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Genome Conservation in Isolates of *Leptospira interrogans*

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Reference strains for each of the 23 serogroups of *Leptospira interrogans* yielded different pulsed-field gel electrophoresis patterns of *Not*I digestion products. This was also the case for the 14 serovars belonging to serogroup Icterohaemorrhagiae (with one exception). The *Not*I restriction patterns of 45 clinical leptospiral isolates belonging to serovar icterohaemorrhagiae were analyzed and compared with those of type strains. No differences were observed between isolates from countries of different continents, namely, France, French Guiana, New Caledonia, and Tahiti. The pattern was indistinguishable from that of the reference strain of serovar icterohaemorrhagiae.

Leptospirosis, caused by *Leptospira interrogans*, is a zooanthroponose which has a worldwide distribution. *L. interrogans* is an immunologically diverse species, in which antigenically related serovars constitute serogroups (15). In total, 202 serovars distributed in 23 serogroups have been described (19). The serovar icterohaemorrhagiae is found worldwide and causes the most severe disease. Recently, Yasuda et al. demonstrated by genomic DNA-DNA hybridization that the different strains of *L. interrogans* constitute not one but at least six distinct species (37).

The diagnosis of leptospirosis usually depends upon the demonstration of serum antibodies. The serological method of choice is the microagglutination test (MAT) (9). An accurate diagnosis of infection may additionally require direct demonstration of the presence of *L. interrogans* in tissues, blood, or urine. This is achieved by culturing the organism and then subjecting it to immunological techniques. The identification of an unknown strain at the serovar level is tedious, requiring the maintenance of reference strains and their corresponding rabbit-immune sera (6), and takes at least 2 months.

To overcome these difficulties in the characterization of *L. interrogans* strains, DNA-based techniques have been studied. For the detection of *L. interrogans* in biological samples, restriction endonuclease techniques (8, 13, 22, 23, 27, 30, 32–34) and nucleic acid probes (14, 25, 26, 31, 35, 38, 39) have been utilized. However, the published methods have shortcomings. Digestion of chromosomal DNA with restriction endonucleases often yields hundreds or even thousands of fragments, and differentiation between resulting electrophoresis patterns is an imposing task. Southern blotting with specific probes provides a specific and simpler banding pattern, but the method is cumbersome and unsuitable for routine application.

Pulsed-field agarose gel electrophoresis (PFGE) of large DNA fragments produced by rare-cutting restriction enzymes offers the advantage of a simple interpretation combined with a rapid result. The genome size of *L. interrogans* was estimated at 5,000 kb by using PFGE after restriction with three different endonucleases, and the pattern of each of the three serovars analyzed was different (3).

In experiments described in this paper, the *Not*I restriction patterns of 45 *L. interrogans* wild-type strains isolated from humans or animals and identified by classical methods as belonging to serovar icterohaemorrhagiae were compared with those of serogroup reference strains and serovars of the Icterohaemorrhagiae serogroup. Surprisingly, the patterns of the low-passage isolates from different parts of the world were identical. Furthermore, they were indistinguishable from the pattern of the icterohaemorrhagiae serovar reference strain.

MATERIALS AND METHODS

Bacterial strains and media. A total of 37 highly passaged strains of *L. interrogans* from the National Reference Center Collection were studied (Table 1). These include the 23 reference strains of each serogroup of *L. interrogans* and the 14 serovars of serogroup Icterohaemorrhagiae. Forty-five clinical isolates from France, French Guiana, New Caledonia, and Tahiti (isolated from blood, cerebrospinal fluid, and urine) were used at passage 10 or lower. In vitro passage is necessary and requires 3 to 20 days.

Each strain was grown in 25 ml of EMJH medium (7, 16) at 30°C with shaking, and growth was verified by counting in a Petroff-Hausser chamber. The yield was between 4×10^7 and 2.5×10^8 cells per ml.

DNA extraction, enzyme digestion, and PFGE. A PFGE instrument from Pharmacia LKB (Uppsala, Sweden) with a hexagonal electrode array was used (Pulsaphor 2015). *Not*I was purchased from Amersham. Restriction analysis and electrophoresis of in situ lysed cells was performed as described previously (2) (DNA from 1.5×10^8 to 20×10^8 cells was loaded per lane). Two different pulse times enabled us to establish the restriction profile of the majority of *L. interrogans* serovars: a 25-s pulse gave optimal separation of the medium-sized fragments (70 to 400 kb), while a pulse setting of 100 s gave optimal separation of the largest *Not*I fragments. Usually, a program was used combining three pulse times (30, 60, and 120 s or 10, 25, and 50 s). The sizes of the DNA fragments were estimated by comparison of band mobilities with concatemerized λ bacteriophage genomes (the size of the λNM1149 used was 48.5 kb [24]) and *Saccharomyces cerevisiae* chromosomes (Bio-Rad Laboratories, Richmond, Calif.) (2,200, 1,600, 1,125, 1,020, 945, 850, 800, 770, 700, 630, 580, 460, 370, 290, and 245 kb). To calculate the genome size of the different *Leptospira* strains, the following strategy was used: the presence of single or double bands was estimated by evaluating ethidium bromide staining, the size of each fragment was estimated by calculating the mean of the different sizes obtained from several

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TABLE 1. List of *L. interrogans* strains (reference type strains or reported strains)

Serogroup	Serovar	Strain	Genomic species[a]
Icterohaemorrhagiae	icterohaemorrhagiae	RGA	*L. interrogans*
Icterohaemorrhagiae	copenhageni	M 20	*L. interrogans*
Icterohaemorrhagiae	gem	Simon	*L. interrogans*
Icterohaemorrhagiae	mankarso	Mankarso	*L. interrogans*[b]
Icterohaemorrhagiae	naam	Naam	*L. interrogans*[b]
Icterohaemorrhagiae	smithi	Smith	*L. interrogans*[b]
Icterohaemorrhagiae	birkini	Birkin	*L. interrogans*[b]
Icterohaemorrhagiae	tonkini	LT 96-68	—[c]
Icterohaemorrhagiae	lai	Lai	—
Icterohaemorrhagiae	dakota	Grand River	—
Australis	australis	Ballico	*L. interrogans*
Autumnalis	autumnalis	Akiyami A	*L. interrogans*
Bataviae	bataviae	Van Tienen	*L. interrogans*
Canicola	canicola	Hond Utrecht IV	*L. interrogans*
Djasiman	djasiman	Djasiman	*L. interrogans*
Hebdomadis	hebdomadis	Hebdomadis	*L. interrogans*
Pomona	pomona	Pomona	*L. interrogans*
Pyrogenes	pyrogenes	Salinem	*L. interrogans*
Javanica	javanica	Veldrat Batavia 46	*L. borgpetersenii*
Mini	mini	Sari	*L. borgpetersenii*
Sejroë	sejroë	M 84	*L. borgpetersenii*
Tarassovi	tarassovi	Perepelycin	*L. borgpetersenii*
Ballum	ballum	Mus 127	*L. borgpetersenii*
Manhao	manhao 2	L 105	*L. borgpetersenii*
Panama	panama	CZ 214	*L. noguchii*
Louisiana	louisiana	LSU 1945	*L. noguchii*
Celledoni	celledoni	Celledoni	*L. weilii*
Sarmin	sarmin	Sarmin	*L. weilii*
Shermani	shermani	1342 K	*L. santarosai*[d]
Cynopteri	cynopteri	3522 C	*L. alstoni*[b]
Grippotyphosa	grippotyphosa	Moskva V	*L. alstoni*[b]
Icterohaemorrhagiae	ndambari	Ndambari	*L. alstoni*[b]
Icterohaemorrhagiae	ndahambukuje	Ndahambukuje	*L. alstoni*[b]
Icterohaemorrhagiae	mwogolo	Mwogolo	*L. alstoni*[b]
Icterohaemorrhagiae	bogvere	Bogvere	*L. alstoni*[b]
Ranarum	ranarum	ICF	*L. meyeri*

[a] As defined by Yasuda et al. (37).
[b] Reported by Kaufmann (17a).
[c] —, not determined.
[d] The strain used was not the reference strain.

TABLE 2. *Not*I fragment sizes from 23 *Leptospira* serogroup reference strains[a]

Autumnalis, autumnalis, Akiyama A	Australis, australis, Ballico	Bataviae, bataviae, Van Tienen	Canicola, canicola, Hond Utrecht IV	Hebdomadis, hebdomadis, Hebdomadis	Pomona, pomona, Pomona	Pyrogenes, pyrogenes, Salinem	Icterohaemorrhagiae, icterohaemorrhagiae, RGA	Diasiman, diasiman, Djasiman	Cynopteri, cynopteri, 3522 C	Grippotyphosa, grippotyphosa, Moskva V
1,840		1,840	1,720		2,500	1,600		1,700		
	1,250						1,300			
	850	940	940	830		1,050	1,000			
760	775			770						
				700					685	685
			630	630	630					660
									605	605
				480						
420	435	445		445				450		
			400	405	395	400	405*	395*	420	410
	370					370	365	370	400	390
	340	355	355		340	330	320	335	340	340
315	315	315*				300		300	320	320
280*	285*					285	285*		290	300
									270	280
260			250		245			245	245	265
220		220				230*	225		225	235
			200	205		200				
				195	185				190	180
185									175*	
	170			170						
			165*		165					160*
		155*				150	150*		150	145
145	145	145			145*			140		
			135							
					110	115	115		115	
100		100								
	95			90				90*		
85	80		85*			80	80			
				70	70					
	50			50				50*		
		40							40	
										30
4,890	5,445	5,025	5,130	5,040	4,930	5,340	5,085	4,610	4,645	5,165

[a] For each restriction fragment, the size (kb) was calculated from data of several gels run at six different pulse times (1, 10, 25, 50, 100, and 200 s) by using digests of low-concentration DNA preparations. An asterisk (*) indicates a doublet. The bottom numbers refer to estimated genome sizes. Column headings indicated serogroup, serovar, and strain.

gels run at different pulse times, and then the total of the fragments was summed.

Identification of serovars by the MAT and a cross-adsorption test. Identification of *L. interrogans* strains was based on serological criteria defined by the MAT used in a cross-adsorption procedure (9). The MAT consists of evaluating in dark-field illumination the agglutination of an *L. interrogans* culture (2×10^8 bacteria) by an equal volume of hyperimmune reference serum (obtained in a rabbit) at increasing dilutions and is done routinely in our reference laboratory.

Serological typing thus entailed an initial screening of the isolate with a group of 23 selected antisera (group sera) encompassing most of the known cross-reactions and served to determine serogroup affinities. The isolate was then tested with antisera to different serovars, which made up one or more serogroups, in order to narrow the number of possibly related serovars. Finally, on the basis of observed cross-reactions, representative strains of serovars were chosen for reciprocal agglutinin-adsorption tests for definitive identification. On the basis of current criteria, two strains were considered to belong to different serovars if, after cross-adsorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remained in at least one of the two antisera in repeated tests (9).

RESULTS

***Not*I restriction patterns specific to each serovar.** Intact genomic DNA from 37 *L. interrogans* strains from the Pasteur Institute collection (Table 1) was digested with *Not*I, a restriction endonuclease previously found to digest *L. interrogans* infrequently (3), and submitted to PFGE. The *Not*I DNA restriction patterns of the 23 serogroup reference strains of the pathogenic species were all different, providing a reliable identification for each reference strain (Fig. 1). Furthermore, within the serogroup Icterohaemorrhagiae, each of the 14 serovars had a unique restriction pattern (Fig. 2) except for serovars copenhageni and icterohaemorrhagiae, which were identical (Fig. 2a, lanes 5 and 6). These two serovars are serotypically also closely related (see Discussion).

Identification of clinical isolates. To test the usefulness of

TABLE 2—*Continued*

Panama, panama, CZ 214	Louisiana, louisiana, LSU 1945	Javanica, javanica, Veldrat Batavia 46	Sejroe, sejroe, M 84	Tarrassovi, tarrassovi, Perepelicyn	Ballum, ballum, Mus 127	Mini, mini, Sari	Celledoni, celledoni, Celledoni	Sarmin, sarmin, Sarmin	Shermani, shermani, 1342 K	Ranarum, ranarum, ICF	Manhao, manhao 2, L 105
1,205											
800	735										
655	670			700			650		770		
	630										
540	530		540*	500	505	500	505			540	
	460	480			490		450	455			
425		435*		455*			440				
400	405	420			400	400		395	385		360
		370			370	370		375		340	350
						360*			350	320	305
335		330	335*		345					280	275
300	320							300		240	255*
270	280									225	245
		265		255				200	255	170	230*
	240*	240	250	245*	245*	245		190	240*	120*	210*
		225	235*	225	230			180*		110	180*
		205	215*			210	225	170*		100*	
205		195	200*	195*	200*	200	210*	160	190	95*	150*
	180				185		195*	150	180	85	
		175	175*		175	175*	175	140	175	75*	145
	165			165*	170*	170	150	130	145	70*	120*
		160	150*		160	150	130*	120*	135	65*	
		140				145	120*	115*	130*	55	110
		130	130	125*	130*	130	110	105*	120*	50	105
		125	120	115*		125*	100	85*	105	45	95
		120	110	105*	110*	115	90*	80	100*	40	75*
105	100*	105	95		100	100	80*	75	85	30*	70*
85	80	85	85	90	90	85	70	70	80*	27*	55
			75		70	70	60	65	75	26*	50*
		65	65	60		60*	55	60*	65*	24	40*
	50*	50	50	50	55*	45	50*	55	60	23	30
		45	40	40*	40	40	40*	50*	40	22	
		35		30			30*	40*	35	20	
25				25*	30	25	25	25*	25	17	
		20		15		20	20	20	20	15	
				10						11	
5,350	5,235	4,855	4,720	4,875	5,010	4,465	4,925	4,365	4,540	3,848	4,655

this method within a serogroup, we chose all of the clinical isolates available to us from the clinically most important serovar, i.e., icterohaemorrhagiae. The *Not*I restriction patterns of 45 clinical isolates from human patients (43 isolates) and animals (2 isolates) in France (5 isolates), French Guiana (1 isolate), New Caledonia (33 isolates), and Tahiti (7 isolates) and identified by the MAT as belonging to serovar icterohaemorrhagiae were compared with those of each serovar of serogroup Icterohaemorrhaghiae. The *Not*I restriction patterns for all 45 strains were identical to that of the reference strain. An example is shown in Fig. 3.

Size of the *L. interrogans* genome. For each reference strain representative of a serogroup, and for each serovar of the Icterohaemorrhaghiae serogroup, the minimum genome size was determined. The values are all between 3,850 kb (serovar ranarum strain ICF) and 5,450 kb (serovar australis strain Ballico) (Table 2). These results are consistent with previous work, undertaken with three different endonucleases (3), showing a size of 5,000 kb for *L. interrogans* serovar icterohaemorrhagiae (strain Verdun), *L. interrogans* serovar grippotyphosa (strain Moskva V), and the saprophytic *Leptospira biflexa* serovar patoc (strain Patoc I).

DISCUSSION

Analysis of the *Not*I restriction patterns of *L. interrogans* DNA by PFGE allowed us to differentiate between each serogroup reference strain. Each of the 23 serogroup reference strains possesses a unique profile which can be considered as a specific genomic fingerprint (Fig. 1 and Table 2). Furthermore, our results suggest that there are major genetic differences among serovars within a serogroup. Each of the 14 serovars belonging to serogroup Icterohaemorrhagiae has its own specific fingerprint apart from the two serovars, icterohaemorrhagiae and copenhageni (Fig. 2), which are very difficult to distinguish by serological methods (20) and which have identical PFGE *Not*I restriction profiles. These two serovars have been reported previously to have identical *Eco*RI or other endonuclease restriction patterns (28, 34) and identical DNA-rRNA hybridization patterns (26). They must therefore be extremely closely related genetically. Thus, the distinction between serovars that we have observed at the DNA level is in agreement with the phenotypic results used by clinicians for many years.

Surprisingly, the *Not*I restriction patterns of each of 45

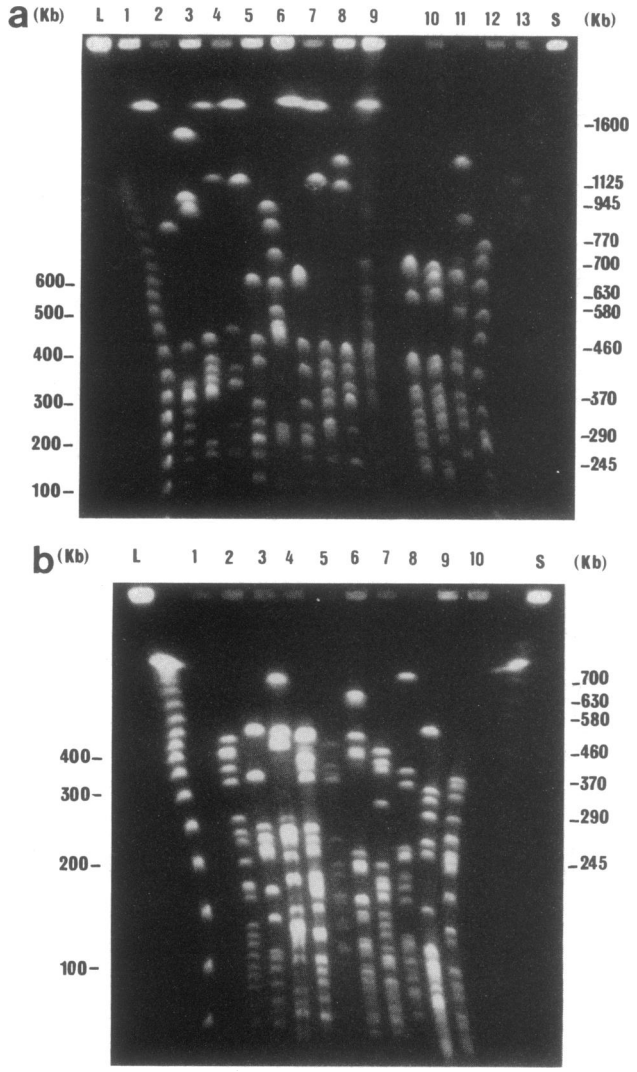

FIG. 1. PFGE of *Not*I restriction fragments from serogroup reference strains. The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× TBE. Lanes L and S are size markers (λ and *S. cerevisiae*, respectively). (a) Lanes 1 to 13, serovars autumnalis, australis, bataviae, canicola, hebdomadis, pomona, pyrogenes, icterohaemorrhagiae, djasiman, cynopteri, grippotyphosa, panama, and louisiana, respectively. The three pulse times were as follows: 30 s, 13 h; 60 s, 13 h; 120 s, 14 h. (b) Lanes 1 to 10, serovars javanica, sejroe, tarassovi, ballum, mini, celledoni, sarmin, shermani, ranarum, and manhao, respectively. The three pulse times were as follows: 10 s, 8 h; 25 s, 12 h; 50 s, 16 h.

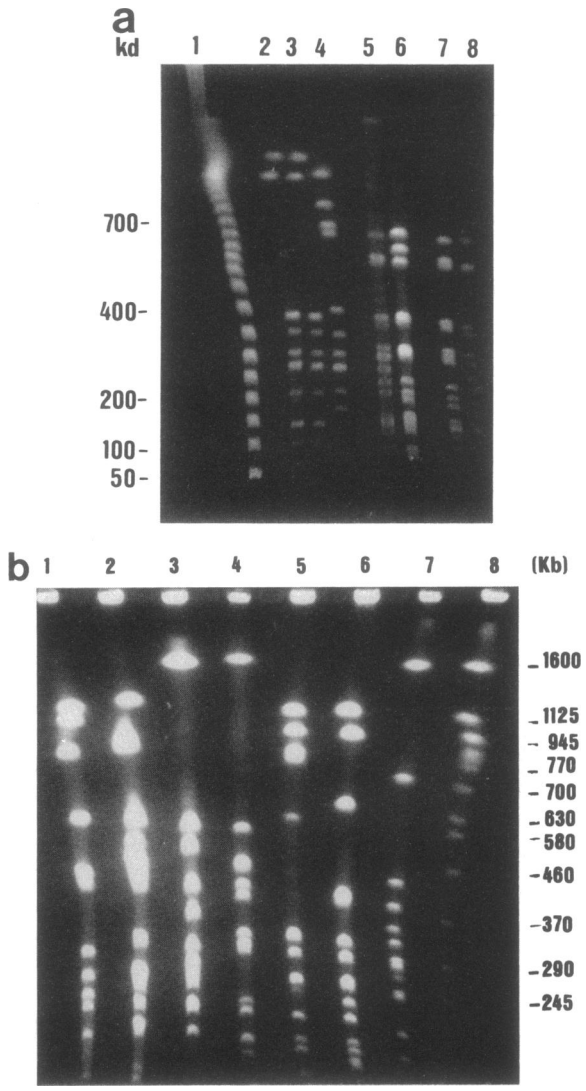

FIG. 2. PFGE of *Not*I restriction fragments from serovars of serogroup Icterohaemorrhagiae. The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× TBE with three pulse times as follows: 30 s, 13 h; 60 s, 13 h; 120 s, 14 h. (a) Lanes 2 to 8, serovars icterohaemorrhagiae, copenhageni, lai, ndambari, bogvere, ndahambukuje, and mwogolo, respectively. Lane 1, size markers (λ). (b) Lanes 1 to 7, serovars gem, tonkini, dakota, mankarso, naam, smithi, and birkini, respectively. Lane 8, size markers (*S. cerevisiae*).

clinical isolates of human or animal origin available from different endemic countries, identified by classical methods as belonging to serovar icterohaemorraghiae, were found to be identical. The pattern corresponds to that obtained with the reference strain of serovar icterohaemorrhagiae. This result suggests that very little genetic rearrangement had occurred in nature within this serovar. Similar genomic stability within two other *L. interrogans* serovars of economic importance in livestock, pomona and hardjobovis, has been suggested by Marshall et al. (23) and Thiermann et al. (33, 34) by using restriction endonuclease analysis and fixed-field gel electrophoresis.

It seems that gene organization can be quite stable within certain bacterial populations (21). For example, strains of *Mycobacterium leprae* isolated from three different continents have essentially identical genomes: no polymorphisms were detected among restriction endonuclease digests of these genomes by using various probes (5). In contrast, other bacteria such as *Streptomyces* and *Rhizobium* spp. are variable because of the presence of plasmids, insertion elements, and repeated sequences (1, 4, 10–12, 17, 18). *L. interrogans* probably falls between these extremes, with more than 200 serovars (19), each one analyzed thus far being very stable (data not shown). Stability could be due to the fastidious nature and long generation time (estimated to be approximately 12 h in vitro) of *L. interrogans*, whereas variability could be attributed to the presence of plasmids

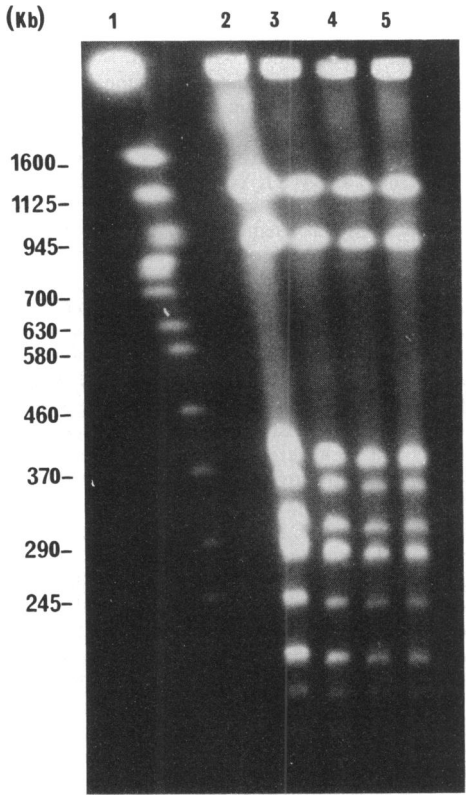

FIG. 3. PFGE of *Not*I restriction fragments from clinical strains belonging to serovar icterohaemorrhagiae (lanes 3 to 5) compared with serovar icterohaemorrhagiae reference strain RGA (lane 2). The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× TBE with three pulse times as follows: 30 s, 13 h; 60 s, 13 h; 120 s, 14 h. Lane 1, size markers (*S. cerevisiae*).

(29) and repetitive sequences (38). The current state of knowledge about the genetics (no availability of gene transfer) of *L. interrogans* is too limited to allow us to do more than speculate on the answers to these questions.

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