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***Leptospira*: A Spirochete with a Hybrid Outer Membrane**

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Summary

Leptospira is a genus of spirochetes that includes organisms with a variety of lifestyles ranging from aquatic saprophytes to invasive pathogens. Adaptation to a wide variety of environmental conditions has required leptospires to acquire a large genome and a complex outer membrane with features that are unique among bacteria. The most abundant surface-exposed outer membrane proteins are lipoproteins that are integrated into the lipid bilayer by amino terminal fatty acids. In contrast to many spirochetes, the leptospiral outer membrane also includes lipopolysaccharide and many homologues of well-known beta-barrel transmembrane outer membrane proteins. Research on leptospiral transmembrane outer membrane proteins has lagged behind studies of lipoproteins because of their aberrant behavior by Triton X-114 detergent fractionation. For this reason, transmembrane outer membrane proteins are best characterized by assessing membrane integration and surface exposure. Not surprisingly, some outer membrane proteins that mediate host-pathogen interactions are strongly regulated by conditions found in mammalian host tissues. For example, the leptospiral immunoglobulin-like (Lig) repeat proteins are dramatically induced by osmolarity and mediate interactions with host extracellular matrix proteins. Development of molecular genetic tools are making it possible to finally understand the roles of these and other outer membrane proteins in mechanisms of leptospiral pathogenesis.

Introduction

Elucidating the features of the leptospiral outer membrane is the key to understanding its unique biogenesis, structure, and function. *Leptospira* spp. share many features with other spirochetes, including a double membrane architecture and subsurface, periplasmic endoflagella that coil back around the helical peptidoglycan cell wall and are anchored in subterminal polar bodies. In addition, leptospires share the characteristic of abundant surface lipoproteins with the *Borreliae*. However, in other respects, as shown in Figure 1, the leptospiral outer membrane presents a very different surface from that found in other

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invasive spirochetes. Unlike *Treponema pallidum* and *Borrelia spp.*, leptospires have lipopolysaccharide (LPS) and a rich diversity of amphipathic β -sheet transmembrane outer membrane proteins (OMPs), many of which have clear homologues in enteric Gram-negative bacteria. While much has been learned about the expression, structure, and function of leptospiral outer membrane components, little is known about their roles in pathogenesis. The goal of this review is to summarize our understanding of the leptospiral outer membrane, offer new insights, and suggest approaches for addressing key challenges facing the field, without attempting to provide an exhaustive review. Readers are referred to other sources for more comprehensive reviews of spirochetal lipoproteins and OMPs (Cullen *et al.*, 2004, Haake, 2000).

Host Susceptibility and Lipopolysaccharide Structure

From the spirochetal perspective, LPS could be seen as a bad idea because it facilitates innate immune recognition and clearance by the host. For example, *T. pallidum* is considered to be a stealth spirochete that persists in host tissues by producing no LPS, relatively few surface protein antigens and altering those that are present to evade the immune response. Leptospires also persist in the host by disseminating through the bloodstream to the kidney where they adhere to the luminal surface of proximal tubular epithelium. Located just downstream from the nutrient rich glomerular filtrate, this is an ideal niche for leptospires to multiply and be shed into the urine for dissemination to new hosts. Pathogenic leptospires are highly adapted to their reservoir hosts, as indicated by their ability to colonize the kidney for long periods, without causing a significant local inflammatory response. This commensal relationship avoids damage to the reservoir host, which is obviously to the leptospires advantage. However, if infection occurs in an accidental host, an intense inflammatory response can result, leading to a generalized sepsis-like syndrome, multiorgan failure, and occasionally, death. Why *L. interrogans* can colonize one host (frequently rats and mice) without causing disease and yet produce a lethal infection in humans is a subject of intense interest.

A key factor contributing to the susceptibility of humans to leptospiral infection is the inability of the human innate immune system to detect leptospiral LPS via TLR4, the canonical LPS receptor (Werts *et al.*, 2001). The reason for failure of human TLR4 to recognize leptospiral LPS may be due to its unusual structure of its lipid A component. Leptospiral lipid A has a unique methylated phosphate not found in lipid A of any other bacteria (Que-Gewirth *et al.*, 2004). Unlike murine macrophages, human cells are unable to recognize the lipid A component of leptospiral LPS (Nahori *et al.*, 2005). Human cells can detect intact leptospiral LPS, but this occurs via TLR2 rather than TLR4 (Werts *et al.*, 2001). In contrast, leptospiral LPS is detected by both murine TLR2 and TLR4 (Nahori *et al.*, 2005), with TLR2 also required for recognition of lipoproteins such as LipL32 (Werts *et al.*, 2001). As might be expected, mice with intact TLRs are resistant to leptospiral infection, whereas TLR2/TLR4 double knockout mice are highly susceptible to fulminant leptospiral infection and are appropriate as model of the accidental host (Chassin *et al.*, 2009). Based on these results, it would appear that the ability of the innate immune system to respond efficiently to leptospiral LPS via TLR4 serves a defining characteristic of a reservoir host. If this is true, we urgently need to understand how leptospires expressing LPS are able persist

in the kidney of reservoir hosts without stimulating a vigorous inflammatory response. In this regard, it is interesting to note that a small percentage of persons living in a village in the Peruvian Amazon were found to be asymptomatic carriers of *L. interrogans* (Ganoza *et al.*, 2010). It will be of great interest to examine the LPS of the strains carried by these patients and to test their ability to recognize this LPS via TLR4.

As in enteric Gram-negative bacteria, leptospiral LPS is a major component of the outer membrane and contributes to its structural integrity. As a result, the leptospiral outer membrane is destabilized by chelation of divalent cations involved in bridging between LPS molecules (Haake & Matsunaga, 2002). Random transposon mutagenesis studies found relatively few mutations in the ~100-kb *rfb* LPS biosynthetic locus involved in synthesis of sugars of the O-side chain (Murray *et al.*, 2009a). This observation could indicate either that the *rfb* locus is a transpositional cold spot or that the O antigen is essential for leptospiral viability. Mutations in LPS genes also affect virulence. One of the few LPS transposon mutants (in gene LA 1641) makes a smaller form of LPS and is unable to cause lethal infection in hamsters (Murray *et al.*, 2009a). Variations in O-side chain sugars are thought to account for the hundreds of known leptospiral serovars. The enzymes for lipid A biosynthesis are present both in leptospiral pathogens and the related environmental saprophyte, *L. biflexa*, indicating that these enzymes are part of the core set of leptospiral genes. As in most Gram-negative bacteria, leptospiral LPS presumably provides a number of general benefits that facilitate long-term survival in the environment after the bacteria are shed in the urine of reservoir hosts. Consistent with this concept, the outer membranes of intestinal spirochetes (*Brachyspira* spp.), which must survive outside the host for transmission, and the environmental organism, *Spirochaeta aurantia*, also contain LPS. In contrast, *T. pallidum* and *B. burgdorferi* neither live independently nor have LPS. Thus, having an LPS-containing outer membrane correlates- and is consistent with the host-independent lifestyle of leptospiral pathogens.

Outer Membrane Lipoproteins

New proteomic techniques have made it possible to learn more about the protein composition of *L. interrogans* than any other bacterium (Malmström *et al.*, 2009). Malmström and colleagues adapted liquid chromatography tandem mass spectrometry (LC-MS/MS) to determine the concentration (copy number) of proteins per cell. Abundance estimates were obtained for 1,864 proteins representing 51% of the predicted open reading frames of the *L. interrogans* genome. This feat was accomplished by seeding samples with known amounts of heavy labelled reference peptides, which served as internal standards. Their results demonstrate that, as in *Borrelia* spp., lipoproteins are the predominant leptospiral OMPs (Table 1). The major outer membrane lipoprotein, LipL32, is the most abundant protein of the entire cell at 38,000 copies per cell. Leptospiral lipoproteins are covalently modified by fatty acids that are attached to the amino-terminal cysteine. Lipidation occurs in concert with enzymatic cleavage of the signal peptide by signal peptidase II, which recognizes the lipobox sequence at the end of lipoprotein signal peptides. In *Escherichia coli*, the lipobox consensus sequence is L-X-Y-C, where Y is usually Gly, sometimes Ala, and rarely Ser. Leptospiral SpII substrate specificity is considerably more complex. Gly, Ala, and Ser can also be at position Y in leptospiral lipoboxes, but Asn is the

most frequent amino acid at Y and Gln, Thr, or Cys can also occur. To cope with this complexity, we created “SpLip” a hybrid algorithm that predicts spirochete lipoproteins using a combination of weight matrix scoring (based on a training set of well-characterized spirochetal lipoproteins) and exclusion of charged amino acids in the Y position (Setubal *et al.*, 2006). SpLip predicts over 140 probable or possible lipoproteins in the *L. interrogans* genome.

It should be kept in mind that in leptospires, lipidation may actually be a mechanism for directing proteins to the outer membrane or beyond. This is the case for P31_{LipL45}, which appears only transiently as a full-length 45-kD lipidated protein before its carboxy-terminal region is converted to a 31-kD peripheral outer membrane protein (Matsunaga *et al.*, 2002). The fact that P31_{LipL45} does not integrate into the outer membrane was demonstrated by showing that it can be removed by treating leptospiral membranes with urea or sodium bicarbonate. Another example of lipidation as an export strategy is LigA, which remains attached to the outer membrane until it is released from the surface by an endogenous protease. Leptospiral lipoproteins transit across the periplasm to the outer membrane via the Lol lipoprotein sorting machinery. Most of the components of the Lol system are present in the leptospiral genome. As in *Borrelia burgdorferi*, the leptospiral Lol system appears to transport leptospiral lipoproteins to the outer membrane “by default” when they lack an inner membrane retention signal (Schulze & Zuckert, 2006). Comparison of inner vs. outer membrane lipoprotein sequences indicates that in *Leptospira* spp. the inner membrane retention signal may be negatively charged amino acids following the N-terminal cysteine: inner membrane lipoproteins such as LipL31, LruA, and LruB have negatively charged amino acids in the +2 to +4 positions, while OM lipoproteins have neutral amino acids in these positions (Matsunaga *et al.*, 2006). The only member of the Lol pathway missing from the leptospiral genome and those of other spirochetes is LolB, which is an inward-facing outer membrane lipoprotein that transfers lipoproteins from the periplasmic LolA shuttle protein to the inner leaflet of the outer membrane. The lack of a LolB homologue suggests that spirochetes have a novel mechanism for inserting lipoproteins into their outer membranes. A transmembrane OMP that transfers lipoproteins from LolA into the OM has been proposed for *B. burgdorferi* (Lenhart & Akins, 2009).

Lipoproteins of *Leptospira* and *Borreliae* are unusual in that many are surface exposed, which is not the case for lipoproteins in enteric bacteria. Evidence for surface-exposure of the leptospiral major outer membrane lipoprotein, LipL32, comes from immunoelectron microscopy, whole cell ELISA, biotinylation, and immunofluorescence studies (Haake *et al.*, 2000, Cullen *et al.*, 2005). Once leptospiral lipoproteins are transported to the inner leaflet of the OM via the Lol pathway, they are probably transferred to the surface through an as yet undescribed “flipase” channel. Recent studies indicate that the *B. burgdorferi* outer surface protein, OspA, must be maintained in an unfolded form for it to reach the surface (Schulze *et al.*, 2010). The carboxyterminus of OspA leads the way in passing through the outer membrane to begin the folding process as it emerges on the surface. Folding is an energetically favourable process that “pulls” the protein through the channel, with flipping the lipid anchor from the inner leaflet to the outer leaflet of the outer membrane as the last

step in the process. An alternative scenario is that lipoproteins remain anchored to the inner leaflet of the OM and reach the surface via a transmembrane domain.

Could lipoproteins reach the outer leaflet of the outer membrane via the Type 2 Secretion System (T2SS) (Pugsley, 1993)? Pullulanase of *Klebsiella oxytoca* and the lipoprotein cytochromes MtrC and OmcA of *Shewanella oneidensis* have been shown to reach the outer leaflet of the OM via the T2SS (d'Enfert *et al.*, 1987, Shi *et al.*, 2008). In addition, the T2SS of *Pseudomonas*, secretes a wide variety of proteins that do not share any structural features. So it is certainly possible that some leptospiral lipoproteins could reach the outer leaflet of the OM via this route because *Leptospira* species have an intact type II secretion system and proteomic studies have detected its components (Malmström *et al.*, 2009). In this scenario, lipoproteins are exported across the cytoplasmic membrane, lipidated, folded, recognized by the T2SS secreton, and transported across the OM via the GspD pore. This route would not apply in other spirochetes because the components of T2SS are not found in treponemal, borrelial, or brachyspiral genomes.

A number of outer membrane surface lipoproteins appear to play key roles in leptospiral pathogenesis, including the acquisition of host proteins. For example, the endostatin-like lipoprotein, LenA, may function in invasion and serum resistance by binding plasminogen and Factor H, a key complement regulatory protein (Verma *et al.*, 2006, Verma *et al.*, 2010). Similarly, the Lig (leptospiral immunoglobulin-like repeat) proteins are the focus of considerable ongoing research interest because of their ability to interact with the host proteins including fibrinogen and plasma fibronectin (Choy *et al.*, 2007, Lin & Chang, 2007). Examples of bacterial adhesins with Ig-like domains, such as Invasin of *Yersinia pseudotuberculosis* and Intimin of enteropathogenic *E. coli* are well known. However, the leptospiral Lig proteins are unusual in the number of Ig-like domains that they contain. LigA and LigB are composed of 13 and 12 imperfect tandem Ig-like domains, respectively, and the first six domains and part of the seventh are identical or nearly identical, depending on the strain (McBride *et al.*, 2009). The later domains are related but distinct and unlike LigA, LigB contains a large, unique C-terminal region instead of a 13th Ig-like domain. A fragment of LigB comprising the nonidentical domains and the first seven amino acid residues of the C-terminal region binds fibronectin with high affinity similar to that of FnBPA of *Staphylococcus aureus* (Choy *et al.*, 2007). Although FnBPA and LigB are both composed of Ig-like domains, LigB retains high affinity binding to both the N-terminal domain (NTD) and the gelatin-binding domain (GBD) of fibronectin, whereas the binding affinity of FnBPA to NTD is considerably stronger than to GBD. The differential binding affinity of FnBPA and LigB for GBD indicates that LigB binding to fibronectin involves a unique mechanism of interaction. LigB also binds with high avidity to elastin and tropoelastin using Ig-like domains (Lin *et al.*, 2009). Weak elastin binding sites have been detected in four of the Ig-like domains of LigB, suggesting that the high avidity interaction results from multivalent interactions. Alignment of the four elastin-binding domains reveals a conserved aspartate residue. Mutation of the aspartate in the fourth Ig domain reduces its affinity for elastin without altering the secondary structure of the domain. This result suggests that binding of LigB to elastin is mediated by salt bridges. Both the binding mechanisms and

LigB domains involved in fibronectin and elastin interactions differ, suggesting that each of the Ig-like domains contributes in different ways to its functional characteristics.

In addition to whatever structural roles LipL32 has, it also interacts with fibronectin and the basement membrane proteins laminin and type IV collagen (Hauk *et al.*, 2008, Hoke *et al.*, 2008, Tung *et al.*, 2010). LipL32 is the first leptospiral OMP for which a crystal structure has been determined (Vivian *et al.*, 2009, Hauk *et al.*, 2009). The core of LipL32 forms a β -sandwich structure with an amino-terminal β hairpin that extends 35 Å away from the core, possibly mediating dimerization of the protein. An unusual aspartate-rich segment (DDDDDGDDTYK) at the edge of one of the β strands forms part of a high-affinity calcium-binding pocket (Tung *et al.*, 2010). The calcium ion is also coordinated by residues from loops at the edge of LipL32. Calcium binding causes a dramatic change in the position of two loops at the edge of LipL32, and one of these loops is found in the C-terminal fragment that binds to fibronectin (Hauk *et al.*, 2008, Hoke *et al.*, 2008). These structural data are consistent with the finding that high affinity fibronectin binding by LipL32 is calcium dependent (Tung *et al.*, 2010). A recent proteomic study of *L. interrogans* revealed that a tyrosine and lysine at the end of the aspartate-rich segment were phosphorylated and methylated, respectively, in native LipL32 (Cao *et al.*, 2010). By virtue of their close proximity to the calcium binding region, these post-translational modifications may be important for LipL32 function. The extracellular matrix binding activities of this major protein appear to be redundant because a *lipL32* knock-out mutant retains virulence in hamsters and the ability to colonize the renal tubules of rats (Murray *et al.*, 2009b). The idea that LipL32 is non-essential is surprising both because of the high cost to the cell of producing such an abundant protein and because of its unusually high level of DNA and amino acid sequence conservation across a broad range of pathogenic *Leptospira* spp. (Haake *et al.*, 2004). Taken together, this evidence suggests that the most important benefits of high-level LipL32 expression in the outer membrane may involve some other aspect of the leptospiral lifecycle such as life outside the mammalian host.

Transmembrane OMP Structure & Export

Genome sequencing reveals that leptospires have a rich diversity of transmembrane OMPs. The best described transmembrane OMP is the porin OmpL1, which may account for much of the channel-forming activity of the leptospiral outer membrane. In planar bilayer studies, the profile of measured conductance changes associated with porin insertion events for total leptospiral outer membrane and gel-purified OmpL1 were similar (Shang *et al.*, 1995). OmpL1 is predicted to have ten amphipathic β -strand transmembrane domains forming a barrel or pore similar to the structure described for *E. coli* OMPs (Haake *et al.*, 1993). The transmembrane β -strands of OMPs are amphipathic because hydrophobic amino acids facing the lipid bilayer alternate with hydrophilic amino acids oriented towards the aqueous pore of the barrel. This amphipathic structure so characteristic of transmembrane β -strands that OMP topology prediction algorithms such as PRED-TMBB can be used as a genomic search engine (Bagos *et al.*, 2004). When PRED-TMBB was applied to the *L. interrogans* genome, >100 genes containing multiple amphipathic β -sheet segments were identified (Pinne & Haake, 2009).

One limitation of this approach is that not all transmembrane OMPs traverse the outer membrane using β -strands. The crystal structure of the *E. coli* outer membrane lipoprotein, Wza, has an amphipathic α -helical transmembrane domain at its C-terminus (Dong *et al.*, 2006). Octamers of Wza form the channel that allows *E. coli* to secrete capsular polysaccharides across the outer membrane to its surface. Although α -helical transmembrane OMPs have not been described in spirochetes, this is likely to be the case for Loa22, the second most abundant leptospiral protein. Loa22 is lipidated and appears to traverse the outer membrane based on its predicted OmpA-like peptidoglycan binding domain AND strong experimental evidence for surface-exposure (Ristow *et al.*, 2007). The surface-exposed region is presumably somewhere between the lipidated N-terminal cysteine (residue 21) and the start of the predicted peptidoglycan binding domain (residue 111). The intervening 90 amino acids are very hydrophilic, and lack the ability to form amphipathic β -strands. However, several stretches within the intervening region are α helical and one of these (residues 53-70) is amphipathic with a strongly hydrophobic region on one face of the helix. Structural studies on Loa22 are needed to test this hypothesis. There is a strong rationale for research to understand the structure of Loa22 given that it is required for virulence: a Loa22 transposon mutant grows well in culture but was highly attenuated and unable to produce a lethal infection in hamsters (Ristow *et al.*, 2007). Additional OMPs with potential α helical transmembrane regions await discovery in the leptospiral genome sequence and bioinformatic approaches are needed to identify these genes.

Functional inferences regarding a number of transmembrane OMPs can be drawn either because they are members of transmembrane protein families (pfam) or because they have well-characterized homologues. For example, seven TolC homologues are present in *L. interrogans*, each of which presumably interact with different ABC transporters involved in efflux pathways. Leptospirae have a diverse repertoire of TonB-dependent OMP receptors involved in nutrient uptake: 12 in *L. interrogans* and 8 in *L. biflexa*. The functions of TonB dependent OMP family proteins are difficult to infer bioinformatically but have been examined by systematically knocking out the genes in *L. biflexa*, which is much easier to manipulate genetically than is *L. interrogans*. Examination of the growth dependence on iron sources of *L. biflexa* knockouts revealed a ferrioxamine receptor and a FecA-like receptor for iron salts, which has a close homologue in *L. interrogans* (Louvel *et al.*, 2006). An additional TonB dependent OMP has been designated HbpA (LIC20151), based on its ability to bind hemin (Asuthkar *et al.*, 2007). It can be anticipated that additional substrates for leptospiral TonB dependent OMPs will be discovered, including other metal ions, cofactors, and carbohydrates (Schauer *et al.*, 2008). Nascent transmembrane OMPs are probably inserted into the outer membrane occurs through the activity of the leptospiral BamA-like protein, LIC11623, which is predicted to have four POTRA domains. Periplasmic POTRA domains of BamA are responsible for folding, assembly, and insertion of transmembrane OMPs into the outer membrane (Tomassen, 2007). Efficient β -barrel assembly into the outer membrane requires a number of accessory proteins, but homologues of the BamB, C, D, and E lipoproteins are yet to be identified.

In contrast to the lipoprotein OMPs, relatively little experimental work has been reported on leptospiral transmembrane OMPs. One problem that might partially account for the slow

progress in this area is that Triton X-114 detergent extraction and phase partitioning, a reliable method for localization of spirochetal outer membrane lipoproteins, turns out to be biased against leptospiral transmembrane OMPs. The Triton X-114 detergent extract partitions into a hydrophobic detergent phase and a hydrophilic aqueous phase upon warming to 37°C and hydrophobic integral membrane proteins are expected to fractionate into the detergent phase. We examined the Triton X-114 fractionation behaviour of four proteins predicted to be transmembrane OMPs using TMBB-PRED and validated as OMPs using methods for demonstrating membrane integration and surface exposure (surface immunofluorescence, surface biotinylation, and surface proteolysis) (Pinne & Haake, 2009). Each of these four OMPs had a different Triton X-114 fractionation pattern, and only one of them appeared in the detergent phase. The reason for this seemingly aberrant behaviour isn't understood, but the results suggest that transmembrane OMPs are either poorly soluble in Triton X-114, or are tethered to a subsurface structure such as the cell wall. Because of their unpredictable and potentially misleading behavior in Triton X-114, we propose a new experimental paradigm for identification of leptospiral transmembrane OMPs based on membrane integration and surface exposure rather than detergent fractionation.

Outer Membrane Changes Through the Life Cycle

Leptospira species encounter dramatically different environmental conditions, ranging from a free-living state outside the host to the tissues of carrier host animals. Colonization of the proximal renal tubular lumen affords leptospires the benefit of being bathed in the nutrient-rich glomerular filtrate. Nevertheless, after being shed in the urine of reservoir host animals, *L. interrogans* can also survive for many months in water or moist soil. This remarkable adaptability derives from a large genome equipped with a high number and rich diversity of stimulus-response systems. For example, the *L. interrogans* 4.6 Mb genome is predicted to encode 107 signal transduction proteins, including 47 of the histidine-kinase type. By comparison, *Escherichia coli* has 30 histidine kinase signal transduction proteins and the host-dependent spirochete *Treponema pallidum* has only one (Galperin, 2005). Some of these signal transduction systems are likely to be involved in regulating LPS and OMP expression, because the leptospiral surface undergoes significant changes during the key transitions in its life cycle from outside to inside the mammalian host, and vice versa. It has long been known that *L. interrogans* recovered from kidneys of infected animals were antigenically distinct from cultivated organisms (Faine, 1962). Some of these antigenic changes likely involve LPS carbohydrate side chains; *L. interrogans* recovered from the liver of infected guinea pigs failed to react with an LPS O-antigen-specific monoclonal antibody that was strongly reactive with same organism cultivated in vitro (Nally *et al.*, 2005). In addition, the levels of several OMPs were altered in *L. interrogans* recovered from the guinea pig liver, thereby demonstrating effects of host environment on OMP expression as well (Nally *et al.*, 2007). Subsequent proteomic studies indicate that OMP expression changes are driven in part by temperature and/or iron deprivation (Lo *et al.*, 2009, Eshghi *et al.*, 2009).

Culture conditions that mimic the host environment strongly affect the production levels of a number of OMPs. One striking example of altered OMP expression in response to environmental signals involves LigA and LigB. The Lig proteins were discovered by

screening a bacteriophage lambda expression library with sera from convalescent patients with leptospirosis, indicating that they are expressed during infection of the mammalian host (Matsunaga *et al.*, 2003). Production of LigA and LigB was dramatically enhanced by increasing osmolarity of standard leptospiral culture medium (67 mOsm) to physiologic levels found in the mammalian host (300 mOsm) (Matsunaga *et al.*, 2005, Matsunaga *et al.*, 2007a). These results suggest that *lig* expression is induced when pathogenic leptospires in an aqueous environment encounter an upshift in osmolarity, signalling the presence of mammalian host tissues. Osmoregulation is highly relevant to host tissue colonization because adherence of *L. interrogans* to fibrinogen and the extracellular matrix proteins fibronectin and collagen IV is enhanced when grown at physiologic osmolarity, mediated at least in part by LigA and LigB (Choy *et al.*, 2007, Lin & Chang, 2007).

Whole genome transcriptional microarray studies demonstrated that the *lig* genes are among those most strongly induced by increasing osmolarity from that of EMJH leptospiral culture medium (67 mOsm) to physiologic levels found in the mammalian host (300 mOsm) (Matsunaga *et al.*, 2007a). Many of the osmoregulated genes in *L. interrogans* serovar Copenhageni are also regulated by temperature in the closely related organism, *L. interrogans* serovar Lai. Coregulated genes include *sph2*, which encodes an up-regulated sphingomyelinase released by *L. interrogans* into the culture supernatant, and *lipL36*, encoding a down-regulated outer membrane lipoprotein (Matsunaga *et al.*, 2007b). LipL36 is a prominent outer membrane lipoprotein during cultivation of the pathogen *Leptospira kirschneri*, but is immunohistochemically undetectable during infection (Barnett *et al.*, 1999). Surprisingly, temperature regulation of many OMPs does not correlate with changes in transcript levels, suggesting the importance of post-transcriptional regulation mechanisms involving small RNAs and RNA binding proteins and superiority of proteomic approaches in assessing the effect of environmental signals on protein expression (Lo *et al.*, 2009).

Reflecting the importance of iron in leptospiral physiology, genes encoding four different TonB dependent OMPs are transcriptionally upregulated by iron-deprivation including those involved in uptake of siderophores, iron salts, and hemin (Lo *et al.*, 2010). Random transposon mutagenesis is beginning to yield insights into leptospiral regulatory mechanisms. Transcriptional microarray studies of a knock-out of one of four *fur* homologues found in *L. interrogans* revealed changes in expression of genes for catalase and heme biosynthesis, suggesting that this particular Fur homologue might play a role in responses to oxidative stress and/or metal limitation (Lo *et al.*, 2010).

Conclusions

This review has attempted to summarize the recent explosion in knowledge regarding the leptospiral outer membrane, which has benefitted greatly from genomic and proteomic approaches. We estimate a combined total of over 300 potential lipoprotein and transmembrane OMPs. The vast majority of these await characterization and/or lack well-understood homologues in other organisms. Even where well-characterized homologues do exist, it is increasingly clear that, as in the case of TlyC, a thermolysin homologue that binds fibronectin, laminin, and collagen IV (Carvalho *et al.*, 2009), spirochetes have found new uses for genes borrowed from other bacteria. There is a particularly pressing need for new

approaches to understanding the roles of OMPs in host-pathogen interactions. The recent past has seen a welcome shift towards work on elucidating the function of leptospiral OMPs. A considerable number of putative adhesins have been identified with evidence of protein-protein affinity based on ELISA methods using recombinant proteins. However, in studying leptospiral OMPs, we believe more rigor is necessary to assess the surface exposure of candidate leptospira OMPs - we advocate multiple, complementary approaches. Additionally, the post-translational modifications of LipL32 highlight the tremendous need for improved genetic tools, model systems, and in vivo approaches so that the roles of OMPs defined using recombinant proteins can be confirmed with native proteins. A major advance has been the development of random transposon mutagenesis and, at least in one case (*ligB*), site-directed mutagenesis approaches for knocking out OMP genes (Croda *et al.*, 2008). However, improved site-directed mutagenesis approaches are needed, perhaps by identifying virulent strains with higher transformation efficiency. Genes encoding LipL32 and LigB are only found in pathogens, and there were excellent reasons to anticipate that these proteins had essential roles in pathogenesis, yet *L. interrogans* mutants lacking these genes were virulent in hamsters, as defined by lethality, organ culture, and histopathology (Croda *et al.*, 2008, Murray *et al.*, 2009b). These surprising results might reflect more subtle changes than could be revealed by the readout of hamster mortality, and indicate the need for more nuanced approaches to understanding leptospiral virulence. Many virulence mechanisms are probably either redundant and/or require more sophisticated animal models involving competitive infection methods, quantitative results looking at different stages of infection, organ-specific readouts, and more biologically relevant challenge routes to assess the role of Omps in establishment of infection. Histopathology is inherently subjective and should be supplemented with objective measures of organ (especially liver and kidney) function and immune system activation by measurement of relevant cytokine levels. A still more intriguing result was the finding that, although the gene encoding Loa22 is conserved in the nonpathogenic saprophyte *L. biflexa*, the *L. interrogans* *loa22* mutant is avirulent (Ristow *et al.*, 2007). This result indicates a need to refine our opinions about the roles of OMPs in diverse *Leptospira* species that are either nonpathogenic for mammals or are of intermediate pathogenicity. From a microbiological point of view, life in the aquatic environment outside the host might actually be considerably more challenging than life in the mammalian host. Many of the survival mechanisms important for leptospiral saprophytes may also be relevant during infection. *L. biflexa* genome sequence strain has many of the same protein export and processing systems as *L. interrogans*, is considerably more tractable to genetic manipulation, and will continue to be an excellent surrogate host for questions involving the function and regulation of leptospiral OMPs.

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Fig. 1.

Leptospiral membrane architecture. The leptospiral outer membrane contains a mixture of lipopolysaccharide, surface-exposed lipoproteins, and transmembrane proteins. The most abundant outer membrane lipoproteins are LipL32 and Loa22. Loa22 may contain a novel transmembrane segment connecting its surface-exposed and periplasmic peptidoglycan binding domains. Periplasmic flagella (PF) are located in the periplasm. FecA is a TonB-dependent receptor for ferric citrate and aerobactin. TolC forms an efflux channel with ATPase pumps in the cytoplasmic membrane.

Table 1

Surface-Exposed Outer Membrane Proteins of *Leptospira interrogans*, serovar Copenhageni, strain L1-130^a

Name	Locus Tag	Type ^b	Size (kd) ^c	Copy Number ^d	Knock-out Virulent?	<i>L. biflexa</i> % identity ^e	Putative Function(s), Comment ^f &g	Selected References
LipL32	11352	Lip	32	38,050	Yes	-	Binds Ca ²⁺ , lam, cIV, fn*	(Hoke et al., 2008, Hauck et al., 2009)
Loa22	10191	Lip	22	30,329	No	56%	Binds peptidoglycan	(Koizumi & Watanabe, 2003, Ristow et al., 2007)
LipL41	12966	Lip	41	10,531	-	-	Binds hemin*	(Shang et al., 1996, Asuthkar et al., 2007)
LipL21	10011	Lip	21	8,830	-	46%	-	(Cullen et al., 2003)
OmpL36	13166	TM	36	8,021	-	76%	-	(Pinne & Haake, 2009)
OmpL1	10973	TM	33	5,441	-	-	Porin*	(Haake et al., 1993, Shang et al., 1995)
LipL46	11885	Lip	46	5,276	-	54%	-	(Matsunaga et al., 2006)
OmpL47	13050	TM	47	5,022	-	50%	-	(Pinne & Haake, 2009)
OmpL37	12263	TM	37	924	-	47%	Binds elst (unpublished)*	(Pinne & Haake, 2009)
LigB	10464	Lip	200	914*	Yes	-	Binds fn, fg, cI, cIV, elst, Ca ²⁺ *	(Choy et al., 2007, Lin et al., 2008)
GspD	11570	TM	66.5*	658	-	62%	T2SS channel	(Choy et al., 2007)
LigA	10465	Lip	130	553*	-	-	Binds fn, fg, col-I, col-IV*	(Louvel et al., 2005)
FecA	10714	TM	92.5*	529	-	49%	TBDR for Fe ³⁺ -dicitrate*	(Pinne & Haake, 2009)
OmpL54	13491	TM	54	491	-	44%	-	
OstA	11458	TM	113.5*	145	-	49%	LPS assembly	
Omp85	12254	TM	60*	136*	-	-	-	
OmpA	10258	TM	68*	75*	-	-	Binds peptidoglycan	
BamA	11623	TM	113*	37	-	64%	OMP biogenesis	
TolC	12307	TM	56*	21*	-	48%	Export channel	
HbpA	20151	TM	80	14*	-	58%	TBDR for hemin*	(Asuthkar et al., 2007)
TlyC	13143	TM	50.4*	8*	-	58%	Binds fn, lam, cIV*	(Carvalho et al., 2009)

^aInclusion criteria: 1. Proteomic quantitation(Malmström et al., 2009); and 2. Either a surface-exposed homologue, or published evidence of surface-exposure including appropriate controls (see text for details).

^bType: Lip (lipoprotein), TM (transmembraneOmp), PM (peripheral membrane Omp)

^cSize (kd): Observed or predicted*.

^dCopy number: Estimated by MS or spectral* methods (Malmström et al., 2009).

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^ePercent identity with *L. biflexa* homologue.

^fPutative function: T2SS (type 2 secretion system), TBDR (tonB-dependent receptor), LPS (lipopolysaccharide), Host ligands: fn (fibronectin), fg (fibrinogen), cl (collagen D), cIV (collagen IV), lam (laminin), and elast (elastin).

^gBased on experimental evidence*.