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Presence of Putative Sphingomyelinase Genes among Members of the Family *Leptospiraceae*

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The presence of multiple DNA elements in pathogenic members of the family *Leptospiraceae*, similar to the *sphA* sphingomyelinase gene from *Leptospira borgpetersenii*, was demonstrated by low-stringency hybridization experiments. These DNA elements were designated putative sphingomyelinase genes. Grouping of strains by similarity of hybridization patterns corresponds to the species subdivision of the family *Leptospiraceae* on the basis of genetic characteristics. Therefore, hybridization with the *sphA* gene can be used as a taxonomic tool. These hybridization experiments indicate the presence of two groups of genetically related pathogenic *Leptospira* species.

The family *Leptospiraceae* comprises a collection of bacteria which is distinct from other spirochetes (9) but antigenically and genetically very heterogeneous. Currently, two types of classification, based on genetic and antigenic determinants, are being used. These two classifications do not correspond to each other, and only the genetic classification reflects the phylogenetic relationships among this family (7a, 8, 19). The large genetic diversity observed among members of the family *Leptospiraceae* allows for the discrimination of at least 12 species. Currently, pathogenic *Leptospira* species include the officially recognized *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira weilii*, *Leptospira inadai*, *Leptospira noguchi*, and *Leptospira santarosai* species (19). Among pathogenic leptospires, a seventh group of strains was identified as a possible species but not named (19). Further research has confirmed the existence of this new species, which has been unofficially named "*Leptospira alstoni*" (7a). Saprophytic members of the family *Leptospiraceae* now include the species *Leptospira biflexa*, *Leptospira meyeri*, *Leptospira wolbachii*, and *Leptospira parva* (19). Previously, a sphingomyelinase gene (*sphA*) from a serovar hardjo strain, which was provisionally classified in the species *L. borgpetersenii* (15, 17), was characterized at the molecular level and shown to be homologous to sphingomyelinase genes from gram-positive bacteria (14). As shown by hybridization under stringent conditions, this *sphA* gene is present in a limited number of pathogenic leptospires only (4, 15). However, pathogenic leptospires from the species *L. interrogans* and *L. borgpetersenii* do produce sphingomyelinase (15) and also have DNA elements that hybridize with the *sphA* gene under low-stringency conditions (13). In the present article, the investigation of this type of DNA element among the various members of the family *Leptospiraceae* and their use as a classification tool are described.

Southern blots containing *Eco*RI-digested chromosomal DNA from various *Leptospira* strains (Table 1) were hybridized under high- and low-stringency conditions with a 1,295-bp *Eco*RI-*Ssp*I DNA fragment consisting of the central part of the *sphA* gene as described previously (15). All results are summarized in Table 1, and a relevant selection is shown in Fig. 1. Hybridization under stringent conditions was observed with *L. borgpetersenii*, *L. santarosai*, *L. weilii*, and *L. meyeri* ICF only. Under low-stringency conditions, hybridization was observed with all pathogenic *Leptospiraceae* except for *L. inadai*. So far, there is no proof that the hybridizing DNA fragments represent (parts of) actual genes. Therefore, they will be referred to as putative sphingomyelinase genes (PSGs).

In the strain Sponselee DNA, in addition to the expected 2.0-kb *Eco*RI DNA fragment from the *sphA* gene, four *Eco*RI fragments (15) (Fig. 1c, lane 4) and, with DNA from all *L. interrogans* strains tested, five *Eco*RI fragments hybridize under low-stringency conditions (15) (Fig. 1c, lanes 1 to 3; the signal at approximately 3.4 kb is actually composed of two bands). Considering the 1,295-bp length of the DNA fragment used as a probe and the size of the hybridizing fragments and assuming that each PSG contains at least one *Eco*RI restriction site, *L. borgpetersenii* strains contain, in addition to the *sphA* gene, at least one or two PSGs. By similar reasoning, it can be concluded that *L. weilii* contains at least one or two PSGs, "*L. alstoni*" contains at least two or three PSGs, and *L. noguchi* and *L. interrogans* each contain at least three, but probably more, PSGs (Fig. 1 and Table 1). *L. meyeri* ICF contains eight hybridizing DNA fragments which should theoretically represent at least four PSGs. The other *L. meyeri* strain, Veldrat Semarang 173, did not hybridize in any of our experiments. Since sphingomyelinase genes from gram-positive bacteria do not hybridize under identical conditions (15), all leptospiral PSGs must be more similar to each other than to the sphingomyelinase genes from gram-positive bacteria.

Comparison of all hybridization data indicates that the presence of multiple PSGs is characteristic for pathogenic *Leptospiraceae* and that saprophytic *Leptospiraceae* do not contain PSGs (Fig. 1 and Table 1). However, there are two exceptions. The *L. inadai* species is considered to be patho-

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TABLE 1. Strains of members of the family *Leptospiraceae* used in this study and summary of chromosomal *Eco*RI DNA fragments hybridizing with the *sphA* gene[a]

Species	Serogroup	Serovar	Strain (reference)[b]	Hybridizing fragments (kb)[c]		Minimal no. of PSGs[d]	Lane no. in Fig. 1[e]
				High stringency	Low stringency		
L. interrogans	Icterohaemorrhagiac	icterohaemorrhagiae	Wijnberg (15, 17)		11.4 5.3 4.5 2.7 1.8	3	1
	Canicola	canicola	Hond Utrecht IV (19)		11.4 4.3 3.5 3.4 1.8	3	2
	Pomona	pomona	Pomona (19)		11.4 4.7 3.5 3.4 1.8	3	3
	Icterohaemorrhagiae	icterohaemorrhagiae	RGA (19)		11.4 5.3 4.5 2.7 1.8	3	
	Djasiman	djasiman	Djasiman (19)		11.4 5.3 3.5 3.4 1.8	3	
	Australis	bratislava	Jeź-Bratislava (7a)		11.4 6.0 4.5 2.8 1.8	3	
	Icteroheamorrhagiae	copenhageni	M20 (19)		11.4 5.3 4.5 2.7 1.8	3	
	Australis	fugis	Fudge (7a)		11.4 8.6 7.5 2.7 1.8	3	
L. borgpetersenii	Sejroe	hardjo	Sponselee (15, 17)	2.0	3.6 1.7 1.6 0.7 0.4	2	4
	Ballum	ballum	Mus127 (19)	1.9	3.5 2.6 1.4 0.8	2	5
	Hebdomadis	worsfoldi	Worsfold (7a)	1.0	4.0 0.7	1	
	Tarassovi	tarassovi	Perepelicin (19)	1.9	1.7 1.4 0.7	2	
	Mini	mini	Sari (19)	1.9	1.7 1.4 0.7	2	
L. santarosai	Shermani	shermani	1342K (7a)	2.8 1.0 0.4		2	6
	Grippotyphosa	canalzonae	CZ188 (7a)	4.0 1.9 1.0 0.4		2	7
	Hebdomadis	borincana	HS622 (19)	2.8 1.9 1.0 0.4		2	
	Tarassovi	bakeri	LT79 (19)	1.8 1.1 1.0 0.4		2	
	Pomona	tropica	CZ299 (7a)	1.8 1.7 1.0 0.4		2	
L. noguchii	Pomona	proechimys	1161U (7a)		6.6 1.8 1.7 1.4 1.3	3	8
	Panama	panama	CZ214K (19)		7.5 1.8 1.7 1.35 1.3	3	
	Autumnalis	louisiana	LSU1945 (7a)		8.7 3.5 2.0 1.9 1.5 1.3	3	
	Australis	peruviana	V42 (7a)		8.8 3.5 2.4 1.9 1.5 1.3	3	
	Autumnalis	fortbragg	Fort Bragg (19)		8.7 3.8 3.0 2.0 1.5 1.3	3	
"L. alstoni"	Cynopteri	cynopteri	3522C (19)		9.4 6.1 5.4 4.0	2	9
	Icterohaemorrhagiae	mwogolo	Mwogolo (7a)		9.6 6.1 5.4 4.0	2	
	Pomona	mozdok	5621 (7a)		9.5 6.1 5.4 4.0	2	
	Autumnalis	bim	1051 (7a)		9.5 6.1 5.4 4.0	2	
	Grippotyphosa	grippotyphosa	Moskva V (7a)		9.5 5.4 4.0 2.0 1.0	3	
L. weilii	Celledoni	celledoni	Celledoni (19)	0.95	3.6	1	10
	Sarmin	sarmin	Sarmin (19)	3.4 2.0 0.95		2	
L. inadai	Lyme	lyme	10 (19)			0	11
L. biflexa	Semaranga	patoc	Patoc I (19)			0	12
	Andamana	andaman	CH11 (19)			0	
L. meyeri	Ranarum	ranarum	Iowa City Frog (ICF) (19)	4.4 3.0 2.8	1.5 1.2 1.15 0.8 0.4	4	13
	Semaranga	semaranga	Veldrat Semarang 173 (19)			0	
L. wolbachii	Codice	codice	CDC (19)			0	14
Leptonema illini		illini	3055 (19)			0	15

[a] All *Leptospira* strains used were obtained from the WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis at the Royal Tropical Institute, Amsterdam, The Netherlands, except for strains CDC and 10 which were obtained from the Centers for Disease Control, Atlanta, Ga. For all strains, both the serological and genetic classifications have been indicated.

[b] The strains were classified genetically by the methods of Yasuda et al. (19) or Kaufmann (7a) or provisionally classified pending further hybridization studies with various probes (15, 17). Data were obtained from Fig. 1 and several experiments whose results are not shown.

[c] The sizes of the DNA fragments which hybridize under stringent conditions are given as well as the sizes of the additional DNA fragments hybridizing under low-stringency conditions. The prominent hybridizing fragments have been underscored.

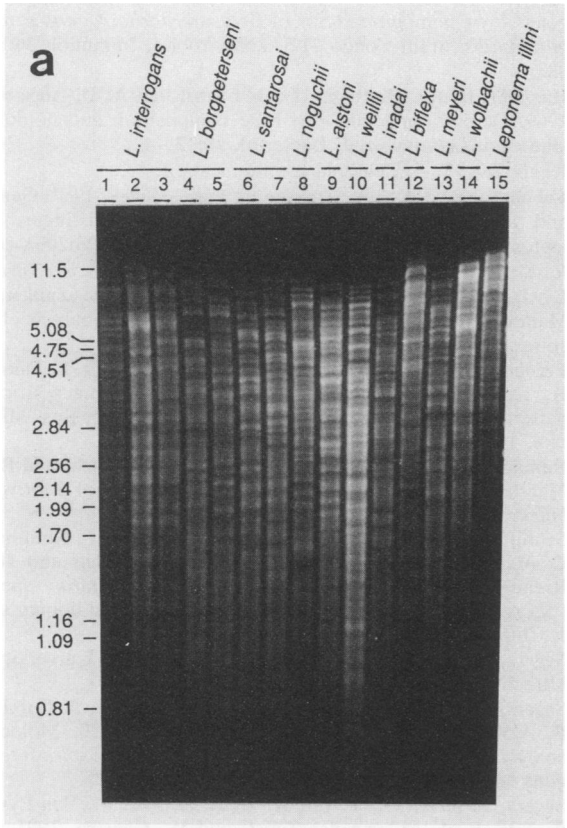
[d] The minimal number of PSGs expected to be present on the basis of the number and size of the hybridizing DNA fragments is indicated.

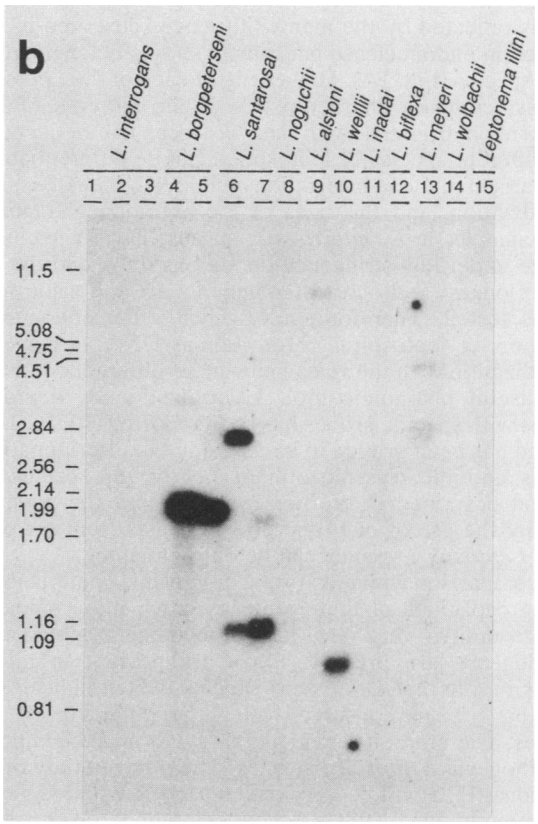
[e] The last column refers to the lane numbers in Fig. 1.

genic (12) but does not contain PSGs, and *L. meyeri* ICF is considered to be saprophytic (5) but contains the highest number of PSGs. However, the taxonomic status of both strains is questionable (13). Moreover, *L. inadai* does not react in a polymerase chain reaction test specific for pathogenic leptospires, whereas strain ICF does (18). As deduced from hybridizations involving the whole genome, both of the *L. meyeri* strains tested are genetically highly related (19). We cannot explain why such genetically similar organisms differ in a whole set of genes which are otherwise conserved within pathogenic *Leptospira* species.

In addition to the *sphA* gene, two other leptospiral hemolysin genes have been cloned from a serovar pomona and a serovar autumnalis strain (3, 6). At least the latter gene seems to be different from the PSGs on the basis of restriction patterns. The hemolysin gene from serovar pomona cannot be compared, since no *Eco*RI DNA restriction digestion map or any further information is available.

So far, it is not known whether any of the PSGs is actively expressed. Since the *sphA* gene used as a probe is a functional sphingomyelinase gene, it is likely that at least the DNA fragments from the *L. borgpetersenii*, *L. weilii*, and *L. santarosai* strains, which hybridize under stringent conditions, represent the same gene and could also encode sphingomyelinase. Some *L. interrogans* strains, like Hond Utrecht IV and Pomona, express sphingomyelinase when cultured in vitro (13). It is likely that one or more of the PSGs encode this activity. Since the presence of multiple types of





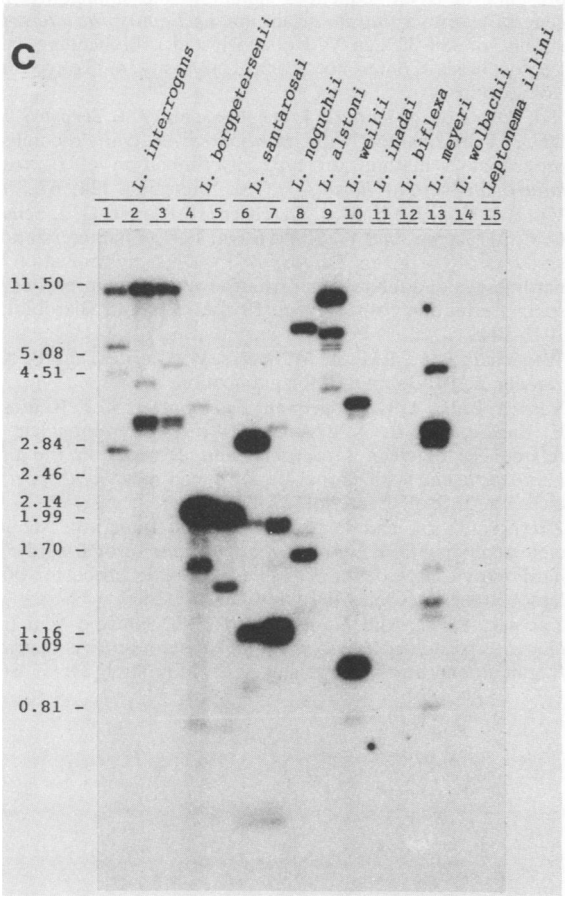


FIG. 1. Presence of PSGs among strains of the family *Leptospiraceae*. Chromosomal DNA fragments from various strains (Table 1) obtained after endonuclease restriction digestion with *Eco*RI were separated on a 1% agarose gel and blotted onto nitrocellulose. The blot was hybridized with a ^{32}P-radiolabeled 1,295-bp insert DNA fragment from pHL2-B3 as described previously (15). Size markers are indicated on the left in kilobases. (a) Ethidium bromide-stained agarose gel; (b) autoradiograph after hybridization under stringent conditions; (c) autoradiograph after hybridization under low-stringency conditions.

sphingomyelinase in a serovar pomona strain was suggested (1), the active expression of multiple PSGs is a distinct possibility.

The family *Leptospiraceae* is genetically very heterogeneous and comprises at least 12 different species (2, 7, 11, 19). As deduced from DNA hybridizations involving the whole genome, the genetic relatedness among the different *Leptospira* species differs considerably. Nevertheless, *L. interrogans*, *L. noguchii*, and "*L. alstoni*" on the one hand and *L. borgpetersenii*, *L. santarosai*, and *L. weilii* on the other hand seem to be more related to each other than to the other pathogenic *Leptospira* species (13, 19). A similar subdivision of the pathogenic leptospires into two groups on the basis of G+C content of the chromosomal DNA, 2,6-diaminopurine resistance, and lipase production was previously reported by Haapala et al. (7). The subdivision of pathogenic leptospires into two groups corresponds to the presence or absence of DNA fragments hybridizing with the *sphA* gene under stringent hybridization conditions (Table 1), since under such conditions PSGs hybridize in *L. borgpetersenii*, *L. santarosai*, and *L. weilii* but not in the other pathogenic *Leptospira* species. Within each species, strains may be genetically different to a considerable degree (19).

This is reflected by the many differences observed in DNA restriction endonuclease patterns in strains belonging to the same species (Fig. 1A). However, for each of the *Leptospira* species, common characteristics of the patterns of DNA fragments hybridizing with the *sphA* gene can be identified, and different species of leptospires can be differentiated by comparison of the hybridization conditions, the intensities of hybridization, and the sizes of the hybridizing fragments. For example, in *L. interrogans* strains, five fragments hybridize under low-stringency conditions only, and the sizes of the longest and shortest fragments are the same for all strains tested. Therefore, *Eco*RI restriction endonuclease digestion of leptospiral chromosomal DNA, followed by hybridization with the *sphA* gene under different conditions, is a useful taxonomic tool. Hybridization of *Leptospira* strains with specific probes has been reported (10, 16, 17, 20, 21) and has been proven to be useful in the discrimination of strains and the classification of *Leptospira* species. The method described in the present study has the advantage that, by the use of only one probe, strains from six pathogenic *Leptospira* species can be differentiated.

In comparison with eukaryotic organisms, bacteria use the coding capacities of their genomes much more efficiently, and generally, very few long noncoding regions and/or pseudogenes are present. PSGs are present in multiple copies in different *Leptospira* species, which indicates that they have been conserved after the diversion of these species. Therefore, it is evident that PSGs must be important to pathogenic *Leptospira* species. The in vivo study of each individual PSG will be very difficult in the whole organism. Separate cloning of PSGs is now being undertaken and will allow further study of these genes, which seem to be members of a gene family.

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