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Determination of Genome Sizes of *Rickettsia* spp. within the Spotted Fever Group, Using Pulsed-Field Gel Electrophoresis

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The chromosome lengths of six spotted fever group *Rickettsia* species (*Rickettsia rickettsii*, *R. conorii*, *R. rhipicephali*, *R. sibirica*, *R. australis*, and *R. akari*) were estimated by pulsed-field gel electrophoresis. The genome size of *R. rickettsii* was about 2,100 kb, but the chromosome lengths of the five other species were, surprisingly, much lower and ranged between 1,200 and 1,300 kb.

The spotted fever group rickettsiae belong to the *Rickettsiaceae* family and consist of arthropod-associated, obligately intracellular, gram-negative bacteria. This family comprises several genera, including *Rickettsia*, which is subdivided into three groups: typhus, scrub typhus, and spotted fever.

Little is known about the genetics of these microorganisms, but studies of the *Rickettsia rickettsii* genome have shown that the DNA is only chromosomal and the genome size is estimated to be about $130 \times 10^7 \pm 10 \times 10^7$ daltons (12). The G+C content of this bacterium is about 32 to 33% (18). Studies of rickettsiae based on sequencing of the 16S rRNA (20) have enabled determination of the classification of the *Rickettsiaceae* family based on biological, ecological, and serological criteria (13). Pulsed-field gel electrophoresis has been shown to be a powerful means of investigating the genomes of microorganisms by accurately measuring chromosome size. In this report, we describe the results of our experiments using pulsed-field gel electrophoresis on spotted fever group rickettsial isolates.

The following reference rickettsial strains were obtained from the American Type Culture collection: *R. conorii* Moroccan strain VR-141, *R. rickettsii* Sheila Smith VR-149, *R. akari* VR-148, and *R. sibirica* 232. *R. australis* and *R. rhipicephali* were provided by G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.

Rickettsial species were propagated on Vero cells by using minimal essential medium (Flow Laboratories, Puteaux, France) supplemented with fetal calf serum (4%) and glutamine (1%). The bacteria were harvested 3 or 4 days after inoculation, when heavy infections could be demonstrated by Gimenez staining (7). Infected cells were pelleted by centrifugation (12,000 × *g* for 10 min) and suspended in K36 buffer (16.5 mM KH_2PO_4, 33.5 mM K_2HPO_4, 100 mM KCl, 15.5 mM NaCl). The rickettsiae were purified by renografin density gradient centrifugation (21), washed, and resuspended in K36.

The bacterial suspension was mixed with 1 volume of 1% Incert low-melting-point agarose (FMC Bioproducts, Rockland, Maine) as described by Schwartz and Cantor (16). Solidified plugs were transferred to a lysis mixture (0.5 M EDTA [pH 8], 1% *N*-lauroyl sarcosine, 1 mg of proteinase K per ml) and then incubated twice for 24 h each time at 50°C. The blocks were washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]) and then incubated twice for 1 h each time at 50°C with TE buffer containing 0.04 mg of phenylmethylsulfonyl fluoride per ml. After washes in TE buffer, the blocks were stored in 0.5 M EDTA at 4°C or digested with the appropriate restriction endonuclease.

Each block was independently digested with 20 U of enzyme three times for 2 h each time and electrophoresed in a 1% agarose gel in 0.5× TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA [pH 8]) at 14°C by using the contour-clamped homogeneous electric field system described by Chu et al. (3) (CHEF-DRII; Bio-Rad Laboratories, Richmond, Calif.). Three molecular weight markers were used: the Low-Range PFG Marker, the Lambda Ladder PFG Marker, and the Yeast Chromosome PFG Marker (New England BioLabs, Inc., Beverly, Mass.). Four conditions of migration were used, depending on DNA size: 190 V with ramped pulse times of 3 to 10 s for 24 h, 180 V with 5 to 20 s for 33 h, 190 V with 3 to 5 s for 25 h, and 150 V with 5 to 120 s for 48 h.

Owing to the low G+C contents of these bacteria (32 to 33%), we tested enzymes expected to recognize only a few sites in the rickettsial genome (11). The endonucleases tested were *Not*I, *Sfi*I, *Spe*I, *Xba*I, *Kpn*I, *Ksp*I, *Nar*I, *Bss*HII, *Eag*I, and *Sma*I. Under our experimental conditions, we did not obtain good digestion with *Ksp*I, *Kpn*I, and *Nar*I. *Sph*eI and *Xba*I were not interesting, since many of the DNA fragments obtained were too small, i.e., the largest size was about 50 kb; *Not*I and *Sfi*I did not always cut the rickettsial chromosome. As a result, we used *Eag*I (CGGCCG), *Bss*HII (GCGCGC) (New England BioLabs, Inc.), and *Sma*I (CCCGGG) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to study the rickettsial DNA (Fig. 1 and 2). Digestion of the chromosome with *Eag*I and *Sma*I showed about 20 bands. For *R. rickettsii*, high bands of about 500 and 880 kb were found after digestion with *Sma*I and *Eag*I, respectively, followed by a set of fragments of between 8 and 350 kb. With the other species, DNA bands ranged between 7 and 270 kb. Several of these bands migrated at the same level, and it was impossible to separate them. *Bss*HII was interesting, because we obtained a low number of DNA fragments, facilitating estimation of the total molecular weight. For these three endonucleases, several conditions of migration were used to improve separation of the bands and motility as a linear function of size. Thus, we tested changes in pulse duration, voltage, and time of migration (2). Gels run with short pulses (3 to 10 s) and high voltages (190 to 200 V) resulted in good resolution of the smallest fragments, while migration over 33 to 48 h with longer pulses and lower voltages (150 to 180 V) resulted in better separation of the

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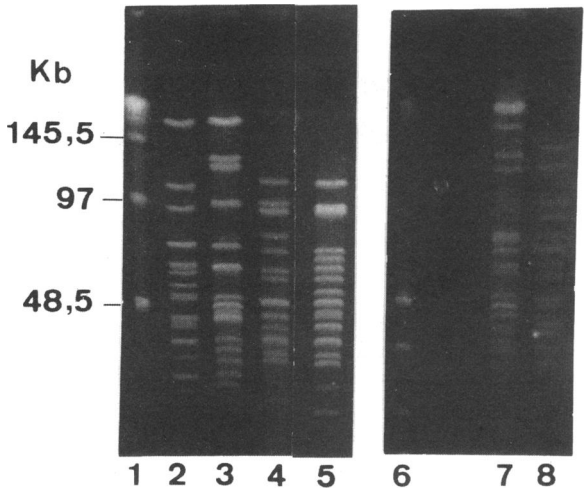

FIG. 1. PFGE of *Sma*I restriction fragments of rickettsial DNAs. Lanes 1, Lambda Ladder PFG Marker; 2 to 5, *R. akari*, *R. australis*, *R. conorii*, and *R. sibirica*; 6, Low Range PFG Marker; 7, *R. rickettsii*; 8, *R. rhipicephali*. Electrophoresis was done at 14°C in a 1% agarose gel in 0.5× TBE with ramped pulse times of 3 to 10 s for 24 h. The bars at the left show the positions of marker bands of the sizes indicated.

largest fragments. Estimation of band size and number of fragments of identical size inside a given band was performed by using The Imager apparatus and the QGEL-1D program (APPLIGENE, Illkirch, France).

Pulsed-field gel electrophoresis is a powerful technique in the study of bacterial DNA. We used the contour-clamped homogeneous electric field system, which gave good separation of the different fragments, resulting in correct evaluation of their molecular weights. Our estimated bacterial genome sizes were 1,221 to 1,276 kb for *R. conorii*, *R. sibirica*, *R. rhipicephali*, *R. australis*, and *R. akari* (Table 1), and we showed that the lengths of these *Rickettsia* genomes are shorter than those of other bacteria, such as *Pseudomonas aeruginosa* (5,900 kb [15]), *Escherichia coli* (4,700 kb [17]), *Staphylococcus aureus* (2,800 kb [19]), and *Haemophilus influenzae* (1,980 kb [9]). The chromosome sizes were also found to be smaller than those of other members of the family *Rickettsiaceae*, such as the genera *Coxiella* (1,600 kb [8]) and *Rickettsiella* (1,700 to 2,100 kb [5]) but were found to be close to those of the genera *Mycoplasma* (900 to 1,200 kb [14]) and *Chlamydia* (1,045 kb [1]) and *Borrelia burgdorferi* (950 kb [4]). On the other hand, the *R. rickettsii* genome is medium sized (about 2,100 kb), close to that of the genus *Rickettsiella*. There is an important difference between the value found for *R. rickettsii* and those of the other spotted fever group species studied here. For this group of bacteria, previous evaluations were made only for *R. rickettsii* and the values were calculated by the renaturation rate method of Gillis and al. (6). Two results were published: 1,500 kb (10) and 1,950 kb (12). Our results are in accordance with the latter value; as for the other species, we obtained concordant evaluations with three restriction endonucleases, so that it is reasonable to think that our results are correct. It is surprising that the genome size of *R. rickettsii* is 70% greater than those of the other rickettsiae of this group, since these bacteria are apparently closely related because they all grow only intracellularly, preferably at 32°C; they all exhibit transmission to humans due to an arthropod and clinical symptoms they cause are universally equivalent, consisting mainly of fever, skin eruption, and headache (except for *R. rhipicephali*, which has an unknown pathogenic role). The current classification is based on a microimmunofluorescence reaction which allows characterization of several subgroups, in particular, one including *R. rickettsii*, *R. conorii*, and *R. sibirica* (13). It will be interesting to determine which bands correspond to the additional part of the *R. rickettsii* chromosome and what information is encoded by these genes. The genome size of the spotted fever group rickettsiae is different from that of other bacteria of the *Rickettsiaceae* family; furthermore, major differences exist within this group. This leads to the conclusion that it is impossible to make generalizations concerning spotted fever

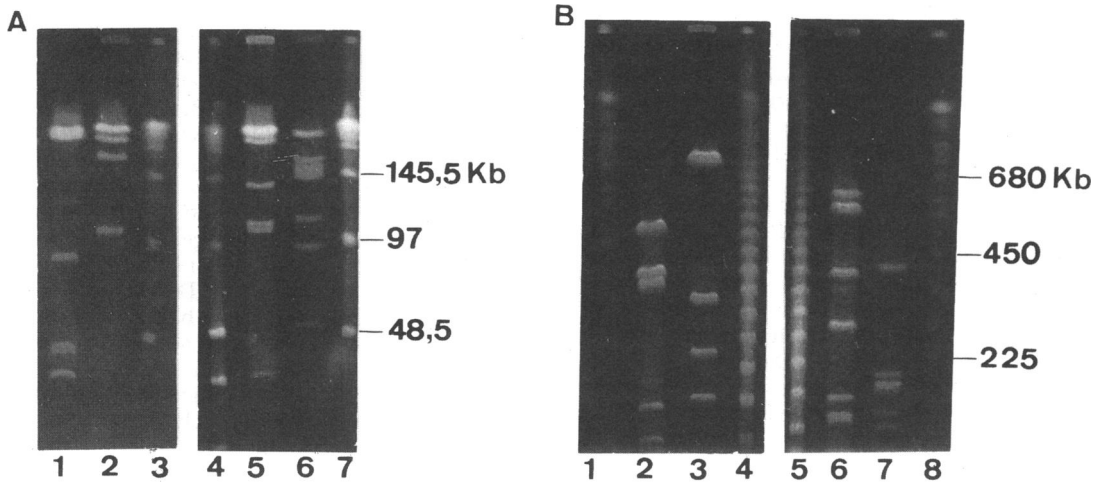

FIG. 2. PFGE of *Bss*HII restriction fragments of rickettsial DNAs. (A) Lanes: 1, *R. australis*; 2, *R. sibirica*; 3 and 7, Lambda Ladder PFG Marker; 4, Low Range PFG Marker; 5, *R. rickettsii*; 6, *R. rhipicephali*. The gel was run at 14°C in 0.5× TBE at 190 V for 24 h with ramped pulse times of 3 to 10 s. (B) Lanes: 1 and 8, Yeast Chromosome PFG Marker; 2, *R. australis*; 3, *R. sibirica*; 4 and 5, Lambda Ladder PFG Marker; 6, *R. rickettsii*; 7, *R. rhipicephali*. The gel was run at 14°C in 0.5× TBE at 150 V for 48 h with ramped pulse times of 5 to 120 s. The bars at the right of both panels show the positions of marker bands of the sizes indicated.

TABLE 1. Restriction fragment and genome sizes[a] of six strains of spotted fever group rickettsiae

R. conorii			*R. rickettsii*			*R. australis*			*R. akari*			*R. rhipicephali*			*R. sibirica*		
A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
270	350	111	884	616	508	229	436	260	184**	450	225	206	388	145	270	720	111
169	334	96	320	560	352	149**	338	130	142	200	114	187	164	128	169	272	96
124	314	93**	300	372	194	129	296	121	119	134	94	181	154	105	126	160	93**
106**	126	80	128	256	139	117	90	96	74	129	78**	174	148	93	111**	106	75
97	102	73	109	136	126	97	44	75	67	75	69	95	143	84	99		71
61	20	64	97	113	84	88	40	66**	56	68	65	81	114	74***	61		66
56		59	61	107	80	81	32	52	51**	60	59	56	95	66	44		60
42		48****	44	25	73	61		48**	43**	44	53**	47	52	61	40**		55
40		42***	40**		66	56		46	39	38	40**	42		47**	32**		51***
25***		34	32		62	52		42***	36	18	38	35		42**	27**		44**
21		28***	29**		50	38		30	33	16	36	29**		32**	20		38**
20		22**	20		42****	30		26	31		28***	23		27**	16		30***
18		21**	16		36**			22	29		23	20		21	8		23
16		17	8		28			20	27		19***	18		18**			22**
13		14			27			16	26		15	16					17
		8			22			8				13					12
					21												8
					20												7
					18												
					12												
					10												
					8**												
1,234	1,246	1,230	2,157	2,185	2,148	1,276	1,276	1,256	1,235	1,232	1,221	1,252	1,258	1,257	1,233	1,258	1,238

[a] Established by comparison with the concatemers of the λ phage after digestion of bacterial DNA with *Eag*I (A), *Bss*HII (B), and *Sma*I (C). The number of asterisks indicates the number of identical-size fragments inside a given band, as revealed by densitometry with The Imager apparatus and the QGEL-1D program (APPLIGENE). The number at the bottom of each column is the genome size. All sizes are in kilobases.

group rickettsiae on the basis of genetic data obtained from a single species of the group.

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