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The Leptospiral Outer Membrane

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Abstract

The outer membrane (OM) is the front line of leptospiral interactions with their environment and the mammalian host. Unlike most invasive spirochetes, pathogenic leptospires must be able survive in both free-living and host-adapted states. As organisms move from one set of environmental conditions to another, the OM must cope with a series of conflicting challenges. For example, the OM must be porous enough to allow nutrient uptake, yet robust enough to defend the cell against noxious substances. In the host, the OM presents a surface decorated with adhesins and receptors for attaching to, and acquiring, desirable host molecules such as the complement regulator, Factor H. On the other hand, the OM must enable leptospires to evade detection by the host's immune system on their way from sites of invasion through the bloodstream to the protected niche of the proximal tubule.

The picture that is emerging of the leptospiral OM is that, while it shares many of the characteristics of the OMs of spirochetes and Gram-negative bacteria, it is also unique and different in ways that make it of general interest to microbiologists. For example, unlike most other pathogenic spirochetes, the leptospiral OM is rich in lipopolysaccharide (LPS). Leptospiral LPS is similar to that of Gram-negative bacteria but has a number of unique structural features that may explain why it is not recognized by the LPS-specific Toll-like receptor 4 of humans. As in other spirochetes, lipoproteins are major components of the leptospiral OM, though their roles are poorly understood. The functions of transmembrane OMPs in many cases are better understood thanks to homologies with their Gram-negative counterparts and the emergence of improved genetic techniques. This chapter will review recent discoveries involving the leptospiral OM and its role in leptospiral physiology and pathogenesis. Readers are referred to earlier, excellent summaries related to this subject (Adler and de la Peña Moctezuma, 2010; Cullen et al., 2004; Haake, 2000; Haake and Matsunaga, 2010; Ko et al., 2009; Raja and Natarajaseenivasan, 2013; Zuerner et al., 2000).

1. Lipopolysaccharide (LPS)

LPS is a major component of the leptospiral OM and its polysaccharides dominate the leptospiral surface. The degree to which LPS is exposed on the leptospiral surface is

reflected in the abundance of electron-dense particles on the surface of *L. interrogans* after incubation with a gold-labeled anti-LPS monoclonal antibody (Fig. 1). Agglutination occurs within minutes in the presence of small concentrations of LPS-specific antibodies. Monoclonal antibodies to LPS mediate macrophage opsonization (Farrelly et al., 1987) and protect animals against challenge with pathogenic leptospires (Jost et al., 1989). LPS-specific immune responses are the basis for the sterilizing immunity elicited by whole cell vaccines (Midwinter et al., 1994). Given the sensitivity of leptospires to LPS-specific antibodies, it is not surprising that there is tremendous selective pressure to undergo genetic changes leading to O-antigen variation. Hundreds of leptospiral serovars have been defined, based on differential reactivity with antibodies or antisera in the microscopic agglutination test (MAT). The simple addition of LPS-antiserum to a leptospiral culture can result in the growth of escape mutants with altered LPS.

Despite its accessibility, LPS is by no means a liability for these organisms. Expression of intact LPS appears to be essential for leptospiral survival both inside and outside the mammalian host. This conclusion is based in part on the finding that the *rfb* locus encoding the enzymes responsible for LPS biosynthesis was relatively spared of insertions in a study of random transposon mutagenesis (Murray et al., 2009a), suggesting that most LPS mutants are nonviable for growth in culture. The rare mutants that did survive transposon insertion into the LPS locus were attenuated for virulence and were rapidly cleared after challenge (Murray et al., 2010). Interestingly, the LPS expressed by one of these LPS mutants, M1352, had little or no change in its molecular mass, suggesting that even subtle changes in LPS can result in a loss of virulence. Mutant M1352 was effective as a live attenuated vaccine, stimulating both homologous and heterologous immunity in the hamster model of leptospirosis (Srikram et al., 2011). Differential detection of organisms in the liver and kidney using serovar-specific monoclonal antibodies suggests that the O-antigen side chains of leptospiral LPS are not static and may undergo antigenic changes during infection (Nally et al., 2005a).

1.1. LPS Structure and Biosynthesis

As in Gram-negative bacteria, leptospiral LPS consists of three components: lipid A, the core, and polysaccharide. The *L. interrogans* genome contains homologs of all the genes required for lipid A biosynthesis (Ren et al., 2003). The structure of leptospiral lipid A has now been fully elucidated and found to contain both similarities with, and striking differences from, typical forms of lipid A (Que-Gewirth et al., 2004). The first key difference is that *L. interrogans* converts the usual GlcNAc (N-acetylglucosamine) disaccharide backbone of lipid A to GlcNAc3N, so that each of the two sugars has two amino groups instead of one. Consequently, there are four amide-linked fatty acids in the *L. interrogans* lipid A instead of two. This is unusual, but has been observed in some environmental bacteria. In addition, the leptospiral fatty acids in leptospiral lipid A differ in length from those typically found in Gram-negative lipid A and some are unsaturated. An even more unusual aspect of *L. interrogans* lipid A involves the phosphate residue. *E. coli* lipid A has two phosphates, one on each end of the disaccharide, whereas leptospiral lipid A has a single phosphate, and that single phosphate is methylated. Methylated phosphates are extremely unusual in biology and have not been previously observed in lipid A.

1.2. Innate Immunity: TLR4 and TLR2

The structural differences between LPS of *E. coli* and *Leptospira* are of great interest because of their differential recognition by TLR4, the Toll-like receptor involved in the innate immune response to LPS. While human TLR 4 reacts with *E. coli* LPS at extremely low concentrations, it is unable to interact with leptospiral LPS (Werts et al., 2001). Failure of human TLR4 to recognize leptospiral LPS may be one reason why humans are accidental hosts in whom leptospirosis occasionally causes overwhelming, lethal infections. In contrast, murine TLR4 is able to recognize leptospiral LPS (Nahori et al., 2005) and mice are natural, reservoir hosts for pathogenic leptospires. This idea is consistent with the observation that while mice with intact Toll-like receptors are resistant to leptospiral infection, young (but not adult) C3H/HeJ mice lacking TLR4 are susceptible to lethal infection with *L. interrogans* (Viriyakosol et al., 2006). Surprisingly, leptospiral LPS is recognized by both human and murine TLR2, the Toll-like receptor primarily involved in lipoprotein recognition. The importance of both TLR2 and TLR4 receptors in mice was highlighted by the finding that only when both of these receptors were knocked out did adult C57BL/6J mice develop lethal infections after leptospiral challenge (Nahori et al., 2005). Murine TLR4 and TLR2 appear to recognize different leptospiral LPS components: TLR4 recognizes leptospiral lipid A while TLR2 recognizes the polysaccharide or 2-keto-3-deoxyoctonoic acid (KDO) portion of leptospiral LPS (Nahori et al., 2005; Werts, 2010).

1.3. LPS Assembly and Transport

Many of the genes involved in LPS export to the OM are present in leptospiral genomes, suggesting that the processes are similar to those in typical Gram-negative bacteria. A number of excellent reviews on the subject of LPS assembly and transport have recently been published (Ruiz et al., 2009; Sperandeo et al., 2009). The lipid A and core components of LPS are assembled on the cytoplasmic surface of the inner membrane. These rough LPS molecules (lacking the O-antigen) are transported to the periplasmic leaflet of the inner membrane by the ABC transporter, MsbA. It has not yet been determined which of the many *L. interrogans* ABC transporters is MsbA. O-antigen is assembled via the Wzy-dependent pathway in which polysaccharides are synthesized on the cytoplasmic surface of the inner membrane, followed by transport across the inner membrane by the Wzx flippase (LIC12135), where they are ligated to rough LPS by Wzy O-antigen ligase (LIC11753). After polysaccharide has been added to the LPS core, full-length (smooth) LPS is transported across the periplasm by LptA to the LPS assembly site on the OM formed by LptD (aka OstA, LIC11458) and LptE (11007). LptD is a porin-like molecule and appears to be involved in translocating LPS to the OM surface. Cryo-electron tomography has shown that the thickness of the *L. interrogans* LPS layer, and presumably the length of its polysaccharide, is 50% greater than that of *L. biflexa* (Fig. 2), which again illustrates the importance of LPS for virulence (Raddi et al., 2012).

2. Outer Membrane Proteins (OMPs)

2.1. General Considerations

In recent years, much has been learned about the identity, expression and functions of OMPs. The picture of the OM that has emerged (Fig. 3) is the result of improved methods

for determining whether proteins are located in the OM and on its surface. A number of cell fractionation methods have been developed, including Triton X-114 fractionation (Haake et al., 1991; Zuerner et al., 1991), isolation of OM vesicles by sucrose density gradient fractionation (Haake and Matsunaga, 2002; Nally et al., 2005b), and membrane fractionation (Matsunaga et al., 2002). Of particular importance are methods to identify surface-exposed OMPs. Multiple assays should be applied, including surface and subsurface controls, before concluding whether a particular protein is surface-exposed. The most accurate methods include surface immunoprecipitation (Haake et al., 1991), surface biotinylation (Cullen et al., 2003), surface proteolysis (Pinne and Haake, 2009), and surface immunofluorescence (Pinne and Haake, 2011).

Particularly useful has been the application of Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) to identification of surface-exposed (Cullen et al., 2005) and OM-associated proteins (Cullen et al., 2002; Nally et al., 2005b). More is now known about the absolute level of expression of leptospiral proteins than in almost any other bacterial species, thanks to the proteome-wide application of MALDI-TOF to identify and quantify leptospiral proteins (Malmström et al., 2009). Absolute quantification was achieved by inclusion of isotope-labelled reference peptides in leptospiral samples. DNA microarrays have been used to examine the response of leptospiral transcript levels to environmental signals including temperature up shift (Lo et al., 2006), osmolarity (Matsunaga et al., 2007a), iron levels (Lo et al., 2010), serum (Patarakul et al., 2010), and macrophage-derived cells (Xue et al., 2010). Proteomic methods have also been used to examine thermoregulation (Lo et al., 2009) and post-translational modification of OMPs (Cao et al., 2010; Eshghi et al., 2012). Proteome arrays have been used to identify fibronectin-binding OMPs (Pinne et al., 2012) and seroreactive OMPs (Lessa-Aquino et al., 2013).

2.2. Lipoprotein OMPs

Bacterial lipoproteins are proteins that have been post-translationally modified by fatty acids (i.e. lipids) at a cysteine residue. This cysteine becomes the amino-terminal residue after the signal peptide has been removed by lipoprotein signal peptidase. Because the fatty acids of lipoproteins are extremely hydrophobic, they become embedded into membrane lipid bilayers and provide an anchor for lipoproteins to be tightly associated with the membrane. Treatments such as salt and urea that remove peripheral membrane proteins from membranes will not remove lipoproteins. This demonstrates that even though lipoproteins are generally not transmembrane proteins (Loa22 appears to be an exception, see below), lipoproteins remain tightly associated with membranes, even after treatment with reagents that remove peripheral membrane proteins (Matsunaga et al., 2002). In contrast to the hydrophobicity of fatty acids, the protein components of most lipoproteins are typically hydrophilic and relatively soluble in aqueous buffers when expressed as recombinant proteins without their signal peptide. As such, the protein components of lipoproteins project out from membranes and decorate their surfaces. The first bacterial lipoprotein to be described was the Murein (or Braun's) lipoprotein, which is integrated into the inner leaflet of the *E. coli* OM by lipids at its amino terminus and covalently attached to peptidoglycan (PG) at its carboxyterminal lysine. Murein lipoprotein is a major OM protein of *E. coli* and serves an important structural role in maintaining cellular integrity by providing a link

between the OM and the PG cell wall. The OM-PG linkage is so important that *E. coli* has a number of other proteins that play similar roles, including OmpA and Pal (peptidoglycan-associated lipoprotein). Leptospires also have a number of OmpA-related proteins, such as Loa22, that are presumed to play similar OM-anchoring roles.

2.3. Lipoprotein Lipidation and Export

The steps involved in lipoprotein lipidation and export are shown in Fig 3. Proteins with amino-terminal signal peptides, including lipoproteins, are exported across the inner membrane by the Sec translocase complex. Orthologs of all essential components of the Sec translocase complex are present in *Leptospira.* Upon reaching the periplasm, lipoproteins of Gram-negative bacteria are processed by a series of three enzymes that remove the signal peptide and modify the new N-terminal cysteine with fatty acids. Each of these three lipoprotein processing enzymes is also present in *Leptospira* (Haake, 2000; Nascimento et al., 2004). The first of these enzymes is lipoprotein diacylglyceryl transferase (Lgt), which attaches a diacyl group containing two fatty acids to the sulfhydryl residue of cysteine via a thioester linkage. Because Lgt is the first enzyme in the series, its active site is presumably responsible for identifying the “lipobox”, which distinguishes the signal peptides of lipoproteins from those of other exported proteins. Lipoprotein signal peptidase (Lsp) is the second enzyme in the series, and is responsible for removing the signal peptide so that cysteine becomes the N-terminal amino acid of the mature lipoprotein. The third enzyme in the series is lipoprotein N-acyl transferase (Lnt), which adds a third and final fatty acid to the now available amino residue of cysteine via an amide bond. Interestingly, most Gram-positive bacteria lack Lnt and their lipoproteins are usually diacylated rather than triacylated (Kovacs-Simon et al., 2011). Perhaps Gram-negative bacteria triacylate their lipoproteins to strengthen the connection between the OM lipid bilayer and lipoproteins involved cell wall anchoring.

Experimental verification of lipidation is important when examining how lipoproteins interact with leptospiral membranes and the host innate immune system. Several methods are available. A commonly used method is to add radiolabeled palmitate to growth medium to demonstrate incorporation of label into the protein, which can be purified by immunoprecipitation. [^{14}C]palmitate labeled at each of its carbons is the preferred form of palmitate because the higher specific activity results in a much shorter time to identification of bands by autoradiography. It should be noted that spirochetes, including leptospires, can digest fatty acids to two-carbon fragments which are incorporated into amino acid biosynthetic pathways. In this way, [^{14}C]palmitate could potentially label any protein. For this reason, it is desirable to take advantage of the acid-labile linkage of the palmitate to the lipoprotein by demonstrating that the label can be removed from the lipoprotein by treatment of the electrophoresis gel containing the protein with acetic acid: immunoblots would remain positive while the autoradiogram would become negative. Historically, globomycin has been used to inhibit lipidation of lipoproteins. However, it should be noted that globomycin selectively inhibits lipoprotein signal peptidase, the second enzyme in the series, so proteins could still become acylated through the previous step mediated by Lgt. Indirect evidence for lipidation can be obtained by Triton X-114 detergent fractionation. This detergent is similar to Triton X-100 except that Triton X-114 has a shorter polyethylene

side chain, giving Triton X-114 a much lower cloud point. As a result, Triton X-114 solutions that occur in a single phase at 4°C partition into two phases upon warming to 37°C: a heavier, detergent-rich “hydrophobic” phase and a lighter, detergent-poor “hydrophilic” phase. Lipoproteins extracted by treatment of bacteria with Triton X-114 on ice should partition into the hydrophobic phase. The combination of sequence analysis plus behavior in Triton X-114 is an argument, albeit indirect, for lipidation.

Significant progress has been made in predicting which leptospiral genes encode lipoproteins. Lipoprotein signal peptides differ from other signal peptides in that they contain a “lipobox” sequence near the carboxyterminal region of the signal peptide. In *E. coli*, the lipobox sequence is typically Leu-Leu-X-Y-Cys. There is relatively little variation in *E. coli* lipobox sequences and substitutions that do occur are with conservative amino acids: X is typically Ala, but can also be Thr or Ser, while Y is typically Gly, but can also be Ala. Based on sequences from experimentally verified lipoproteins, spirochete lipobox sequences are much more variable than those of *E. coli*. As a consequence, the Psort lipoprotein prediction program, based on lipoprotein sequences of *E. coli* and related bacteria has low (17–33%) sensitivity for spirochetal lipoproteins. A later algorithm, LipoP, that utilized hidden Markov model statistical methods had significantly greater (50–81%) sensitivity, but also higher (8–30%) false positivity. To address these problems, we developed a spirochete-specific lipoprotein algorithm called “SpLip” based on sequences from 28 experimentally verified spirochetal lipoproteins. The SpLip algorithm is a hybrid approach of supplementing weight matrix scoring with rules including exclusion of charged amino acids from the lipobox (Setubal et al., 2006).

The inclusion in SpLip of rules based on sequences of experimentally verified lipoproteins reduces false positive hits, but these rules require further validation. For example, one of the SpLip rules is that the only allowed amino acids at the −1 position are Ala, Gly, Ser, Thr, Asn, Gln, or Cys. Now that a large number of leptospiral genomes have been sequenced, researchers at the J. Craig Venter Institute have discovered that newly sequenced homologs of known lipoproteins may have additional amino acids at the −1 position (Daniel Haft, personal communication). This genome sequence analysis will provide much greater confidence regarding the plasticity of amino acids at positions within the lipobox. It is unclear why spirochetal lipobox sequences are so much more variable than those of *E. coli*. One possible explanation for this difference is that *E. coli* growth rates are so much faster than spirochete growth rates. As a result, *E. coli* enzymes, including those involved in lipoprotein processing, must have much higher rates of catalysis than spirochetal enzymes. Higher catalytic rates may require higher substrate fidelity to maintain enzymatic efficiency.

After lipidation occurs, lipoproteins either remain in the outer leaflet of the inner membrane (IM) or undergo trafficking to one or more of four other possible destinations. From inside to outside these are: the inner leaflet of the OM, the outer leaflet of the OM, as a peripheral OM protein, and secretion beyond the cell. In *E coli*, lipoproteins destined for the OM are recognized by the IM ABC transporter-like sortase complex LolCDE (Yakushi et al., 2000) and then presented to periplasmic lipoprotein-binding chaperone LolA (Yokota et al., 1999) for transport to the OM lipoprotein receptor LolB (Yokota et al., 1999). The *L. interrogans* serovar Copenhageni genome appears to contain multiple homologs of a LolADE subset

(LolA-1 and -2, LolD-1 and -2 and LolE-1, -2, and -3) (Fig. 4) (Nascimento et al., 2004). A LolC homolog is missing, as is LolB. LolB homologs so far have only been detected in β- and γ-Proteobacteria, and LolC function might be provided by one of the LolE homologs. While it remains to be determined which of the spirochetal genes are indeed functional Lol orthologs or functionally diverse paralogs, it appears likely that the Lol pathway is involved in shuttling lipoproteins from the IM to the OM.

Even less is known about how lipoproteins travel to the leptospiral surface and beyond. However, it would not be surprising if leptospiral lipoproteins follow the model established for *B. burgdorferi* lipoprotein secretion. In this model, the targeting information of surface lipoproteins was found to be located in the intrinsically disordered N-terminal tether peptides, with sorting signals differing from those in other well-characterized diderm model systems (Kumru et al., 2010; Kumru et al., 2011b; Schulze and Zückert, 2006, Schulze et al., 2010). A surface lipoprotein's periplasmic conformation (or lack thereof) was found to determine its ability to cross the OM, and crossing could be initiated by a disordered C terminus after insertion of the protein to the periplasmic leaflet of the OM (Chen and Zückert, 2011; Schulze et al., 2010). Shown in the context of cell envelope biogenesis (Fig. 4), our evolving working model of the leptospiral lipoprotein transport pathway consists of LolCDE and LolA orthologs being mainly involved in periplasmic sorting, while surface lipoproteins use two additional, so far uncharacterized modules to facilitate translocation, or 'flipping', of surface lipoproteins through the OM: (i) a periplasmic surface lipoprotein 'holding' chaperone functioning like the chaperones guiding transmembrane proteins (TMPs) to the OM (Bos et al., 2007), and (ii) an OM lipoprotein translocase complex functioning similarly to lipid flippases (Pomorski and Menon, 2006) (Fig. 4).

Alternative routes to the leptospiral surface are possible. Sec-dependent or -independent bacterial protein secretion pathways in *Leptospira* are limited to a type 2 secretion system, which might be involved in lipoprotein secretion tracking the *Klebsiella* model (d'Enfert et al., 1987; Sauvonnet and Pugsley, 1996) (Fig. 4), a twin-arginine translocation (Tat) system, which may provide for export of folded proteins from the cytoplasm to the periplasm (Lee et al., 2006), and a type I secretion system (discussed in 2.14).

2.4. LipL32, the Major Outer Membrane Lipoprotein

As is strikingly apparent in protein stains of whole bacteria fractionated by SDS-PAGE, LipL32 is the most abundant protein in pathogenic *Leptospira* spp. Localization of LipL32 to the outer membrane was demonstrated by Triton X-114 fractionation (Haake et al., 2000) and isolation of leptospiral OM vesicles by sucrose density gradient ultracentrifugation (Haake and Matsunaga, 2002; Nally et al., 2005b). One of the early challenges encountered in these studies was that solubilization with Triton X-114 in the presence of EDTA results in degradation of LipL32 and other leptospiral membrane proteins by endogenous protease(s) when the detergent extract is warmed from 4°C to 37°C. This challenge was overcome by Zuerner et. al. (1991) who found that addition of calcium prior to warming the extract prevented LipL32 degradation. The relationship between LipL32 and calcium was further elucidated by the LipL32 crystal structure, which revealed an acidic pocket formed in part by an extraordinary region of the LipL32 sequence in which seven out of eight amino acids

are aspartates (Vivian et al., 2009). Co-crystalization of LipL32 with calcium showed that two of these aspartates are involved in calcium ion coordination (Hauk et al., 2009). When the aspartates in the calcium-binding pocket were mutated to alanines, denaturation of LipL32 in response to heat was similar with or without calcium. This elegant study used circular dichroism and tryptophan fluorescence to show that calcium helps LipL32 resist thermal denaturation (Hauk et al., 2012).

The abundance of LipL32 contributed greatly to its unfortunate misidentification as a surface lipoprotein. The first studies to claim LipL32 surface localization involved surface biotinylation experiments (Cullen et al., 2003; Cullen et al., 2005). This technique involves addition of the biotinylation reagent sulfo-NHS-LC-biotin, which is considered to be "membrane impermeable" if membranes are intact but is a small enough molecule to penetrate through damaged membranes. This issue is problematic for spirochetes for which the outer membrane is fragile and subject to disruption if organisms are not handled carefully. In this context it is worth noting that in the second of these biotinylation studies, the cytoplasmic protein GroEL and the periplasmic protein FlaB1 were also found to be biotinylated (Cullen et al., 2005). Surface immunoelectron microscopy studies with LipL32 antibodies showed increased labeling of leptospiral cells compared to control antibodies. However, the number of gold particles (10.8 particles per cell) was far below what would have been expected for such an abundant protein. Seemingly confirmatory whole cell ELISA studies added to the confusion. The LipL32 surface protein dogma was recently overturned when more careful surface immunofluorescence studies were performed, including a number of controls including antisera to positive and negative control antigens and comparisons of intact and methanol fixed organisms (Pinne and Haake, 2013). Studies were performed in parallel on intact and fixed organisms and fluorescence microscopy images were obtained using identical exposure times to ensure that they were truly comparable. LipL32 immunofluorescence of intact organisms was mostly negative, but occasionally showed irregular staining patterns, particularly if organisms were disrupted by shear force. However, the homogeneous staining observed with methanol fixed organisms did not occur with intact organisms. These immunofluorescence studies were supplemented with surface proteolysis studies showing that treatment with Proteinase K could digest LipL32 only if organisms were disrupted. Treatment of organisms with Proteinase K had previously been shown to be a reliable method for identifying surface proteins (Pinne and Haake, 2009). In conclusion, LipL32 appears to be located in the periplasmic leaflet of the OM, a location shared by LipL36 (Shang et al., 1996). Based on Triton X-114 fractionation, other subsurface lipoproteins including LipL31 (Haake and Matsunaga, 2002) and LruB (Verma et al., 2005) appear to be restricted to the periplasmic leaflet of the inner membrane. Interestingly, despite its location, LruA (also known as LipL71) modulates interactions with mammalian apolipoprotein A–I (Zhang et al., 2013).

If LipL32 is not on the leptospiral surface to any significant extent, what is its function? This is an important question given that pathogenic leptospires devote such a large amount of their protein-synthetic resources to expression of LipL32. Based on the Triton X-114 and OM vesicle evidence that LipL32 is an OM protein, it would be logical to conclude that the protein is located in the inner leaflet of the outer membrane. Considering the known size of a LipL32 molecule (29 Å × 50 Å) as determined crystallographically (Vivian et al., 2009), the

average length (10 μM) and diameter (0.1 μM) of leptospiral cells, and the quantitation of 38,000 copies of LipL32 molecules per cell (Malmström et al., 2009), it can be estimated that LipL32 occupies an extraordinary 20% of the leptospiral OM inner surface. Perhaps LipL32 serves some structural role, for example in OM stabilization? One possible function is as a calcium sink. Calcium is well known to be important for membrane integrity generally and chelation of divalent cations with EDTA is essential for release of the OM from leptospiral cells (Haake et al., 1991; Haake and Matsunaga, 2002; Nally et al., 2005b). However, LipL32 does not appear to be essential for OM integrity, given that a Himar transposon mutant of *L. interrogans* serovar Manilae lacking LipL32 had normal morphology and growth rate compared to the wild type (Murray et al., 2009b).

Aside from serving as a large calcium sink for leptospiral cells, the function of LipL32 not understood. There is strong evidence that LipL32 is expressed during infection, given that there is intense staining by immunohistochemistry for LipL32 in the kidneys of infected animals (Haake et al., 2000) and that LipL32 is one of the most dominant seroreactive antigens recognized during acute and convalescent leptospirosis (Lessa-Aquino et al., 2013). On the other hand, LipL32 is not essential for infection given that the *lipL32* transposon mutant was able to cause acute, lethal infections in hamsters and chronic infections in rats that were indistinguishable from those caused by the wild type organism (Murray et al., 2009b). Nevertheless, given the large amount of LipL32 expressed by pathogenic leptospires, this protein has the potential to play a critical role in stimulating the host inflammatory response during infection. Purified, native (and therefore lipidated) LipL32 stimulates an innate immune response through TLR 2 (Werts et al., 2001). Inflammation in the kidney, a major target organ during leptospirosis, is manifested by interstitial nephritis. LipL32 induces interstitial nephritis in kidney proximal tubule cells (Yang et al., 2002) and the inflammation induced by LipL32 is mediated by TLR 2 (Yang et al., 2006). For reasons that remain obscure, LipL32 is one of the most highly conserved leptospiral OMPs among pathogenic leptospires, suggesting that it might be a favorable vaccine target for induction of cross-protective immunity. However, results obtained by immunization with a large variety of different LipL32 constructs remain largely negative or at best indeterminate, which may be related in part to its subsurface location. Readers interested in more information on this subject and other aspects of LipL32 are referred to the excellent, recent review by Murray (2013).

2.5. Loa22 and Other OmpA-like Proteins

The second most abundant OM protein is Loa22 (Malmström et al., 2009). While there remains some uncertainty as to whether Loa22 is a lipoprotein, it is covered here because of experimental evidence of lipidation: Expression of Loa22 in *E. coli* resulted in labeling with [^{3}H]palmitate (Koizumi and Watanabe, 2003). This result is somewhat surprising because of the unusual Loa22 lipobox: SFTLC. As mentioned above, virtually all amino acids found in the −1 position relative to cysteine have been relatively small amino acids, and we are unaware of any documentned examples of a large, hydrophobic amino acid like leucine in that location. For this reason, Loa22 is not predicted to be a lipoprotein by the SpLip algorithm. However, it is predicted to be a lipoprotein by the LipoP algorithm. While the [^{3}H]palmitate-labeling data should be considered more convincing than the bioinformatic

data, they would have been more conclusive if the experiment also had been performed in *L. interrogans* and if the label had been shown to be acid labile.

Lipoprotein or not, Loa22 represents a conundrum because it is both surface exposed and binds peptidoglycan via a carboxyterminal OmpA domain. OmpA domains are peptidoglycan binding domains found in proteins that, like OmpA, link membranes to the cell wall situated beneath the OM. In the case of Loa22, the OmpA domain begins at amino acid 111 and occupies more than half the protein. There are strong immunofluorescence data showing that Loa22 is surface exposed (Ristow et al., 2007). One possible explanation for these data is that Loa22, like *E. coli* murein lipoprotein, exists in both peptidoglycan-bound and –free forms. The peptidoglycan-free form of murein lipoprotein has been found to be surface exposed (Cowles et al., 2011). The second explanation is that in the 90 amino acid segment between the signal peptide and the OmpA domain, Loa22 crosses the outer membrane at least once. This 90 amino acid segment is hydrophilic and lacks the amphipathic beta sheets typically found in transmembrane OM proteins. Instead, as shown in Fig. 5A, there is an alpha-helical stretch with a strongly hydrophobic region on one face of the helix. This suggests that Loa22 is similar to the *E. coli* OM lipoprotein Wza, which forms large channels for export of the high molecular weight capsular polysaccharides. Wza forms octamers (Fig. 5B) in which the hydrophobic faces of the Wza monomers interact with the hydrophobic interior of the OM, while the hydrophilic faces form the walls of the channel (Dong et al., 2006). Although the role of Loa22 in the OM remains uncertain, that role appears to be essential for virulence; a *Himar* transposon mutant lacking Loa22 expression was unable to cause lethal infections in hamsters and guinea pigs, although it was able to cause bacteremia and renal colonization (Ristow et al., 2007). It is interesting to note that a homolog of the *loa22* gene with 56% sequence identity is present in *L. biflexa*, indicating that just because a gene is present or not in leptospiral saprophytes does not predict whether it is likely to be required for virulence in leptospiral pathogens.

Loa22 belongs to a family of seven leptospiral OmpA-like proteins. The other members of the family differ from Loa22 in multiple ways. They do not appear to be lipoproteins, they tend to be much larger proteins, and appear to be more typical transmembrane OM proteins along the lines of the *E. coli* version of OmpA. For example, LIC10050 has a signal peptidase 1 cleavage site and is predicted to be a 78-kD protein with 22 beta-sheet transmembrane segments. However, in all cases, leptospiral OmpA-like proteins are probably important in linking the OM to the peptidoglycan cell wall.

2.6. Outer Membrane Lipoprotein LipL41

LipL41 is the third most abundant OM lipoprotein (Malmström et al., 2009). Levels of *lipL41* transcript (Matsui et al., 2012) and LipL41 protein (Cullen et al., 2002; Nally et al., 2001b) are remarkably unaffected by temperature, osmolarity and other environmental factors. The stability of LipL41 expression is useful as a control when studying the effects of growth conditions on the expression of other genes and proteins. For example, LipL41 antiserum is frequently included in immunoblots to compare the loading of bacteria per lane (Matsunaga et al., 2013). Although it is treated as one, it would be incorrect to call *lipL41* a “housekeeping gene” until more is known about its function. Although too preliminary to be

conclusive, a clue to the function of LipL41 is that it was identified as a potential hemin-binding protein in hemin-agarose affinity chromatography (Asuthkar et al., 2007). King et. al. (2013) were unable to confirm hemin binding activity. However, a subsequent study documented a sub-micromolar hemin-LipL41 dissociation constant and identified amino acids involved in hemin binding (Lin et al., 2013). Interestingly, the same study found that LipL41 forms a supramolecular assembly consisting of 36 molecules (Lin et al., 2013).

The *lipL41* gene is located immediately upstream of a smaller gene, with which it is co-transcribed. For this reason, the smaller gene has been designated *lep* for *l*ipL41 *e*xpression *p*artner (King et al., 2013). Even though *lipL41* transcript levels were unaffected in a lep transposon mutant, LipL41 levels were greatly reduced. Because Lep expression appeared to be required for stable expression of LipL41, perhaps by acting as a chaperone, researchers examined whether Lep bound to LipL41. Lep molecules were found to bind to LipL41 molecules at a molar ratio of 2:1 (King et al., 2013). Neither a *lipL41* nor a *lep* mutant was attenuated for virulence in hamsters. Interestingly, Lep was not detected by whole organism MALDI-TOF (Malmström et al., 2009), indicating that Lep is only required in small amounts transiently during export of LipL41 to the outer membrane.

2.7. The Lig Family of OM Lipoproteins

The Lig family of OM lipoproteins was discovered by screening *L. kirschneri* and *L. interrogans* expression libraries with convalescent human leptospirosis sera. This approach identified GroEL, and DnaK, and LipL41, and three novel genes encoding a series of bacterial immunoglobulin (Ig)-like domains. The proteins encoded by these novel genes were designated as **L**eptospiral **Ig**-like proteins LigA, LigB, and LigC (Matsunaga et al., 2003). The Lig proteins consist of a lipoprotein signal peptide followed by a series of 12–13 Ig-like domains and, in the case of LigB and LigC, a large carboxyterminal domain. The region upstream of *ligA* and *ligB*, as well as the first six Ig-like domains of LigA and LigB, are virtually identical, indicating that the *ligA* gene resulted from a partial gene duplication event. This event likely occurred relatively late in leptospiral evolution, as *ligA* is found only found in stains of *L. kirschneri* and *L. interrogans* (McBride et al., 2009). In contrast, LigB is found in all pathogenic *Leptospira* species. LigC is also widely distributed but is a pseudogene or absent in some strains. Sequence comparison revealed a surprising degree of mosaicism, indicating genetic rearrangements involving *ligB* gene fragments of *L. interrogans* and *L. kirschneri* (McBride et al., 2009). OMP mosaicism can confer a survival advantage in the face of antigenic pressure.

Temperature and osmolarity are key environmental signals that control the expression of the Lig proteins. In the process of examining the interaction of leptospires with cells in tissue culture, Matsunaga et al., (2005) observed that the addition of EMEM tissue culture medium to leptospiral culture medium induced LigA and LigB expression and caused a substantial increase in released LigA. Sodium chloride was primarily responsible for these effects. All other EMEM components, including iron, bicarbonate, and oxygen concentrations, had no effect on Lig expression. As shown in Fig. 6, addition of sodium chloride, potassium chloride, or sodium sulfate to leptospiral medium (EMJH) to the level of osmolarity found in the mammalian host (~300 mOsm/L) induced expression of both cell-associated LigA and

LigB, and release of LigA into the culture supernatant. Osmolarity affects both *lig* transcript and Lig protein levels (Matsunaga et al., 2007b). In addition to its effects on Lig protein expression, osmolarity increases the transcription of the leptospiral sphingomyelinase, Sph2 (Matsunaga et al., 2007b), the putative adhesin, LipL53 (Oliveira et al., 2010), and a number leptospiral lipoproteins and OMPs (Matsunaga et al., 2007a). These results suggest that leptospires upregulate a defined set of OMPs when they encounter mammalian host tissues and sense an increase in osmolarity. The sensory transduction proteins involved in osmoregulation have not yet been defined.

More recently, it was discovered that expression of the *lig* genes is also regulated by temperature. The long 175 nucleotide 5′ untranslated region is predicted to contain secondary structure that includes and obscures the ribosome binding site and start codon, preventing binding to the ribosome and initiation of translation (Fig. 7). Toe-print experiments showed binding of ribosomes to the *lig* transcript was poor unless most of the left stem of predicted structure 2 (Fig. 7) was removed. In *E. coli*, a *lig'-'bgaB* translational fusion transcribed from a heterologous promoter was regulated by temperature, demonstrating the ability of the *lig* sequences to exert post-transcriptional control by temperature. Mutations on the left or right stem of structure 2 partially relieved inhibition of β-galactosidase expression; inhibition was regained when the mutations were combined to restore base pairing, providing evidence that base-paired RNA is a component of the inhibitory element. These results are consistent with a model in which structure 2 functions as a thermolabile "thermometer," trans-acting factors may also have a dominant role in melting the inhibitory stem.

The upregulation of LigA and LigB by osmolarity and temperature suggests that these proteins are expressed early during mammalian host infection and may be involved in critical bacterial-host interactions. Various lines of evidence support these conclusions. Patients with leptospirosis have a strong antibody response to the Lig Ig-like repeat domains, suggesting that recombinant Lig repeats would be useful serodiagnostic antigens, confirming that Lig proteins are expressed during infection (Croda et al., 2007). Lig proteins are expressed on the leptospiral surface based on immunoelectron microscopy (Matsunaga et al., 2003) and LigA is released from leptospiral cells (Matsunaga et al., 2005). Osmotic induction of Lig expression resulted in *L. interrogans* becoming more "sticky", with increased adherence to several different extracellular matrix proteins, including fibronectin, fibrinogen, and collagens I and IV (Choy et al., 2007). Heterologous expression of LigA and LigB in *L. biflexa* increased adherence to eukaryotic cells and fibronectin (Figueira et al., 2011). We advocate this "gain of function" approach when studying potential leptospiral adhesins as a way to evaluate the significance of protein-protein interaction assays. LigB binds more avidly to fibronectin and fibrinogen than LigA and the LigB binding activity was localized to 3 of the 12 LigB Ig-like domains; domains 9–11 were both necessary and sufficient to reproduce the binding activity of LigB (Choy et al., 2011). A remarkable aspect of these studies is the range of different proteins to which LigB is able to bind with high avidity. LigB not only binds to complement components and the complement regulatory protein, Factor H, but also inhibits complement activity (Castiblanco-Valencia et al., 2012;

Choy, 2012). These results suggest that a role of LigB is to coat the leptospiral surface with a variety of circulating host proteins and protect leptospires from host defense mechanisms.

Leptospiral vaccines are discussed in Chapter 10, while their use in humans and animals is described in Chapters 5 and 6. Nevertheless, it should be mentioned in this context that when *L. interrogans* sv Copenhageni is the challenge strain, immunization of hamsters with LigA converts a lethal infection into sublethal kidney colonization. The initial studies showed that the unique part of LigA (Ig-like domains 7–13) was most effective as a vaccine (Silva et al., 2007). Subsequent studies localized the Ig-like domains involved in immunoprotection (Coutinho et al., 2011). There was an absolute requirement for LigA domains 11 and 12. However, these two domains were not sufficient for immunoprotection; a third, flanking domain (either domain 10 or 13) was needed. This requirement for three contiguous Ig-like domains near the carboxy-terminal end of the molecule is highly reminiscent of the finding that LigB domains 9–11 are required for binding activity (see previous paragraph). LigA immunization is effective not only when injected subcutaneously as a purified, recombinant protein, but also when expressed in a lipidated form in *E. coli* that is administered orally (Lourdault et al., 2013). Some important caveats are in order. LigA does not provide sterilizing immunity and because the immunoprotective region of the LigA molecule is subject to variation (McBride et al., 2009), cross-protective immunity may be limited. Additionally, no homologous protection was elicited following immunization of hamsters with LigA from *L. interrogans* serovars Manilae (Deveson Lucas et al., 2011) or Canicola (N. Bomchil, personal communication). An important goal of future studies is to understand why LigA appears to protect against challenge by some serovars but not others.

2.8. More Outer Membrane Lipoproteins

As summarized in Table 1, quantitative MALDI-TOF data reveal that after LipL32, Loa22, and LipL41, the next most abundant outer membrane lipoproteins are LipL36, LipL21, and LipL46 (Malmström et al., 2009). Although LipL36 is an outer membrane protein, it is not surface exposed, being restricted to the inner leaflet of the outer membrane (Haake et al., 1998). Based on serological evidence (Haake et al., 1998), immunohistochemistry data (Barnett et al., 1999) and downregulation of LipL36 expression at physiologic osmolarity (Matsunaga et al., 2007), LipL36 appears to be expressed only when leptospires are outside the mammalian host. In contrast to LipL36, LipL21 and LipL46 are both surface-exposed and expressed during infection (Cullen et al., 2003, Matsunaga et al., 2006). While not quite as abundant as originally thought, LipL21 is highly expressed during infection based on immunoblot analysis of organisms harvested from infected guinea pigs (Nally et al., 2007) and immunohistochemistry of liver from infected hamsters (Eshghi et al., 2009). LipL46 can also be detected immunohistochemically in a variety of organs during infection (Matsunaga et al., 2006).

A fundamental difference between leptospiral saprophytes and pathogens is that saprophytes are serum sensitive while pathogens are serum resistant. A common serum resistance mechanism shared by many bacterial pathogens is binding the complement regulators factor H and factor H protein-1. Using a ligand blot approach, *L. interrogans* was found to have two factor H-binding proteins with molecular masses of 25- and 50-kDa. The 25-kDa factor

H-binding protein was initialy referred to as LfhA (leptospiral factor H-binding protein) (Verma et al., 2006). A subsequent study identified the same protein as a laminin binding adhesin and applied the designation Lsa24 (leptospiral surface adhesin 24-kD) (Barbosa et al., 2006). Structural analysis revealed that LfhA/Lsa24 was a member of a family of six leptospiral adhesins that share structural similarities with endostatin (Stevenson et al., 2007). For this reason, LfhA/Lsa24 was renamed LenA. In addition to binding Factor H, LenA was subsequently found to bind plasminogen (Verma et al., 2010). Binding of LenA to plasminogen facilitated conversion to plasmin, which in turn degraded fibrinogen, suggesting a role for LenA in penetration through, and/or escape from, fibrin clots. Several other leptospiral OMPs have also been implicated in plasminogen binding and activation (Fernandes et al., 2012; Vieira et al., 2012).

2.9. Transmembrane Outer Membrane Proteins

Transmembrane OMPs are defined as integral outer membrane proteins that contain strands that traverse the lipid bilayer of the outer membrane. Such proteins can be visualized by freeze-fracture electron microscopy (FFEM), a technique that separates the two leaflets of membranes, exposing transmembrane OMPs as studs in a sea of lipid. When applied to spirochetes, FFEM revealed that pathogenic spirochetes, including leptospires, have transmembrane OMPs in far fewer numbers than typical Gram-negative bacteria (Haake et al., 1991; Radolf et al., 1989; Walker et al., 1991). Transmembrane OMPs are essential for OM-containing bacteria because of their unique ability to form pores or channels that allow bacteria to acquire nutrients and to export toxins and waste products. For researchers interested in bacterial surface antigens, transmembrane OMPs are of great interest because their surface-exposed loops represent potential targets of a protective immune response.

Transmembrane OMPs have an amino-terminal signal peptide, which facilitates their secretion across the inner membrane to the periplasm by the Sec translocase complex. After removal of the signal peptide by signal peptidase I, transmembrane OMPs are shuttled across the periplasm to the OM by the chaperone SurA (Sklar et al., 2007). LIC12922 of *L. interrogans* serovar Copenhageni has been identified by X-ray crystallography to have both the parvulin and peptide-binding domains of SurA (Giuseppe et al., 2011). The peptide-binding domain allows SurA to keep transmembrane OMPs in an unfolded form until they are delivered to the OMP assembly complex, which consists of the transmembrane OMP, BamA, and several accessory lipoproteins. *L. interrogans* has a BamA homologue with four POTRA domains that are involved in the folding, assembly and insertion of transmembrane OMPs in the OM (Tommassen, 2007).

2.10. Discovery of the Porin OmpL1

OmpL1 was one of the first porins to be described in a spirochete, preceded only by the 36.5 kD porin of *Spirochaeta aurantia* (Kropinski et al., 1987). The discovery of OmpL1 resulted from experiments aimed at identifying surface-exposed OMPs. Using a technique called “surface immunoprecipitation”, antibodies raised to whole *L. kirschneri* bacteria were added to intact bacteria followed by gentle washing to remove unbound antibodies. The antibody-antigen complexes were solubilized using Triton X-100 detergent and then purified using Protein A beads. In addition to LPS, the surface immunoprecipitate was found to contain

three proteins with molecular masses of 33-, 41-, and 44-kD (Haake et al., 1991). The amount of the 33-kD protein was increased in a highly-passaged strain of *L. kirchneri*, correlating with the density of transmembrane particles visualized by FFEM. Isolation of the gene encoding the 33-kD protein revealed a series of porin-like transmembrane segments (see next section), and henceforth the protein was called OmpL1 (Haake et al., 1993). The other two proteins were subsequently identified as LipL41 and LipL46 (see above). Confirming its role in the leptospiral OM, OmpL1 was later found to have several other properties typical of porins, including: 1. Heat-modifiable electrophoretic mobility; 2. Cross-linkable trimers; and 3. The ability to form channels in lipid bilayers (Shang et al., 1995).

Bacterial porins are of great interest because of their surface exposure and potential to serve as targets of a protective immune response. Like most porins, OmpL1 is hydrophobic and requires detergent for solubilization. Recombinant OmpL1 expressed in *E. coli* with a His6 tag can be purified by nickel chromatography under denaturing conditions. Unfortunately, this denatured form of OmpL1 proved to be ineffective as a vaccine (unpublished results). However, when hamsters were immunized with OmpL1 expressed in *E. coli* as a membrane protein, this resulted in partial protection from lethal and sublethal infection, particularly when combined with a lipidated form of LipL41 (Haake et al., 1999). The *ompL1* gene is present and moderately well conserved (~90% deduced amino acid sequence identity) across a broad range of pathogenic *Leptospira* species. Interestingly, comparison of sequences from a number of *Leptospira* strains revealed that 20% of strains carried mosaic *ompL1* genes composed of segments with multiple leptospiral ancestries arising from horizontal DNA transfer and genetic recombination (Haake et al., 2004). These sequence variations, of course, could limit cross-protection from an OmpL1-based vaccine. Other leptospiral genes that have been found to undergo mosaicism include *ligA* and *ligB* (McBride et al., 2009).

2.11. Beta-Barrel Structure of Transmembrane OMPs

As mentioned in the previous section, OmpL1 has a series of trans-membrane segments characteristic of channel-forming porins. The transmembrane segments of a number of OMPs from a variety of Gram-negative bacteria have been determined by X-ray crystallography to have a beta-sheet conformation, such that the orientation of amino acid side chains is 180° opposite of those of adjacent amino acids. This allows the side chains of alternating amino acids to interface with the lipid bilayer or with the aqueous pore of the channel. As these transmembrane segments thread their way back and forth across the lipid bilayer, they form the walls of a cylinder or barrel, and such proteins are called “beta barrels”. The beta-sheet conformation in these transmembrane strands is the basis for transmembrane OMP prediction programs such as TMBB-PRED (Bagos et al., 2004) and TMBETA-NET (Gromiha and Suwa, 2005).

Screening of the *L. interrogans* serovar Copenhageni genome for OMPs by querying the TMBB-PRED webserver revealed 84 genes that met the relatively stringent cutoff score of ≤ 2.965. As a positive control, the TMBB-PRED algorithm gave OmpL1 a score of 2.900, the sixth best score of any leptospiral protein. A useful feature of TMBB-PRED is that the output includes a plot of the probability of transmembrane membrane beta-strands. As shown in Fig. 8, the TonB-dependent receptor, HbpA (see below) received a score of 2.939

and was predicted to have 22 transmembrane beta strands. Using homology-based annotation and sequence-based criteria (signal peptide + ≤3 alpha helices + ≥6 transmembrane beta strands) a list of 184 possible transmembrane OMPs was derived (Pinne et al., 2012). These putative transmembrane OMPs and 177 predicted lipoproteins were expressed by in vitro transcription/translation to construct an OMP proteome array to screen for adherence to fibronectin. 14 novel leptospiral fibronectin binding proteins were identified, including Lsa66, a previously identified OmpA-like adhesin (Oliveira et al., 2011). Adherence function was confirmed by expression of proteins in *L. biflexa*, conferring dramatically increased fibronectin-binding activity on this surrogate host.

2.12. Experimental Validation of Transmembrane OMPs

A new paradigm has emerged for experimental confirmation of transmembrane OMPs. Originally, Triton X-114 detergent extraction and phase partitioning was thought to be a more or less definitive test for localization of leptospiral proteins (Haake et al., 1991). OMPs were expected to be found, in whole or in part, in the Triton X-114 detergent phase, while cytoplasmic and inner membrane proteins remained in the protoplasmic cylinder fraction and periplasmic proteins fractionated to the aqueous phase. Although many outer membrane components, including lipopolysaccharide and many OMPs, were found in the Triton X-114 detergent phase, it is now clear that a number of transmembrane OMPs do not behave as expected in this detergent (Pinne and Haake, 2009).

We now advocate a multistep strategy for defining transmembrane OMPs. The first step is sequence analysis. The sequence of transmembrane OMPs should begin with a signal peptide and signal peptidase I cleavage site but lack a lipobox (see above). The sequence of the mature protein should contain multiple beta-sheet transmembrane segments (predicted using an algorithm such as TMBB-PRED) and should not contain a hydrophobic, membrane spanning alpha helix. Of course, OMPs such as Loa22 with alpha-helical transmembrane domains are an exception to this rule. The second step is to test whether the protein is an integral membrane protein by treating total leptospiral membranes with reagents, such as high salt, urea, or sodium bicarbonate, that remove membrane-associated proteins (Matsunaga et al., 2002; Pinne and Haake, 2009). The third step is to test for surface exposure. Conclusions should not be based on a single method. Several complementary methods are available: surface immunofluorescence, surface proteolysis, and surface biotinylation. In each of these methods, it is essential to include controls. In the case of surface immunofluorescence, control experiments with pre-immune sera to show that antibody binding to the leptospiral surface is a result of immunization with the protein of interest must be included. In negative control experiments, it is important to counter-stain the slide with DAPI (4′,6-diamidino-2-phenylindole) to show that organisms are present. OmpL1 as a positive control for surface exposure and the endoflagellar protein FlaA1 as a subsurface control must be included. Relatively abundant periplasmic proteins such as FlaA1 are preferred as subsurface controls because these would more readily become surface exposed as a result of outer membrane disruption than cytoplasmic proteins such as GroEL. Information about obtaining antisera for surface and subsurface control antigens is available on our website: http://id-ucla.org/sharing.php.

Using this strategy, four novel leptospiral transmembrane OMPs were defined: OmpL36, OmpL37, OmpL47, and OmpL54 (Pinne and Haake, 2009). Each of these four proteins was found to have a signal peptide and signal peptidase I cleavage site and at least 6 membrane-spanning beta strands. Although OmpL36 and OmpL37 were partially removed from total membrane fractions by sodium bicarbonate, none was removed by high salt or urea. All four proteins were found to be surface exposed by surface immunofluorescence, surface proteolysis, and surface biotinylation. except for OmpL36, which was not digested by the highest concentration of proteinase K. It should be noted that OmpL47 (also known as Q8F8Q0) had previously been identified by surface biotinylation as a component of the leptospiral "surfaceome" (Cullen et al., 2005) and is annotated as a glycosyl hydrolase. The behavior of these proteins in Triton X-114 cell fractionation experiments was surprising in that only OmpL54 was found in the Triton X-114 detergent phase. OmpL36 was not extractable with Triton X-114 and was found entirely in the protoplasmic cylinder fraction, which is consistent with the subsequent finding that this protein is a flagellar component (Wunder et al., 2013). While OmpL37 and OmpL47 were partially or completely extracted with Triton X-114, these proteins fractionated into the aqueous phase rather than the detergent phase. These results suggest that localization by Triton X-114 fractionation alone may be unreliable for some types of proteins, especially transmembrane OMPs.

2.13. OMPs Involved in Import Pathways

Pathogenic and saprophytic leptospires appear to have a full complement of TonB-dependent receptors (TB-DRs). TB-DRs are beta-barrel OMPs that function as high affinity receptors and channels for uptake of substrates such as vitamin B_{12} (cobalamin), iron, and other heavy metals. Uptake is energy- dependent and requires interactions between TB-DRs in the OM and TonB in the IM. *L. interrogans* has 12 genes encoding TB-DRs and 3 genes encoding TonB. Thanks to the elegant work of Picardeau and colleagues on TB-DRs of *L. biflexa*, the function of several leptospiral TB-DRs is now known (Louvel et al., 2006). For example, the *L. biflexa* mutant lacking gene LEPBIa2760 was unable to grow on the siderophore desferrioximine as a source of iron, thereby indicating that this gene encodes the siderophore uptake receptor CirA. Because many TB-DRs are highly conserved across leptospiral species, this information is relevant to pathogenic leptospires. The amino acid sequence of LEPBIa2760 is 77% identical with that of LIC11694. Likewise, LEPBIa1883 and LIC10714 encode the Fe^{3+}-dicitrate receptor FecA. As shown in Fig. 9, both LIC11694 (CirA) and LIC10714 (FecA) have paralogs that presumably perform similar, if not redundant, functions. LIC20151 has been shown to bind hemin, and represents a third TB-DR class. Three additional TB-DR classes remain to be characterized, but presumably are involved in uptake of vitamin B12, copper, or nickel (Schauer et al., 2008). Leptospires also have OM proteins involved in TonB-independent import pathways, such as FadL (LIC12524), the long-chain fatty acid transporter.

2.14. OMPs Involved in Export Pathways (TolC and GspD)

Leptospires have at least two different OMP-mediated export pathways: Type 1 secretion involving TolC and Type 2 secretion involving GspD. Type 1 secretion is Sec-independent, meaning that substrates can be exported directly from the cytoplasm. In the case of proteins (e.g. hemolysins), this means that a signal peptide is not required. Type I secretion can also

be involved in efflux of drugs or toxins, such as heavy metals. TolC is the OMP component of the Type 1 secretory apparatus and forms a beta barrel channel in the OM and spans the periplasm to the IM where it engages with a translocase to form a contiguous passage from the cytoplasm to the exterior of the cell. *L. interrogans* encodes seven TolC homologs, presumably to accommodate different types of translocases and substrates. One of these TolC proteins, LIC12575, is expressed at high levels in cultivated cells. Type 2 secretion is Sec-dependent, meaning that proteins exported via this pathway must have a signal peptide and be secreted first to the periplasm before exiting the cell. As discussed above, Type 2 secretion represents a potential pathway for lipoprotein export in *Leptospira* species, as has been demonstrated in *Klebsiella* (d'Enfert et al., 1987; Sauvonnet and Pugsley, 1996). Possible substrates include potential lipoproteins LigA and Sph2, which are released from *L. interrogans* in response to elevated osmolarity and/or temperature (Matsunaga et al., 2005; 2007b).

2.15. LipL45 and Related Peripheral Membrane Proteins

LipL45 was first identified as a protein, designated Qlp42, whose expression was upregulated when *L. interrogans* cultures were shifted from 30° to 37°C (Nally et al., 2001a). Subsequent studies revealed that Qlp42 was initially expressed as a 45-kD lipoprotein, the carboxyterminal portion of which was removed to become a 31-kD peripheral membrane protein, designated $P31_{LipL45}$ (Matsunaga et al., 2002). Peripheral membrane proteins are membrane-associated proteins that are not integrated into the lipid bilayer and can be removed by treating membranes with a variety of reagents such as high salt, urea, or sodium bicarbonate. The latter two reagents removed $P31_{LipL45}$ from *L. interrogans* membranes, but had no effect on LipL41. Interestingly, in addition to upregulation of expression at higher temperatures, $P31_{LipL45}$ was dramatically increased in stationary phase cultures of *L. interrogans*. The function, membrane location(s) and surface exposure of $P31_{LipL45}$ remain to be determined. Genome sequencing has revealed that LipL45 belongs to a large family of leptospiral proteins; *L. interrogans* has 11 LipL45-related genes, most of which are predicted to be lipoproteins. Although LipL45 itself is the most highly expressed member of the family in cultivated cells (Malmström et al., 2009), two other family members are expressed at comparable levels, which probably explains why $P31_{LipL45}$ appears as a doublet in many strains of pathogenic leptospires (Matsunaga et al., 2002).

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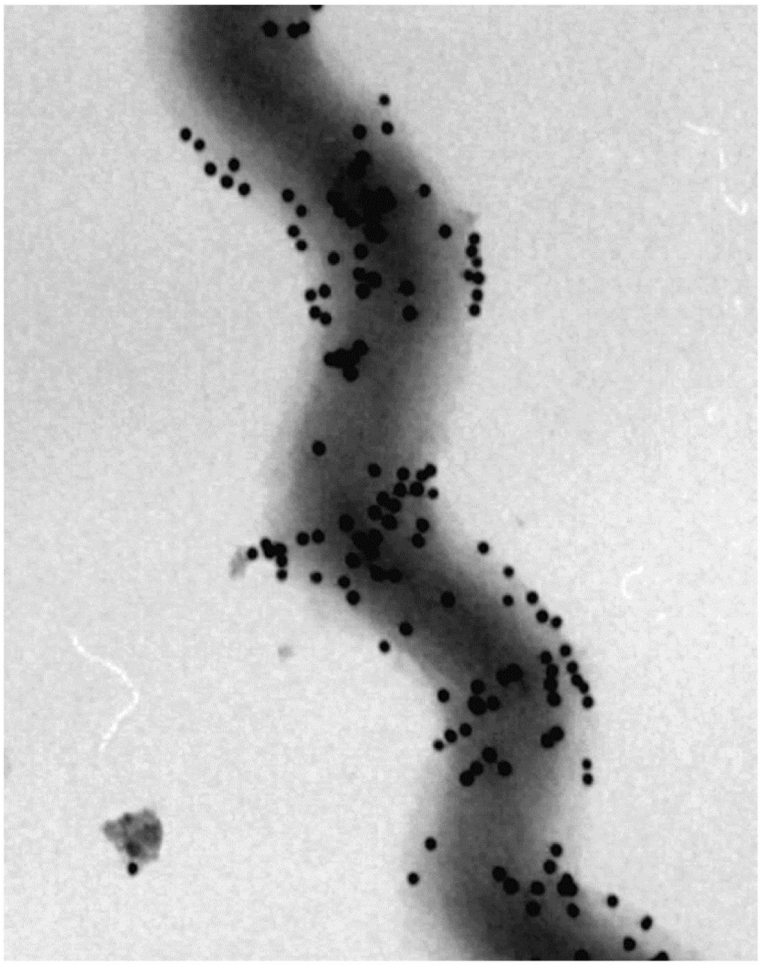

Fig. 1. *Leptospira interrogans* coated with gold-labeled anti-LPS monoclonal antibodies
The number of electron-dense particles reflects the level of LPS exposure on the leptospiral surface.

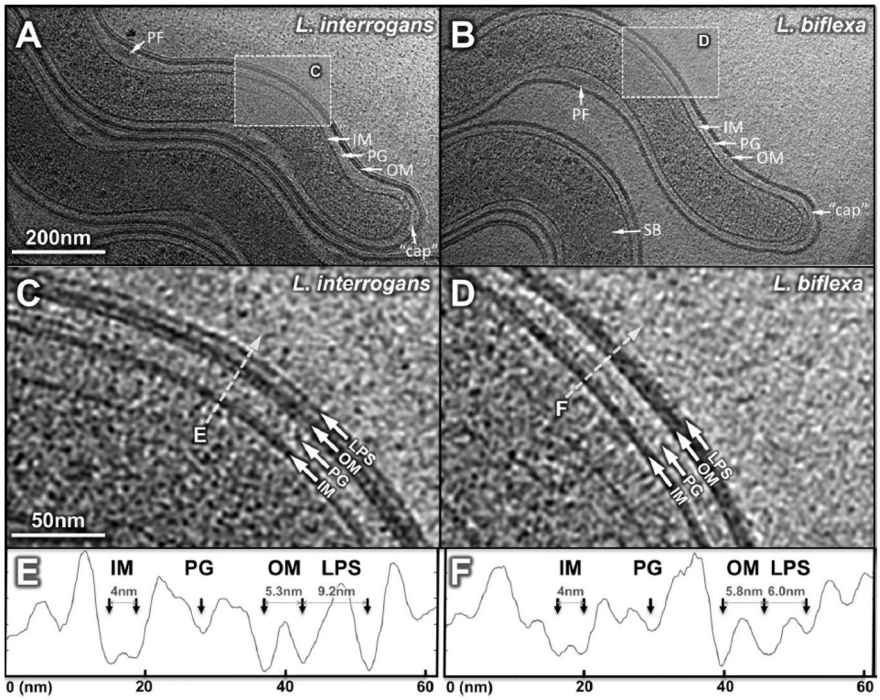

Fig. 2. Cryoelectron tomography of *L. interrogans* vs *L. biflexa*

The thickness of the LPS layer of *L. interrogans* is 9.2 nm vs. 6.0 nm for *L. biflexa*. The increased thickness of the *L. interrogans* LPS layer is probably important for virulence. Reproduced from Raddi et al (2012).

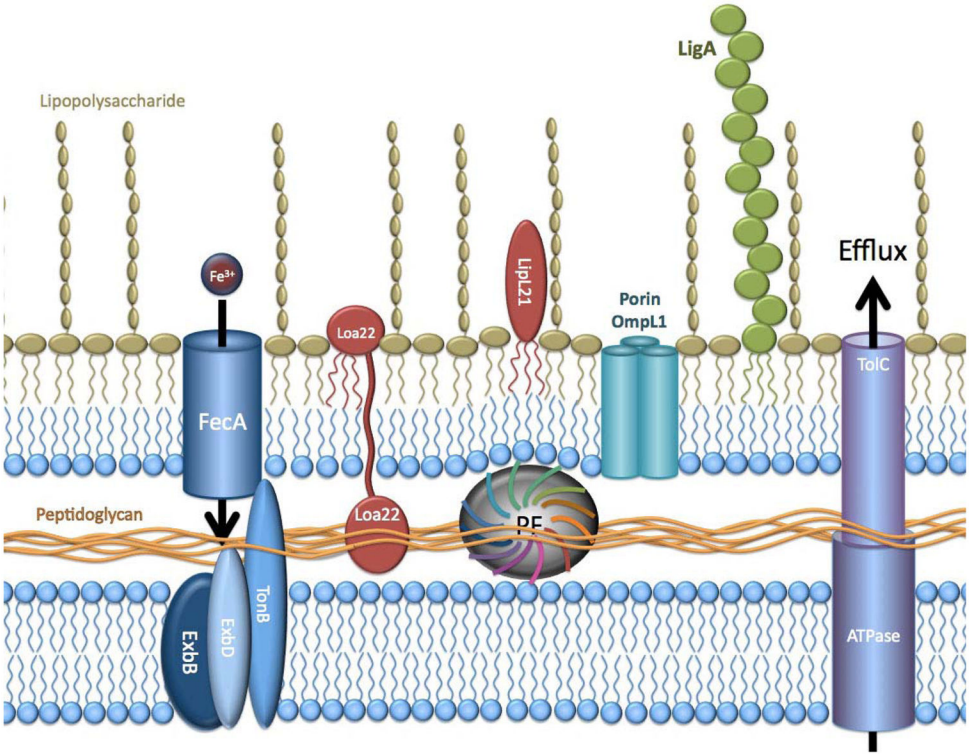

Fig. 3. Membrane architecture of *L. interrogans*

The outer membrane contains lipopolysaccharide, lipoproteins such as LipL21, Loa22, and LigA, and transmembrane proteins such as FecA, OmpL1, TolC, and possibly Loa22. Peptidoglycan and the periplasmic flagella (PF) are located in the periplasm.

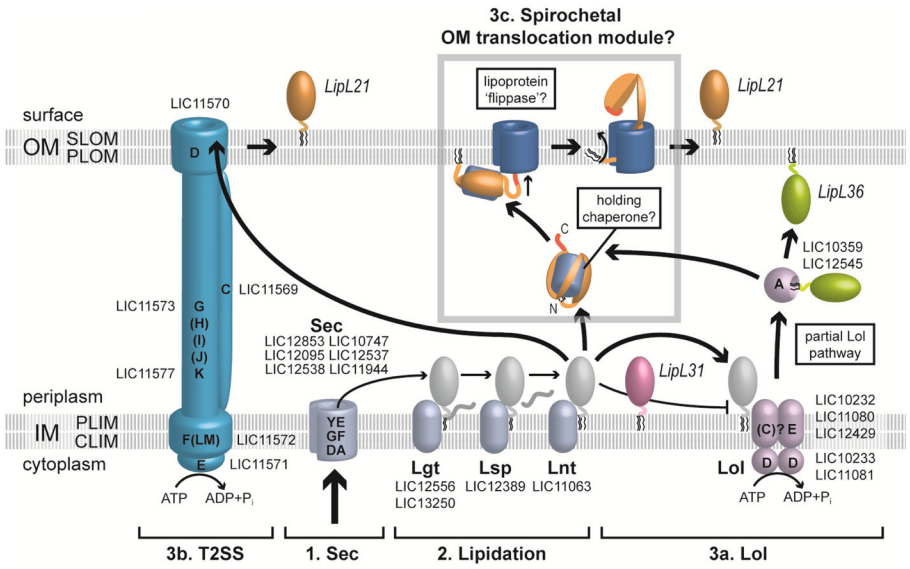

Fig. 4. Leptospiral lipoprotein export

Lipoproteins are exported via the Sec pathway (Step 1) from the cytoplasm to the periplasmic leaflet of the inner membrane (PLIM) where they are lipidated (Step 2). After lipidation, export to the periplasmic leaflet of the outer membrane (PLOM) occurs via the Lol pathway (Step 3a). Export to the surface leaflet of the outer membrane (SLOM) could occur either by the Type II Secretion System (T2SS, Step 3b) or by a lipoprotein flippase.

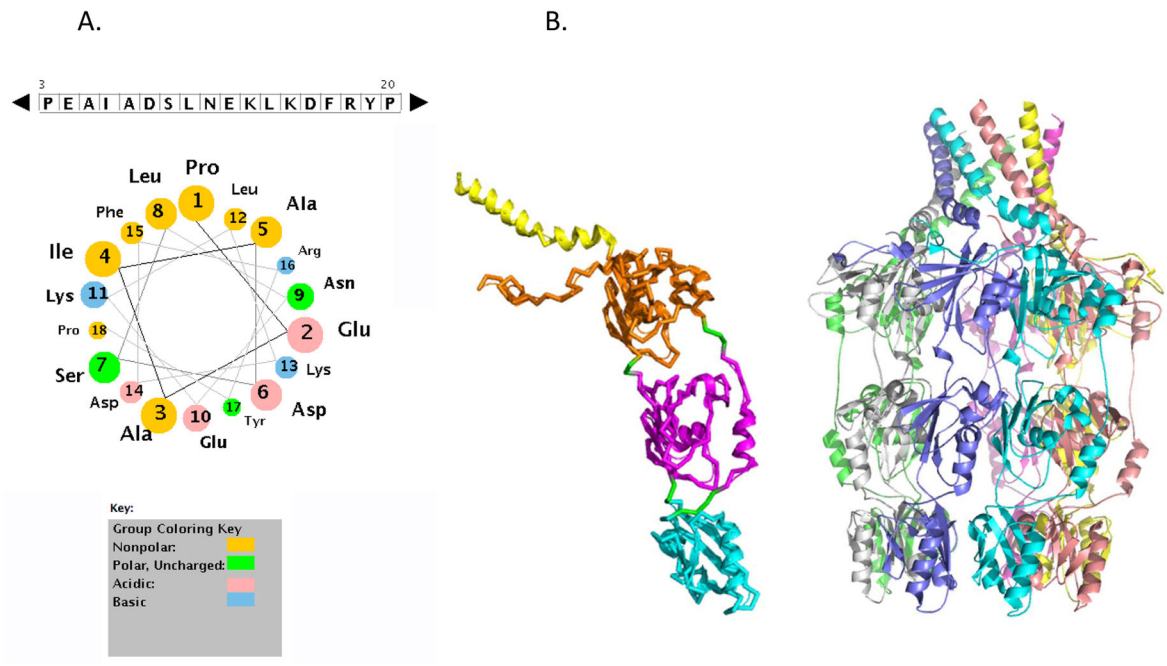

Fig. 5. Loa22 as an alpha-helical transmembrane outer membrane protein

Panel A shows a helical wheel for the putative alpha-helical transmembrane domain of Loa22. The collection of nonpolar residues on one face indicates that the transmembrane helix could be amphipathic. Panel B shows the monomeric and octameric forms of Wza, which serves as a model for how Loa22 crosses the outer membrane. Reproduced from Dong et al (2006).

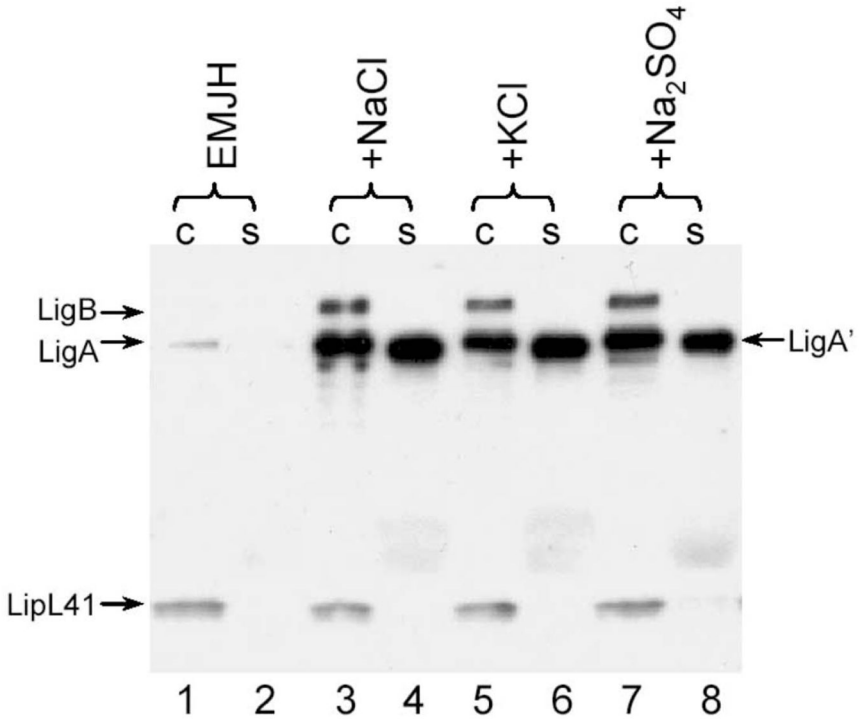

Fig. 6. Induction of Lig expression by osmolarity

Expression of LigA and LigB is strongly induced by addition of salt to Ellinghau-sen-McCullough-Johnson-Harris (EMJH) medium. LigA is found in both the cellular (c) and supernatant (s) fractions. A variety of salts are effective, indicating that induction of Lig expression is mediated by osmolarity rather than any particular salt component.

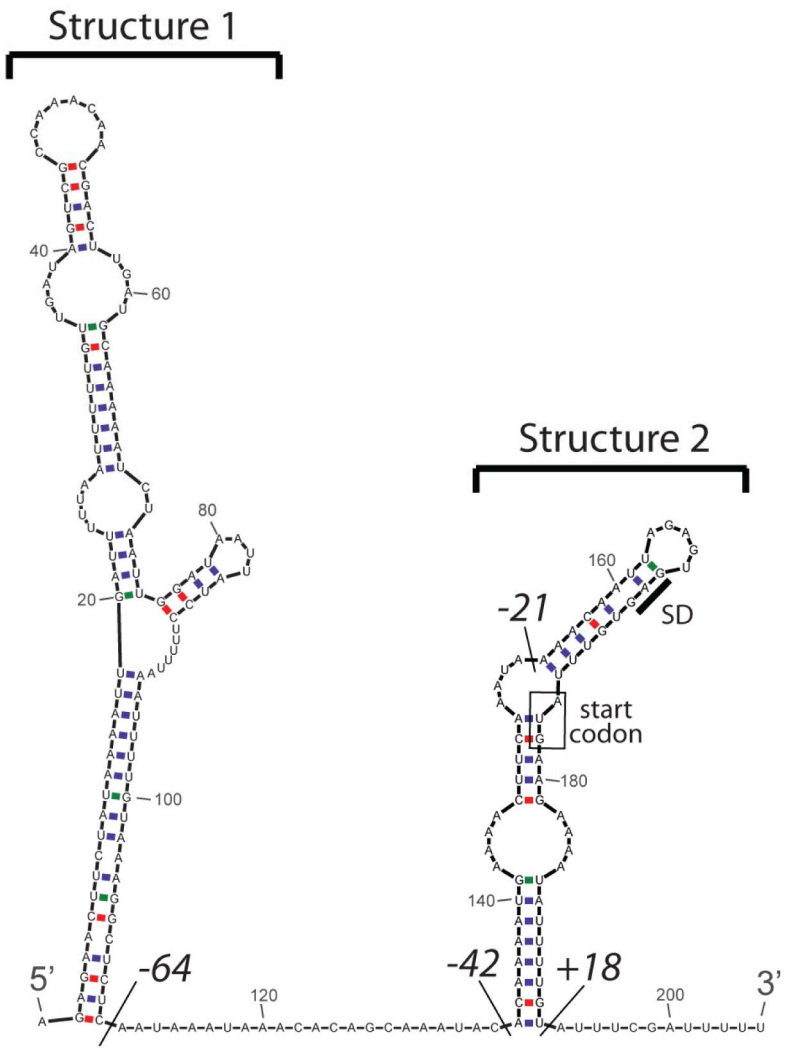

Fig. 7. Secondary structure of the 5′ untranslated region of the *lig* genes

The mRNA of the lig genes has an unusually long 5′ untranslated region which is predicted to form two stem-loop structures. Structure 2 obscures the ribosome binding site (SD) and start codon and must be unfolded for translation to occur.

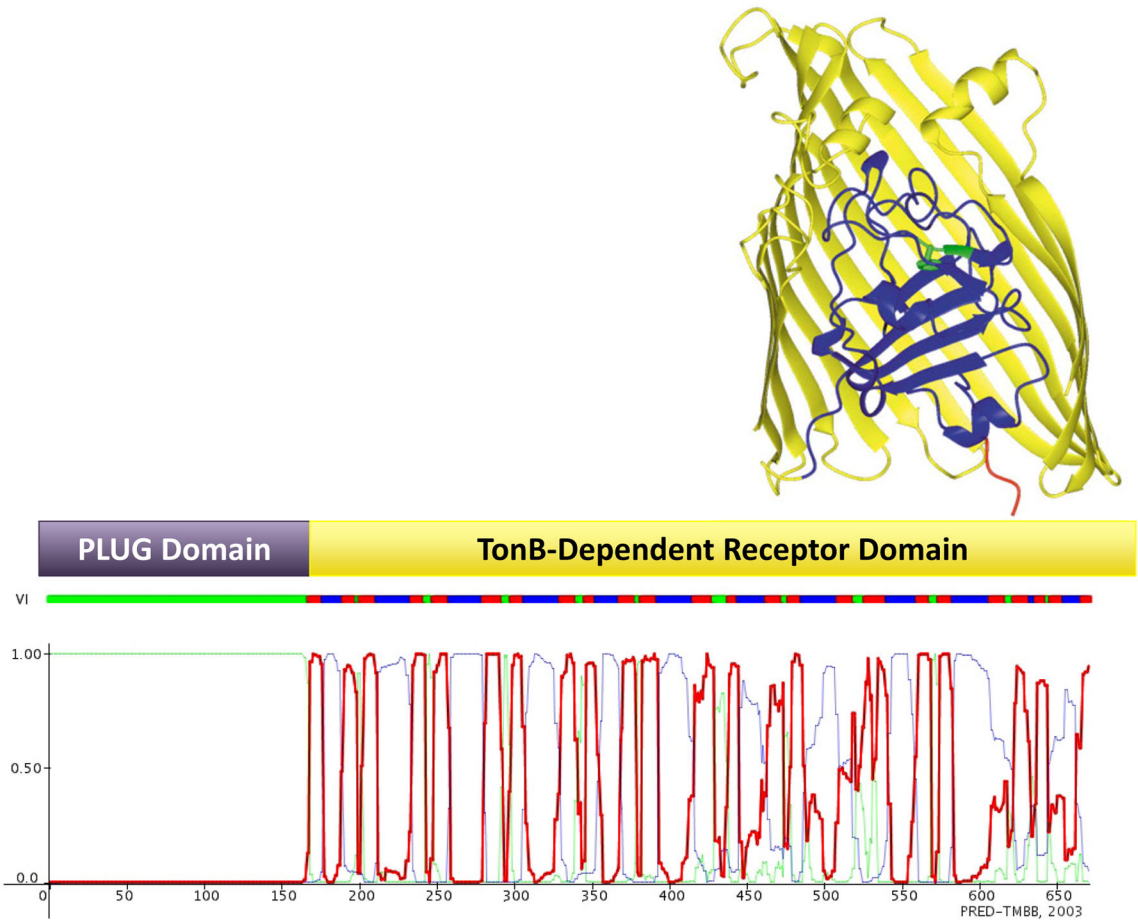

Fig. 8. Topology of TonB-dependent receptor HbpA

Hemin binding protein A (HbpA, LIC20151) is predicted to have a PLUG domain and a TonB Dependent Receptor (TBDR) domain. The PLUG domain sits inside the beta-barrel formed by the TBDR domain, reproduced from Oke et al (2004). The beta-barrel structure is predicted using the TMBB-PRED algorithm.

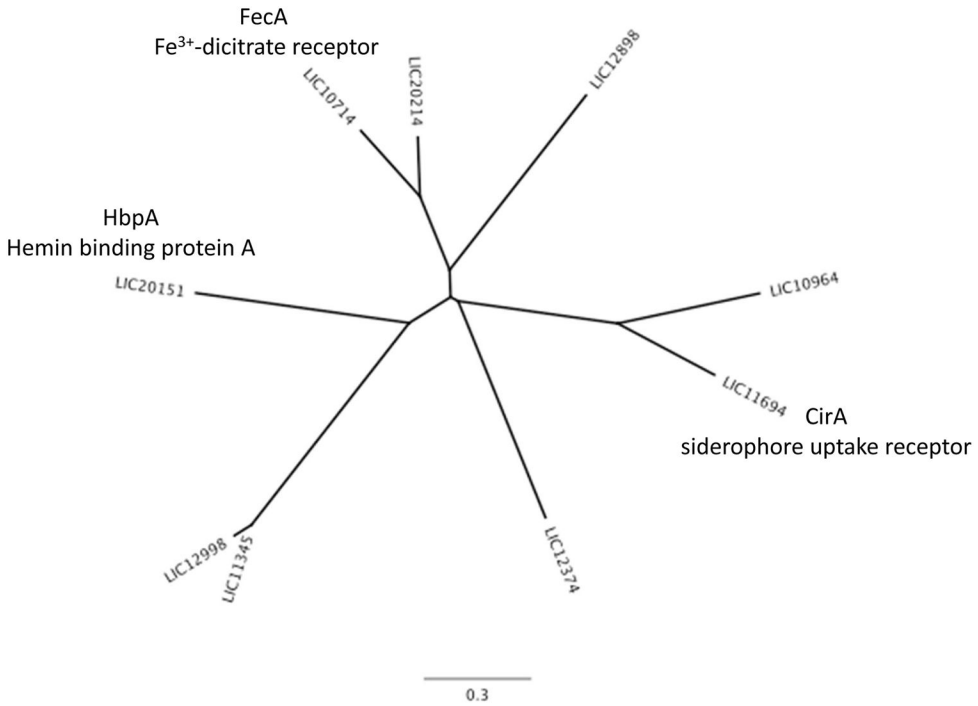

Fig. 9. Relatedness tree for leptospiral TonB dependent receptors (TBDRs)

The *L. interrogans* serovar Copenhageni strain L1-130 genome is predicted to contain 9 TBDR genes involved in uptake of vitamin B12, iron and other metals. The functions of the three TBDR genes that have been elucidated are shown.

Table 1

Candidate and known leptospiral OM proteins

Name	Locus tag[a]	Type[b]	Size (kd)[c]	Copy number[d]	Knockout virulent?	*L. biflexa* % identity[e]	Putative function(s), comment[f]
LipL21	10,011	Lip	21	8,830	–	46	–
OmpA	10,050	TM	78*	216*		44	Contains OmpA domain
Loa22	10,191	Lip	22	30,329	No	56	Binds peptidoglycan
OmpA	10,258	TM	68*	75*	–	28	Binds peptidoglycan
LigB	10,464	Lip	200	914*	Yes	–	Binds fn, fg, cI, cIV, elst, Ca2+*
LigA	10,465	Lip	130	553*	–	–	Binds fn, fg, col-I, col-IV*
OmpA	10,592	TM	52	118*		39	Also known as Omp52
FecA	10,714	TM	92.5*	529	–	49	TonB DR for Fe^{3+}-dicitrate*
CirA	10,964	TM	86.6*	ND		31	Siderophore uptake receptor
OmpL1	10,973	TM	33	5,441	–	–	Porin*
LipL32	11,352	Lip	32	38,050	Yes	–	Binds Ca2+, lam, cIV, fn*
OstA	11,458	TM	113.5*	145	–	49	LPS assembly
GspD	11,570	TM	66.5*	658	–	62	T2SS channel
BamA	11,623	TM	113*	37	–	64	OMP biogenesis
CirA	11,694	TM	92*	ND		60	Siderophore uptake receptor
LipL46	11,885	Lip	46	5,276	–	54	–
Omp85	12,254	TM	60*	136*	–	–	–
OmpL37	12,263	TM	37	924	–	47	Binds elst*
TolC	12,307	TM	56*	21*	–	48	Export channel
FadL	12,524	TM	52	61*	–	51	Long-chain fatty acid transporter
TolC	12,575	TM	60*	1,064		53	Export channel
TolC	12,693	TM	63.5*	377		50	Export channel
LenA	12,906	Lip	30	ND		–	Binds Factor H, fn, lam
LipL41	12,966	Lip	41	10,531	Yes	–	Binds hemin*
OmpL47	13,050	TM	47	5,022	Yes	50	–
LipL36	13,060	Lip	36	14,100		–	Not expressed during infection
TlyC	13,143	TM	50.4*	8*	–	58	Binds fn, lam, cIV*
OmpL54	13,491	TM	54	491	–	44	–

Name	Locus tag[a]	Type[b]	Size (kd)[c]	Copy number[d]	Knockout virulent?	*L. biflexa* % identity[e]	Putative function(s), comment[f]
HbpA	20,151	TM	80	14*	Yes[g]	58	TonB DR for hemin*
FecA	20,214	TM	100*	ND	Yes	52	TonB DR for Fe^{3+}-dicitrate

[a] Locus tag for *Leptospira interrogans* serovar Copenhageni, strain L1-130

[b] Type: Lip (lipoprotein), TM (transmembrane Omp), PM (peripheral membrane Omp)

[c] Size (kd): Observed or predicted*

[d] Copy number: Estimated by MS or spectral* methods (Malmström et al. 2009). ND None detected

[e] Percent identity with L. biflexa homologue

[f] Putative function: T2SS (type 2 secretion system), TonB DR (TonB-dependent receptor), LPS (lipopolysaccharide). Host ligands: fn (fibronectin), fg (fibrinogen), cI (collagen I), cIV (collagen IV), lam (laminin), and elst (elastin). Based on experimental evidence*

[g] Virulent in hamsters but renal colonization deficient in mice

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