

Review

Multiple leptospiral sphingomyelinases (or are there?)

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Culture supernatants of leptospiral pathogens have long been known to haemolyse erythrocytes. This property is due, at least in part, to sphingomyelinase activity. Indeed, genome sequencing reveals that pathogenic *Leptospira* species are richly endowed with sphingomyelinase homologues: five genes have been annotated to encode sphingomyelinases in *Leptospira interrogans*. Such redundancy suggests that this class of genes is likely to benefit leptospiral pathogens in their interactions with the mammalian host. Surprisingly, sequence comparison with bacterial sphingomyelinases for which the crystal structures are known reveals that only one of the leptospiral homologues has the active site amino acid residues required for enzymic activity. Based on studies of other bacterial toxins, we propose that leptospiral sphingomyelinase homologues, irrespective of their catalytic activity, may possess additional molecular functions that benefit the spirochaete. Potential secretion pathways and roles in pathogenesis are discussed, including nutrient acquisition, dissemination, haemorrhage and immune evasion. Although leptospiral sphingomyelinase-like proteins are best known for their cytolytic properties, we believe that a better understanding of their biological role requires the examination of their sublytic properties as well.

Introduction

Sphingomyelinases are of great interest because of their potential to mediate key aspects of leptospiral pathogenesis. Leptospirosis is most prevalent in tropical countries where moist conditions favour environmental survival of pathogenic *Leptospira* species excreted by animal carriers of the spirochaete. Transmission occurs when contaminated soil or water comes into contact with cutaneous lacerations or mucous membranes of the mouth, eyes and nose (WHO, 2003). Leptospirosis is an invasive infection manifested by a broad spectrum of symptoms that are often mistaken for other infections. The disease is usually self-limiting but can progress to a severe form characterized by renal failure, haemorrhagic diathesis and jaundice. Pulmonary haemorrhage is a feared complication caused by damage to the endothelial lining of blood vessels (Dolhnikoff *et al.*, 2007), possibly caused by a toxin as leptospire are often not detected at the site of the lesion (Miller *et al.*, 1974). Another

occasional complication is haemolytic anaemia (Feigin *et al.*, 1975). Through their action on host cell membranes, leptospiral sphingomyelinases are potentially involved in aspects of pathogenesis, including tissue invasion, endothelial damage, immune evasion and nutrient acquisition.

Sphingomyelinases are enzymes that catalyse the hydrolysis of sphingomyelin into ceramide and phosphorylcholine. Biochemically, sphingomyelinases are classified as either acidic, neutral or alkaline, depending on their pH optimum for activation. Most of the neutral sphingomyelinases of bacteria and mammals form a family defined by a set of conserved catalytic core residues and overall sequence relatedness (Clarke *et al.*, 2011). Mammalian members of the neutral sphingomyelinase family are membrane-associated, whereas the bacterial members are secreted. Mammalian sphingomyelinases act on the sphingomyelin present on the membranes and release ceramide, which controls cellular functions by acting as a signalling molecule and by altering the biophysical

properties of the membrane (Hannun & Obeid, 2008). Ceramide is also the central hub of the sphingolipid signalling network, which includes other bioactive sphingolipids such as sphingosine and sphingosine-1-phosphate. The levels of ceramide and other sphingolipids are therefore tightly controlled (Breslow & Weissman, 2010), and their dysregulation contributes to the patho-biology of numerous infectious and non-infectious disease processes (Zeidan & Hannun, 2007). For example, cellular infection by diverse pathogens, including *Neisseria gonorrhoea*, rhinovirus and *Cryptosporidium parvum*, involves activation of the host acid sphingomyelinase by translocation of the enzyme from the endolysosomal to the plasma membrane (Grassmé *et al.*, 2005; Zeidan & Hannun, 2007). Hydrolysis of sphingomyelin in the plasma membrane by acid sphingomyelinase leads to assembly of ceramide-enriched membrane platforms, which may be necessary to concentrate receptors to facilitate intracellular signal transduction and microbial internalization (Lafont & van der Goot, 2005).

Sphingomyelinases produced by *Bacillus cereus*, *Staphylococcus aureus* and *Listeria (List.) ivanovii* are the best characterized among the bacterial sphingomyelinases. As most bacteria do not synthesize sphingomyelin, bacterial sphingomyelinases probably target the sphingomyelin in the external leaflet of the host cell's plasma membrane. Their inactivation in *S. aureus* and *List. ivanovii* diminished their infectivity in animal models (Bramley *et al.*, 1989; González-Zorn *et al.*, 1999). *List. ivanovii* sphingomyelinase enables the intracellular pathogen to escape from phagocytic vacuoles in epithelial cells by rupturing the membrane of the vacuole (González-Zorn *et al.*, 1999). The sphingomyelinase activity of *S. aureus* β -toxin promotes excessive inflammation and vascular leakage in the lungs by inducing shedding of the ectodomain of the proteoglycan syndecan-1 in a mouse model of pneumonia (Hayashida *et al.*, 2009). The response does not occur when the catalytic residues of β -toxin are altered, highlighting the importance of the enzymic activity of the toxin in triggering uncontrolled inflammation. In this review, we examine the evidence that sphingomyelinase-like proteins are involved in mechanisms of leptospiral pathogenesis.

Discovery of many leptospiral genes encoding sphingomyelinase-like proteins

Sphingomyelinase activity was first detected in *Leptospira* cultures in the 1960s (Kásarov & Addamiano, 1969), yet cloning of a sphingomyelinase gene was not reported until 1989 (del Real *et al.*, 1989), when a genomic expression library of *Leptospira (Lept.) borgpetersenii* serovar Hardjo was screened for haemolytic activity. Haemolytic and sphingomyelinase activities were expressed from a single gene that was later designated *sphA* (del Real *et al.*, 1989; Segers *et al.*, 1992). The sphingomyelinase encoded by *sphA* shared significant similarity to those found in *S. aureus* and *Bacillus subtilis* (Segers *et al.*, 1990). Multiple sphingomyelinase sequences were detected in pathogenic members of

Leptospira by low stringency Southern hybridization using *Lept. borgpetersenii sphA* as a probe (Segers *et al.*, 1992).

SphH, one of the sphingomyelinase homologues in the genome of serovar Lai, was identified from a genomic library using *sphA* as the probe (Lee *et al.*, 2000). The protein showed 75% similarity to SphA. However, the clone failed to express sphingomyelinase (or phospholipase) activity, although the partially purified recombinant protein lysed sheep erythrocytes (Lee *et al.*, 2000, 2002). The haemolytic activity of SphH was neutralized with rabbit antiserum raised against SphH, eliminating the possibility that haemolysis was due to the cryptic haemolysin of *E. coli*. Transmission electron microscopy of sheep erythrocytes incubated with the SphH preparation revealed pores in the membrane, suggesting that the haemolytic activity of SphH was due to pore-forming ability (Lee *et al.*, 2002). However, another group was unable to confirm the haemolytic activity of a purified preparation of rSphH (Carvalho *et al.*, 2010), possibly due to improper refolding of the insoluble recombinant protein.

Genome sequencing uncovered the multiple sphingomyelinase-like proteins encoded in several pathogenic *Leptospira*. The Lai, Copenhageni, Manilae and Pomona strains each carried genes annotated as *sph1*, *sph2*, *sph3*, *sph4* and *sphH* (Bulach *et al.*, 2006b; Nascimento *et al.*, 2004; Ren *et al.*, 2003) (B. Adler, personal communication). In contrast, the genomes of two *Lept. borgpetersenii* strains harboured only *sphA*, *sphB* and *sph4* (Bulach *et al.*, 2006b). The non-pathogen *Leptospira biflexa* lacks *sph* coding sequences (Picardeau *et al.*, 2008).

Domains of leptospiral sphingomyelinase-like proteins

Multi-sequence alignment of all available leptospiral sphingomyelinase-like sequences reveals the modular nature of the proteins (Fig. 1). In addition to signal sequences, there are N-terminal and C-terminal extensions flanking the central enzymic domain. The region of sequence similarity among the proteins comprises the enzymic domain and C-terminal extensions

Enzymic domain

The crystal structures of the sphingomyelinases of *List. ivanovii* (Openshaw *et al.*, 2005), *B. cereus* (Ago *et al.*, 2006) and *S. aureus* (Huseby *et al.*, 2007) have been determined. These structures revealed the active site configuration of the conserved residues shown to be crucial for sphingomyelinase activity in mutagenesis studies (Huseby *et al.*, 2007; Obama *et al.*, 2003a, b). The active site of *B. cereus* sphingomyelinase contained the divalent metal cation necessary for catalytic activity (Ago *et al.*, 2006). Using the numbering for *B. cereus* sphingomyelinase, essential residues include Glu-53, His-151, Asp-195 and His-296, the metal-binding and catalytic functions of which are shown in Fig. 2(a). Surprisingly, the multi-sequence alignment shows that

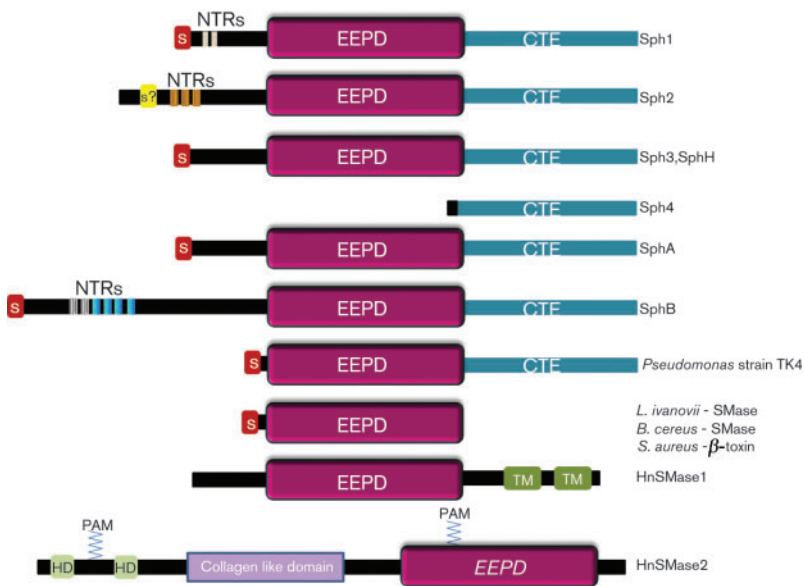


Fig. 1. Schematic representation of leptospiral sphingomyelinase-like proteins. NTRs, N-terminal repetitive sequences; S, signal peptide; EEPD, exo-endo phosphatase domain; CTE, C-terminal extension; TM, transmembrane domain; HD, hydrophobic domain; PAM, palmitate.

only *Lept. borgpetersenii* SphA and *Leptospira interrogans* Sph2 possess these four amino acid residues (Fig. 2b). In contrast, Sph1 and Sph3 of *Lept. interrogans* and SphB of *Lept. borgpetersenii* have non-conservative amino acid substitutions for three or all four of these critical residues. This raises the possibility that these latter Sph proteins are not true sphingomyelinases, despite their overall sequence similarity with other bacterial sphingomyelinases. This observation is consistent with the finding that SphH lacks sphingomyelinase activity (Lee *et al.*, 2002). Although one study reported sphingomyelinase activity for recombinant Sph1, Sph3 and Sph4 expressed in *E. coli* (Zhang *et al.*, 2005), their conclusions are in doubt for several reasons. First of all, Sph4 lacks the entire enzymic domain (Fig. 1) and therefore should not have exhibited any sphingomyelinase activity. Secondly, their results are difficult to interpret because data from the negative control experiment were not presented. Thirdly, the observed reduction of the sphingomyelinase peak as measured by HPLC could have resulted from the activity of *E. coli* lipases in the extract. This is possible because of the high protein concentrations of the crude extracts (100 mg ml^{-1}) in their assays (Zhang *et al.*, 2005). In conclusion, we propose that pathogenic *Leptospira* species have only one true sphingomyelinase (Sph2 or SphA) and that all of the sphingomyelinase-like proteins may possess additional molecular functions.

What, then, could be the additional functions of the 'enzymic' domain of the leptospiral sphingomyelinase-like proteins? Their non-catalytic function may target host sphingomyelin on membrane surfaces for attachment of the protein. For example, the *Helicobacter pylori* toxin VacA uses sphingomyelin as a receptor to enter the target cell (Gupta *et al.*, 2008). The domain may also possess surfaces that bind other host receptors. This is reminiscent of the leptospiral haemolysin-like protein TlyC, which lacks haemolytic activity yet binds to extracellular matrix

proteins fibronectin, collagen IV and laminin (Carvalho *et al.*, 2009). A novel role for sphingomyelinase has been described for the *S. aureus* β -toxin. In the process of biofilm formation, β -toxin covalently interacts with extracellular DNA, forming insoluble nucleoprotein complexes. Biofilm assembly occurred even when the two histidine residues responsible for catalytic activity were altered by mutation, indicating that the residues involved in biofilm formation are distinct from the ones involved in catalysis (Huseby *et al.*, 2010).

The crystal structures of the sphingomyelinases of *B. cereus*, *List. ivanovii* and *S. aureus* revealed a protruding hydrophobic β -hairpin and a second external hydrophobic loop adjacent to the active site. The surface hydrophobic loops may be important in properly positioning the catalytic site in relation to the sphingomyelin substrate in the target membrane. Replacement of the hydrophobic residues in the β -hairpin with alanine in *B. cereus* sphingomyelinase impaired its binding to sphingomyelin liposomes and disrupted its sphingomyelin hydrolytic activity (Ago *et al.*, 2006; Narayanavari *et al.*, 2012). The leptospiral sphingomyelinases lack the hydrophobic β -hairpin (Openshaw *et al.*, 2005). Hence the initial interaction of the leptospiral sphingomyelinase-like proteins with the target membrane may involve sequences located outside of the enzymic domain.

C-terminal extension

The leptospiral sphingomyelinase-like proteins and *Pseudomonas* strain TK4 sphingomyelinase have a carboxy-terminal extension of approximately 186 aa that is missing in the other bacterial sphingomyelinases (Narayanavari *et al.*, 2012; Sueyoshi *et al.*, 2002). The role of the C-terminal extension in the *Pseudomonas* sphingomyelinase has been examined. Deletion of 186 aa from the C-terminal end of

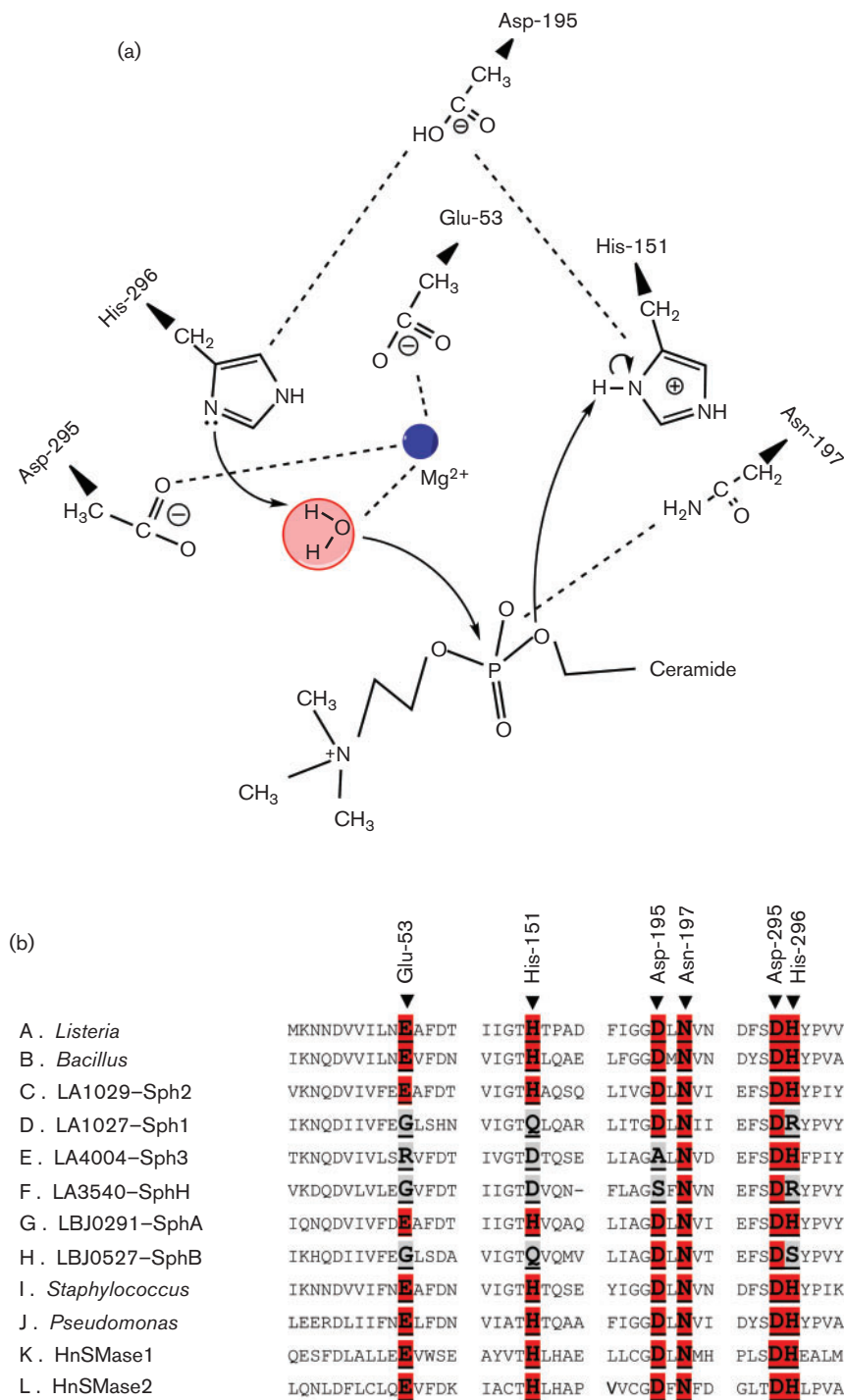


Fig. 2. Catalytic site functions and multi-sequence alignment of the active-site amino acid residues required for sphingomyelinase activity. (a) The proposed function of amino acids at the catalytic site of *B. cereus* sphingomyelinase (adapted from Obama *et al.*, 2003a). Asn-197 interacts with the phosphate group of sphingomyelin, and Glu-53 and Asp-295 coordinate a divalent cation. His-296 and His-151 function as the acid-base catalytic residues; His-296 and the metal ion activate the water molecule that attacks the phosphorus of sphingomyelin, resulting in its hydrolysis to phosphocholine and ceramide. Asp-195 maintains the appropriate spatial arrangement of the catalytic histidine residues. (b) Multi-sequence alignment showing six of the amino acids (highlighted in red) conserved in all members of the extended neutral sphingomyelinase family, including the two human neutral sphingomyelinases. Note that Glu-53, His-151, Asp-195 and/or His-296 are not conserved (highlighted in grey) in Sph1, Sph3, SphH and Sph4.

Pseudomonas sphingomyelinase completely abolished the haemolytic activity without affecting the sphingomyelinase activity, indicating that the C-terminal extension is indispensable for haemolytic activity (Sueyoshi *et al.*, 2002). This observation suggests that the function of the C-terminal extension is to interact with the target host membrane to position the enzymic domain near the sphingomyelin substrate (Sueyoshi *et al.*, 2002).

Export and secretion signals

Sphingomyelinase activity has been detected in the culture fluids of several strains of pathogenic *Leptospira* (Bernheimer & Bey, 1986). The secreted sphingomyelinase is most likely to be SphA or Sph2 because only these enzymes possess the essential catalytic residues. Sph2 has been detected in the culture supernatant with specific antiserum (Carvalho *et al.*, 2010; Matsunaga *et al.*, 2007). However, the mechanism by which Sph2 is secreted is unknown because the protein appears to lack an amino-terminal signal peptide (Fig. 1). In contrast, Sph1, Sph3, SphB and SphH are predicted to have a cleavable amino-terminal signal peptide, suggesting that they are exported out of the cytoplasm to an unknown destination. *Lept. interrogans* also releases sphingomyelinase in membrane vesicles under some culture conditions (Velineni *et al.*, 2009).

Transport of Sph2 and SphA out of the leptospiral cell could involve either the type I or type II secretion pathway (Bulach *et al.*, 2006a). Recently a 63 kDa TolC homologue (LA0957) was immunoprecipitated from an outer membrane preparation of *Lept. interrogans* with antiserum raised against the enzymic domain of Sph3 (Velineni *et al.*, 2009). Although further experimentation is necessary to confirm the association of the proteins, this observation suggests that at least one of the sphingomyelinase-like proteins is secreted via the TolC-based type I secretory pathway (Jenewein *et al.*, 2009). Another TolC homologue (LA3927/LIC13135) was also noted as potentially functioning in sphingomyelinase secretion (Louvel *et al.*, 2006).

N-terminal repeats

Analysis of the sequences attached to the N-termini of the enzymic domain using RADAR (Heger & Holm, 2000) revealed between two and seven short N-terminal imperfect repeats (NTRs) in Sph1, Sph2 and SphB (Table 1). The repeats are enriched in disorder-promoting amino acids (Tompa, 2005). Based on the known functions of intrinsically disordered sequences, the NTRs may harbour proteolytic sites, function as a flexible linker between the signal peptide and the enzymic domain, or bind macromolecules or small ligands (Tompa, 2005).

Phylogenetic analysis of leptospiral sphingomyelinase-like proteins

A phylogenetic tree was constructed from a multi-sequence alignment of the amino acid sequences of the leptospiral

sphingomyelinase-like proteins from four strains of *Lept. interrogans* and two strains of *Lept. borgpetersenii* (Fig. 3). Sph4 was excluded from the analysis because it lacks the enzymic domain. The dendrogram shows that the leptospiral sphingomyelinase-like proteins can be grouped into six clusters. The *Lept. interrogans* and *Lept. borgpetersenii* proteins form separate clusters. The genes encoding Sph1 and Sph2 in *Lept. interrogans* appear to have arisen from a relatively recent duplication event, consistent with *sph1* and *sph2* being located next to each other on the *Lept. interrogans* chromosome. In contrast, only one copy of *sphA* is present in the same genomic position in *Lept. borgpetersenii*.

Expression of leptospiral sphingomyelinase-like proteins during infection

Clear evidence for expression of a sphingomyelinase-like protein during a natural leptospiral infection came from a study of equine leptospirosis. Sera from mares infected with *Lept. interrogans* serovar Pomona strongly recognized recombinant Sph2 protein (Artiushin *et al.*, 2004). A more recent study showed that IgG antibodies present in the sera of leptospirosis patients recognized recombinant Sph2 but not Sph1, Sph4 or SphH (Carvalho *et al.*, 2010). Moreover anti-Sph2 and anti-SphH antisera reacted with renal tubular epithelium of laboratory hamsters infected with *Lept. interrogans*. These results indicate that Sph2 and possibly SphH are expressed during infection.

The expression of *sph2* can be regulated by simulating host-like conditions. Except in several strains of serovar Pomona, Sph2 was not detected by Western blot analysis in *Lept. interrogans* strains cultivated in the standard leptospiral culture medium EMJH (Artiushin *et al.*, 2004; Carvalho *et al.*, 2010; Matsunaga *et al.*, 2007). When sodium chloride or sucrose was added to raise the osmolarity of the culture medium to equal that found in the mammalian host, Sph2 was detected in the *Lept. interrogans* strain Fiocruz L1-130 cell lysates and in a processed form in the culture supernatant fluid, suggesting that the increase in osmolarity experienced by leptospire entering the host triggers *sph2* expression (Matsunaga *et al.*, 2007).

Possible roles of leptospiral sphingomyelinase-like proteins in leptospirosis

A role in nutrient acquisition has been proposed for the leptospiral sphingomyelinases (Bulach *et al.*, 2006a). *Leptospira* depend on β -oxidation of fatty acid to meet their carbon and energy needs *in vitro* (Henneberry & Cox, 1970). Inside the host, cell membranes could provide a rich source of fatty acids as nutrients. However, sphingomyelinase would seem to be an inefficient means for obtaining fatty acid. Since the genomes of pathogenic *Leptospira* do not encode a ceramidase homologue, a host ceramidase would be necessary to release fatty acid molecules from ceramide for utilization by *Leptospira*. *Leptospira* also

Table 1. N-terminal repeats

Protein	Locus tag	Species/serovar	Signal peptide*	No. of repeats	Repetitive sequences†
Sph1	LA1027	<i>List. interrogans</i> serovar Lai	Yes (39–40)	2	60–70 (NVNEKIEDSTN) 76–86 (NVNEEDENSIN)
	LIC12632	<i>List. interrogans</i> serovar Copenhageni	Yes (38–39)	2	59–69 (NVNEKIEDSTN) 75–85 (NVNEEDENSIN)
	LIP0979	<i>List. interrogans</i> serovar Pomona	Yes (38–39)	2	59–69 (NVNEKIEDSTN) 75–85 (NVNEEDENSTN)
	LiL49501006	<i>List. interrogans</i> serovar Manilae	No	2	59–69 (NVNEENENVTN) 75–85 (NVNEKEDENATN)
Sph2	LA1029	<i>List. interrogans</i> serovar Lai	No	3	49–67 (NQVNSVSINNDPANPNPVN) 74–92 (NQVNAVPEPNDPANLNPVN) 99–117 (NQVNAAPENGSPADPNPAN)
	LIC12631	<i>List. interrogans</i> serovar Copenhageni	No	3	49–67 (NQVNSVSINNDPANPNPVN) 74–92 (NQVNAVPEPNDPANLNPVN) 99–117 (NQVNAAPENGSSADPNPAN)
	LIP0980	<i>List. interrogans</i> serovar Pomona	No	4	55–77 (SINNDPANPNPNVPASANNQVN) 80–102 (PENDNPANLNPVNPASANSQVN) 105–127 (PENDNPANLNPVNPASANSQVN) 130–152 (PENGSPDPNPANLASANNQVN)
	LiL49501008	<i>List. interrogans</i> serovar Manilae	No	3	27–48 (DPTNPNPVNPASATSNQVNAV) 52–73 (DPANPNPNVPASANNQVNAV) 77–98 (NPADPNPANSASANNQVNAV)
Sph3	LA4004	<i>List. interrogans</i> serovar Lai	Yes (38–39)	–	–
	LIC13198	<i>List. interrogans</i> serovar Copenhageni	Yes (38–39)	–	–
	LIP0774	<i>List. interrogans</i> serovar Pomona	No	–	–
	LiL49503485	<i>List. interrogans</i> serovar Manilae	No	–	–
SphH	LA3540	<i>List. interrogans</i> serovar Lai	No	–	–
	LIC10657	<i>List. interrogans</i> serovar Copenhageni	No	–	–
	LIP2950	<i>List. interrogans</i> serovar Pomona	Yes (44–45)	–	–
	LiL49503095	<i>List. interrogans</i> serovar Manilae	No	–	–
SphB	LBJ 0527	<i>Lept. borpetersenii</i> serovar Hardjobovis strain JB197	Yes (38–39)	6	71–90 (GYDPISGSPASPTSpAGGPG) 92–110 (DLDPSPNPDANSSS–TNSGS) 112–130 (NSSSTSSGSANSSS–TSSGS) 142–160 (NSSSTSSGSANSSS–TSSGS) 162–180 (NSSSTSSGSANSSS–TSSGS) 182–199 (NSSSTSSGSANSSS–KAPP)
	LBL 2552	<i>Lept. borpetersenii</i> serovar Hardjobovis strain L550	Yes (38–39)	7	79–109 (PASPTSPAgpgpGDLDPSPNPDANSSSTSSG) 110–139 (SANPDTAN–sssTSSGSANPDTANSSSTSSG) 140–169 (SANPDTAN–sssTSSGSANPDTANSSSTNSG) 170–184 (SANPDTAN–SSSTSSG) 185–214 (–ANPDTAN–sssTNSGSANPDTANSSSTSSG) 215–244 (SANPDTA–sssTNSGSANPDTANSSSTSSG) 245–274 (SANPDTA–sssTNSGSANPDTANSSSTSSG)
SphA	LBJ 0291	<i>Lept. borpetersenii</i> serovar Hardjobovis strain JB197	Yes (26–27)	–	–
	LBL 2785	<i>Lept. borpetersenii</i> serovar Hardjobovis strain L550	Yes (26–27)	–	–

*The number in parentheses represents the amino acids flanking the putative signal peptidase cleavage site.

†The number represents the amino acid position in the protein sequence. Lower case characters are used for amino acid residues that are not aligned. Gaps are represented by –.

express phospholipases that yield fatty acid from abundant glycerophospholipids directly, seemingly rendering sphingomyelinases unnecessary for acquisition of fatty acid (Kasárov, 1970).

Cell lysis by sphingomyelinase or the pore-forming activity of SphH may also be important in iron acquisition. Haem released from damaged erythrocytes is a potential source of iron for *Leptospira* during infection. Expression of the

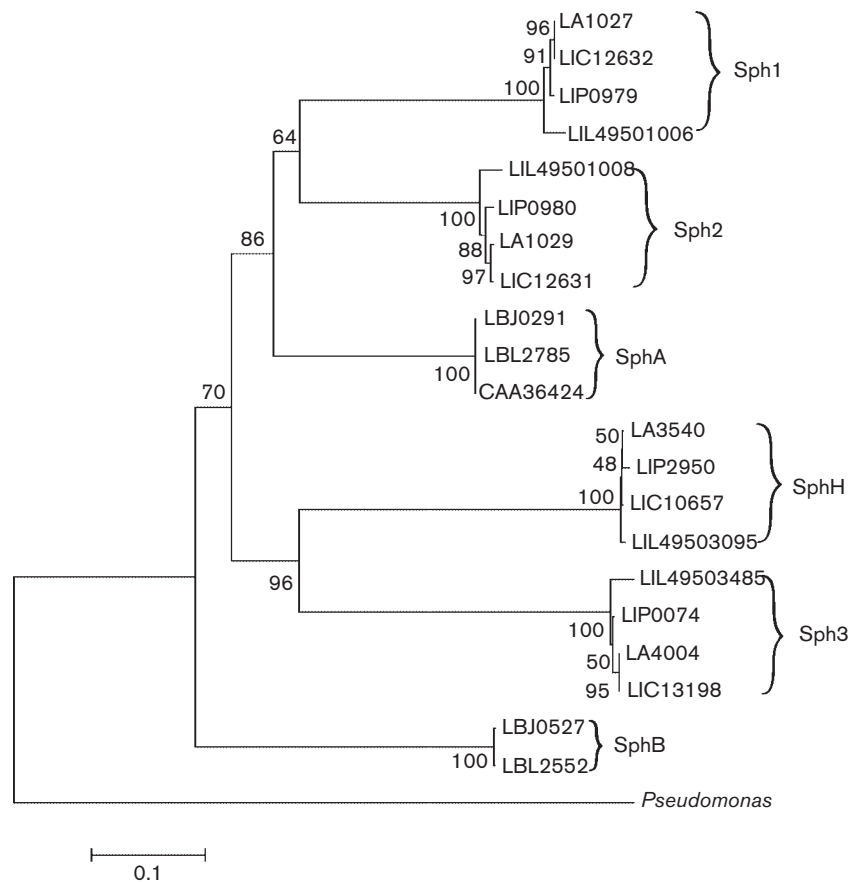


Fig. 3. Phylogenetic analysis of leptospiral sphingomyelinase-like proteins. Multi-sequence alignment was performed using Geneious software utilizing the BLOSUM62 score matrix. The phylogenetic tree was constructed using MEGA tool version 5 utilizing the neighbour-joining method. The robustness of the tree was determined using bootstrapping with 500 replicates. The tree was rooted with the *Pseudomonas* species TK4 sphingomyelinase (GenBank accession no. BAB69072.1).

haemin-binding protein HbpA, identified in *Lept. interrogans* (Sritharan *et al.*, 2005) is induced upon iron limitation and acquires iron from haemin (Asuthkar *et al.*, 2007). Although the expression and release of a 42 kDa sphingomyelinase-like protein in outer membrane vesicles in the presence of the chelator EDDA may support a role for an Sph protein in iron acquisition by *Lept. interrogans* serovar Lai (Velineni *et al.*, 2009), microarray analysis with a strain of serovar Manilae failed to show changes in *sph* transcript levels when iron was depleted with 2,2'-dipyridyl (Lo *et al.*, 2010). The different strains or chelators selected for the studies may account for the discrepancies in the results.

Another case where membrane damage may be critical to leptospiral survival is immune evasion. Although *Leptospira* is primarily an extracellular pathogen, it is able to escape from the phagosome of cultured mouse macrophages (Toma *et al.*, 2011). As observed for several *Listeria* species, escape from the phagocytic vacuole may require the cooperation of lipases and pore-forming activities (González-Zorn *et al.*, 1999; Schnupf & Portnoy, 2007), which may be provided by

the sphingomyelinase activity of Sph2 and the pore-former SphH.

Sphingomyelinases may also have a role in cytotoxicity as part of the pathogenesis of leptospirosis. Recombinant Sph2 was cytotoxic towards mouse lymphocytes and macrophages (Zhang *et al.*, 2008). Some evidence suggests that the immune cells undergo a proinflammatory form of apoptosis when exposed to Sph2 *in vitro* (Zhang *et al.*, 2008). Additionally, damage to the vascular endothelium may be responsible for the haemorrhage observed during severe disease (Carvalho & Bethlem, 2002). Recombinant Sph2 (Lk73.5) from a Pomona strain of *Lept. interrogans* was cytotoxic to equine pulmonary endothelial cells (Artiushin *et al.*, 2004). However, disruption of endothelial cell layer integrity by *Lept. interrogans* crossing the monolayer did not affect the viability of the cells (Martinez-Lopez *et al.*, 2010). Thus, the evidence accumulated to date does not support a cytotoxic role for sphingomyelinases in leptospiral dissemination or haemorrhage.

The true relevance of sphingomyelinase in leptospiral pathogenesis may lie in sublytic effects that do not damage

the host cell membrane. For example, alteration of vascular permeability is caused in part by generation of ceramide by acid sphingomyelinase (Göggel *et al.*, 2004), which may explain the ability of sphingomyelinase-producing *Leptospira* to cross the endothelial layer without cytolytic effects. Excessive ceramide production induced by leptospiral sphingomyelinase could also explain the pulmonary oedema observed in some cases of severe leptospirosis. Alterations of sphingolipid homeostasis and lipid rafts have also been linked to altered renal function (Zager, 2000). The activity of the renal Na^+/H^+ NH_3 transporter, whose levels are diminished in the proximal tubule of severe leptospirosis patients, depends on formation of lipid rafts (Araujo *et al.*, 2010; Murtazina *et al.*, 2006). Finally, the novel non-catalytic role of *S. aureus* sphingomyelinase in biofilm formation described earlier may also be an important function of leptospiral sphingomyelinase-like proteins during infection (Huseby *et al.*, 2010). β -Toxin also promoted biofilm formation *in vivo* in a rabbit model of *S. aureus* endocarditis (Huseby *et al.*, 2010). Pathogenic leptospires have been shown to form biofilms *in vitro* (Ristow *et al.*, 2008), and biofilm formation may be essential for long-term leptospiral survival in the renal tubules of the reservoir host.

The pore-forming activity of SphH may also have profound biological consequences. The pore-forming proteins α -toxin of *S. aureus* and pneumolysin of *Streptococcus pneumoniae* activate the metalloprotease ADAM10, which cleaves E-cadherin, an intercellular protein essential for epithelial barrier function (Inoshima *et al.*, 2011). ADAM10 is required by α -toxin to disturb the alveolar barrier function in the mouse model of pneumonia (Inoshima *et al.*, 2011). These results raise the possibility that SphH promotes the acute lung injury that is observed in many cases of severe leptospirosis.

Conclusion

In this review, we have examined a number of potential roles for sphingomyelinase and its non-enzymic homologues in leptospirosis. In *Lept. interrogans*, only Sph2 retains all of the active-site amino acid residues essential for catalysis. Because the other sphingomyelinase homologues lack at least three of the residues, experimental studies are still needed to settle the fundamental issue of whether Sph1, Sph3 and SphH have sphingomyelinase activity. Irrespective of their catalytic activity, the proteins may dock onto sphingomyelin or some other host molecule as a prelude to performing their effector function, which may include the type of pore-forming activity described for SphH. Even in the case of Sph2, sphingomyelin hydrolysis is likely to be relevant to pathogenesis in ways that go beyond mere host cell membrane damage. Previous studies that addressed the biological functions of leptospiral Sph2 have focused on its cytotoxic potential. However, disruption of sphingolipid homeostasis by leptospiral sphingomyelinase activity also has the potential

to alter cellular functions in ways that do not necessarily kill the host cell. Future studies should therefore also seek non-cytotoxic effects of Sph2 on host cells. We hope that by broadening our view of the potential biological activities of the Sph proteins, we can acquire the evidence we need to truly understand the role of leptospiral sphingomyelinases and sphingomyelinase-like proteins in leptospiral pathogenesis.

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