DNA Relatedness among *Erysipelothrix rhusiopathiae* Strains Representing All Twenty-Three Serovars and *Erysipelothrix tonsillarum*

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The levels of relatedness among strains of *Erysipelothrix rhusiopathiae* (serovars 1 through 23 and type N) were estimated by performing DNA-DNA hybridization experiments with the type strains of *E. rhusiopathiae* and *Erysipelothrix tonsillarum*, which are the two *Erysipelothrix* species that have been described. Two distinct DNA relatedness groups were identified. The group 1 strains, representing serovars 1, 2, 4 through 6, 8, 9, 11, 12, 15 through 17, 19, and 21 and type N, exhibited more than 73% hybridization with the type strain of *E. rhusiopathiae* but less than 24% hybridization with the type strain of *E. tonsillarum*. Group 2 included serovar 3, 7, 10, 14, 20, 22, and 23 strains, and these strains exhibited more than 66% hybridization with the type strain of *E. tonsillarum* but less than 27% hybridization with the type strain of *E. rhusiopathiae*. Strains representing serovars 13 and 18 exhibited low levels of hybridization (16 to 47%) with both of the type strains, indicating that these serovars may be members of a new genomic species. The members of the *E. rhusiopathiae* and *E. tonsillarum* groups resembled each other in many phenotypic characteristics, but differed in their ability to produce acid from saccharose and in their pathogenicity for swine. Our results confirm that the genus *Erysipelothrix* contains two main genomic species, *E. rhusiopathiae* and *E. tonsillarum*, which can be differentiated into serovars.

Erysipelothrix rhusiopathiae is an organism which has a major economic impact, causing a variety of diseases in animals and birds (ranging from septicemia to urticaria), as well as erysipeloid, a skin disease of humans (23). This organism has also been isolated from the tonsils of apparently healthy slaughter swine, from slime on fish, and from the environment. At the present time, the strains of *E. rhusiopathiae* are classified into 23 serovars and the type N strains, which do not produce any precipitating antibody against homologous and heterologous heat-stable extracts in rabbits (9, 17). Heat-stable antigens that are derived from cells by hot aqueous extraction are the basis for dividing the species into serovars (3).

Most isolates obtained from diseased swine belong to serovars 1 and 2 (22), and the genus *Erysipelothrix* was once thought to be monospecific (12).

A cluster of avirulent serovar 7 strains that were isolated from porcine tonsils were found to be genetically distinct from the other *E. rhusiopathiae* strains. We have previously described these strains as new species, *Erysipelothrix tonsillarum* (13, 14). However, whether genotypic *E. tonsillarum* strains occur in serovars other than serovar 7 is still unclear. The purposes of this study were to examine the levels of relatedness among organisms belonging to serovars 1 through 23 and type N by performing DNA-DNA hybridization experiments with the type strains of *E. rhusiopathiae* and *E. tonsillarum* and to clarify the taxonomic relationships among these serovars and the species in the genus *Erysipelothrix*.

MATERIALS AND METHODS

Bacterial strains. Details concerning the test strains and their sources are shown in Table 1. The strains were maintained on Bacto Stock Culture Agar (Difco Laboratories, Detroit, Mich.).

Cultural and biochemical tests. The strains were characterized biochemically by using the tests listed in Table 2. The production of H₂S was tested in triple sugar iron agar (Difco). Cytochrome oxidase test strips (Eiken Chemical Co., Ltd, Tokyo, Japan) were used to detect the presence of this enzyme. Plasma-clotting activity was detected by mixing a 24-h broth culture with fresh citrated rabbit plasma (19). All of the other biochemical test procedures which we used have been described previously (21). The medium used to test for production of acid from carbohydrates was nutrient broth supplemented with 1% Andrade's indicator and 10% horse serum (20). Antibiotic susceptibility was tested by using an agar dilution method (15).

Test used to determine serovars. The serotypes of the strains were determined by using a previously described method (5, 6, 17). 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 at 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 by using an agar gel double-diffusion precipitation system against typing sera (rabbit origin) representing serovars 1 through 23 and type N of *E. rhusiopathiae*.

Pathogenicity tests. For pathogenicity tests, we used

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TABLE 1. Serovars, origins, and levels of pathogenicity of *E. rhusiopathiae* and *E. tonsillarum* strains used in this study

Strain	Serovar or type	Origin ^a	Pathogenicity for mice (log LD ₅₀) ^b	Pathogenicity for swine	
				Size of erythema ^c (cm) ^c	Systemic ^d
<i>E. rhusiopathiae</i> strains					
ATCC 19414 ^T	2	Pig with endocarditis	0.3	Generalized	+
ME-7	1a	Unknown	1.0	Generalized	+
Sayo-SP	1a	Chicken	1.1	Generalized	+
422/1E1	1b	Porcine spleen	1.0	Generalized	+
R32E11	2	Unknown	1.5	Generalized	+
NF4E1	2	Porcine spleen	1.3	Generalized	+
Witlling	3	Fish	0.5		—
Doggerscharbe	4	Fish	>7.0		—
Pécs 67	5	Porcine tonsil	1.4	15 by 17	—
Dolphin S-1	5	Dolphin	2.2		—
Tuzok	6	Bustard	0.8		—
Dolphin E-1	6	Dolphin	0.4		—
Goda	8	Godwit	0.6	10 by 10	—
Kaparek	9	Fish	>6.9		—
Lengyel-P	10	Squirrel	0.2		—
IV12/8	11	Porcine tonsil	0.9	16 by 17	—
Pécs 9	12	Porcine tonsil	0.7	Generalized	+
Pécs 56	13	Porcine tonsil	>6.5		—
Iszap-4	14	Mud of zoo pond	0.8		—
Pécs 3597	15	Porcine tonsil	1.2		—
Tanzania	16	Parrot	1.4		—
545	17	Porcine spleen	>6.5		—
715	18	Porcine spleen	1.1	18 by 20	—
2017	19	Porcine spleen	0.9	12 by 16	—
2553	20	Porcine spleen	1.5	10 by 12	—
Báno 36	21	Sheep dip	0.8	14 by 18	—
Báno 107	22	Sheep dip	>5.9		—
KS20A	23	Pig slurry	1.4		—
MEW 22	N	Unknown	1.0		—
<i>E. tonsillarum</i> strains					
ATCC 43339 ^T	7	Porcine tonsil	>5.3		—
ATCC 43338	7	Porcine tonsil	>5.8		—
P-43	7	Fish	1.8		—
L1-3	7	Chopped pork	0.9		—
L1-4	7	Chopped pork	0.8		—
Nairiku-21	7	Porcine lymph node	0.8		—
NG-37	7	Pig slurry	1.0		—
T-131	7	Porcine tonsil	1.2		—

^a Strains ATCC 19414^T (T = type strain), ATCC 43339^T, and ATCC 43338 were received from the American Type Culture Collection, Rockville, Md. Strains Sayo-SP, Dolphin S-1, Dolphin E-1, L1-3, L1-4, Nairiku-21, and NG-37 were preserved in our laboratory. The other strains, including the serovar reference strains representing serovars 1 through 23 and type N, were provided by K. Hashimoto, National Institute of Animal Health, Tokyo, Japan, R. L. Wood, National Animal Disease Center, Ames, Iowa, and V. Nørrung, State Veterinary Serum Laboratory, Copenhagen, Denmark.

^b Mice were inoculated subcutaneously with serial dilutions of a broth culture of each strain. The LD₅₀ values are expressed as the numbers of viable bacteria per mouse.

^c Maximum size of erythema at the skin injection site, which was observed for 2 weeks after intradermal inoculation with 0.1 ml of a broth culture (approximately 10⁷ CFU) of each strain.

^d +, depression and anorexia; —, no response.

4-week-old female outbred strain ddY mice (Nippon SLC Co., Ltd., Hamamatsu, Japan).

Female and castrated male Yorkshire swine, which were purchased from the Minano Agricultural Cooperative Association for Laboratory Animals, Saitama, Japan, were used when they were 3 to 4 months old. These swine were conventionally farrowed and raised in confinement. The sera of the swine had growth agglutination titers (11) of <1:8.

The levels of strain pathogenicity for mice and swine were determined by using a previously described method (16, 18). A portion (0.1 ml) of one of a series of 10-fold dilutions of a 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, the colonies in the agar were counted. To determine the 50% lethal doses (LD₅₀), mortality rates were recorded 14 days after exposure. The LD₅₀ values were determined by using the method of Kärber (4).

One pig was inoculated intradermally with 0.1 ml of a BI broth culture (approximately 10⁷ CFU per pig) of each strain. Clinical signs were observed every day for 14 days after exposure.

Preparation of DNA. To prepare DNA, bacterial cells grown in BI broth containing 0.1% Tween 80 were harvested in the logarithmic phase and were washed twice with 0.15 M NaCl–0.1 M EDTA (pH 8.0). The DNA was isolated by using a modification of the procedure of Marmur (7). The purity and the amount of DNA were estimated by measuring the hyperchromic shift during thermal denaturation (1).

TABLE 2. Differential phenotypic characteristics of *Erysipelothrix* species

Characteristic	% of strains having a positive reaction		
	<i>E. rhusiopathiae</i> (21 strains)	<i>E. tonsillarum</i> (14 strains)	Others (2 strains)
H ₂ S production (triple sugar iron agar)	100	100	100
Test tube brush in gelatin	100	100	100
Catalase activity	0	0	0
Oxidase activity	0	0	0
Acid produced from:			
Saccharose	0	93	0
Glycerol	0	0	0
Maltose	100	100	100
Salicin	0	14	50
Inositol	0	0	0
Galactose	100	100	50
Levulose	100	100	100
Dulcitol	0	0	0
Rhaffinose	0	0	0
Mannite	0	0	0
Mannose	100	43	100
Lactose	100	100	50
Glucose	100	100	100
Rhamnose	0	0	0
Trehalose	0	0	50
Inulin	0	0	0
Xylose	0	0	0
Sorbitol	0	0	0
Arabinose	0	0	0
Clotting of citrated plasma	100	100	100
Susceptibility to:			
Penicillin G (0.1 U/ml)	100	100	100
Ampicillin (0.1 µg/ml)	100	100	100
Kanamycin (100 µg/ml)	0	0	0
Sulfadimethoxine (100 µg/ml)	0	0	0
Pathogenicity for swine:			
Generalized erythema ^a	33	0	0
Localized erythema ^b	24	7	50
No clinical signs ^c	43	93	50

^a Induced generalized erythema with depression and anorexia in swine after intradermal inoculation with 0.1 ml of a broth culture (approximately 10⁷ CFU) of each strain.

^b Induced localized erythema at the skin injection site.

^c Induced no clinical signs of erysipelas in swine.

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. (10).

DNA base composition. The guanine-plus-cytosine contents of DNAs were determined by the thermal melting point method (8), using an automatic recording spectrophotometer (Komatsu Electronics, Tokyo, Japan). Calf thymus DNA was included in each set as a standard.

DNA homology experiments. DNA homology experiments were performed by using the S1 nuclease procedure, as described by Johnson et al. (2). S1 nuclease digestion was performed 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 model 3330 liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

TABLE 3. Levels of DNA relatedness among *E. rhusiopathiae* and *E. tonsillarum* strains

Strain	Serovar or type	Guanine-plus- cytosine content of DNA (mol%)	% Homology with [³ H]DNA from:	
			Strain ATCC 19414 ^T	Strain ATCC 43339 ^T
<i>E. rhusiopathiae</i> strains				
ATCC 19414 ^T	2	38	100	16
ME-7	1a	36	89	14
Sayo-SP	1a	37	97	16
422/1E1	1b	37	92	24
R32E11	2	40	97	18
NF4E1	2	37	102	17
Doggerscharbe	4	36	76	17
Pécs 67	5	39	99	21
Dolphin S-1	5	37	86	21
Tuzok	6	37	73	20
Dolphin E-1	6	37	77	17
Goda	8	36	101	18
Kaparek	9	37	95	17
IV12/8	11	36	89	15
Pécs 9	12	37	87	14
Pécs 3597	15	38	86	16
Tanzania	16	36	103	15
545	17	37	96	17
2017	19	39	91	16
Báno 36	21	40	85	17
MEW 22	N	40	93	21
<i>E. tonsillarum</i> strains				
ATCC 43339 ^T	7	35	25	100
Witlling	3	37	21	82
ATCC 43338	7	37	21	93
P-43	7	36	20	87
L1-3	7	38	24	82
L1-4	7	38	21	79
Nairiku-21	7	38	23	66
NG-37	7	36	24	77
T-131	7	37	18	75
Lengyel-P	10	37	26	78
Iszap-4	14	36	21	80
2553	20	36	26	74
Báno 107	22	38	27	80
KS20A	23	38	26	81
Other strains				
Pécs 56	13	35	19	24
715	18	38	47	16

RESULTS

The results of the pathogenicity tests are shown in Table 1. In swine, seven strains belonging to serovars 1, 2, and 12 induced generalized urticarial lesions with depression and anorexia after intradermal inoculation. Seven strains belonging to serovars 5, 8, 11, and 18 through 21 induced local urticarial lesions at the site of inoculation. The remaining strains, which belonged to serovars 3, 4, 6, 7, 10, 12 through 17, 22, and 23 and type N, induced no clinical signs.

Of the 37 strains tested, 30 (belonging to serovars 1 through 3, 5 through 8, 10 through 12, 14 through 16, 18 through 21, and 23 and type N) were highly virulent for mice (LD₅₀ values, <10^{2.2} CFU), and the remaining strains had LD₅₀ values of more than 10^{5.9} CFU.

The DNA base compositions of the strains tested and the results of the DNA-DNA hybridization experiments are shown in Table 3. The strains had guanine-plus-cytosine contents ranging from 35 to 40 mol% but were separated into

three groups on the basis of the percentages of DNA-DNA reassociation with the type strains of *E. rhusiopathiae* and *E. tonsillarum*. Clearly, the strains belonging to *E. rhusiopathiae* serovars hybridized at levels of 73% or more with the type strain of that species, and the strains belonging to *E. tonsillarum* serovars hybridized at levels of 66% or more with the type strain of *E. tonsillarum*. In each case the nonhomologous values were less than <30%. There was a minor group containing two strains which exhibited minimal association with each type strain.

The differential biochemical and pathogenic characteristics of the *Erysipelothrix* species are shown in Table 2. Except for the ability to produce acid from saccharose, the biochemical characteristics of the *Erysipelothrix* species were nearly identical; 13 (93%) of the 14 strains which belonged to *E. tonsillarum* serovars produced acid from saccharose, whereas none of the 21 strains which belonged to *E. rhusiopathiae* serovars did so. In the pathogenicity tests, 12 (57%) of the 21 strains which belonged to *E. rhusiopathiae* serovars induced generalized or localized erythema in swine after intradermal inoculation, whereas 13 (93%) of the 14 strains which belonged to *E. tonsillarum* serovars did not induce any clinical sign of erysipelas in swine.

DISCUSSION

In previous study we described a cluster of avirulent serovar 7 strains that were isolated from porcine tonsils and were genetically distinct from the other *E. rhusiopathiae* isolates and suggested that these serovar 7 strains should be transferred to a new species, *E. tonsillarum* (13, 14). In contrast to the original description of *E. tonsillarum*, in this study we found that *E. tonsillarum* strains can be members of several of the serovars (other than serovar 7) formerly associated with *E. rhusiopathiae*. Both *E. rhusiopathiae* and *E. tonsillarum* contain a number of serovars, but none of the strains which we tested is common to both species. *E. tonsillarum* is distinguished from the other species by the fermentation of saccharose and by a lack of pathogenicity for swine, as well as by serology. Therefore, we suggest that the serovar 3, 10, 14, 20, 22, and 23 strains that have been identified phenotypically as *E. rhusiopathiae* previously should be transferred to *E. tonsillarum* along with the serovar 7 strains.

The low DNA relatedness values (16 to 47%) between the serovar 13 or 18 strains and the type strains of two genomic species indicate that these minor serovar groups may be members of a new genomic species of the genus *Erysipelothrix*. However, more strains will have to be collected to support this hypothesis.

The virulence factor of the organisms is not clearly understood. The serovars of the *Erysipelothrix* species are based on antigenic characteristics of soluble peptidoglycans of the cell wall (3). In this study, we clarified that the serologically distinguished strains which are highly virulent for swine are capable of inducing generalized urticaria lesions with depression and anorexia and can be assigned to the *E. rhusiopathiae* group. Our results from pathogenicity tests confirmed our previous results (13, 14); *E. rhusiopathiae* strains are pathogenic for swine, but *E. tonsillarum* strains are not. Our genetic data, in conjunction with differences in some phenotypic properties, confirmed the heterogeneity of the groups of organisms and led to recognition of the two *Erysipelothrix* species.

On the basis of these findings, we confirmed that the genus *Erysipelothrix* contains two species, *E. rhusiopathiae* and *E.*

tonsillarum. These species are serologically distinct, belong to separate DNA-DNA hybridization groups, and can be distinguished by whether they ferment saccharose.

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