INFECTION AND IMMUNITY, July 1990, p. 2177–2185
0019-9567/90/072177-09$02.00/0

Vol. 58, No. 7

Molecular Analysis of a Sphingomyelinase C Gene from *Leptospira interrogans* Serovar hardjo

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Received 29 January 1990/Accepted 10 April 1990

A thermolabile hemolysin from *Leptospira interrogans* serovar hardjo, strain Sponselee, was shown to specifically degrade sphingomyelin. Nucleotide sequence determination revealed that sphingomyelinase activity was encoded by an open reading frame of 1,668 nucleotides. Although a putative signal sequence could be identified, no evidence for protein export in either *L. interrogans* or *Escherichia coli* was obtained. The apparent molecular mass of the expression product in *E. coli* minicells was 41.2 kilodaltons, whereas open reading frame 1 encoded a protein of 63,268 daltons. The observed difference may be explained by processing at the carboxy-terminal part of the hemolysin in *E. coli*. A high degree of similarity on the DNA and protein levels with *Staphylococcus aureus* β-hemolysin and sphingomyelinase C from three *Bacillus cereus* strains was observed. The presence of various sphingomyelinase genes within the *L. interrogans* species is demonstrated.

Leptospira interrogans is the etiologic agent of leptospirosis, which is a worldwide zoonosis. The bacteria of this species are divided into 19 serogroups and subdivided into more than 180 serovars on the basis of a microscopic agglutination test (23). Members of the serovar hardjo (belonging to the serogroup Sejroe) cause leptospirosis in dairy cattle, resulting in serious economic losses due to agalactia and abortion (11, 36). The infection can be transmitted by the urine to humans, resulting in dairy fever, characterized by headache, severe fever, meningitis, and icterus. In spite of the medical and economical importance of leptospirosis, very little is known about the virulence factors involved in pathogenesis. Hemolysins are involved in the pathogenesis of infections by *Escherichia coli* (16), *Staphylococcus aureus* (5), *Listeria monocytogenes* (7, 26), and *Streptococcus pneumoniae* (4) and have been claimed to be important in leptospiral infections (2, 35, 38, 39). Hemolysis by leptospirae is caused by phospholipases (38); both phospholipase A and sphingomyelinase C activities have been demonstrated (3, 6). Whereas pathogenic *L. interrogans* and nonpathogenic *Leptospira biflexa* strains have phospholipase A activity, sphingomyelinase C activity has only been demonstrated in strains of *L. interrogans*. A DNA fragment containing a sphingomyelinase C gene has been cloned from a Dutch field strain, causing bovine leptospirosis (10). In this study the molecular properties of the gene and its expression product are analyzed.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and transformation. *L. interrogans* strains were obtained from the World Health Organization/Food and Agricultural Organization Collaborating Center for Reference and Research on Leptospirosis at the Royal Tropical Institute in Amsterdam, The Netherlands, and were grown as described previously (10). Bluescript SK M13$^+$ (pBS) was used as plasmid cloning vehicle, and all experiments with pBS, unless stated otherwise, were performed with XL1-Blue (Stratagene, La Jolla, Calif.) as the *E. coli* host strain. *E. coli* cells were grown in Luria-Bertani (LB) medium or on LB agar plates (28) containing 100 μg of ampicillin per ml. Competent *E. coli* cells were prepared by the $CaCl_2$ method (28). Plasmid pHL2-B3 has been described previously (10). Plasmid pHL2-B4 contains the same DNA fragment as pHL2-B3 in the opposite orientation.

Production of mutants and sequence analysis. DNA from clones pHL2-B3 and pHL2-B4 was digested with restriction enzymes *Xba*I and *Sst*I, and unidirectional deletion mutants were produced by exonuclease III digestion with the Erase-a-Base kit (Promega Biotec, Madison, Wis.). Single-stranded DNA from these clones was prepared as described by the manufacturers of pBS (Stratagene). Nucleotide sequences were determined by the dideoxy-chain termination method of Sanger et al. (33). Nucleotide sequences were analyzed with the Beckman Microgenie (release 6.0; Beckman Instruments, Palo Alto, Calif.) and the PC/Gene (release 6.0; Genofit S.A., Geneva, Switzerland) computing programs.

The FASTA program, release 1.0, April 1988 (30), was used to compare nucleotide and amino acid sequences with the following data bases: EMBL (release 19.0), NBRF/PIR (release 21.0), NBRF/NEW (release 39.0), Swiss-prot (release 11.0), and Brookhaven (July 1989). Similar sequences were aligned by using the Clustal computer program (19, 20).

Hemolysin plate assay. Colonies of *E. coli* harboring recombinant plasmids were streaked on plates containing LB broth, 1% agar, 20% (vol/vol) fresh sheep erythrocytes (washed twice in 0.9% NaCl), 25 mM $MgCl_2$, and 100 μg of ampicillin per ml. Cells were grown for 18 h at 37°C, and hemolytic zones appeared after an additional incubation of 24 h at room temperature. Omission of $MgCl_2$ from the agarplates resulted in much smaller hemolytic zones.

Phospholipase assay and analysis by thin-layer chromatography. Sphingomyelinase activity was tested in a biphasic system, essentially as described previously (24), consisting of an ether-methanol (9:1, vol/vol) organic phase containing 2 mg of sphingomyelin isolated from bovine brain (Sigma Chemical Co., St. Louis, Mo.) per ml and a water phase containing 10 mM Tris hydrochloride (pH 7.4), 25 mM $MgCl_2$, and (sonicated) bacteria and/or culture medium. The

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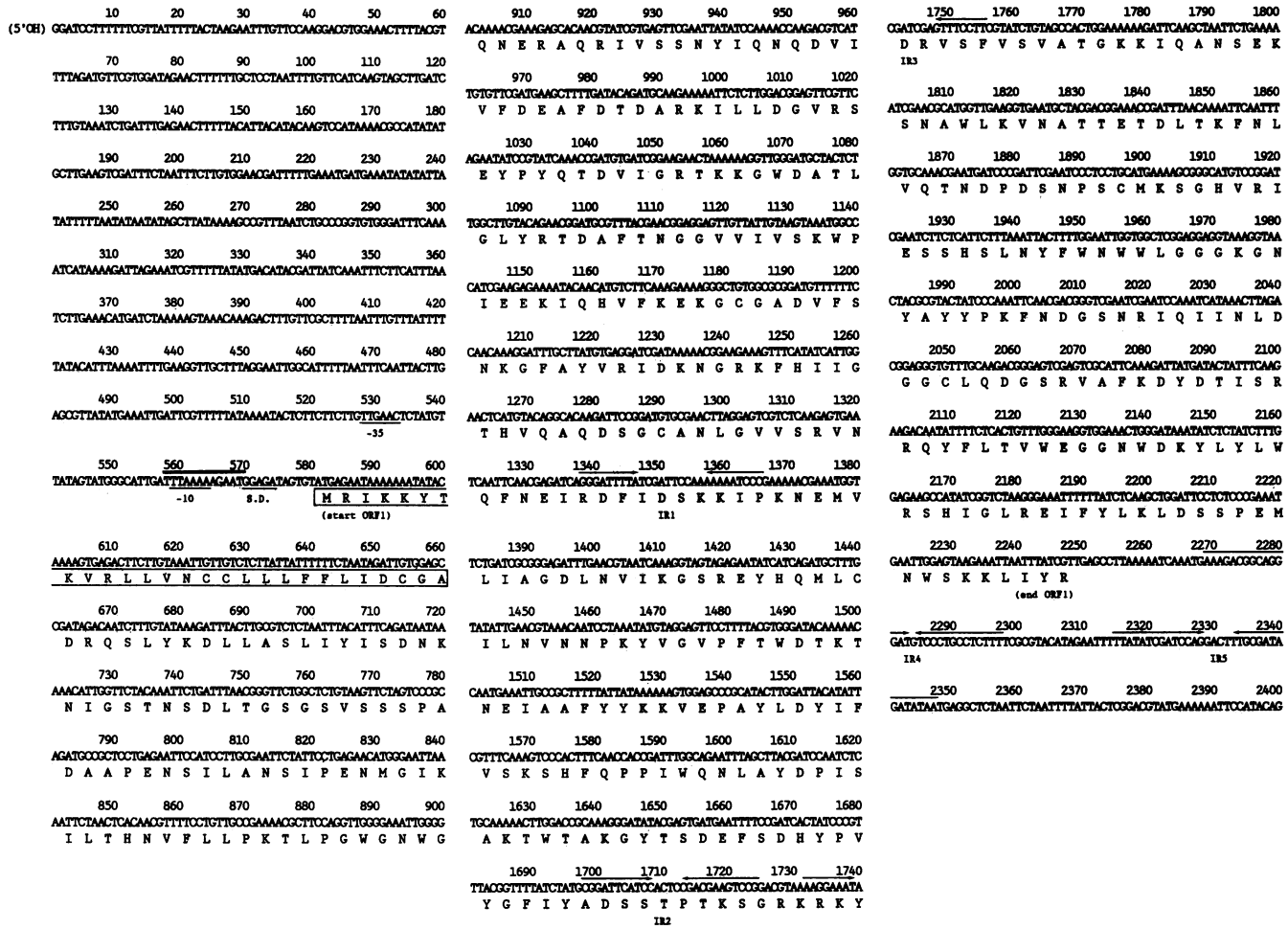

FIG. 1. Nucleotide and derived amino acid sequences from the 3,987-bp *Bam*HI insert in pHL2-B3. The putative signal sequence is boxed, and −10 and −35 transcription signals and putative ribosome-binding sites (RBS) are underlined. Inverted repeats (IRs) are numbered and overlined by arrows. Gibbs free energy differences were calculated by using the Microgenie program and are indicated in kilocalories (1 kcal is equal to ca. 4.184 kJ) per mole: IR1, −9.6; IR2, −8.8; IR3, −9.6; IR4, −25.4; IR5, −10.8; IR6, −8.0. Sequences similar to the potential regulatory sequence in *L. biflexa* (42) are overlined with a boldface bar. These data have been submitted to the EMBL Data Library under accession no. X52176.

samples were vigorously shaken for 4 h at 37°C, and 2 μl of the organic phase was applied on a silica gel-60-coated glass plate (E. Merck AG, Darmstadt, Federal Republic of Germany). When $MgCl_2$ was omitted from the reaction mixture, sphingomyelinase activity was much lower. The chromatogram was developed with a chloroform–methanol–water–25% ammonia (58:35:3.5:3.5, vol/vol) mixture as the mobile phase. (Phospho)lipids were visualized by spraying the plates with 30% sulfuric acid, followed by heating at 110°C for 5 min. Purified sphingomyelinase C (0.08 U) from *S. aureus* (Sigma) was used as a positive control. Sonicated *E. coli* cells containing pBS were used as a negative control. Degradation of other phospholipids was performed as described above, chromatograms were developed with mobile-phase mixtures as previously described (24, 25), and reaction products were visualized as described above. Degradation of L-α-lysophosphatidylcholin (egg yolk; Sigma), L-α-phosphatidylethanolamine (bovine brain; U.S. Biochemical Corp., Cleveland, Ohio), phosphatidyl-L-serine (bovine brain; U.S. Biochemical), L-α-phosphatidic acid (egg yolk; U.S. Biochemical), and L-α-lecithin (U.S. Biochemical) as substrates was tested.

RESULTS

Nucleotide sequence analysis. The nucleotide sequence of a cloned 3,987-base-pair (bp) *Bam*HI DNA fragment, which was shown to code for sphingomyelinase activity (10), was determined (Fig. 1; data submitted to EMBL Data Library under accession no. X52176). For this purpose, a number of deletion clones (Fig. 2C) were produced. After translation of the nucleotide sequence, three open reading frames (ORFs) were identified (Fig. 2A). The percentage of G+C in the whole fragment (35.9%) as well as in the individual ORFs (Fig. 2A) was within the range of 34.1 to 39.1%, which corresponds to the known G+C percentage of genomic DNA from *L. interrogans* (23) and from *L. interrogans* serovar hardjo strains (27).

Upstream of ORF1 and ORF2, putative −10 and −35 transcription and Shine-Dalgarno translation-initiation sequences similar to those in *E. coli* were identified. In ORF2 a second putative translation initiation site was identified at bp 3264 (Fig. 1). Sequences upstream of ORF1 and ORF2 and the internal transcription initiation site in ORF2 were similar to the previously reported potential regulatory se-

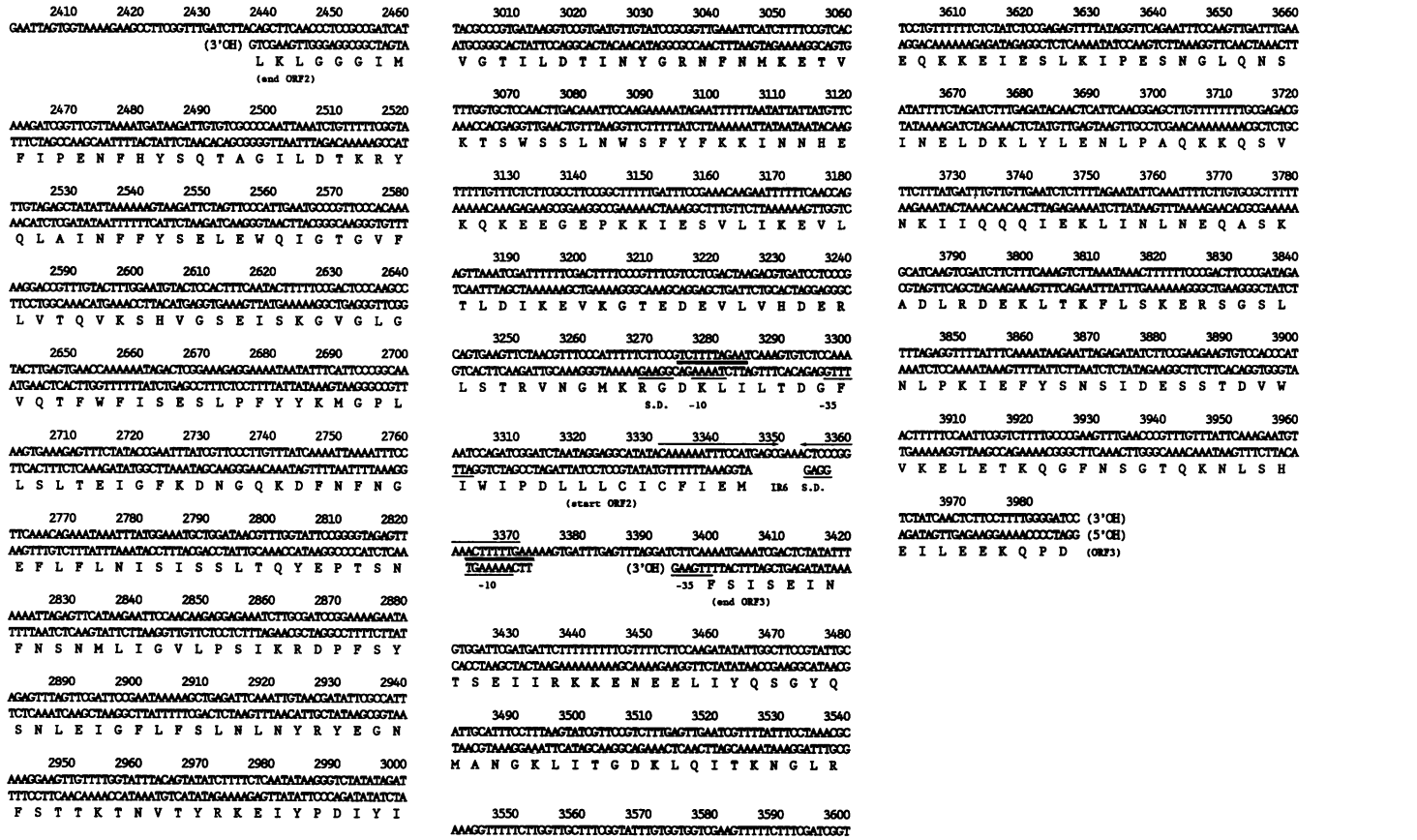

FIG. 1—*Continued*

quence TCAAAAT/CGAAT, found upstream of *L. biflexa trpE* and *trpG* genes (42) (Fig. 1). In ORF1 and ORF2 this sequence was closer to the putative translation initiation site than was found with the *trp* genes. Inverted repeats that could function as rho-independent transcription termination signals were located directly downstream of ORF1 and ORF3. The first inverted repeat downstream of ORF2 was after 80 bp. The initiation methionine codon and the regulatory sequences of ORF3 were not located on clone pHL2-B3. Therefore we only sequenced the coding region for the carboxy-terminal part of the protein.

Localization of region encoding hemolytic and sphingomyelinase activity. A number of deletion mutants (Fig. 2C) were tested for hemolytic activity in blood agar plates and for sphingomyelinase C activity (Fig. 3). In all clones tested in both assays, the presence of sphingomyelinase activity coincided with the presence of hemolytic activity. In both experiments, pHL2-B4-51 was the clone with the smallest DNA insert, still expressing hemolytic and sphingomyelinase C activities at the same level as clones pHL2-B3 and pHL2-B4. *E. coli* containing recombinant pHL2-B4-53, coding for 45.6 kilodaltons (kDa) of the amino-terminal part of the protein, lost both enzymatic activities. Recombinant pHL2-B4-06 contained the coding region of ORF1 except for the last 10 amino acids; *E. coli* cells harboring this recombinant still contained both enzymatic activities, but at lower levels (data not shown). Therefore the whole coding region of ORF1 is required for full expression of hemolytic and sphingomyelinase activities in *E. coli*. Since *E. coli* containing pHL2-B3-05 was not hemolytic and had no sphingomyelinase activity, expression also required the presence of at least a portion of the 580-bp noncoding DNA region upstream of ORF1. A DNA sequence controlling the expression of sphingomyelinase activity may be located on this DNA region.

Temperature sensitivity and substrate specificity. Sphingomyelinase activity in lysates from *E. coli* cultures harboring pHL2-B3 and *L. interrogans* Sponselee cultures was destroyed by heating the samples for 10 min at 56°C. Lysates from clone pHL2-B3 were also tested for the ability to degrade L-α-lysophosphatidylcholin, L-α-phosphatidylethanolamine, phosphatidyl-L-serine, L-α-phosphatidic acid, and L-α-lecithin, but no activity was detected.

Protein sequence analysis and homology with other sphingomyelinases. At the amino terminus of ORF1, the first 27 amino acids have the consensus of a procaryotic signal sequence (40): a basic amino-terminal region, central hydrophobic region, and a polar carboxy-terminal region. The cleavage site of signal peptides is always preceded by a small amino acid residue at the −1 position and a small uncharged amino acid residue at the −3 position (40). Therefore, cleavage is most likely to occur after the alanine residue at position 27.

Comparison of nucleotide and amino acid sequences of ORF1 with those in data bases revealed homology with a β-hemolysin from *S. aureus* (32) and three sphingomyelinases from different *B. cereus* strains (14, 22, 41) on both the DNA level (data not shown) and the protein level (Fig. 4).

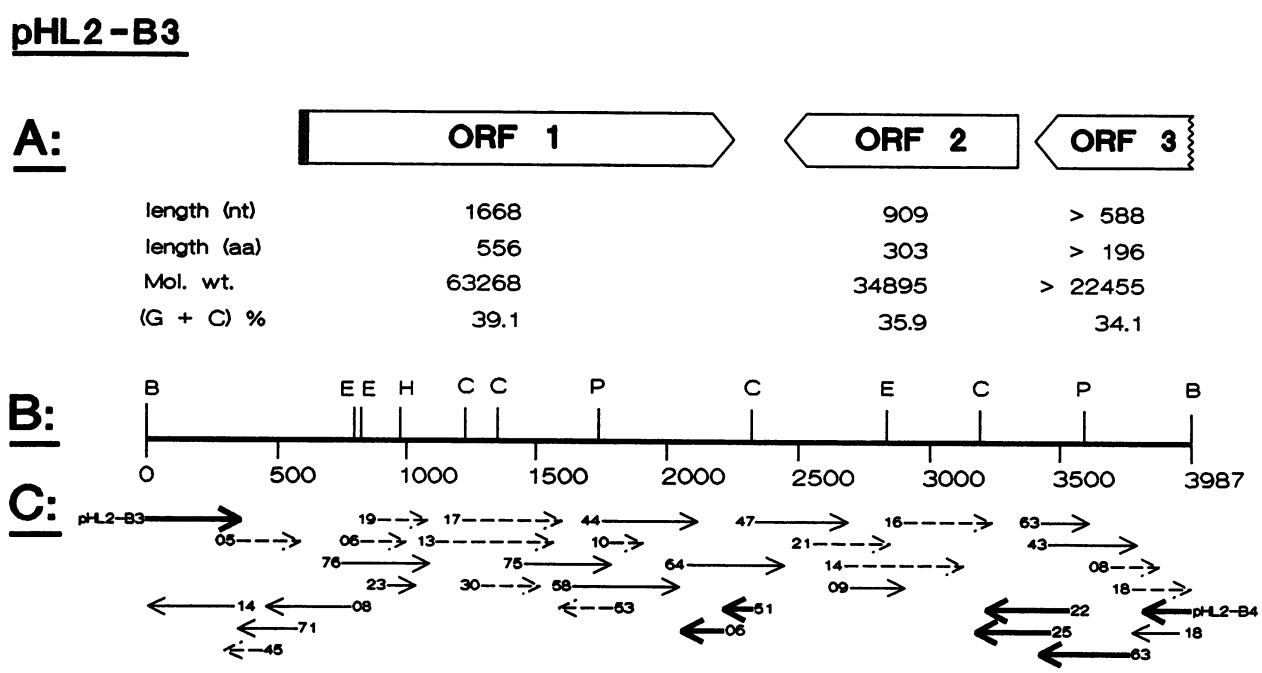

FIG. 2. Restriction map of pHL2-B3 and sequence strategy. (A) Properties of the three ORFs deduced from the nucleotide sequence analysis. The hatched bar indicates a putative signal peptide. (B) Restriction map of the 3,987-bp *Bam*HI insert in pHL2-B3 (pHL2-B4 contains the same insert in the opposite orientation) (B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*I). (C) Deletion clones generated by exonuclease III digestion and used for nucleotide sequence analysis and localization of hemolytic and sphingomyelinase activity. Arrows pointing to the right indicate clones derived from pHL2-B3; the 3,987-bp *Bam*HI insert was digested by exonuclease III exclusively from the side of bp 1, and the remaining insert DNA extends from the base of the arrow up to bp 3987. Arrows pointing to the left are derived from pHL2-B4; the 3,987-bp *Bam*HI insert was digested by exonuclease III exclusively from the side of bp 3987, and the remaining insert DNA extends from the base of the arrow up to bp 1. The length of the arrow represents the part of which the nucleotide sequence was determined. Deletion clones are named after the clone from which they were derived (i.e., pHL2-B3 or pHL2-B4), followed by the number indicated above. A number of clones were tested for hemolytic activity; boldface and broken-line arrows indicate the presence and absence of hemolytic activity, respectively. The other arrows indicate clones that were not tested.

Both on the DNA and protein levels, the similarity was present in the middle part of the gene and protein, respectively. The amino termini differed considerably, and the carboxy termini could not be aligned because of the difference in molecular weight. No sequences homologous to ORF2 or ORF3 were detected during the homology searches.

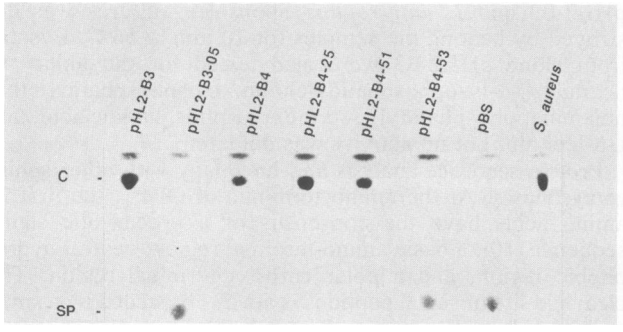

FIG. 3. Assay of sphingomyelinase activity of various recombinant DNA clones to localize the region on pHL2-B3 encoding enzyme activity. Sonicated *E. coli* cells containing the plasmids indicated at the top were tested for sphingomyelinase activity; the reaction products were analyzed by thin-layer chromatography. Purified sphingomyelinase C from *S. aureus* and *E. coli* containing the pBS vector were used as positive and negative controls, respectively. The sphingomyelin substrate and ceramide degradation products are indicated on the left by SP and C, respectively.

Codon usage. The codons used in *L. interrogans* for the expression of the ORFs of pHL2-B3 are shown in Table 1 and compared with the codon usage in *L. biflexa trpE* and *trpG* genes (42), *E. coli* (average values for 407 genes calculated from the data from Aota et al. [1]), *S. aureus* β-hemolysin (32), and *B. cereus* sphingomyelinase C genes (average values compiled from three published sequences [14, 22, 41]). Codon usage in the *L. interrogans* genes was quite different from the codon usage in *E. coli* genes for the amino acids arginine, asparagine, cysteine, glutamine, glycine, proline, and leucine. The codon usage was more similar to that of *L. biflexa*, although there were differences for asparagine, arginine, isoleucine, and proline. Comparison of the codon usage in the different sphingomyelinase genes revealed the frequent use of the arginine codon AGA and the proline codon CCC in the leptospiral sphingomyelinase gene, whereas these codons were hardly or not at all used in the other four genes. The frequencies of bases in the third position of a codon were calculated and were similar to what has been shown for *L. biflexa* (42), reflecting the overall base composition of the organism.

Sphingomyelinase activities of different *Leptospira* strains. Total culture, pellet, and supernatant fractions of four *L. interrogans* strains belonging to different serogroups were compared for the ability to degrade sphingomyelin (Fig. 5). The results indicated that all four strains contained sphingomyelinase activity. In strains Sponselee and Mus 127, sphingomyelinase activity was associated with the cellular fraction, whereas the sphingomyelinase activity from strains

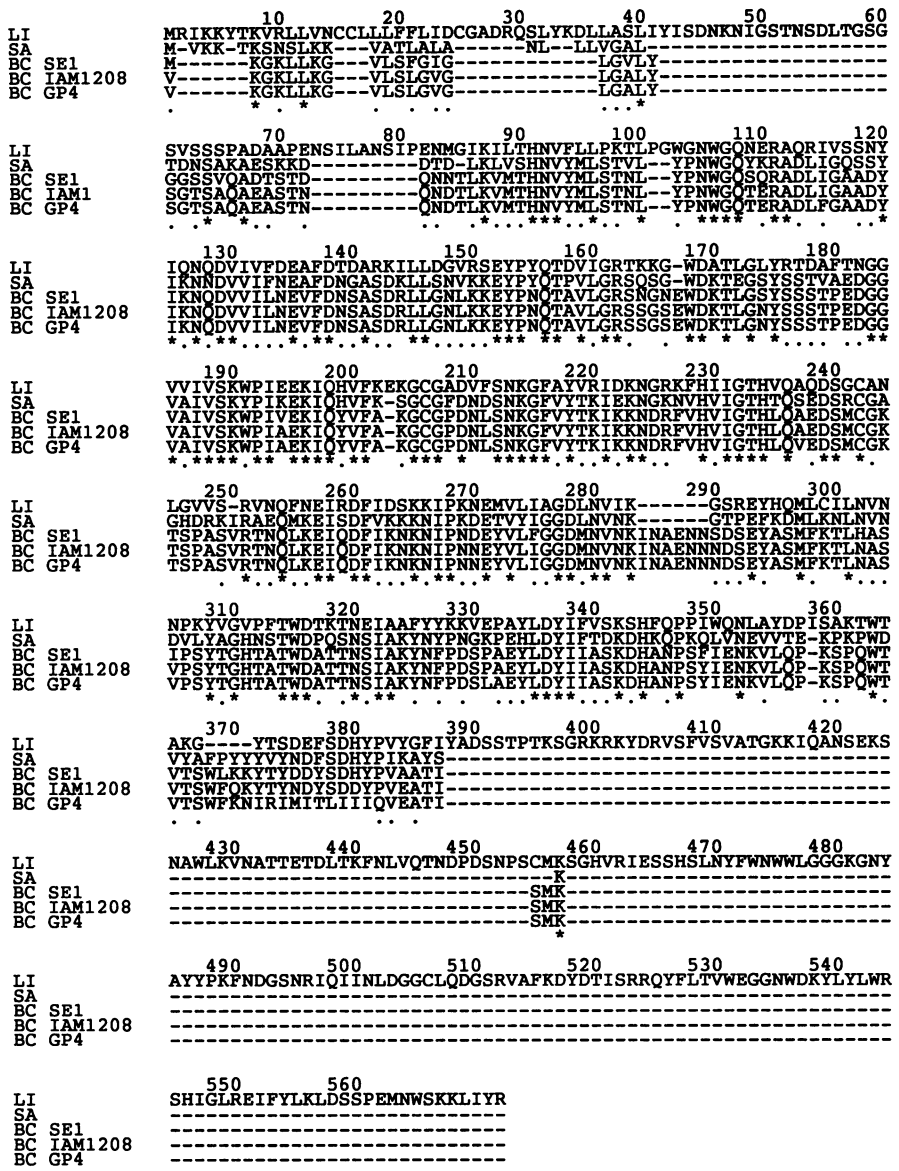

FIG. 4. Alignment of the amino acid sequences from sphingomyelinases from *L. interrogans* (LI) Sponselee (this work), *S. aureus* β-hemolysin (SA) (32), and *B. cereus* (BC) SE1 (22), IAM1208 (41), and GP4 (14). Identical residues are marked with an asterisk, and conservative mutations are marked with a dot, according to the log-odds amino acid similarity matrix of Dayhoff (9). Strokes indicate a gap introduced into the sequence for alignment purposes.

Pomona and Hond Utrecht IV seemed to be secreted. When sonication was omitted, the cellular fraction of strain Sponselee still had sphingomyelinase activity (data not shown but identical to those in Fig. 5). Therefore the enzyme is probably located in the outer envelope. No sphingomyelinase activity could be demonstrated in strains Wijnberg and M20 (belonging to serogroup Icterohaemorrhagiae) and the apathogenic PatocI strain (*L. biflexa*).

DISCUSSION

The nucleotide sequence analysis of a 3,987-bp DNA fragment of *L. interrogans*, encoding sphingomyelinase, revealed the presence of three ORFs. These are the first *L. interrogans* protein-encoding genes for which the nucleotide sequence has been determined. The molecular mass of the product of ORF1 (63,268 Da), corresponds to the apparent molecular mass of 64 kDa from a hemolysin cloned from *L. interrogans* serovar pomona (8; A. A. Dain, M. N. Rozinov, and Y. G. Chernukha, VI Joint Meeting of Leptospira Workers, abstr. no. 7.7, 1988). It has previously been shown that from the DNA insert of pHL2-B3, only one smaller protein of 39.2 kDa (41.6 kDa, including the 2.4-kDa signal peptide) is expressed in *E. coli* (10). Indeed, ORF1 has a putative signal peptide with a calculated molecular mass of 3,175 Da. Moreover, ORF1 is the only reading frame large enough to encode such a protein and has been shown here to code for hemolysin and sphingomyelinase activities. We therefore conclude that the 39.2-kDa protein is the mature expression product of ORF1 in *E. coli* minicells and probably has sphingomyelinase activity. However, we cannot exclude the possibility that sphingomyelinase activity is expressed as a larger, short-lived protein, and that the

TABLE 1. Codon usage in *L. interrogans*, *L. biflexa*, *E. coli*, *S. aureus*, and *B. cereus*[a]

Amino acid	Codon	Frequency of codon usage (%) in:						
		L. interrogans			*L. biflexa*	*E. coli*	*S. aureus*	*B. cereus*
		ORF1	ORF2	ORF3				
Ala	GCT	8 (28)	1 (50)	1 (25)	16 (45)	(20)	3 (18)	14 (21)
Ala	GCC	4 (13)	0 (0)	0 (0)	3 (9)	(23)	3 (18)	4 (6)
Ala	GCA	9 (31)	0 (0)	3 (75)	10 (29)	(22)	8 (46)	37 (54)
Ala	GCG	8 (28)	1 (50)	0 (0)	6 (17)	(35)	3 (18)	13 (19)
Arg	CGT	4 (16)	1 (13)	1 (20)	7 (25)	(59)	2 (40)	16 (100)
Arg	CGC	0 (0)	2 (25)	0 (0)	2 (7)	(37)	1 (20)	0 (0)
Arg	CGA	3 (12)	1 (13)	1 (20)	10 (36)	(4)	1 (20)	0 (0)
Arg	CGG	1 (4)	1 (13)	1 (20)	2 (7)	(6)	0 (0)	0 (0)
Arg	AGA	13 (52)	3 (37)	2 (40)	5 (18)	(2)	1 (20)	0 (0)
Arg	AGG	4 (16)	0 (0)	0 (0)	2 (7)	(2)	0 (0)	0 (0)
Asn	AAT	20 (51)	13 (62)	13 (76)	24 (89)	(37)	21 (81)	63 (70)
Asn	AAC	19 (49)	8 (38)	4 (24)	2 (11)	(63)	5 (19)	27 (30)
Asp	GAT	32 (86)	7 (58)	5 (71)	24 (83)	(57)	19 (73)	57 (92)
Asp	GAC	5 (14)	5 (42)	2 (29)	5 (17)	(43)	7 (27)	5 (8)
Cys	TGT	7 (88)	1 (50)	0 (0)	3 (60)	(42)	2 (100)	1 (17)
Cys	TGC	1 (12)	1 (50)	0 (0)	2 (40)	(58)	0 (0)	5 (83)
Gln	CAA	15 (83)	8 (100)	14 (93)	19 (90)	(30)	9 (90)	27 (75)
Gln	CAG	3 (17)	0 (0)	1 (7)	2 (10)	(70)	1 (10)	9 (25)
Glu	GAA	15 (75)	18 (78)	19 (76)	49 (86)	(71)	13 (87)	25 (60)
Glu	GAG	5 (25)	5 (22)	6 (24)	8 (14)	(29)	2 (13)	17 (40)
Gly	GGT	10 (25)	1 (4)	1 (13)	20 (32)	(41)	11 (52)	22 (33)
Gly	GGC	4 (10)	6 (25)	0 (0)	2 (3)	(41)	5 (24)	5 (7)
Gly	GGA	22 (55)	14 (58)	6 (74)	31 (50)	(7)	3 (14)	27 (40)
Gly	GGG	4 (10)	3 (13)	1 (13)	9 (15)	(11)	2 (10)	13 (20)
His	CAT	7 (70)	3 (75)	1 (100)	12 (75)	(49)	8 (89)	13 (76)
His	CAC	3 (30)	1 (25)	0 (0)	4 (25)	(51)	1 (11)	4 (24)
Ile	ATT	16 (40)	13 (48)	5 (28)	32 (64)	(44)	5 (33)	37 (62)
Ile	ATC	18 (45)	9 (33)	3 (33)	12 (24)	(51)	6 (40)	7 (12)
Ile	ATA	6 (15)	5 (19)	7 (39)	6 (12)	(5)	4 (27)	16 (26)
Leu	TTA	10 (24)	7 (22)	2 (10)	18 (29)	(10)	11 (52)	43 (61)
Leu	TTG	10 (24)	6 (19)	9 (43)	14 (22)	(11)	3 (14)	14 (20)
Leu	CTT	8 (20)	8 (25)	3 (14)	17 (28)	(9)	4 (19)	3 (6)
Leu	CTC	6 (15)	2 (6)	3 (14)	9 (13)	(9)	1 (5)	1 (1)
Leu	CTA	4 (10)	5 (16)	4 (29)	5 (8)	(3)	2 (10)	8 (11)
Leu	CTG	3 (7)	4 (12)	0 (0)	0 (0)	(58)	0 (0)	1 (1)
Lys	AAA	35 (80)	19 (83)	20 (83)	39 (81)	(76)	36 (88)	61 (74)
Lys	AAG	9 (20)	4 (17)	4 (17)	9 (19)	(24)	5 (12)	21 (26)
Met	ATG	6 (100)	6 (100)	1 (100)	15 (100)	(100)	4 (100)	19 (100)
Phe	TTT	16 (64)	18 (78)	3 (75)	32 (84)	(47)	5 (50)	17 (63)
Phe	TTC	9 (36)	5 (22)	1 (25)	6 (16)	(53)	5 (50)	10 (37)
Pro	CCT	4 (20)	3 (33)	3 (50)	11 (31)	(14)	7 (50)	9 (23)
Pro	CCC	8 (40)	0 (0)	1 (17)	8 (22)	(8)	0 (0)	0 (0)
Pro	CCA	3 (15)	0 (0)	0 (0)	13 (36)	(18)	7 (50)	25 (62)
Pro	CCG	5 (25)	6 (67)	2 (33)	4 (11)	(60)	0 (0)	6 (15)
Ser	TCT	15 (33)	9 (38)	5 (20)	13 (28)	(21)	5 (19)	25 (28)
Ser	TCC	11 (24)	5 (21)	1 (5)	7 (14)	(18)	1 (4)	1 (1)
Ser	TCA	6 (13)	3 (13)	3 (15)	8 (16)	(10)	9 (35)	25 (28)
Ser	TCG	4 (9)	1 (4)	6 (30)	9 (18)	(13)	1 (4)	6 (7)
Ser	AGT	8 (17)	3 (12)	2 (10)	11 (22)	(11)	6 (23)	20 (22)
Ser	AGC	2 (4)	3 (12)	3 (15)	1 (2)	(27)	4 (15)	13 (14)

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TABLE 1—*Continued*

Amino acid	Codon	Frequency of codon usage (%) in:						
		L. interrogans			*L. biflexa*	*E. coli*	*S. aureus*	*B. cereus*
		ORF1	ORF2	ORF3				
Thr	ACT	9 (32)	7 (35)	3 (25)	5 (25)	(21)	6 (38)	23 (34)
Thr	ACC	4 (14)	3 (15)	4 (33)	5 (25)	(46)	1 (6)	0 (0)
Thr	ACA	5 (18)	3 (15)	2 (17)	7 (35)	(10)	8 (50)	24 (35)
Thr	ACG	10 (36)	7 (35)	3 (25)	3 (15)	(22)	1 (6)	21 (31)
Trp	TGG	15 (100)	5 (100)	1 (100)	2 (100)	(100)	4 (100)	18 (100)
Tyr	TAT	20 (67)	9 (64)	3 (75)	15 (60)	(50)	15 (71)	45 (87)
Tyr	TAC	10 (33)	5 (36)	1 (25)	10 (40)	(50)	6 (29)	7 (13)
Val	GTT	11 (31)	4 (25)	0 (0)	12 (31)	(31)	14 (52)	19 (28)
Val	GTC	6 (17)	5 (31)	1 (33)	4 (11)	(18)	3 (11)	1 (2)
Val	GTA	9 (26)	5 (31)	1 (33)	13 (33)	(18)	7 (26)	26 (38)
Val	GTG	9 (26)	2 (13)	1 (33)	10 (25)	(33)	3 (11)	22 (32)

[a] Codons used in the three ORFs of *L. interrogans* were compared with those of *L. biflexa trpE* and *trpG* genes (41), *E. coli* (average values for 407 genes calculated from the data from Aota et al. [1]), *S. aureus* β-hemolysin (32), and *B. cereus* sphingomyelinase C genes (average values compiled from three published sequences [14, 22, 41]). The frequency of codon usage is indicated, followed by a percentage, representing the number of times this codon is used to encode its amino acid.

39.2-kDa protein, generated by degradation or processing, has no enzymatic activity. The molecular mass of 39.2 kDa is comparable to the observed molecular mass of the four homologous sphingomyelinases and corresponds very well to the calculated molecular mass of 38,660 Da of the N-terminal part of the protein, starting after the signal sequence, up to the point where homology with the other sphingomyelinases ends (Fig. 4). How do we explain the discrepancy between the size of ORF1 and the experimentally detected product? Inverted repeats IR2 and IR3, which could be involved in transcription or translation termination, are located immediately downstream of the DNA region coding for this part of the protein (Fig. 1). Premature transcription or translation termination, however, is unlikely to occur, since the whole coding region of ORF1 is needed for optimal sphingomyelinase activity in *E. coli*. More likely, the 39.2-kDa protein would be the result of posttranslational processing of the complete 63-kDa expression product of ORF1. Since in *E. coli* minicells processing occurs before cleavage of the amino-terminal signal sequence, the former processing can only take place at the carboxy terminus of the protein (10). This is supported by the homology data presented in Fig. 4. Similar posttranslational processing at the carboxy terminus of a protein has previously been reported for the immunoglobulin A protease from *Neisseria gonorrhoeae* (31), serine protease from *Serratia marcescens* (29), and activation of aerolysin from *Aeromonas hydrophila* (21).

The codon usage in the *L. interrogans* sphingomyelinase gene is quite different from what is normally observed in *E. coli* for genes with an average expression level. Since the expression rate of genes in *E. coli* is known to be related to their codon usage (15, 34), the leptospiral genes will probably have a low level of expression in *E. coli*. Indeed, no difference could be detected between Coomassie-stained polyacrylamide gels containing lysates from *E. coli* containing pBS or pHL2-B3 (data not shown). Alternatively, the low expression level of the enzyme could be the result of weak promoter activity. On both the DNA and protein levels, the middle part of the leptospiral sphingomyelinase shares a high degree of similarity with sphingomyelinases from the distantly related bacteria *S. aureus* and *B. cereus*. Obviously this part is important for enzyme activity. The differences in the amino termini of the proteins could reflect differences in transport, since *B. cereus* and *S. aureus* sphingomyelinase are extracellular enzymes, whereas the sphingomyelinase C activity of *L. interrogans* Sponselee seems to be cell bound. ORF2 and ORF3 are not necessary for sphingomyelinase activity (Fig. 3); no similar nucleotide or amino acid sequences were found in the data banks, and the functions of the proteins encoded by ORF2 and ORF3 remain unknown. Unlike the case in *B. cereus* GP-4 and IAM1208 (14, 41), in which a phospholipase C gene is located directly downstream of the sphingomyelinase C gene, no such gene was found downstream of the leptospiral sphingomyelinase gene. Since it has previously been shown that five different clones, containing over 10 kilobases of genomic

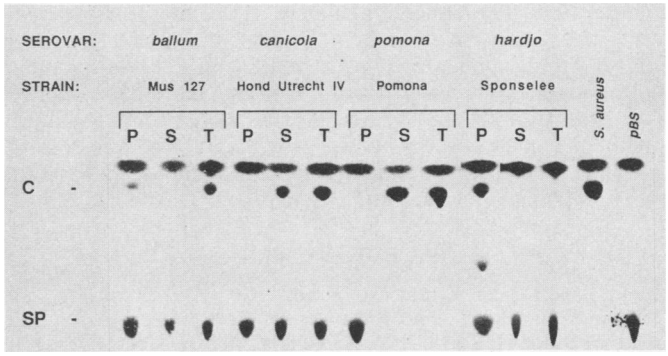

FIG. 5. Sphingomyelinase activity of four different *L. interrogans* strains. Bacterial cultures were harvested in the logarithmic growth phase, and a total (T) culture sample was separated into pellet (P) and supernatant (S) fractions by centrifugation for 10 min at 6,000 × *g*. After mild sonication, the samples were tested for their ability to degrade sphingomyelin, which was monitored by thin-layer chromatography. The sphingomyelin substrate and ceramide degradation products are indicated on the left by SP and C, respectively. Purified sphingomyelinase C from *S. aureus* and *E. coli* containing the pBS vector were used as positive and negative controls, respectively. The following *L. interrogans* strains were tested; Sponselee (serogroup Sejroe, serovar hardjo); Mus127 (serogroup Ballum, serovar ballum); Hond Utrecht IV (serogroup Canicola, serovar canicola); Pomona (serogroup Pomona, serovar pomona).

DNA on which the sphingomyelinase gene is located, were negative in a phospholipase C assay (10), it is unlikely that such a gene is located nearby on the *L. interrogans* genome.

Among strains of *L. interrogans*, various sphingomyelinases are produced. Strain Mus127 (serovar ballum) seems to contain a gene similar to that present in pHL2-B3 (10). Strains Hond Utrecht IV and Pomona, however, which do not cross-hybridize with the sphingomyelinase gene from strain Sponselee under stringent conditions (10), do degrade sphingomyelin. Moreover, the sphingomyelinase activities of strains Hond Utrecht IV and Pomona are predominantly found in the supernatant, whereas those of strain Sponselee and Mus127 are cell associated. Possibly, the sphingomyelinase is a contact hemolysin in strains Mus127 and Sponselee and an excreted hemolysin in strains Pomona and Hond Utrecht IV. Strains belonging to the pomona and ballum serovars were also reported to have different hemolytic properties (37). Bovine erythrocytes are preferentially lysed by pomona strains, and hamster erythrocytes are preferentially lysed by ballum strains. It is not known whether this difference is caused by a difference in substrate specificity of the sphingomyelinase. Contrary to the Dutch field strain Sponselee, *L. interrogans* strains of the serovar hardjo, isolated in New Zealand, do not lyse ovine erythrocytes (18). Therefore genetic variation seems to occur within the serovar hardjo. This is supported by the presence of different genotypes within the serovar hardjo, based on DNA restriction endonuclease analysis (12), and differences in the G+C percentage of the genomic DNA (27). Although the presence of multiple sphingomyelinase genes within the *L. interrogans* species indicates the importance of this enzyme for the bacterium, the involvement of the sphingomyelinase in pathogenesis is not known. However, several speculations can be made. The homologous β-hemolysin from *S. aureus* significantly increases recovery of bacteria from experimentally infected mice, compared with that of the β-hemolysin-negative mutant, and therefore contributes to virulence in vivo (5). Second, leptospires cannot grow without iron (13) and use free fatty acids as the main carbon and energy source (23). The sphingomyelinase could therefore play a role in obtaining iron and fatty acids from lysed erythrocytes. Third, sphingomyelinase may be an important factor in pathogenesis without lysing erythrocytes; sphingolipids like sphingomyelin and their degradation products affect many pharmalogical responses, growth factor action, receptor functions, and phorbolester-induced responses and have been implicated as second messengers (17).

ACKNOWLEDGMENTS

R.P.A.M.S. thanks Intervet International B.V. (Boxmeer, The Netherlands) for a research grant. Use of the services and facilities of the Dutch National NWO/SURF Expertise Center CAOS/CAMM, under grant numbers SON 326-052 and STW NCH99.1751, is gratefully acknowledged.

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