Pulsed-Field Gel Electrophoresis in Differentiation of *Erysipelothrix* Species Strains

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Received 29 May 2001/Returned for modification 16 July 2001/Accepted 3 September 2001

We report here the first analysis of *Erysipelothrix* spp. using pulsed-field gel electrophoresis (PFGE). Seventy strains of *Erysipelothrix* spp. were analyzed. *Sma*I, *Asc*I, and *Not*I were tested for the ability to cleave the DNA extracted from those strains, and among them, *Sma*I was the most reliable enzyme. Sixty-three distinct PFGE patterns were produced, and no DNA degradation was observed, allowing the identification of all of the strains. Based on these results and on those of a previous analysis using randomly amplified polymorphic DNA and ribotyping, PFGE with *Sma*I might be considered to be more sensitive than those methods and to be the best method for epidemiological studies of strains of this genus.

Erysipelothrix rhusiopathiae is a gram-positive, slender, and straight or slightly curved rod that causes a wide spectrum of diseases in animals, birds, and humans (14, 49). This bacterium has been isolated in most parts of the world, not only from sick and healthy animals but even from pork, seafood, retail game meat, and chicken meat (11, 19, 26, 27, 38, 40, 44). Human infections with this bacterium are usually related to occupational exposure (33). However, infection after consumption of undercooked pork and infections of patients with no history of contact with animals or skin lesions have been reported, and in many cases, the source of infection has not been identified (6, 13, 20, 28, 37). Moreover, potential errors in the recognition of this organism isolated from human infections due to unusual clinical presentations and the possibility of underdiagnosed infections have been reported (3, 10, 34). PCR-based assays for the rapid diagnosis of *Erysipelothrix* species have been described (22, 39, 42). However, to proceed with an epidemiological study and identify the source of infection, it is necessary to be able to identify each strain isolated from a case or outbreak, as well as the relatedness among the strains isolated from the possible source.

During the last few years, molecular biological methods such as randomly amplified polymorphic DNA (RAPD), ribotyping, and pulsed-field gel electrophoresis (PFGE) have been demonstrated to be reliable tools for the differentiation of species and strains of one genus and for use in epidemiological studies of several pathogenic bacteria (9, 15, 16, 24, 32, 46–48). Although PFGE has been considered to be the “gold standard” among these methods (30), studies have shown that this method can be less sensitive than ribotyping and PCR-based methods with regard to the ability to differentiate between bacterial strains of some species (5, 36, 45). Moreover, there is no standard and universal PFGE protocol for all species of bacteria and it is necessary to adapt the procedures and choose

a suitable enzyme for each genus or species. However, the use of this method for strains of the genus *Erysipelothrix* has not been reported. Therefore, we describe herein the first analysis of a large collection of *Erysipelothrix* species strains by PFGE.

MATERIALS AND METHODS

Bacterial strains. Seventy strains, including 55 of *E. rhusiopathiae* and 12 of *E. tonsillarum*, as well as 2 strains of serovar 13 and 1 strain of serovar 18 that have been considered to be members of a possible new species (41), were chosen from our previous study (29). The sources for and details regarding each strain are shown in Table 1.

DNA preparation. Chromosomal DNAs from the strains were prepared by using the CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad Laboratories, Richmond, Calif.) with some modifications of the manufacturer's instructions. All of the buffers and solutions, except those for which the manufacturer are identified, were supplied with the DNA Plug Kit. The strains were inoculated in 50 ml of tryptose phosphate broth (Difco Laboratories) and cultured overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation, suspended in 1 ml of phosphate-buffered saline (8.45 mM NaH₂PO₄, 5.12 mM KH₂PO₄, 0.12 M NaCl [pH 7.2]), transferred to 2-ml Eppendorf tubes, washed once, resuspended, and then diluted with the same buffer to an optical density at 600 nm of 0.8 to 1.0. Chloramphenicol was added to a final concentration of 180 µg/ml, and the suspension was incubated at 37°C for 1 h. One milliliter of the suspension was centrifuged, and the harvested cells were resuspended in 50 µl of cell suspension buffer. The suspension was heated to 50°C in warm water and combined with the same amount of melted 2% CleanCut agarose, also equilibrated to 50°C. The mixture was transferred to disposable plug molds and allowed to solidify at 4°C for 10 min. Each plug was removed from the molds and placed in 250 µl of lysozyme solution, prepared by adding 200 µl of the lysozyme stock (25 mg/ml) and 100 µl of *N*-acetylmuramidase (1 mg/ml; Seikagaku Corp., Tokyo, Japan) to 2.5 ml of lysozyme buffer, and then incubated for 48 h at 37°C in a water bath. The lysozyme solution was removed, and the plugs were rinsed once with sterile deionized water, after which they were replaced in 250 µl of proteinase K reaction buffer containing 10 µl of proteinase K stock, yielding a final proteinase K concentration of 100 µg/ml. After further incubation for 24 h at 50°C, the plugs were washed four times in 1× wash buffer for 1 h each time at room temperature. Phenylmethylsulfonyl fluoride (Sigma) was added to a final concentration of 1 mM at the second wash. Plugs were stored in 1 ml of 1× wash buffer until enzyme treatment.

Restriction enzymes and PFGE. DNA plugs were sliced, and DNAs were digested with *Sma*I (Takara Co. Ltd., Tokyo, Japan), *Asc*I (New England Bio-Labs), and *Not*I (Takara). A slice of each plug was placed twice in 0.1× wash buffer and incubated for 1 h each time at room temperature. After removal of the wash buffer, the DNAs were digested with 10 U of each enzyme in the respective reaction mixture in accordance with the manufacturer's instructions. The DNA fragments were separated in 1% agarose NA gel (Amersham Pharmacia) that

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TABLE 1. PFGE patterns of 70 strains of *Erysipelothrix* spp. produced by *Sma*I

Species and serovar	Strain	Source	PFGE pattern	RAPD pattern ^d	Ribo pattern ^e
<i>E. rhusiopathiae</i>					
1a	ME-7 ^a	Unknown	P1	a	B
	E176	Fish	P2	b	
	E157	Fish	P3	a	
1b	422/1E1 ^a	Porcine spleen	P4	a	A
	E019	Fish	P5	g	
	K040	Wild boar meat	P6	a	
	K075	Wild boar meat	P7	a	
2	ATCC 19414 ^b	Pig with endocarditis	P8	c	A
	R32E11 ^a	Unknown	P9	d	
	NF4E1	Porcine spleen	P10	a	A
	115	Chicken	P11	g	
	17.2a	Chicken	P12	a	
	10.2a	Chicken	P12	a	
	E037	Fish	P13	a	
	K003	Wild boar meat	P14	e	
	N026	Chicken	P15	d	
4	Doggerscharbe ^a	Fish	P16	a	
	212	Chicken meat	P17	g	
	213	Chicken meat	P17	g	
	E127	Fish	P18	f	
5	Pécs 67 ^a	Porcine tonsil	P19	d	C
	AKO	Chicken	P20	a	
	2.2a	Chicken	P21	g	
	K059	Wild boar meat	P22	g	
6	Tuzok ^a	Bustard	P23	g	
	36.4a	Chicken	P24	g	
	136	Chicken	P25	f	
	20.4a	Chicken	P25	f	
	K002	Wild boar meat	P26	g	
8	Goda ^a	Godwit	P27	d	A
	47	Chicken	P28	g	
	N008	Chicken	P28	g	
	r4.1a	Chicken meat	P28	g	
	r6.1a	Chicken meat	P28	g	
	E024	Fish	P29	g	
9	Kaparek ^a	Fish	P30	a	B
	E112	Fish	P31	h	
	280	Chicken	P32	g	
	K052	Wild boar meat	P33	f	
10	K024 ^f	Wild boar meat	P34	g	
11	IV12/8 ^a	Porcine tonsil	P35	g	
	K021	Wild boar meat	P36	a	
12	Pécs 9 ^a	Porcine tonsil	P37	a	
	88	Chicken meat	P38	g	
	97	Chicken meat	P38	g	
	E146	Fish	P39	g	
15	Pécs 3597 ^a	Porcine tonsil	P40	d	A
16	Tanzania ^a	Parrot	P41	d	A
17	545 ^a	Porcine spleen	P42	d	A
19	2017 ^a	Porcine spleen	P43	g	A
	E053	Fish	P44	d	
	E051	Fish	P45	g	
	K031	Deer meat	P46	d	
21	Báno 36 ^a	Sheep dip	P47	a	C
N	MEW22 ^a	Unknown	P48	a	
<i>E. tonsillarum</i>					
3	Wittling ^a	Fish	P49	j	I
7	ATCC 43339 ^c	Porcine tonsil	P50	j	E
	ATCC 43338	Porcine tonsil	P51	j	E
	P-43	Fish	P52	j	E
	K015	Wild boar meat	P53	j	
10	Lengyel-P ^a	Squirrel	P54	j	E
14	Iszap-4 ^a	Zoo pond mud	P55	j	E
15	E073 ^g	Fish	P56	i	
16	K037 ^g	Wild boar meat	P57	j	
20	2553 ^a	Porcine spleen	P58	k	E
22	Báno 107 ^a	Sheep dip	P59	l	E
23	KS20A ^a	Pig slurry	P60	j	E
<i>Erysipelothrix</i> spp.					
13	Pécs 56 ^a	Porcine tonsil	P61	m	
13	Shiribeshi17	Pigpen litter	P62	m	
18	715 ^a	Porcine spleen	P63	n	H

^a Serovar reference strain.^b *E. rhusiopathiae* type strain.^c *E. tonsillarum* type strain.^d RAPD pattern obtained by Okatani et al. (29).^e Ribopattern obtained by Ahrné et al. (1).^f Strain classified as *E. rhusiopathiae* by PCR-based methods used by Okatani et al. (29).^g Strain classified as *E. tonsillarum* by PCR-based methods used by Okatani et al. (29).

FIG. 1. PFGE patterns produced from four strains of *Erysipelothrix* spp. by *Sma*I, *Asc*I, and *Not*I. Lanes: 1, *E. rhusiopathiae* strain ME-7, serovar 1a; 2, *E. rhusiopathiae* strain ATCC 19414, serovar 2; 3, *E. tonsillarum* strain ATCC 43339, serovar 7; 4, *Erysipelothrix* species strain 715, serovar 18; M, CHEF DNA Size Standard Lambda Ladder (Bio-Rad).

was prepared in 0.5× Tris-borate-EDTA buffer (50 mM Tris base, 50 mM boric acid, 2 mM EDTA) on a Gene Navigator (Pharmacia Biotech). Electrophoresis was carried out for 24 h at 12°C and 200 V with pulse times of 1 to 35s. The CHEF DNA Size Standard Lambda Ladder (Bio-Rad) was used as a DNA size marker. Thereafter, the gels were stained with ethidium bromide for 1 h, de-

stained in distilled water, and photographed under UV light. PFGE patterns were inspected visually, each PFGE pattern that differed by one or more DNA fragment bands was identified, and the relatedness among the patterns was analyzed based on the guidelines described by Tenover et al. (43) and Maslow et al. (24).

RESULTS

Of the three restriction enzymes tested, only *Sma*I produced several bands in repeated screening tests and was able to clearly differentiate between strains. Thus, *Sma*I was used for further analysis of all 70 strains. The PFGE patterns produced from four strains with the three enzymes are shown in Fig. 1. To determine the optimal electrophoresis protocol, a series of trials changing the pulse and electrophoresis times was carried out (data not shown). Of the protocols tested, the one described above was best able to discern the high- and even low-molecular-weight DNA fragment bands produced by *Sma*I.

From the 70 strains analyzed, 63 distinct PFGE patterns with 8 to 27 DNA fragment bands were produced (Fig. 2; Table 1). Twelve strains showed five distinct PFGE patterns with no DNA fragment band difference observed among strains sharing the same PFGE pattern. These were strains 47, N008, r4.1a, and r6.1a, which shared pattern P28 and strains 17.2a and 10.2a, 212 and 213, 136 and 20.4a, and 88 and 97, which shared, respectively, patterns P12, P17, P25, and P38 (Table 1). Single and distinct PFGE patterns were produced for 58 strains. Among them, patterns P50 and P51 of strains ATCC 43339 and ATCC 43338 differed by two bands, patterns P4 and P36 of strains 422/1E1 and K021 differed by three bands, patterns P32 and P39 of strains 280 and E146 differed by four bands, and patterns P19 and P20 of strains Pécs 67 and AKO differed by five bands. Patterns P24 of strain 36.4a and P25 of strains 136 and 20.4a differed by six bands from pattern P26 of strain K002, as well as patterns P34 and P45 of strains K024 and E051. The remaining 45 patterns differed by at least seven bands (Fig. 2). Although some similar bands were observed among many

FIG. 2. Schematic representation of the 63 PFGE patterns produced from the 70 *Erysipelothrix* spp. strains studied by using *Sma*I. *E. spp.*, *Erysipelothrix* species. The serovar and PFGE pattern designation of each strain are given, respectively, in parentheses. Lanes: 1, ME-7 (1a, P1); 2, E176 (1a, P2); 3, E157 (1a, P3); 4, 422/1E1 (1b, P4); 5, E019 (1b, P5); 6, K040 (1b, P6); 7, K075 (1b, P7); 8, ATCC 19414 (2, P8); 9, R32E11 (2, P9); 10, NF4E1 (2, P10); 11, 115 (2, P11); 12, 17.2a (2, P12); 13, E037 (2, P13); 14, K003 (2, P14); 15, N026 (2, P15); 16, Doggerscharbe (4, P16); 17, 212 (4, P17); 18, E127 (4, P18); 19, Pécs 67 (5, P19); 20, AKO (5, P20); 21, 2.2a (5, P21); 22, K059 (5, P22); 23, Tuzok (6, P23); 24, 36.4a (6, P24); 25, 136 (6, P25); 26, K002 (6, P26); 27, Goda (8, P27); 28, 47 (8, P28); 29, E024 (8, P29); 30, Kaparek (9, P30); 31, E112 (9, P31); 32, 280 (9, P32); 33, K052 (9, P33); 34, K024 (10, P34); 35, IV 12/8 (11, P35); 36, K021 (11, P36); 37, Pécs 9 (12, P37); 38, 88 (12, P38); 39, E146 (12, P39); 40, Pécs 3597 (15, P40); 41, Tanzania (16, P41); 42, 545 (17, P42); 43, 2017 (19, P43); 44, E053 (19, P44); 45, E051 (19, P45); 46, K031 (19, P46); 47, Băno 36 (21, P47); 48, MEW22 (N, P48); 49, Wittling (3, P49); 50, ATCC 43339 (7, P50); 51, ATCC 43338 (7, P51); 52, P-43 (7, P52); 53, K015 (7, P53); 54, Lengyel-P (10, P54); 55, Izsap-4 (14, P55); 56, E073 (15, P56); 57, K037 (16, P57); 58, 2553 (20, P58); 59, Băno 107 (22, P59); 60, KS20A (23, P60); 61, Pécs 56 (13, P61); 62, Shiribeshi 17 (13, P62); 63, 715 (18, P63); M, CHEF DNA Size Standard Lambda Ladder (Bio-Rad).

strains, no characteristic PFGE patterns related to the species or even serovar could be differentiated (Fig. 2). The PFGE analysis was repeated twice, and the same results were obtained.

DISCUSSION

Distinct and reproducible PFGE patterns were produced by using *Sma*I, and this allowed us to differentiate 63 PFGE patterns among the 70 strains analyzed. Studies have shown that for some bacterial species, the PFGE method is less sensitive than ribotyping and PCR-based methods. In addition, a high incidence of DNA degradation, which leads to a decrease in typeability by this method, has been cited (17, 23). Although DNA degradation should be avoided by addition of thiourea to the gel buffer (8, 35), no DNA degradation was observed with the strains used in this study and PFGE patterns were obtained for all of the strains analyzed. In a previous study using the same strains, we determined that the RAPD method is able to differentiate between species and to distinguish strains of this genus (29). By that method, 14 distinct amplification profiles were obtained (Table 1). Arh et al. (1) have analyzed 39 strains by ribotyping and identified nine different patterns. Of those 39 strains, 23 producing six different patterns by ribotyping were also analyzed in our study and unique PFGE patterns were produced for each strain (Table 1). Although analysis including more detailed epidemiological data and comparing the PFGE patterns of more strains of one species and one serovar and the inclusion of human isolates may be desirable, based on those previous results and on the results of this study, in which all of the strains studied were typed by PFGE and distinct PFGE patterns were obtained for 90% of the strains analyzed, it might be concluded that this method is better than RAPD analysis and ribotyping for epidemiological studies of strains of this genus.

Of the 70 strains, 12 were clustered in five PFGE patterns and no band differences were observed among strains classified into the same cluster. Since this study did not include an epidemiological study, only the sources from which the strains were isolated are shown in Table 1. However, although those strains that shared the same PFGE patterns were isolated in our laboratory at different times and from different samples, all of the strains were isolated from chickens or chicken meat from the same abattoir or processing plant, and each group was composed of strains sharing the same serovar. Moreover, by the guidelines for the interpretation of PFGE patterns of bacterial isolates in an epidemiological study proposed by Tenover et al. (43), isolates might be considered genetically indistinguishable when their PFGE patterns have the same numbers of bands and the corresponding bands are the same apparent size. Therefore, it might be considered that these strains are possibly members of the same clonal line. According to the same guidelines, isolates might be considered closely related when PFGE patterns differ by one to three bands, which is consistent with a single genetic event (e.g., a point mutation resulting in the loss or gain of a restriction site, an insertion, a deletion, or a chromosomal inversion); possibly related when PFGE patterns differ by four to six bands; and different when they differ by seven or more bands. Among the PFGE patterns obtained in this study, two pairs of strains showed PFGE patterns differing by three or fewer DNA fragment bands. The PFGE

analysis of strains ATCC 43339 and ATCC 43338 showed, respectively, patterns P50 and P51, which differed by two bands, and that of strains 422/1E1 and K021 showed, respectively, patterns P4 and P36, which differed by three bands. The difference between the PFGE patterns of the first pair would be explained by a single genetic event, as described by Tenover et al. (43). Therefore, it might be that one strain is a subtype of the other and corroborate the results obtained by RAPD analysis and ribotyping since both of the strains showed, respectively, the same RAPD pattern (j) and ribopattern (E) (Table 1). However, the differences between the patterns of the second pair do not match any pattern differences proposed in those guidelines. Maslow et al. (24) reported that when there are a very limited number of band differences that are not explained by a single genetic event, the isolates may be closely related but distinct. Thus, we believe that although these two strains are closely related, they should be classified as distinct strains. Similarly, and as described elsewhere (12), the other strains that differed by fewer than seven bands but by four or more were classified as distinct strains. In an epidemiological study using PFGE, a common pattern must be identified and the comparisons among PFGE patterns must be done based on that pattern (43). Some common bands could be identified among the strains analyzed in this study. However, few strains showed nearly identical patterns. Based on results of previous studies, in which many distinct PFGE patterns were produced from strains of only one species (2, 21, 23), the high variability of PFGE patterns observed in this study is not surprising since we included not only the two species of the genus *Erysipelothrix* but also numerous strains from many sources, strains representing the 23 serovars and type N, as well as serovars 13 and 18, which are considered possible members of new and separate species (41).

*Sma*I, *Asc*I, and *Not*I have been described as effective enzymes in producing a clear and reliable number of DNA fragments producing distinct PFGE patterns for several bacterial species (4, 7, 18, 25, 31). However, with DNAs of strains of this genus, few fragments, such as two or three, were produced with *Asc*I and *Not*I (Fig. 1). In the same set of guidelines for the interpretation of DNA restriction patterns generated by PFGE described above, Tenover et al. (43) emphasized the need for at least 10 distinct DNA fragment bands for reliable analysis of the relationship among strains. Thus, *Asc*I and *Not*I were considered to be inappropriate for analysis of strains of this genus.

To avoid the labor necessary to prepare the buffers and solutions required to obtain DNA from the strains studied, a commercial kit was used. Although DNA could not be obtained by using the protocol described by the manufacturer, reliable results were obtained with small modifications such as an increase in the lysozyme stock amount supplied with the kit and the addition of *N*-acetylmuramidase, which has been used for DNA extraction from strains of this genus (22, 29), to the lysis solution, as well as an increase in the incubation time.

In this report, we have described a protocol for the performance of PFGE with strains of the genus *Erysipelothrix*, demonstrating that PFGE performed with *Sma*I might be more sensitive than RAPD and ribotyping and also that this method might be a useful and reliable tool for epidemiological studies of strains of this genus. Moreover, as this was the first study to apply this method to strains of this genus, without disregarding the need to screen other enzymes, we expect that the proce-

ture will be of value as a basis for new approaches using this method with strains of *Erysipelothrix* species.

ACKNOWLEDGMENT

We thank Toshio Takahashi (National Veterinary Assay Laboratory, Tokyo, Japan) for kindly providing us with *Erysipelothrix* strains.

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