopathiae strains belonging to serotypes 2 (six strains), 6 (one strain), 11 (one strain), 12 (one strain), and 16 (one strain) induced generalized or local urticarial lesions after intradermal inoculation, except for strain T-312 of serotype 12, which showed 50% lethal dose values of ≤2.5 X 10^6 CFU in mice. The deoxyribonucleic acid base composition of E. tonsillarum is 36 to 40 mol% guanine plus cytosine. Strains of this species have little deoxyribonucleic acid homology (15 to 43%) with the type strain of E. rhusiopathiae (ATCC 19414). The type strain of E. tonsillarum is T-305^T (ATCC 43339).

Erysipelothrix rhusiopathiae is a causative agent of swine erysipelas, a disease that occurs in acute and chronic forms and causes economic loss in pig production. In addition, E. rhusiopathiae causes polyarthritis in lambs and calves, septicemia in turkeys and ducks, septicemia and urticaria in dolphins, and cutaneous lesions in humans, a disease which is known as erysipeloid (20). In addition, the organisms are found in the tonsils of apparently normal swine and in the slime on the bodies of fish. The genus Erysipelothrix consists of a single species which is biochemically and morphologically identified as E. rhusiopathiae (1). At present, strains of E. rhusiopathiae are classified into 22 serotypes (15). Heat-stable antigens derived from the cell by hot aqueous extraction form the basis for division of the species into serotypes (6). Most isolates from swine with clinical erysipelas are serotypes 1 and 2 (19). No difference in the deoxyribonucleic acid (DNA) base composition was observed among some serological types of E. rhusiopathiae (3). Further attempts on chemotaxonomic studies of the Erysipelothrix species have not been made in spite of the wide range of hosts available to the organisms and their wide variety of serological and pathogenic characteristics (16). During a study of the characteristics of E. rhusiopathiae from tonsils of apparently healthy slaughter pigs, we found a cluster of virulent strains of serotype 2 and a cluster of avirulent strains of serotype 7 existing predominantly in the tonsils (T. Takahashi, T. Sawada, M. Muramatsu, Y. Tamura, T. Fujisawa, Y. Benno, and T. Mitsuoka, J. Clin. Microbiol., in press).

DNA base composition and DNA-DNA homology were used to compare and classify these isolates from porcine tonsils. The avirulent organisms of serotype 7 were genetically distinct from the E. rhusiopathiae strains characterized to date, indicating the transfer of these avirulent serotype 7 organisms to a new species, for which the name Erysipelothrix tonsillarum sp. nov. is proposed.

The 17 strains studied were from pigs raised on different farms in four prefectures. They are listed in Table 1. Of these, 16 strains designated with a T number were isolated from tonsils of apparently healthy slaughter pigs in our laboratory; E. rhusiopathiae ATCC 19414 was obtained from the American Type Culture Collection, Rockville, Md. The strains were maintained on stock culture agar (Difco Laboratories, Detroit, Mich.).

Biocatalytic characterization of the strains was made on the basis of the pattern of acid production from 19 fermentable carbohydrates, growth in gelatin, reaction in litmus milk, ability to hydrolyze esculin, production of H2S in triple sugar iron agar (Difco Laboratories), production of catalase, and production of oxidase. Cytochrome oxidase test strips (Eiken Chemical Co., Ltd., Tokyo, Japan) were used to detect the presence of this enzyme. All other biochemical testing procedures have been described previously (18). The tests for carbohydrate utilization were conducted by using Andrade base with 10% horse serum (17). Antibiotic susceptibility was tested by an agar dilution method (13).

Serotyping of the strains was performed by the method described previously (7, 8, 15). Colonies from a 48-h agar plate of each strain were inoculated into beef infusion (BI) broth (pH 7.6, prepared in our laboratory) containing 0.1% Tween 80. After incubation for 48 h at 37°C, the broth culture was centrifuged 12,000 × g for 20 min. The bacterial cells were washed three times with physiological saline and suspended in distilled water to 1/30 of the original volume. The bacterial suspension was autoclaved for 1 h at 121°C, cooled, and clarified by centrifugation. The supernatant fluid was tested in an agar gel double-diffusion precipitation system against typing sera (rabbit origin) representing serotypes 1 through 22 of E. rhusiopathiae.

For pathogenicity tests, 4-week-old female mice of the outbred ddY strain were used. They were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan.

Female and castrated male Yorkshire swine, purchased from the Minano Agricultural Cooperative Association for Laboratory Animals, Saitama, Japan, were used when they

* Corresponding author.
were 3 to 4 months old. They were conventionally farrowed and raised in confinement. The sera of the swine had a growth agglutination titer (12) of 8 or below.

Strain pathogenicities for mice and swine were determined by using the method described previously (14, 16). A portion (0.1 ml) of serial 10-fold dilutions of BI broth culture of each strain was injected subcutaneously into each of five mice. At the same time, 0.1 ml of each dilution was spread onto two petri plates and mixed with BI agar medium containing 0.75% agar. After 48 h of cultivation at 37°C, colonies in the agar were counted. For determination of the 50% lethal dose (LD50), mortality rates were recorded 14 days after exposure. The LD50 values were determined by the method of Kärber (5).

One pig was inoculated intradermally with 0.1 ml of BI broth culture (approximately 10^7 CFU per pig) of each strain. Clinical signs were observed every day for 14 days after exposure.

Colonial morphology was determined with 48-h cultures on BI agar (pH 7.6, prepared in our laboratory). Detailed cellular morphology was determined with selected strains grown in BI broth for 24 h. The cells were suspended in sterile physiological saline, negatively stained with 2% phosphotungstic acid, and examined with a transmission electron microscope (JEM 100s; JEOL, Tokyo, Japan) at 80 kV.

For the preparation of DNA, bacterial cells grown in BI broth containing 0.1% Tween 80 were harvested in the logarithmic phase and washed twice with 0.15 M NaCl-0.1 M ethylenediaminetraacetic acid (pH 8.0). DNA was isolated by modification of the procedures of Marmur (9). The purity and the amount of DNA were estimated by measuring the hyperchromic shift during thermal denaturation (2). Tritium-labeled DNA was prepared by using a nick translation system (New England Nuclear Corp., Boston, Mass.) adapted from the procedure of Rigby et al. (11).

The guanine-plus-cytosine (G+C) contents of the DNAs were determined by the thermal melting point method (10), using an automatic recording spectrophotometer (Komatsu Electronics, Tokyo, Japan). Calf thymus DNA was included in each set as a standard.

DNA homology experiments were performed by the S1 nuclease procedure, as described by Johnson et al. (4). S1 nuclease digestion was conducted with 0.5 U of S1 nuclease (Seikagaku, Kogyo Co., Tokyo, Japan). After incubation for 15 min at 37°C, an equal volume of 10% trichloroacetic acid was added to each tube. The tubes were cooled to 4°C for at least 1 h, and the precipitates were collected on nitrocellulose membrane filters (type HA; Millipore Corp., Bedford, Mass.). The membranes were dried, and the radioactivity was measured in a toluene-based scintillation fluid with a liquid scintillation counter (model 3330; Packard Instrument Co., Inc., Rockville, Md.).

Serotypes and pathogenicities for mice and swine of bacterial strains used in this study are shown in Table 1. Seven strains that belonged to serotype 7 were avirulent in swine, except for strain T-334, which induced a local urticarial lesion at the site of inoculation. The LD50 values in subcutaneously inoculated mice ranged from 1.0 CFU to 10^5 CFU. Of 10 E. rhusiopathiae strains, 6 strains belonged to serotype 2, and 1 strain each belonged to serotypes 6, 11, 12, and 16. All of the E. rhusiopathiae strains of serotype 2 induced generalized urticarial lesions with depression and anorexia after intradermal inoculation, and the LD50's in mice were ≥2.1 × 10^4 CFU. The E. rhusiopathiae strains of serotypes other than 2 induced local urticarial lesions at the site of inoculation in swine (except for strain T-312 of serotype 12, which induced no clinical signs), and the LD50s in mice were ≥2.5 × 10^4 CFU.

Electron microscopic examination failed to reveal any differences between cells of E. tonsillae and E. rhusiopathiae. The DNA base compositions of the test strains and the results of DNA-DNA hybridization experiments are shown in Table 1.
TABLE 2. DNA relatedness among *E. tonsillae* and *E. rhusiopathiae* strains

<table>
<thead>
<tr>
<th>Strain (serotype)</th>
<th>G+C content of DNA (mol%)</th>
<th>% Homology with [IH] DNA from strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 19414^T (2)</td>
<td>T-305^T ATCC 19414^T</td>
</tr>
<tr>
<td>T-181 (2)</td>
<td>36</td>
<td>18  100</td>
</tr>
<tr>
<td>T-183 (2)</td>
<td>36</td>
<td>25  107</td>
</tr>
<tr>
<td>T-191 (2)</td>
<td>36</td>
<td>20  105</td>
</tr>
<tr>
<td>T-194 (2)</td>
<td>40</td>
<td>38  93</td>
</tr>
<tr>
<td>T-205 (11)</td>
<td>40</td>
<td>15  49</td>
</tr>
<tr>
<td>T-209 (2)</td>
<td>35</td>
<td>15  41</td>
</tr>
<tr>
<td>T-312 (12)</td>
<td>35</td>
<td>17  40</td>
</tr>
<tr>
<td>T-184 (16)</td>
<td>36</td>
<td>18  86</td>
</tr>
</tbody>
</table>

The homology results indicate that, although a cluster of avirulent strains isolated from tonsils of apparently healthy slaughter pigs is morphologically and biochemically indistinguishable from *E. rhusiopathiae*, it is genetically distinct from *E. rhusiopathiae* characterized to date. Therefore, we propose the name *Erysipelothrix tonsillarum* sp. nov. for these strains and designate strain T-305^T (ATCC 43339) as the type strain.

**Description of *Erysipelothrix tonsillarum* sp. nov.**

*Erysipelothrix tonsillarum* (ton. sil. la'rum. L. gen. noun, of the tonsils). Cells are gram-positive, nonmotile, non-sporeforming rods approximately 0.3 μm wide by 1.0 to 1.5 μm long.

Surface colonies on BI agar after 2 days of incubation are punctiform to approximately 1 mm in diameter, circular, entire, convex, colorless, transparent, and soft.


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