 DOI 10.1099/mic.0.2007/007948-0

Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of *Leptospira interrogans*: differential effects on LigA and Sph2 extracellular release

James Matsunaga,[1,2] Marco A. Medeiros,[3] Yolanda Sanchez,[1]† Kristian F. Werneid[1] and Albert I. Ko[4,5]

[1]VA Greater Los Angeles Healthcare System, Research Service, Los Angeles, CA 90073, USA

[2]UCLA David Geffen School of Medicine, Los Angeles, CA 90095, USA

[3]Biomanguinhos, Oswaldo Cruz Foundation, Brazilian Ministry of Health, Rio de Janeiro, Brazil

[4]Division of International Medicine and Infectious Diseases, Weill Medical College of Cornell University, New York, NY 10021, USA

[5]Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Brazilian Ministry of Health, Salvador, Brazil

Correspondence
James Matsunaga
jamesm@ucla.edu

The life cycle of the pathogen *Leptospira interrogans* involves stages outside and inside the host. Entry of *L. interrogans* from moist environments into the host is likely to be accompanied by the induction of genes encoding virulence determinants and the concomitant repression of genes encoding products required for survival outside of the host. The expression of the adhesin LigA, the haemolysin Sph2 (Lk73.5) and the outer-membrane lipoprotein LipL36 of pathogenic *Leptospira* species have been reported to be regulated by mammalian host signals. A previous study demonstrated that raising the osmolarity of the leptospiral growth medium to physiological levels encountered in the host by addition of various salts enhanced the levels of cell-associated LigA and LigB and extracellular LigA. In this study, we systematically examined the effects of osmotic upshift with ionic and non-ionic solutes on expression of the known mammalian host-regulated leptospiral genes. The levels of cell-associated LigA, LigB and Sph2 increased at physiological osmolarity, whereas LipL36 levels decreased, corresponding to changes in specific transcript levels. These changes in expression occurred irrespective of whether sodium chloride or sucrose was used as the solute. The increase of cellular LigA, LigB and Sph2 protein levels occurred within hours of adding sodium chloride. Extracellular Sph2 levels increased when either sodium chloride or sucrose was added to achieve physiological osmolarity. In contrast, enhanced levels of extracellular LigA were observed only with an increase in ionic strength. These results indicate that the mechanisms for release of LigA and Sph2 differ during host infection. Thus, osmolarity not only affects leptospiral gene expression by affecting transcript levels of putative virulence determinants but also affects the release of such proteins into the surroundings.

Received 8 March 2007
Revised 30 May 2007
Accepted 6 June 2007

INTRODUCTION

Spirochaetes of the genus *Leptospira* are a major cause of morbidity and mortality in developing nations (McBride *et al.*, 2005). *Leptospira interrogans* is one of 14 genomospecies of pathogenic *Leptospira* and is the cause of endemic disease and large outbreaks of leptospirosis worldwide (Brenner *et al.*, 1999; Levett *et al.*, 2006). Outbreaks involving *L. interrogans* have been associated with flooding in rat-infested urban slums (Ko *et al.*, 1999). *L. interrogans* resides chronically in the lumen of rat kidney tubules and is released within urine into the environment, where it can survive for weeks (Crawford *et al.*, 1971; Gordon Smith & Turner, 1961; Ryu & Liu, 1966). Humans exposed to *Leptospira* in the environment can be infected through breaks in skin or mucous membranes. After breaching the epithelium or mucosa, leptospires migrate to the bloodstream and disseminate to numerous organs. Flu-like symptoms including fever and myalgia characterize early acute-phase infection. Severe symptoms including

Abbreviation: ECM, extracellular matrix.

†Present address: UC Davis School of Veterinary Medicine, Davis, CA 95616, USA.

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jaundice, renal failure and pulmonary haemorrhage are subsequently observed in 5–10 % of cases (Bharti *et al.*, 2003; McBride *et al.*, 2005).

Survival of *L. interrogans* inside and outside of the host is likely to require the expression of different sets of gene products. Little is known about which environmental cues signal *L. interrogans* to modify its gene expression patterns during infection. At least four components of pathogenic *Leptospira* have been shown to be differentially expressed when leptospires are transferred from culture medium into a host animal: LigA, Sph2 (Lk73.5), LipL36 and lipopolysaccharide (LPS) O antigen. The extracellular matrix (ECM)-binding proteins LigA and LigB, the haemolysin Sph2, and LPS are potential virulence determinants (Artiushin *et al.*, 2004; Matsunaga *et al.*, 2003; Nally *et al.*, 2005; Choy *et al.*, 2007). Initial studies of LigA and Sph2 failed to detect either protein in *L. interrogans* lysates (Artiushin *et al.*, 2004; Palaniappan *et al.*, 2002), although later studies revealed low levels of both in lysates and culture supernatant fluids (Matsunaga *et al.*, 2005; Zhang *et al.*, 2005). However, infected humans and horses, respectively, produced antibody to these proteins, indicating that expression of these proteins occurs in the host. Additionally, LigA was detected in kidneys of hamsters infected with *L. interrogans* but not in cultured leptospires (Palaniappan *et al.*, 2002). In contrast, LipL36, an abundant outer-membrane lipoprotein in *in vitro*-cultured leptospires, was not detected in leptospires residing in the kidney tubule of an experimentally infected hamster (Haake *et al.*, 1998). Similarly, *L. interrogans* isolated from the liver of an acutely infected guinea pig did not contain detectable levels of O antigen, which is found on the outer surface of cultivated leptospires (Nally *et al.*, 2005).

The lack of efficient tools for genetic manipulaton of pathogenic *Leptospira* and intrinsic difficulties in characterizing leptospiral gene expression during infection of experimental animals have impeded elucidation of the role of host-adapted leptospiral determinants in virulence. Furthermore, little progress has been made in identifying culture conditions that reproduce the changes in leptospiral gene expression observed during infection. The diminished LipL36 levels observed *in vitro* by temperature upshift and iron limitation may explain the inability to detect LipL36 in the kidneys of infected hamsters (Cullen *et al.*, 2002; Haake *et al.*, 1998; Nally *et al.*, 2001b). Recent microarray and proteomic experiments have revealed changes in the levels of numerous transcripts and several outer-membrane proteins in response to different culture conditions (Cullen *et al.*, 2002; Lo *et al.*, 2006; Nally *et al.*, 2001b; Qin *et al.*, 2006). However, whether these genes behave similarly during infection of a mammalian host is unknown.

Leptospira outside of the host is typically found in environments such as fresh water or moist soil, where osmolarity is lower than that found within mammals (Kratz *et al.*, 2004; Miller & Wood, 1996). We recently demonstrated that levels of cellular LigA and LigB and extracellular LigA are increased when the ionic strength of leptospiral culture medium is increased to achieve the osmolarity found in the host (Matsunaga *et al.*, 2005). The change in osmolarity that occurs as *L. interrogans* enters the host from a freshwater environment may be a signal to the leptospires to increase expression of *ligA*, *ligB*, and other putative virulence genes and reduce expression of gene products necessary for survival outside of the host. In this study, we systematically examined expression of leptospiral genes known to be regulated by mammalian host signals. We found that osmolarity, irrespective of whether ionic or non-ionic solutes were used, influenced cellular levels of LigA, LigB, Sph2 and LipL36. In contrast, the amount of LigA and Sph2 detected in culture supernatant fluids was differentially affected by the method used to raise the osmolarity. Thus, osmolarity can affect expression of leptospiral genes at different steps in gene expression.

METHODS

Bacterial strain and cultivation. *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 is a clinical isolate obtained during an outbreak of leptospirosis in Salvador, Brazil (Ko *et al.*, 1999; Nascimento *et al.*, 2004a). Virulence was maintained by infection and reisolation of the strain from Golden Syrian hamsters. *L. interrogans* was maintained in EMJH medium supplemented with 1 % rabbit serum and 100 μg 5-fluorouracil ml^{-1} at 30 °C (Ellis & Thiermann, 1986). Cell density was determined by direct counts of motile bacteria by darkfield microscopy. Unless indicated otherwise, all experiments were performed with *L. interrogans* grown to 2–8×10^{8} ml^{-1}.

Plasmid DNA. The portion of the *sph2* gene encoding codons 27 through 190 was cloned into an expression vector as follows. The forward primer 5′-CACCGAAAAAGAATCCTCATATAAGGATT-TATTTACTTCG-3′ and the reverse primer 5′-TCATACGTAATC-TGATTTTGAAATTCGTTTTGC-3′ were used to amplify the *sph2* gene fragment from *L. interrogans* genomic DNA by PCR with *Pfu* DNA polymerase (Finnzymes). The PCR product was inserted directionally into pET151/D-TOPO (Invitrogen) to generate the plasmid pTOPO-Sph2(27-190). Similarly, the portion of *sph2* corresponding to codon 27 through the stop codon was cloned into pET151/D-TOPO to generate pTOPO-Sph2(27-623). The sequence of the reverse primer used with the forward primer described above was 5′-TTAGCGATAAATAAGATCCGCACTCCA-3′.

Antisera. Lig, LipL36, and LipL41 rabbit antisera have been described (Haake *et al.*, 1998; Matsunaga *et al.*, 2003; Shang *et al.*, 1996). Sph2 rabbit antiserum was raised as follows. Plasmid pTOPO-Sph2(27-190) was transformed into *Escherichia coli* BLR(DE3)/pLysS (EMD Biosciences), and expression of the His_6-Sph2(27-190) protein was induced with 0.5 mM IPTG. Purification of the recombinant protein and immunization of New Zealand rabbits were performed as previously described (Matsunaga *et al.*, 2005). The immunization protocol was approved by the VA Greater Los Angeles Institutional Animal Care and Use Committee.

Immunoblot analysis. Culture supernatant fluid was collected following centrifugation of 5×10^{8}–1×10^{9} leptospires for 4 min at 9000 ***g*** in a Beckman Coulter Microfuge 18 centrifuge. Leptospiral proteins were isolated from the culture supernatant by immunoprecipitation

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with specific rabbit antisera, as described previously (Matsunaga *et al.*, 2005). Cell pellets and immunoprecipitated protein were subjected to immunoblot analysis as described (Matsunaga *et al.*, 2005). Lig, Sph2, LipL41 and LipL36 antisera were used at titres of 1 : 2000, 1 : 1000, 1 : 10 000 and 1 : 1000, respectively.

RNA extraction. *L. interrogans* cultures were chilled in a dry ice-ethanol bath and centrifuged at 10 000 r.p.m. for 15 min in a Sorvall SS34 rotor. RNA was extracted from the bacteria with TRIzol reagent (Invitrogen) and treated with 2 U Turbo-DNase (Ambion) in a final volume of 100 μl for 30 min at 37 °C, as directed by the manufacturer. An additional 2 U Turbo-DNase was then added, and incubation was continued for another 30 min. DNase was removed by phenol/chloroform extraction followed by ethanol precipitation.

RT-PCR. Two micrograms of leptospiral RNA was hybridized to random nonamer primers (Sigma) and cDNA was synthesized with Omniscript reverse transcriptase, as specified by the manufacturer (Qiagen). The cDNA was amplified with *Taq* DNA polymerase (Qiagen) with the gene-specific primer pairs shown in Table 1. Primers were designed with Primer Premier 5 (Premier Biosoft International). PCR reactions were examined by electrophoresis in 1.5 % agarose gels. The 1 kb DNA ladder was obtained from Gene Choice and Promega.

RESULTS

Incubation of leptospires at physiological osmolarities was found to increase cell-bound levels of LigA and LigB, irrespective of whether ionic (NaCl) or non-ionic (sucrose, glucose) solutes were used to supplement the culture media (Fig. 1). Western blot analysis of *L. interrogans* grown overnight in EMJH supplemented with 120 mM sodium chloride demonstrated an increase in LigA and LigB (Fig. 1, lane 1 vs 3) above levels observed in *L. interrogans* grown in EMJH, as demonstrated previously (Matsunaga *et al.*, 2005). Similarly, when *L. interrogans* was incubated in EMJH with 240 mM glucose or sucrose to attain physiological osmolarity, LigA and LigB levels increased (Fig. 1, lanes 5 and 7), demonstrating for the first time that Lig protein levels respond to the osmolarity and not the ionic strength of the environment.

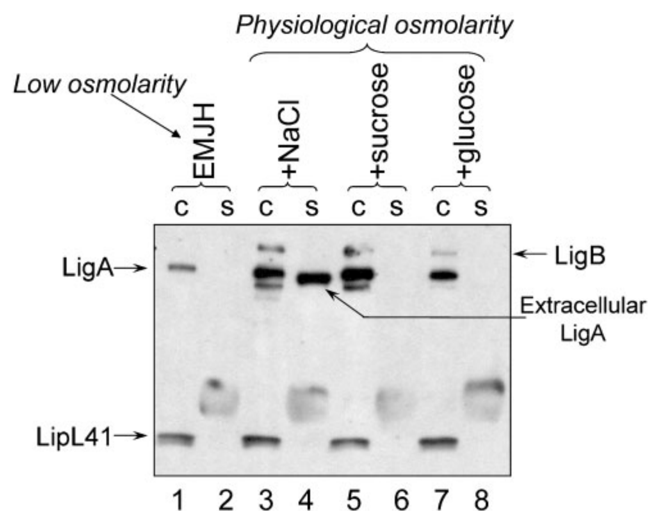

Fig. 1. Effect of osmolarity on Lig protein levels. *L. interrogans* was grown for 20 h in EMJH (lanes 1, 2) or in EMJH supplemented with 120 mM NaCl (lanes 3, 4), 240 mM sucrose (lanes 5, 6), or 240 mM glucose (lanes 7, 8). Cell lysates and culture supernatant were analysed by Western blotting with Lig and LipL41 antisera. c, bacterial cells; s, culture supernatant.

In contrast, levels of extracellular LigA significantly increased in response to sodium chloride (Fig. 1, lane 4) but not to glucose or sucrose (Fig. 1, lanes 6 and 8). LigA was immunoprecipitated with Lig antiserum prior to Western blot analysis to avoid distortion of the gel caused by the high concentration of albumin in EMJH. A control experiment was performed to rule out the possibility that glucose and sucrose inhibited immunoprecipitation of LigA. *L. interrogans* was incubated overnight in EMJH with 120 mM sodium chloride to provide a source of LigA. Culture supernatants were dialysed against EMJH and supplemented with sucrose or glucose at a final concentration of 240 mM. Sucrose and glucose had no effect on the ability to immunoprecipitate LigA (data not shown),

Table 1. Oligonucleotides used for RT-PCR

Gene	Primer name	Orientation	Nucleotide sequence (5′–3′)	Amplicon size (bp)
ligA	ligA-19F	forward	ATTACAGAGCAAGTCACCTGGAAG	399
	ligA-20R	reverse	TACATTTCCCAATGTCGCTTTAAT	
ligB	ligB-17F	forward	ATCCGAAGTGGCATAACTCTCCTCAT	440
	ligB-10R	reverse	ATTTTCAAGATTTGTTCTCCAGATTT	
sph2	lic12631-3F	forward	GGTTTCTAAACAAACTTGGACCGTAT	400
	lic12631-4R	reverse	TTGATTGGAAGCGTCATTAAACTTAG	
lipL36	lipL36-1F	forward	TGCTGCAACGACACTTAAAGTACA	403
	lipL36-2R	reverse	TGCTAAAGCTGCAGATTGAATAGC	
lipL41	lipL41-5F	forward	TCGGTGAAGGTTCCAGTTTTATTGAT	404
	lipL41-6R	reverse	TACTTCTCCGGTTTCTACTTTGATGA	

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indicating that the reduction of extracellular LigA signal was not due to the inability to immunoprecipitate the protein in the presence of non-ionic solutes. LipL41 levels were not affected by osmolarity, as demonstrated previously (Matsunaga *et al.*, 2005).

Another leptospiral gene induced by host signals is *sph2*, which encodes a sphingomyelinase (Artiushin *et al.*, 2004; Zhang *et al.*, 2005). To determine whether *sph2* could be induced by osmolarity, RT-PCR was performed to examine *sph2* transcript levels in *L. interrogans* grown overnight in EMJH and in EMJH supplemented with 100 mM sodium chloride or 200 mM sucrose. The *sph2* mRNA was not detected in *L. interrogans* grown in EMJH (Fig. 2, lane 7). However, *sph2* mRNA was detected in *L. interrogans* grown in salt- or sucrose-supplemented EMJH (Fig. 2, lanes 8 and 9). Transcripts for *ligA* and *ligB* also increased when EMJH was supplemented with sodium chloride or sucrose (Fig. 2, lanes 1 vs 2 and 3, 4 vs 5 and 6). The control *lipL41* transcript was detected under all growth conditions (Fig. 2, lanes 10–12). When reverse transcriptase was omitted from the reaction, PCR products were not detected, demonstrating adequate digestion of genomic DNA (data not shown).

To determine whether expression of *sph2* at the protein level was also induced by ionic and non-ionic solutes at physiological osmolarities, rabbit antiserum was raised against recombinant Sph2. To reduce cross-reactivity with the four additional sphingomyelinase-like proteins encoded by *L. interrogans*, the recombinant protein contained only amino acid residues 27 through 190 of Sph2, a region where sequence similarities to the other four leptospiral sphingomyelinase sequences are minimal (Zhang *et al.*, 2005). The antiserum strongly recognized full-length recombinant Sph2 protein, which has an apparent molecular mass of 89 kDa, larger than the calculated molecular mass of 71.7 kDa (Fig. 3a, lane 4). However, immunoblots of *L. interrogans* grown overnight in EMJH probed with Sph2 antiserum revealed a much smaller species of 63 kDa (Fig. 3a, lane 1). On the other hand, when the bacteria were incubated in EMJH supplemented with sodium chloride or sucrose, a band that co-migrated with the recombinant Sph2 protein appeared (Fig. 3a, lanes 2 and 3). The 63 kDa band is likely to be one of the other sphingomyelinase-like proteins expressed by *L. interrogans* (Zhang *et al.*, 2005). There was no effect on LipL41 levels, as expected (Fig. 3b, lanes 1–3).

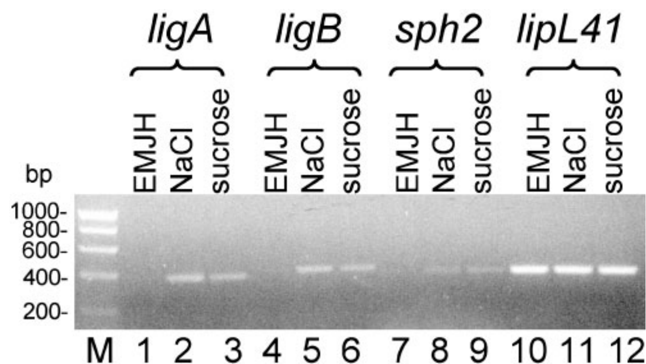

Fig. 2. Induction of *lig* and *sph2* transcripts by osmotic upshift. *L. interrogans* was grown for 22 h in EMJH (lanes 1, 4, 7 and 10), EMJH supplemented with 100 mM NaCl (lanes 2, 5, 8 and 11), and EMJH with 200 mM sucrose (lanes 3, 6, 9 and 12). RNA was extracted and *ligA* (lanes 1–3), *ligB* (lanes 4–6), *sph2* (lanes 7–9) and *lipL41* transcripts were analysed by RT-PCR. Twenty-eight cycles of PCR were performed on 50 ng total RNA. The results are representative of four independent experiments.

Culture supernatant fluid from *L. interrogans* grown in EMJH and EMJH with 100 mM sodium chloride was probed in immunoblots with Sph2 antiserum. Although a reactive band was not observed at the expected size for full-length Sph2 in leptospires cultured in EMJH, two faint bands were found with apparent molecular masses of 68 kDa and 76 kDa (Fig. 3c, lane 1). These bands appear to represent a processed version of the 89 kDa cell-associated Sph2 since the 63 kDa band detected in cell lysates was too small to be a precursor of the two bands. The level of expression of these species was significantly increased in supernatants from leptospires treated with sodium chloride (Fig. 3c, lane 2). Culture supernatant from *L. interrogans* grown in EMJH with sucrose was also examined for induction of extracellular Sph2. In contrast to the minimal change observed with LigA (Fig. 3d, lane 1 vs 3), sucrose treatment induced the release of extracellular Sph2 at similar levels to that observed in supernatants from salt-treated leptospires (Fig. 3c, lane 3). LipL41 was not detected in the culture supernatant, demonstrating that harvesting the bacteria was not accompanied by cell lysis (Fig. 3d, lanes 4–6).

The kinetics of induction of Lig and Sph2 protein expression by sodium chloride was examined. A culture of *L. interrogans* growing in EMJH was transferred to EMJH supplemented with 100 mM sodium chloride at time zero. Cells and culture supernatant were collected 1, 2, 4, 6 and 23 h after osmotic upshift. An increase in LigA, LigB and Sph2 was detected 1 h following osmotic upshift (Fig. 4a and b, lane 1 vs 2). Maximum levels of LigA and LigB were reached after 6 h (Fig. 4a, lane 5). Sph2 levels also increased over 6 h but diminished by 23 h (Fig. 4b, lane 6). There was no effect of sodium chloride on LipL41 levels or the 63 kDa band that cross-reacted with the Sph2 antiserum. Culture supernatant fluid collected at each time point was also examined for LigA and Sph2 signal by immunoprecipitation and subsequent Western blot analysis. Both signals exhibited a gradual increase over several hours following osmotic upshift. A separate experiment demonstrated that Sph2 levels were regulated by growth phase in EMJH with 80 mM sodium chloride (Fig. 4c), which may explain the diminished levels of Sph2 at 23 h in Fig. 4(b).

The outer-membrane lipoprotein LipL36 was examined since its expression is downregulated in the mammalian

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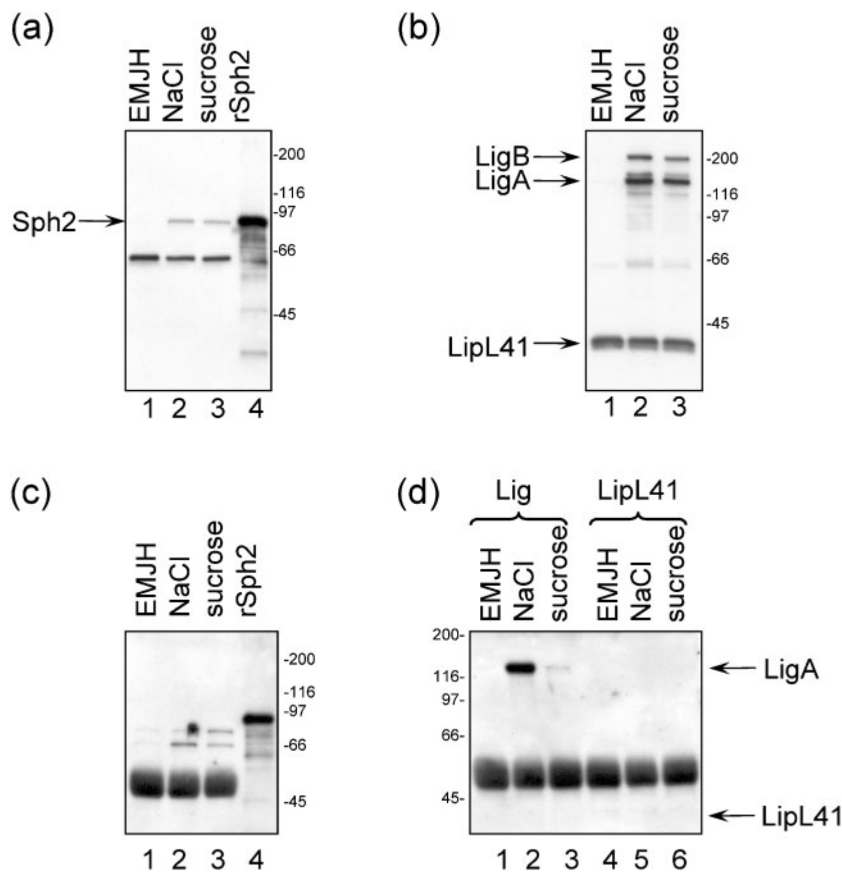

Fig. 3. Effect of osmotic upshift on Sph2 levels. *L. interrogans* was incubated for 22 h in EMJH (lanes 1), EMJH with 100 mM NaCl (lanes 2), or EMJH with 200 mM sucrose (lanes 3). Cell lysates were probed in Western blots with Sph2 (a) or Lig and LipL41 antisera (b). Sph2 (c), LigA and LipL41 (d) were immunoprecipitated from the culture supernatants and analysed by Western blotting. Lane 4 in (a) and (c) was loaded with 1 ng purified His_6-Sph2 recombinant protein (rSph2).

host (Haake *et al.*, 1998). Five *L. interrogans* cultures having different starting cell densities were initiated in EMJH containing 80 mM sodium chloride and allowed to reach a density of $4–5 \times 10^8$ ml^{-1} following 4, 24, 48, 96 and 144 h of incubation. Under these conditions, a gradual decrease in LipL36 levels was observed, whereas LipL41 levels were unaffected (Fig. 5a). In a separate experiment, when *L. interrogans* was allowed to double twice in EMJH containing 100 mM sodium chloride or 200 mM sucrose, LipL36 levels dropped compared to that in leptospires incubated in EMJH alone (Fig. 5b, lane 1 vs 2 and 3). Levels of the abundant outer-membrane proteins LipL21 (Cullen *et al.*, 2003), LipL32 (Haake *et al.*, 2000) and LipL46 (Matsunaga *et al.*, 2006) were not affected by the increase in osmolarity (data not shown).

Levels of *lipL36* transcript in *L. interrogans* grown overnight at low and high osmolarities were assessed by RT-PCR. Osmotic upshift with sodium chloride and sucrose caused diminished *lipL36* transcript levels (Fig. 6, lanes 1–3). As expected, no effect of osmolarity on *lipL41* transcript levels was detected (Fig. 6, lanes 4–6).

DISCUSSION

In this study, we demonstrated that increasing the osmolarity from the low level found in leptospiral culture medium to near the physiological levels found in the mammalian host resulted in significant changes in expression not only of the two ECM-binding Lig proteins but also of the haemolysin Sph2 and the outer-membrane lipoprotein LipL36. Expression of these proteins is regulated by mammalian host signals (Artiushin *et al.*, 2004; Haake *et al.*, 1998; Matsunaga *et al.*, 2003; Palaniappan *et al.*, 2002), suggesting that *L. interrogans* senses osmolarity to regulate expression of *ligA*, *ligB*, *sph2* and *lipL36* during transition from a freshwater environment into a host. Both sodium chloride and sucrose regulate expression of these genes, indicating that the true signal sensed by *L. interrogans* is osmolarity, not ionic strength. Such a mode of gene regulation is ideal for a pathogen whose life cycle involves environments with considerable osmotic differences. To our knowledge, osmotically regulated genes have not been identified in other spirochaetes. We have recently used whole-genome microarrays to identify additional leptospiral genes induced by an upshift to physiological osmolarity (Matsunaga *et al.*, 2007).

In our time-course study, we demonstrated the accumulation of the Lig and Sph2 proteins within hours following osmotic upshift (Fig. 4). Rapid accumulation of Lig proteins is consistent with the detection of IgM antibody against Lig during the acute stage of leptospirosis in 92 %

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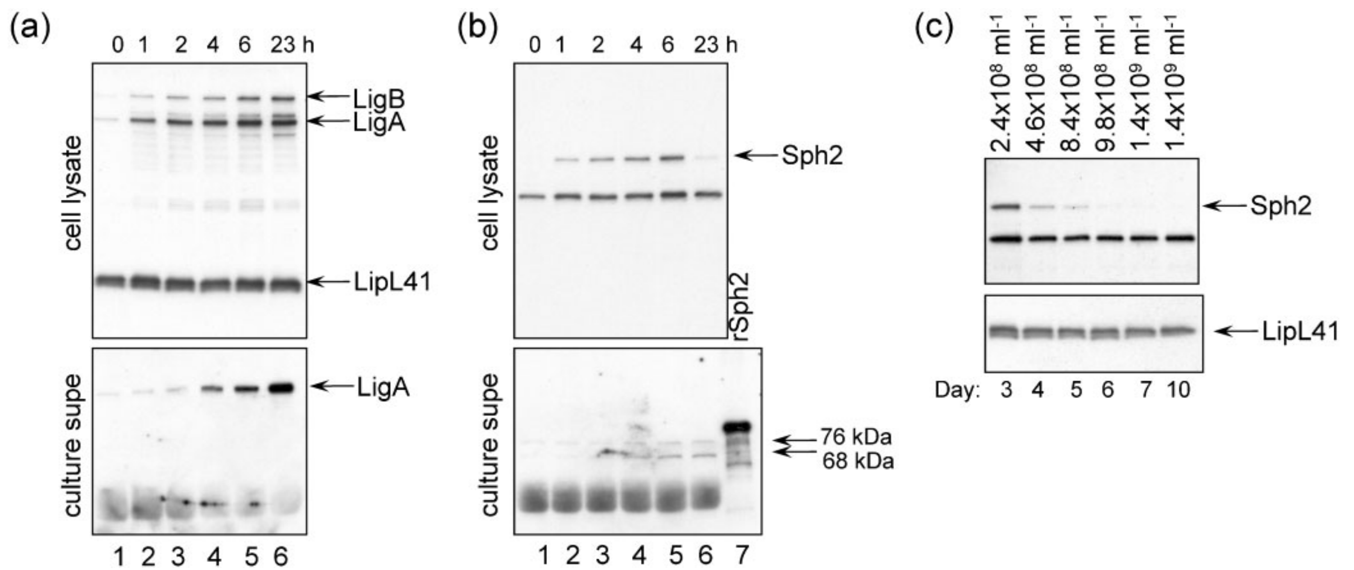

Fig. 4. Kinetics of Lig and Sph2 induction following salt upshift. (a, b) At time 0, *L. interrogans* was placed in EMJH containing 100 mM NaCl at a starting density of 4×10^{8} ml^{-1}. Culture samples were collected at 1, 2, 4, 6 and 23 h. Cell lysates were analysed by Western blotting with Lig and LipL41 antisera (a, top panel) or with Sph2 antiserum (b, top panel). LigA and Sph2 were obtained from the culture supernatant by immunoprecipitation and analysed by Western blotting with Lig antiserum (a and b, bottom panels). The apparent molecular masses of Sph2-derived extracellular species are shown in (b). (c) A culture of *L. interrogans* was initiated in EMJH supplemented with 80 mM NaCl at a density of 2×10^{8} ml^{-1} on day 0. Samples of the culture were collected on the days indicated below each lane for Western blot analysis with Sph2 and LipL41 antisera. Culture densities on the days bacteria were collected are shown above each lane.

of patients (Croda *et al.*, 2007). Previous studies have examined the effects of environmental changes on leptospiral gene expression at longer time points following shift in culture conditions (Cullen *et al.*, 2002; Lo *et al.*, 2006; Matsunaga *et al.*, 2005; Nally *et al.*, 2001a, b; Qin *et al.*, 2006). Our results suggest that rapid accumulation of the putative virulence determinants LigA, LigB and Sph2 may be involved in the initial stages of host infection.

Similar to the results of Artiushin *et al.*, (2004), we were unable to detect Sph2 by immunoblot analysis of *L. interrogans* grown in EMJH (Fig. 3a). The requirement for growth of *L. interrogans* at physiological osmolarity for *sph2* induction is a likely explanation of the inability of Artiushin *et al.* to detect the sphingomyelinase. In contrast, Zhang *et al.* (2005) detected Sph2 in *L. interrogans* lysates. It is possible that the 10 % rabbit serum in the Korthof

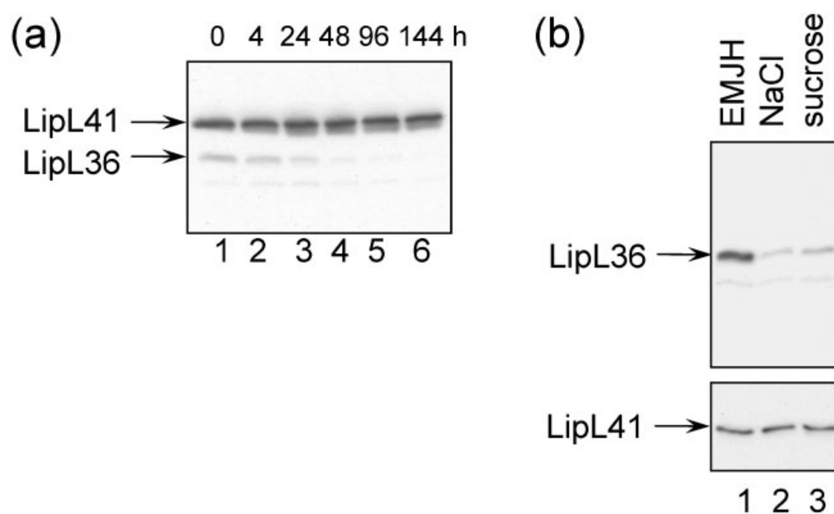

Fig. 5. Effect of osmotic upshift on LipL36 levels. (a) Five cultures of *L. interrogans* were incubated in EMJH with 80 mM NaCl for the time indicated. All cultures were grown to a density of 4–5×10^{8} ml^{-1}. Starting culture densities were adjusted to account for the incubation time. LipL36 and LipL41 levels were examined by immunoblot analysis. (b) *L. interrogans* was incubated in EMJH (lane 1), EMJH supplemented with 100 mM NaCl (lane 2), or EMJH with 200 mM sucrose (lane 3) for 4 days. Cell lysates were probed with LipL36 and LipL41 antisera. Lysates were diluted 10-fold for detection of LipL41.

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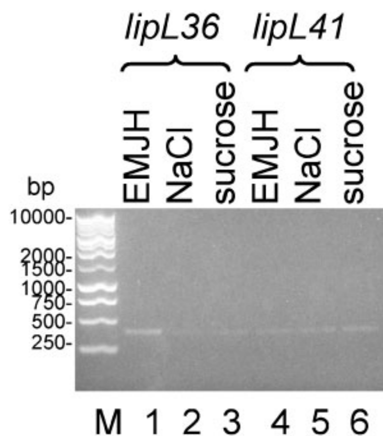

Fig. 6. Reduction of *lipL36* transcript levels following osmotic upshift. *L. interrogans* was incubated for 22 h in EMJH (lanes 1 and 4), EMJH supplemented with 100 mM NaCl (lanes 2 and 5) and EMJH with 200 mM sucrose (lanes 3 and 6). RNA was extracted for RT-PCR analysis of *lipL36* (lanes 1–3) and *lipL41* (lanes 4–6). Twenty cycles of PCR were performed on 4 ng total RNA. The results are representative of three independent experiments.

medium used by Zhang et al. (2005) to generate some of their data increased the osmolarity of the medium sufficiently to raise Sph2 to detectable levels. The cross-reacting 63 kDa band (Fig. 3a) could be SphH, which retains sequence similarity with the Sph2 fragment used to generate the antiserum. In addition, microarray analysis showed that *sphH* transcripts exhibited by far the strongest signal among the sphingomyelinase-like genes in *L. interrogans* grown in EMJH, again consistent with SphH being the 63 kDa species (Matsunaga *et al.*, 2007). Despite its extensive sequence similarity to Sph2, SphH lacks sphingomyelinase activity and instead forms pores in the mammalian cytoplasmic membrane (Lee *et al.*, 2000, 2002).

Earlier studies revealed leptospiral proteins in the culture supernatant, including LigA, sphingomyelinase and Hap1 (LipL32) (Branger *et al.*, 2005; Matsunaga *et al.*, 2005; Zhang *et al.*, 2005). Several of these extracellular proteins elicited a humoral immune response during infection (Zuerner *et al.*, 1991). The osmotically regulated 68 and 76 kDa bands detected in culture supernatant fluids with Sph2 antiserum (Fig. 3) are too large to be derived from the constitutively expressed 63 kDa cell-associated band that cross-reacted with the antiserum. Further, RT-PCR (data not shown) and microarray analysis demonstrated that among the five genes encoding sphingomyelinase-like proteins, only transcript from *sph2* was regulated by salt (Matsunaga *et al.*, 2007). Thus, the 68 and 76 kDa bands most likely derive from Sph2.

Among the five sphingomyelinase-like genes, only *sph2* transcript levels are affected by salt (Matsunaga *et al.*, 2007), suggesting that the genes are regulated by different environmental signals. The different sphingomyelinases may perform distinct functions and exhibit tissue-specific expression during infection. For example, the osmotic induction of *sph2* may have a role in protecting *L. interrogans* from osmotic upshifts, since choline, a potential byproduct of phosphocholine produced by sphingomyelinase activity, is converted by many bacteria into the osmoprotectant glycine betaine (Shortridge *et al.*, 1992). Although a gene encoding the choline transporter has not been found in the *L. interrogans* genome, genes encoding homologues of enzymes that convert choline to glycine betaine are present (Nascimento *et al.*, 2004a; Ren *et al.*, 2003).

L. interrogans harbours numerous genes encoding homologues of signal transduction proteins, including 48 sensors and 38 response regulators that are members of two-component regulatory systems, 12 membrane-bound adenylate cyclases, and 11 extracytoplasmic sigma factors (Galperin, 2005, 2006; Nascimento *et al.*, 2004b). As shown in other bacteria, some of these signalling proteins may be involved in transducing signals to the genetic regulatory apparatus following osmotic upshift (Heusipp *et al.*, 2003; Jubelin *et al.*, 2005; Kimura *et al.*, 2002, 2005). Other than the *kdpDE* genes encoding the two-component regulatory system that regulates expression of a high-affinity potassium transporter, *L. interrogans* lacks obvious homologues of osmotically controlled signal transduction proteins found in other bacteria (Nascimento *et al.*, 2004a; Ren *et al.*, 2003).

The post-transcriptional effect of osmolarity is a novel finding among spirochaetes. Enhanced LigA release occurred when the osmolarity was increased with sodium chloride but not with non-ionic solutes (Fig. 1). Because most of the osmolarity of tissue fluids is accounted for by sodium chloride, the results observed with sodium chloride are more relevant to host infection. Release of LigA at physiological osmolarity was not due to general proteolytic release of surface proteins, as LigB and LipL41 were not released. We therefore hypothesize that large amounts of LigA are released during infection. It is possible that ionic strength affects the conformation of LigA, a putative LigA protease, or both, permitting cleavage when the salt concentration is high. The failure to observe an increase of LigA levels following addition of non-ionic solutes raises the possibility that LigA release is regulated by unknown factors in the variety of niches found in the host. For example, protease inhibitors, which are abundant in human plasma (Travis & Salvesen, 1983), may affect LigA release *in vivo*. In contrast to the results observed for LigA, Sph2 release occurred whether the osmolarity was increased with sodium chloride or sucrose. These results suggest that the mechanisms of LigA and Sph2 release into the environment differ, although release of both involves proteolytic cleavage. Possible roles of adhesin release include enhancing dissemination of bacteria and control of immune cell activity (Abramson *et al.*, 2001; Coutte *et al.*, 2003; McGuirk & Mills, 2000).

LipL36 is subject to multiple environmental controls, including temperature, iron availability and growth phase

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(Cullen *et al.*, 2002; Haake *et al.*, 1998; Nally *et al.*, 2001b). An increase in incubation temperature caused a reduction in LipL36 levels (Nally *et al.*, 2001b) without affecting *lipL36* transcript levels (Lo *et al.*, 2006). In contrast, osmotic upshift caused diminished *lipL36* expression at both the RNA and protein levels. Hence, regulation of *lipL36* expression appears to be molecularly complex.

In conclusion, we have demonstrated that raising the osmolarity of leptospiral growth medium to near-physiological levels reproduced the changes in levels of Lig, Sph2 and LipL36 that are observed when *L. interrogans* infects a host mammal. This observation supports the notion that the ability to sense external osmolarity is important in correctly adjusting gene expression levels during the early stages of infection. The rapid increase in the levels of the putative virulence determinants LigA, LigB and Sph2 observed following upshift to physiological osmolarity *in vitro* may reflect events that occur at the early stages of *L. interrogans* infection of a susceptible host. Finally, osmolarity can mediate its effects via post-transcriptional mechanisms, as observed for extracellular LigA.

ACKNOWLEDGEMENTS

This work was supported by VA Medical Research Funds (J. M.), grant 09224-7 from the Oswaldo Cruz Foundation (A. I. K.), grants 300.861/96 and 420067/05 from the Brazilian National Research Council (A. I. K.), and grants AI052473 and TW00919 from the National Institutes of Health (A. I. K.). We thank Jane Babbitt for critical review of the manuscript.

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