The Molecular Basis of Leptospiral Pathogenesis

Gerald L. Murray

Abstract The mechanisms of disease pathogenesis in leptospirosis are poorly defined. Recent developments in the application of genetic tools in the study of *Leptospira* have advanced our understanding by allowing the assessment of mutants in animal models. As a result, a small number of essential virulence factors have been identified, though most do not have a clearly defined function. Significant advances have also been made in the in vitro characterization of leptospiral interaction with host structures, including extracellular matrix proteins (such as laminin, elastin, fibronectin, collagens), proteins related to hemostasis (fibrinogen, plasmin), and soluble mediators of complement resistance (factor H, C4b-binding protein), although none of these in vitro findings has been translated to the host animal. Binding to host structures may permit colonization of the host, prevention of blood clotting may contribute to hemorrhage, while interaction with complement resistance mediators may contribute to survival in serum. While not a classical intracellular pathogen, the interaction of leptospires and phagocytic cells appears complex, with bacteria surviving uptake and promoting apoptosis; mutants relating to these processes (such as cell invasion and oxidative stress resistance) are attenuated in vivo. Another feature of leptospiral biology is the high degree of functional redundancy and the surprising lack of attenuation of mutants in what appear to be certain virulence factors, such as LipL32 and LigB. While many advances have been made, there remains a lack of understanding of how *Leptospira* causes tissue pathology. It is likely that leptospires have many novel pathogenesis mechanisms that are yet to be identified.

Contents

G.L. Murray (✉)
Department of Microbiology, Monash University, Melbourne, Australia
e-mail: gerald.murray@monash.edu

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1 Introduction to the Pathogenesis of Leptospirosis

The molecular basis of leptospiral pathogenesis remains poorly understood. Leptospires lack classical virulence factors due to the large phylogenetic distance to well-studied, prototypic, bacterial pathogens. This indicates that *Leptospira* likely has novel virulence mechanisms, a notion supported by the over representation of "hypothetical" open reading frames in the group of genes specific to pathogenic *Leptospira interrogans*; 78 % of pathogen-specific genes have no defined function, compared to 40 % of the whole genome (Adler et al. 2011).

Recent advances in genetics, including the construction of the first defined mutants by transposon mutagenesis (Bourhy et al. 2005), and directed mutagenesis (Croda et al. 2008), combined with the increase in available genomic sequences (see the chapter by M. Picardeau, this volume) have led to progress in the identification and characterization of virulence factors. Virulence factors that are required for disease in animal models, identified through mutagenesis, are summarized in Tables 1 and 2. An overview of the stages of acute infection is illustrated in Fig. 1; the virulence factors essential for acute disease and their probable role in disease are indicated. Clearly, most disease processes occur by mechanisms that are yet to be fully defined.

This chapter covers recent research into known and predicted virulence factors, redundancy of virulence mechanisms, molecular mechanisms of damage to the

Table 1 *Leptospira interrogans* virulence factors with a confirmed role in acute disease[a]

Name[b]	Mutated gene[c]	Description/function	Possible role in virulence	Distribution[d]	Serovar	Animal model	Renal colonization of acute host[e]	References[f]
Loa22*	*la0222*	Outer membrane lipoprotein, OmpA domain	Unknown	P, I, S	Lai	Guinea pig, hamster	Yes	Ristow et al. (2007)
HemO	*lb186*	Heme oxygenase, degrades heme	Iron acquisition	P, I, S	Manilae	Hamster	Yes	Murray et al. (2009b)
FliY	*la2613*	Flagellar motor switch, motility	Dissemination	P, I, S	Lai	Guinea pig	NA	Liao et al. (2009)
LPS†	*la1641*	LPS synthesis	Unknown	P	Manilae	Hamster	No	Marcsisin et al. (2013), Murray et al. (2010)
LPS†	*lman_1408* HQ127382	Potential methyltransferase, LPS synthesis	Unknown	P, I	Manilae	Hamster	No	Marcsisin et al. (2013), Murray et al. (2010)
ClpB*	*la1879/ lic12017*	Molecular chaperone	Resist heat and oxidative stress, nutrient restriction	P, I	Kito	Gerbil	NA	Lourdault et al. (2011)
FlaA2	*la3380*	Probable flagellar sheath protein, motility	Dissemination	P, I, S	Manilae	Hamster	No	Lambert et al. (2012a)
KatE†	*la1859*	Catalase, degrades hydrogen peroxide	Resist oxidative stress	P, I	Pomona, Manilae	Hamster	NA	Eshghi et al. (2012)
Mce**	*la2055*	Mce, cell adhesion and entry	Cell entry	P, I, S	Lai	Hamster	Yes	Zhang et al. (2012)

(continued)

Table 1 (continued)

Name[b]	Mutated gene[c]	Description/function	Possible role in virulence	Distribution[d]	Serovar	Animal model	Renal colonization of acute host[e]	References[f]
LruA	*la3097*	Lipoprotein, may be involved in leptospiral interaction with apolipoprotein A-I	Unknown	P, I, S	Manilae	Hamster	Yes	Zhang et al. (2013)
HtpG**	*lb058*	Probable chaperone, function not defined	Unknown	P, I, S	Manilae	Hamster	Yes	King et al. (2013), Marcsisin et al. (2013)
ColA**	*la0872*	Collagenase	Dissemination, tissue damage	P	Lai	Hamster	Yes	Kassegne et al. (2014)
LB139	*lb139*	Sensor protein	Motility, protein expression	P	Manilae	Hamster	No	Eshghi et al. (2014)

[a] Based on animal survival. Tissue pathology such as lung hemorrhage and kidney colonization by *Leptospira* generally still observed. LPS mutants were attenuated by both intraperitoneal injection and conjunctival infection. All other mutants were tested by intraperitoneal infection route only

[b] Partial (*) or full (**) restoration of virulence after complementation, attenuation found in independent mutants of the same gene or pathway (†)

[c] Locus tag from Lai and/or Copenhageni indicated where available. GenBank accession number indicated in parentheses, if appropriate

[d] Distribution of genes across species determined by BLASTp search (NCBI) and/or analysis of gene synteny. P, found in pathogenic strains (*L. kirschneri*, *L. noguchii*, *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. santarosai*, *L. alexanderi*, *L. kmetyi*, *L. alstonii*); I, found in intermediate pathogenicity species (*L. broomii*, *L. licerasiae*, *L. inadai*, *L. fainei*, *L. wolffii*); S, found in saprophytic species (*L. biflexa*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*)

[e] *NA* Not assessed

[f] Further references relating to these factors may be found in the text

Table 2 *Leptospira interrogans* virulence factors with a confirmed role in carrier host colonization

Name[a]	Mutated gene[b]	Description/function	Possible role in virulence	Distribution[c]	Serovar	Renal colonization of mouse	Attenuated in acute model[d]	References[e]
LPS†	*la1641*	LPS synthesis	Unknown	P	Manilae	No	Yes	Marcsisin et al. (2013), Murray et al. (2010)
LPS†	*lman_1408* HQ127382	Potential methyltransferase, LPS synthesis	Unknown	P, I	Manilae	No	Yes	Marcsisin et al. (2013), Murray et al. (2010)
HbpA	*lb191*	TonB-dependent receptor, binds hemin	Iron acquisition	P, I, S	Manilae	No	No	Marcsisin et al. (2013)
LruA	*la3097*	Lipoprotein, may be involved in leptospiral interaction with apolipoprotein A-I	Unknown	P, I, S	Manilae	No	Yes	Zhang et al. (2013)
HtpG	*lb058*	Probable chaperone, function not defined	Unknown	P, I, S	Manilae	No	Yes	King et al. (2013), Marcsisin et al. (2013)
LB194	*lb194*	Hypothetical protein, iron utilization locus	Unknown	P, I, S	Manilae	No	No	Marcsisin et al. (2013)

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Table 2 (continued)

Name[a]	Mutated gene[b]	Description/function	Possible role in virulence	Distribution[c]	Serovar	Renal colonization of mouse	Attenuated in acute model[d]	References[e]
LA2786	*la2786*	Hypothetical	Unknown	P	Manilae	No	No	Marcsisin et al. (2013)
LA0589	*la0589*	Hypothetical, family of paralogous proteins	Unknown	P	Manilae	No	No	Marcsisin et al. (2013)

[a] Attenuation found in independent mutants of the same gene or pathway (†)

[b] Locus tag from Lai and/or Copenhageni indicated where available. GenBank accession number indicated in parentheses, if appropriate

[c] Distribution of genes across species determined by BLASTp search (NCBI) and/or analysis of gene synteny. P, found in pathogenic strains (*L. kirschneri*, *L. noguchii*, *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. santarosai*, *L. alexanderi*, *L. kmetyi*, *L. alstonii*); I, found in intermediate pathogenicity species (*L. broomii*, *L. licerasiae*, *L. inadai*, *L. fainei*, *L. wolffii*); S, found in saprophytic species (*L. biflexa*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*)

[d] Based on animal survival. Tissue pathology such as lung hemorrhage and kidney colonization by *Leptospira* generally still observed. LPS mutants were attenuated by both intraperitoneal injection and conjunctival infection. All other mutants were tested by intraperitoneal infection route only

[e] Further references relating to these factors may be found in the text

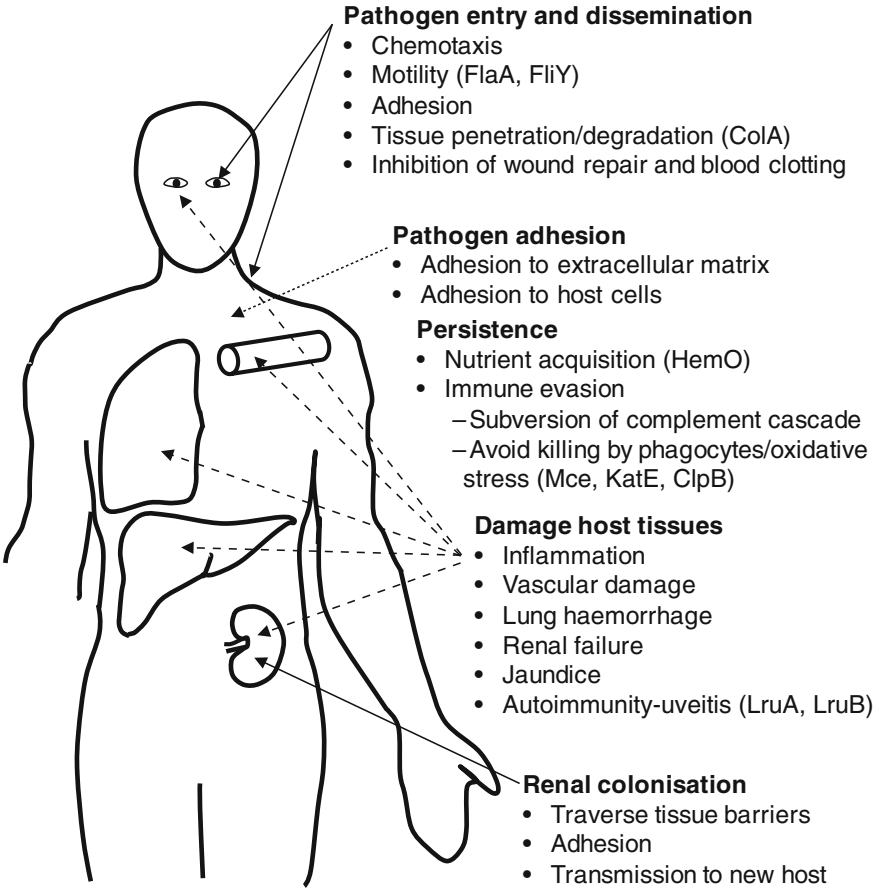

Fig. 1 Stages of the leptospirosis infection process. Probable virulence mechanisms are indicated along with associated virulence factors experimentally confirmed to be required for disease (see text for references). The pronounced lack of confirmed virulence factors for various aspects of infection highlights our limited understanding of the pathogenesis of leptospirosis. Additional virulence factors, without defined function, include Loa22, LPS, LB139, and the heat shock protein HtpG. LruA is also essential for virulence (unrelated to the role in *Leptospira*-induced uveitis)

host, and the molecular basis of host specificity. The term virulence factor is used to describe proteins, structures (e.g., LPS), or phenotypes (e.g., motility) that are required to cause disease, or have been demonstrated to interact with host proteins in a way that may potentiate disease.

1.1 Methods for the Identification and Characterization of Virulence Factors

Virulence factors can be predicted bioinformatically or identified experimentally. Bioinformatics approaches include identification of sequences similar to known virulence factors in other species, and genomic comparisons, especially between pathogenic and saprophytic species (Adler et al. 2011). Few confirmed leptospiral virulence factors have been identified by bioinformatics, with the exception of catalase, collagenase, heme oxygenase, and Mce (Table 1). Genomes of pathogenic leptospires also encode sphingomyelinases and phospholipase, other proteases and TlyABC-like hemolysins, though a definitive role in virulence for these has not been established (Nascimento et al. 2004). Pathogenic leptospiral genomes also encode an unusually large number of leucine-rich repeat proteins, containing a motif often associated with pathogen–host interaction. Notably, leptospires lack recognized systems for translocation of effectors into host cells such as non-flagellar type III, and types IV and VI secretion systems (Nascimento et al. 2004). In vitro experimental approaches for identification of virulence factors include prospecting for interactions between a substrate and leptospiral proteins by ligand blots (Hoke et al. 2008; Verma et al. 2006), "pull down" or column extraction experiments (Asuthkar et al. 2007), analysis of leptospiral cells that have interacted with host proteins (Zhang et al. 2013), protein arrays (Pinne et al. 2012), and phage display (Ching et al. 2012). Potential virulence factors have also been inferred through comparison of the genomic and transcriptional changes between a virulent strain of *L. interrogans* and a culture-attenuated derivative (Zhong et al. 2011; Lehmann et al. 2013; Toma et al. 2014).

Characterization of putative virulence factors can be conducted in vitro or in vivo. In vitro demonstration of interaction between recombinant leptospiral protein and host proteins is commonly used, though this does not prove a role in vivo and is subject to artifacts that may be introduced in vitro, such as protein misfolding and possible lack of appropriate post-translational modifications. Whole, live cells can also be used in in vitro assays such as binding experiments; interactions can be blocked with specific antibodies or by competitive inhibition with the protein of interest (Choy et al. 2007), but it may be difficult to isolate the role of a particular factor due to functional redundancy (Murray et al. 2009c). "Gain of function" studies involve transfer of genes from pathogens to saprophytes and measurement of virulence characteristics such as adhesion (Figueira et al. 2011). In these experiments, a protein is more likely to be expressed with "normal" conformation, post-translational modifications and context, such as lipidation or membrane insertion, and also have the advantage of excluding functionally analogous proteins of pathogens that may confound results.

The only definitive method to determine that a factor is required for virulence is by mutagenesis followed by testing in vivo; factors essential for virulence identified by this methodology are detailed in Tables 1 and 2. It is important to stress that the majority of putative virulence factors have not been shown to have a role in the

host. Notably, mutagenesis and in vivo testing may not identify virulence factors with redundant function. The readout for these experiments is usually animal survival, though as a crude measure of virulence this may not identify subtle attenuation (Adler et al. 2011). Other useful readouts include tissue pathology (e.g., frequency and severity of macroscopic lung hemorrhage, histopathology of various tissues), renal colonization, and bacterial burden in tissues (measured by quantitative PCR) (King et al. 2014; Lambert et al. 2012a). Recently, a high throughput method for screening mutants for attenuation was described (Marcsisin et al. 2013). Tables 1 and 2 should be viewed with the following caveats. Only mutants in *L. interrogans* have been tested, mainly in serovars Manilae and Lai. This may not be representative of pathogenic strains generally, and what is true for one strain may not necessarily be extrapolated to other strains [e.g., the *clpB* mutant is avirulent in serovar Kito but retains virulence in serovar Manilae (Lourdault et al. 2011)]. The intraperitoneal challenge route does not test aspects of disease such as host entry, meaning that factors with a key role in these aspects of disease may not be identified. For some of the attenuated mutants, the challenge strain retained sufficient virulence to cause pathology and kidney colonization, and with sufficient dose, host death. Finally, the majority of mutants have been tested in the acute models of gerbils, hamsters, and guinea pigs, neglecting carrier hosts which are the reservoir from which humans are infected.

Complementation is a cornerstone of microbiological studies that rely on mutagenesis to prove phenotype (Falkow 1988); however, due to the lack of replicating plasmids for pathogenic leptospires this is difficult and few mutants have been successfully complemented (Table 1). This has been achieved by transforming bacteria a second time with the *himar*1 transposon with an alternative selective marker and intact gene with promoter (King et al. 2014; Lourdault et al. 2011; Ristow et al. 2007), or through integrating an intact copy of the gene onto the chromosome by homologous recombination (Zhang et al. 2012; Kassegne et al. 2014). In the absence of complementation use of a second, independent mutant in the gene or pathway of interest (Eshghi et al. 2012; Murray et al. 2010; Lambert et al. 2012a) or whole genome sequencing (Zhang et al. 2013) can be used to rule out other attenuating mutations.

It should be noted that many "virulence factors" are also found in the saprophytes. In some cases, a role in virulence overlaps with normal cell metabolism; for example, heme oxygenase is presumably useful for the degradation of heme from endogenous or exogenous sources in both saprophyte and pathogen (Guégan et al. 2003). Other "virulence factors" in saprophytes may have roles in environmental survival; for example, saprophytes and pathogens can degrade the lipids in cell membranes (Kasărov 1970) which could be used to obtain lipids from environmental organisms as well as animal hosts.

1.2 Redundancy of Leptospiral Virulence Mechanisms

Pathogenic leptospires possess extensive genetic and functional redundancy. This may be a result of a process of genomic expansion through gene duplication (Bulach et al. 2006, Chap. 4). Groups of functionally similar, paralogous genes such as the *lig* and *len* families abound; LigA and LigB both bind collagen, laminin, fibrinogen, fibronectin, and numerous soluble regulators of the complement system (Choy et al. 2007), while LenABCDEF all bind laminin and fibronectin (Barbosa et al. 2006; Stevenson et al. 2007). There is also considerable functional overlap between proteins without sequence similarity, particularly adhesins and proteins that bind complement regulatory proteins. For example, LipL32, LigA, LenABCDEF, TlyC are among more than 25 proteins reported to bind to laminin (Carvalho et al. 2009; Hoke et al. 2008; Stevenson et al. 2007, Table 3).

The maintenance of redundant factors in the leptospiral genome is difficult to explain. Functionally redundant proteins may operate at different stages of disease, in different tissues, or work synergistically. Multiple leptospiral receptors targeting a particular host substrate may also permit leptospires to infect a diverse repertoire of mammalian hosts where the target molecule may vary in structure. It is plausible that the numerous receptors for soluble proteins such as fibronectin and plasminogen (Tables 3 and 4) may coat the surface of leptospires with host proteins in a form of immune evasion, masking the underlying antigens.

The flipside of functional redundancy is the surprising lack of attenuation for specific mutants. There are several proteins, such as LipL32, LipL41, and LigB that appear to be obvious virulence factors by way of in vitro functional characterization, conservation, and expression profiles. However, mutants in genes encoding these proteins retain full virulence (Croda et al. 2008; King et al. 2013; Murray et al. 2009c). Notably, LipL32 and LigB mutants retained virulence in both acute disease and animal colonization models. Other notable mutants that retained virulence include *L. interrogans* serovar Manilae mutants in *ligC*, *lenB*, and *lenE* (Murray et al. 2009a). Presumably the loss of these putative virulence factors is covered by other functionally related proteins (Adler et al. 2011).

2 Pathogen Entry

Human infection with *Leptospira* occurs upon contact with contaminated environmental reservoirs (water, soil) or animal sources (urine, animal tissues). Bacteria breach mucosal membranes or enter transdermally through wet or abraded skin (Adler and de la Peña Moctezuma 2010). The molecular mechanisms by which entry occurs are currently unknown.

Table 3 Leptospiral proteins that interact with extracellular matrix proteins

Name(s)	Locus tag	Description	Collagen I	Collagen III	Collagen IV	Collagen V	Elastin	Tropoelastin	Heparan sulfate	Laminin	Fibronectin[a]	Location[b]	Function verified[c]	References
EF-Tu	LIC12875, LA0737	Elongation factor (protein synthesis)	Y		Y		Y			Y	Y	OM		Wolff et al. (2013)
LenA/ Lsa24/ LfhA	LIC12906, LA0695	Endostatin-like protein								Y	Y	OM	Y	Barbosa et al. (2006), Stevenson et al. (2007), Verma et al. (2006, 2010a)
LenB	LIC10997, LA3103	Endostatin-like protein								Y	Y	NA		Stevenson et al. (2007)
LenC	LIC13006, LA0563	Endostatin-like protein								Y	Y	NA		Stevenson et al. (2007)
LenD	LIC12315, LA1433	Endostatin-like protein								Y	Y	OM		Stevenson et al. (2007)
LenE	LIC13467, LA4324	Endostatin-like protein								Y	Y	NA		Stevenson et al. (2007)
LenF	LIC13248, LA4073	Endostatin-like protein								Y	Y	NA		Stevenson et al. (2007)
LIC12976	LIC12976, LA0602	NAD/FAD-binding protein								Y		NA		Lima et al. (2013)
LigA	LIC10465	Bacterial immunoglobulin-like repeat protein	Y		Y					Y	Y	OM	Y	Choy et al. (2007), Figueira et al. (2011)
LigB	LIC10464, LA3778	Bacterial immunoglobulin-like repeat protein	Y	Y	Y		Y	Y	Y	Y	Y	OM	Y	Ching et al. (2012), Choy et al. (2007, 2011), Figueira et al. (2011), Lin et al. (2009), Toma et al. (2014)

(continued)

Table 3 (continued)

Name(s)	Locus tag	Description	Collagen I	Collagen III	Collagen IV	Collagen V	Elastin	Tropoelastin	Heparan sulfate	Laminin	Fibronectin[a]	Location[b]	Function verified[c]	References
LipL32	LIC11352, LA2637	Major outer membrane protein	Y		Y	Y				Y	Y	OM	Y	Hauk et al. (2008), Hoke et al. (2008), Vieira et al. (2010a)
LipL53	LIC12099, LA1691	Hypothetical protein			Y					Y	Y	I		Oliveira et al. (2010)
LMB216	LB216, LIC20172										Y	OM		Toma et al. (2014)
Lp30	LIC12880, LA0730	Hypothetical protein										I		Oliveira et al. (2011)
Lp95	LIC12690, LA0962	Hypothetical protein								Y	Y	NA		Atzingen et al. (2009)
Lsa20	LIC11469, LA2496	Hypothetical protein								Y		OM	Y	Mendes et al. (2011)
Lsa21	LIC10368, LA0419	Hypothetical protein			Y					Y	Y	OM		Atzingen et al. (2008)
Lsa25	LIC12253, LA1508	Hypothetical protein								Y		OM	Y	Domingos et al. (2012)
Lsa27	LIC12895, LA0710	Hypothetical protein								Y		I		Longhi et al. (2009)
Lsa30	LIC11087, LA2975									Y	Y	OM		Souza et al. (2012)
Lsa33	LIC11834, LA2083	LipL45-related protein, FecR domain								Y		I	Y	Domingos et al. (2012)

(continued)

Table 3 (continued)

Name(s)	Locus tag	Description	Collagen I	Collagen III	Collagen IV	Collagen V	Elastin	Tropoelastin	Heparan sulfate	Laminin	Fibronectin[a]	Location[b]	Function verified[c]	References
Lsa63	LIC10314, LA0365	Hypothetical protein			Y					Y		OM	Y	Vieira et al. (2010b)
Lsa66, MFn8	LIC10258, LA0301	OmpA-related protein								Y	Y	I	Y	Oliveira et al. (2011), Pinne et al. (2012)
MFn1	LIC11612, LA2330	Hypothetical protein									Y	OM	Y	Pinne et al. (2012)
MFn10	LIC11755, LA2167	Hypothetical protein									Y	NA		Pinne et al. (2012)
MFn11	LIC11028, LA3067	Tol transport system component									Y	NA		Pinne et al. (2012)
MFn12	LIC12952, LA0635	Hypothetical protein									Y	NA		Pinne et al. (2012)
MFn13	LIC11893, LA2014	CreD-like protein									Y	NA		Pinne et al. (2012)
MFn14, LruB	LIC10713, LA3469	Lipoprotein LruB									Y	NA		Pinne et al. (2012)
MFn15	LIC13066, LA3834	Hypothetical protein, beta propeller domains									Y	NA		Pinne et al. (2012)
MFn2	LIC10714, LA3468	TonB-dependent receptor									Y	NA		Pinne et al. (2012)
MFn3, Sph3	LIC13198, LA4004	Sphingomyelinase 3									Y	NA		Pinne et al. (2012)
MFn4, Sph2	LIC12631, LA1029	Sphingomyelinase 2									Y	NA	Y	Pinne et al. (2012)

(continued)

Table 3 (continued)

Name(s)	Locus tag	Description	Collagen I	Collagen III	Collagen IV	Collagen V	Elastin	Tropoelastin	Heparan sulfate	Laminin	Fibronectin[a]	Location[b]	Function verified[c]	References
MFn5	LIC13135, LA3927	TolC family protein									Y	NA		Pinne et al. (2012)
MFn6	LIC11051, LA3028	Leucine-rich repeat protein									Y	NA		Pinne et al. (2012)
MFn7	LIC11436, LA2537	LipL45-related protein									Y	OM	Y	Pinne et al. (2012)
MFn9	LIC10537, LA3685	OmpA family protein									Y	OM		Pinne et al. (2012)
OmpL1	LIC10973, LA3138	Transmembrane protein								Y	Y	OM	Y	Fernandes et al. (2012)
OmpL37	LIC12263, LA1495	Transmembrane protein					Y			Y	Y	OM		Pinne et al. (2010)
OmpL47	LIC13050, LA0505	Transmembrane protein		Y			Y			Y	Y	OM		Pinne et al. (2010)
TlyC	LIC13143, LA3937	Hemolysin-like protein			Y					Y	Y	OM	Y	Carvalho et al. (2009)

[a] Indicates binding to cellular fibronectin, plasma fibronectin, or both

[b] Location confirmed experimentally. *OM* Located in outer membrane and/or surface exposed; *I* Inconclusive evidence of surface exposure; *NA*, Data not available

[c] Function(s) verified by one or more of the following: independent study, inhibition of binding by antibodies or purified protein, gain of function after expression in *L. biflexa*. See text for further details

Table 4 Leptospiral proteins that interact with plasma proteins and soluble mediators of complement resistance

Name	Locus tag	Fibrinogen	Plasminogen	Factor H	Factor H-related protein 1	Factor H-like protein 1	C4b-binding protein	Location[a]	References
Enolase	LIC11954, LA1951		Y					OM	Nogueira et al. (2013)
EF-Tu	LIC12875, LA0737	Y	Y	Y				OM	Wolff et al. (2013)
LcpA	LIC11947, LA1957						Y	OM	Barbosa et al. (2010)
LenA/ Lsa24/ LfhA	LIC12906, LA0695		Y	Y	Y			OM	Stevenson et al. (2007), Verma et al. (2006, 2010a)
LenB	LIC10997, LA3103			Y				NA	Stevenson et al. (2007)
LIC10494	LA3735		Y					I	Vieira et al. (2010a)
LIC11360	LA2626	Y						NA	Oliveira et al. (2013)
LIC11975	LA1931	Y						NA	Oliveira et al. (2013)
LIC12238	LA1523	Y	Y					I	Oliveira et al. (2013), Vieira et al. (2010a)
LIC12730	LA0913		Y					I	Vieira et al. (2010a)
LigA	LIC10465	Y		Y	Y	Y	Y	OM	Castiblanco-Valencia et al. (2012), Choy et al. (2007)
LigB	LIC10464, LA3778	Y		Y	Y	Y	Y	OM	Castiblanco-Valencia et al. (2012), Choy et al. (2007)
LipL32	LIC11352, LA2637		Y					OM	Vieira et al. (2010a)

(continued)

Table 4 (continued)

Name	Locus tag	Fibrinogen	Plasminogen	Factor H	Factor H-related protein 1	Factor H-like protein 1	C4b-binding protein	Location[a]	References
LipL40	LIC10091, LA0103		Y					I	Vieira et al. (2010a)
Lp29	LIC12892		Y					OM	Vieira et al. (2010a)
Lp30	LIC12880, LA070		Y					I	Oliveira et al. (2011)
Lp49	LIC10793, LA3370		Y					I	Vieira et al. (2010a)
Lsa20	LIC11469, LA2496		Y					OM	Mendes et al. (2011)
Lsa25	LIC12253, LA1508	Y					Y	I	Domingos et al. (2012), Oliveira et al. (2013)
Lsa30	LIC11087, LA2975	Y	Y				Y	OM	Oliveira et al. (2013), Souza et al. (2012)
Lsa33	LIC11834, LA2083	Y	Y				Y	I	Domingos et al. (2012); Oliveira et al. (2013)
Lsa66	LIC10258, LA0301		Y					I	Oliveira et al. (2011)
MPL36	LIC10054, LA0061		Y					I	Vieira et al. (2010a)
OmpL1	LIC10973, LA3138	Y	Y					OM	Fernandes et al. (2012), Oliveira et al. (2013)
OmpL37	LIC12263, LA1495	Y						OM	Pinne et al. (2010)
OmpL47	LIC13050, LA0505	Y						OM	Pinne et al. (2010)

[a] Location confirmed experimentally. *OM*, Located in outer membrane and/or surface exposed; *I* Inconclusive evidence of surface exposure; *NA* Data not available

2.1 Motility

Leptospires are highly motile as a result of two periplasmic flagella that are inserted subterminally and wrap around the protoplasmic cylinder (see the chapter by C.E. Cameron, this volume); loss of flagella results in loss of motility (Picardeau et al. 2001). Flagella are thought to comprise a core containing FlaB (encoded by four *flaB* genes), a sheath composed of FlaA (encoded by two *flaA* genes), and possibly other proteins yet to be identified (Lambert et al. 2012a). Leptospiral motility is more effective in viscous substrates (Berg and Turner 1979; Kaiser and Doetsch 1975), which may allow penetration of substrates such as collagen and hyaluronic acid found in tissues that would stall externally flagellated bacteria (reviewed in Charon and Goldstein 2002).

In the initial stages of leptospirosis, motility is most likely necessary to breach the mucosal membranes or enter the tissues through damaged skin, though this has not been directly demonstrated. An undefined motility mutant of *L. interrogans* (with defective translational motility, loss of hooked ends) was attenuated in hamsters (Faine and van der Hoeden 1964). Similar motility mutants had reduced adhesion to primary murine renal epithelial cells and a murine fibroblast cell line (Ballard et al. 1986; Vinh et al. 1984). More recently two defined mutants, in *fliY* and *flaA2*, that lack motility were attenuated in acute models of infection. Together these studies indicate that once inside the host motility is essential for disease.

A *fliY* (flagella motor switch) mutant exhibited reduced motility, although polar effects on the expression of multiple downstream flagellar genes were noted (Liao et al. 2009). The *fliY* mutant was attenuated in guinea pigs and showed reduced adhesion to macrophages and reduced induction of macrophage apoptosis (Table 1). The authors speculate that this may result from reduced export of adhesins and toxins through the flagella apparatus (Liao et al. 2009). As an alternative explanation, lack of motility may reduce encounters between leptospires and macrophages, giving the appearance of a less adhesive strain and reduced apoptosis; attenuation in vivo maybe a consequence of reduced dissemination in the host.

A *flaA2* transposon mutant has been described which did not express FlaA1 nor FlaA2 (Lambert et al. 2012a). This mutant had altered flagella structure (loss of helical shape), altered cell morphology (loss of hooked/helical cell ends), and lacked translational motility, similar to motility mutants described earlier (Faine and van der Hoeden 1964). The *flaA2* mutant was highly attenuated in hamsters, which survived $>10^5$ LD_{50} with no detectable kidney colonization 25 days after infection (Table 1). Interestingly, the *flaA2* mutant was present in far lower numbers or undetectable in liver and kidney 5 days post infection compared to very high numbers of WT bacteria (Lambert et al. 2012a), suggesting that motility is necessary for the ubiquitous tissue distribution of leptospires found in acute hosts (Faine 1957).

A mutant in a gene encoding a putative sensor protein, *lb139*, showed down regulation of 115 genes; of these, genes encoding regulatory proteins, putative

secreted proteins, and motility and chemotaxis proteins were over represented (Eshghi et al. 2014). The mutant was highly attenuated in the hamster model of infection by both conjunctival and intraperitoneal routes. While the down regulated secreted proteins may have a direct role in virulence, reduced motility (observed in plate and video microscope assays) may also explain the attenuation of this strain.

2.2 Chemotaxis

Leptospires possess the majority of the key chemotaxis genes found in other bacteria, with approximately 12 methyl-accepting chemotaxis proteins (MCPs) encoded in the genomes of pathogens (Nascimento et al. 2004; Ren et al. 2003). This indicates that leptospires respond to a wide range of chemical stimuli, though the ligand for each receptor is unknown. One suggested chemical attractant is hemoglobin (Yuri et al. 1993), although hemoglobin is too large to cross the outer membrane and be detected in the periplasm by an MCP (Lambert et al. 2012b); perhaps in these experiments bacteria were attracted to a smaller, readily diffusible breakdown product. Nevertheless, this result indicates chemotaxis toward blood, suggesting that leptospires are attracted to the host at the site of injury where tissue barriers have already been degraded.

Other leptospiral chemoattractants include glucose, sucrose, pyruvate, and Tween 80 (a source of oleic acid) (Lambert et al. 2012b). As a synthetic compound, Tween 80 would have no role in leptospirosis per se, although chemotaxis toward Tween 80 may indicate a tendency to move toward nutritional sources of fatty acids, even though the level of lipids such as triglycerides in blood is low (approximately 1 mM). Likewise, the significance of chemotaxis toward glucose is unknown as leptospires do not utilize this sugar as an energy source, and the concentration of glucose in blood is around 5 mM, lower than the tested concentration (100 mM).

The importance of chemotaxis in leptospiral infection has not been thoroughly investigated. Mutants in putative chemotaxis genes *cheB* and *cheX* were not attenuated in hamsters when infected intraperitoneally (Murray et al. 2009a), although a role for these proteins in leptospiral chemotaxis is yet to be established and there are multiple *cheB* genes encoded in leptospiral genome (Dong et al. 2010). Additionally, it is possible that chemotaxis is not required once the host has been invaded; therefore mutants should also be tested via "natural" routes of infection (conjunctiva, dermal abrasion). It is tempting to speculate that chemotaxis is important for tissue tropisms, but apparent tissue tropisms such as localization in the renal tubules may alternatively be the result of immune clearance of leptospires from some sites but not others.

2.3 Crossing Host Tissue Barriers

In order to disseminate throughout the host, leptospires must cross many barriers including extracellular matrix, basement membranes, and cell layers. The mechanisms by which leptospires cross tissue layers occurs remain unknown, but motility is likely to play a key role (Lambert et al. 2012a).

Treponema pallidum is thought to cross cell layers through cell junctions (Thomas et al. 1988), while *Borrelia burgdorferi* had been reported to cross cell layers by invading the cell cytoplasm (Comstock and Thomas 1989) or at cell junctions (Moriarty et al. 2008; Szczepanski et al. 1990). In a mouse infection model, leptospires were observed to cross into the kidney lumen between cells (Marshall 1976). In contrast, two studies examining transcytosis of leptospires across the polarized Madin-Darby canine kidney (MDCK) cell line found evidence of transit through cells (Barocchi et al. 2002; Thomas and Higbie 1990); leptospires crossed layers rapidly without major disruption of tight junctions and were observed intracellularly, presumably in transit across the cell layer. Intracellular bacteria were sometimes surrounded by a host cell membrane, but were also free in the cytoplasm. Many pathogens such as *Salmonella* spp. and *Yersinia* spp. enter host cells through specific interactions that cause perturbations in cellular architecture. By contrast, during leptospiral "invasion" of the monolayer, cells remained intact and there was no evidence of cytoskeletal rearrangements. The results suggest a novel mechanism of cell invasion as a means of crossing tissue barriers. Interestingly, *Leptospira biflexa* was also observed to cross layers (though less efficiently than pathogenic leptospires) (Barocchi et al. 2002); perhaps transcytosis is a result of the thin, helical morphology and vigorous motility shared between pathogen and saprophyte rather than a specific molecular mechanism. Experiments could be repeated with motility mutants to test this theory.

Proteases may also contribute to the crossing of cell layers. Transcytosis across human umbilical vein endothelial cell (HUVEC) monolayers was enhanced when leptospires were coated with plasminogen or plasmin, suggesting a proteolytic mechanism (Vieira et al. 2013). A collagenase mutant also had reduced ability to cross HUVEC and human renal tubular epithelial cell line (HEK293) cell layers, and in vivo the same mutant had reduced distribution in tissues in hamsters, indicating that collagenase may assist with bacterial dissemination (Kassegne et al. 2014).

3 Pathogen Adhesion and Dissemination

3.1 Adhesion to Host Cells

Adhesion to host surfaces is an important step in bacterial pathogenesis (Kline et al. 2009). In animals, close association of leptospires with microvilli of proximal renal tubules has been observed in hamsters and sheep, but generally without obvious

cytopathology (Faine et al. 1999; Marshall 1974). In vitro, adhesion to various host cells has been observed, including MDCK and primary dog kidney cell lines (Thomas and Higbie 1990; Tsuchimoto et al. 1984), mouse fibroblast cells (Vinh et al. 1984), mouse renal tubular epithelial cells (Ballard et al. 1998), human umbilical vein endothelial cells, and porcine kidney epithelial cells (Thomas and Higbie 1990). Adhesion levels correlated with strain virulence (Tsuchimoto et al. 1984). Leptospiral adherence to cells is diminished after pretreatment of monoloayers with proteases, indicating probable protein receptors (Breiner et al. 2009; Thomas and Higbie 1990). Cellular fibronectin and glycosaminoglycans are potential host receptors participating in this binding.

3.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are unbranched, long polymers of disaccharides that may be sulfated. GAGs form part of the extracellular matrix, and when bound to proteins, they constitute proteoglycans and are located on the surface of cells. *Leptospira* binds to GAGs, chondroitin sulfate B and C, though specific unknown receptors (Breiner et al. 2009); in the absence of GAGs significant binding to host cells occurred, implying that additional adhesin targets exist. The authors speculated that GAGs present on cells at mucosal surfaces may be involved in initial host colonization, while GAGs expressed in the renal tubule and released in the urine may facilitate renal colonization and shedding (Breiner et al. 2009). In a phage display experiment, LigB was found to bind to the heparin sulfate, which could mediate binding to host cells (Ching et al. 2012), although a *ligB* mutant bound to MDCK cells at the same rate as WT leptospires (Croda et al. 2008).

During the hematogenous spread, pathogenic leptospires most likely adhere to the endothelium of the blood vessel under fluid shear forces and then penetrate the cell layer to enter tissues. The mechanism by which this occurs is unknown, but studies in *B. burgdorferi* may provide clues. *B. burgdorferi* exits from post-capillary venules through a sequence of interactions with the vessel endothelium: transient interactions, dragging interactions, adhesion, then transmigration into surrounding tissues, mainly through cell junctions (Moriarty et al. 2008). The *B. burgdorferi* protein BBK32 is thought to play a role in this process by mediating direct and indirect interaction via fibronectin with GAGs of endothelial cells (Moriarty et al. 2012). A similar process of escape from the microvasculature may occur in leptospirosis, facilitated by direct interaction with GAGs or indirect interaction via numerous fibronectin receptors with different affinity for fibronectin.

3.3 Adhesion to Extracellular Matrix

The extracellular matrix (ECM) is a complex mixture of fibrous proteins and other components such as GAGs that supports the architecture of tissues, as well as contributing to cell viability, development, differentiation, and motility. Components include 28 types of collagen (with I, III, IV, and VI being most prominent), laminin, fibronectin, and proteoglycans (Batzios et al. 2013). Early studies identified that pathogenic leptospires can bind to ECM components, including fibronectin, collagen, laminin, and hyaluronic acid (Ito and Yanagawa 1987). Adhesion to ECM molecules is enhanced after incubation at physiological osmolarity, simulating the transition from environment to host (Matsunaga et al. 2007).

Proteins that bind to host structures are often termed Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs). The first indication of a specific leptospiral protein that binds ECM components was the finding that a 36-kDa outer membrane protein binds fibronectin, though the identity of this protein is unknown (Merien et al. 2000). Subsequently, a very large number of leptospiral proteins have been reported to bind to components of the ECM (Table 3). Despite the identification of many potential adhesins, none has been shown to be essential for virulence, a possible consequence of functional redundancy.

The majority of studies characterizing the interaction of leptospiral proteins with ECM components have used recombinant protein in in vitro assays. It is therefore difficult to translate the meaning of these findings to natural infection. This difficulty is compounded when the exposure of the protein of interest on the leptospiral cell is either not investigated (Lima et al. 2013), is inconclusive, or cannot be verified (Oliveira et al. 2011; Pinne et al. 2012, Table 3). The use of recombinant protein also presents a number of problems. Some leptospiral proteins undergo post-translational modification, such as lipidation, sialylation, glycosylation, phosphorylation, methylation, and proteolysis (Cao et al. 2010; Cullen et al. 2002; Ricaldi et al. 2012). Recombinant proteins produced in *Escherichia coli* are unlikely to have appropriate modifications. Furthermore, the majority of putative leptospiral membrane proteins are insoluble when expressed in *E. coli* in high quantities (Murray et al. 2013). Refolding of proteins is difficult and may result in formation of soluble multimers of protein which may not produce obvious solution turbidity but participate in non-specific ionic interactions (Burgess 2009). It is unlikely that leptospires require more than 25 laminin-binding proteins and more than 30 fibronectin-binding proteins, leading to the conclusion that some findings may be in vitro artifacts.

In some studies, protein function has been verified through alternative assays (Table 3), adding confidence to the results of the study. For example, the binding of leptospiral cells to host protein substrates was competitively inhibited by the addition of recombinant leptospiral proteins including LigA and LigB (inhibited leptospiral binding to fibronectin) (Choy et al. 2007), TlyC (ECM) (Carvalho et al. 2009), enolase (plasminogen) (Nogueira et al. 2013), Lsa20, Lsa25, and Lsa33

(laminin and plasminogen) (Domingos et al. 2012; Mendes et al. 2011), Lsa66 (ECM and plasminogen) (Oliveira et al. 2011), Lsa63 (collagen IV and laminin) (Vieira et al. 2010b), and OmpL1 (laminin, plasminogen, fibronectin) (Fernandes et al. 2012). In a similar confirmatory process, leptospiral attachment to laminin was blocked by antibody to Lsa24/lenA/LfhA (Barbosa et al. 2006), and binding to plasminogen was reduced by antibodies to enolase (Nogueira et al. 2013). In each of these studies, *Leptospira*–substrate interaction was only partially inhibited by antibody or recombinant protein, supporting the notion of multiple, redundant adhesins sharing the same substrate specificity. In some studies, the use of specific antibodies or excess recombinant protein resulted in no inhibition. For example, leptospiral binding to ECM was not inhibited by specific antibody to LipL32 (Hoke et al. 2008). In the case of OmpL37, cell adhesion to elastin was not inhibited by excess recombinant protein and, surprisingly, was enhanced in the presence of specific antiserum (Pinne et al. 2010). In another approach to confirmation of protein function, the ability of LigA and LigB to bind to fibronectin and laminin and the properties of fibronectin-binding proteins Mfn1, Mfn4, and Mfn7 were confirmed through gain of function studies using the saprophyte *L. biflexa* (Figueira et al. 2011; Pinne et al. 2012; Toma et al. 2014). In contrast, the mutants in *lipL32* and *ligB* displayed normal binding to ECM and MDCK cells, respectively (Croda et al. 2008; Murray et al. 2009c).

3.3.1 Fibronectin-Binding Proteins

Fibronectin exists both as a major component of the extracellular matrix and in soluble form in plasma. A large number of leptospiral proteins have fibronectin-binding properties in in vitro binding experiments (Table 3). Binding interactions with different affinities play a part in the slowing and exit of *B. burdorferi* from blood vessels (Moriarty et al. 2012); hence multiple proteins with different affinities may potentially be involved in the attachment, dragging, and arrest of leptospires in the blood vessel endothelium. Leptospiral fibronectin binding may also mediate binding to host cells via the I domain of the CR3 complement receptor (found on polymorphonuclear leucocytes, mononuclear phagocytes, and natural killer cells), potentially increasing phagocytosis in the absence of specific opsonins (Cinco et al. 2002).

The identification of fibronectin-binding proteins highlights how different methods of analysis do not always correlate. Merien et al. (2000) found one fibronectin-binding protein by ligand blot, yet subsequent studies have found more than 30 such proteins in *Leptospira* (Table 3). When a protein array comprising 401 predicted leptospiral outer membrane proteins was used to screen for fibronectin-binding proteins (Pinne et al. 2012), of the top 15 fibronectin-binding proteins only one had previously been identified (Lsa66). Notable fibronectin-binding proteins LigB (repeat domains 8–12) had 34th highest affinity for fibronectin, while LipL32 was 169th on the list. Regardless of this, fibronectin binding was validated by ligand blot for six of the top 15 proteins, and expression of three proteins in

L. biflexa conferred the ability to bind soluble fibronectin (Pinne et al. 2012). While different experimental approaches may have contributed to the different outcomes from these and other studies, the lack of correlation is surprising and suggests that some binding affinities identified in vitro are artifacts.

3.3.2 Laminin-Binding Proteins

Laminin is an important component of basement membranes of epithelial and endothelial surfaces. Ability to bind to laminin may enhance the ability of leptospires to invade and cross tissue layers. A remarkably large number of leptospiral proteins have been found to bind laminin in in vitro assays. One group of proteins is the Len protein paralogs; all of the Len proteins bind to laminin with varying degrees of affinity (Stevenson et al. 2007).

3.3.3 Elastin-Binding Proteins

Elastin fibers composed of the soluble protein tropoelastin confer elasticity to tissues, and are found in ECM of numerous tissues including the lung, skin, arteries, uterus, and placenta. Leptospirosis has an impact on many of these tissues; hence elastin-binding properties of LigB and OmpL37 and OmpL47 (Lin et al. 2009; Pinne et al. 2010) may assist with the initial stages of colonization in the skin, or facilitate pathogen adherence and damage to the lungs and blood vessel endothelium resulting in lung hemorrhage and vessel damage, or contribute to abortion. Interestingly, all three of these proteins bind specifically to numerous other host ligands (Table 3). LigB was observed to bind to elastin, and binding was localized to certain LigB domains (Lin et al. 2009). LigB also bound tropoelastin, potentially inhibiting tissue repair by preventing formation of elastin fibers (Lin et al. 2009). OmpL37 had high affinity for skin elastin. Rather than inhibit adhesion, antibodies to OmpL37 enhanced OmpL37 binding to elastin but not to other ECM proteins, suggesting that the host immune response to this protein may specifically promote adhesion to elastin (Pinne et al. 2010).

3.4 Disruption of Hemostasis and Wound Repair

3.4.1 Leptospiral Binding of Fibrinogen

Leptospirosis is characterized by thrombocytopenia, hemorrhage, and vascular injury. Some of these pathologies may be explained by the ability of leptospires to bind fibrinogen (Choy et al. 2007) with subsequent inhibition of fibrin formation (Oliveira et al. 2013). This may assist bacterial dissemination and contribute directly to hemorrhage. Numerous fibrinogen-binding proteins have been identified

(Table 4). Of these, LigB, Lsa33, LIC12238, LIC11975, and OmpL1 inhibited thrombin-catalyzed fibrin formation in vitro (Choy et al. 2011; Lin et al. 2011; Oliveira et al. 2013). LigB binding to the C-terminal αC domain of fibrinogen also inhibited platelet adhesion and aggregation in vitro (Lin et al. 2011). Some of the leptospiral proteins caused a slight decrease in the binding of leptospiral cells to fibrinogen in a competitive binding assay (Oliveira et al. 2013). However, none of these proteins has a confirmed role in disease, and as stated above, a *ligB* mutant retains normal virulence (Croda et al. 2008).

Notably, while other fibrinogen-binding proteins such as *Streptococcus epidermidis* SdrG completely inhibit fibrin formation (Davis et al. 2001), inhibition by leptospiral proteins was incomplete. This may indicate that leptospiral proteins are more important as adhesins, or work in concert for an additive effect. LigA also binds fibrinogen and is released from the leptospiral cell; this may inhibit blood coagulation beyond the immediate proximity of the leptospiral cell, though this has not been demonstrated (Choy et al. 2007). In addition to a role in prevention of hemostasis, LigB binds to collagen type III, fibroblast fibronectin and tropoelastin, which are all involved in tissue repair (Choy et al. 2011; Lin et al. 2009). This may allow leptospires to attach to a fresh wound for initial colonization, and may potentiate the formation of lesions and hemorrhage during systemic disease.

It should be noted that in one study, *L. biflexa* serovar Patoc also bound fibrinogen to about 75 % of the level seen in a virulent *L. interrogans* strain; *L. biflexa*-bound fibrinogen was able to inhibit thrombin-dependent fibrin formation to the same degree as *L. interrogans* strains (Oliveira et al. 2013). This finding questions the relevance of in vitro fibrinogen binding to pathogenesis.

3.4.2 Leptospiral Binding of Plasminogen

Plasminogen is a proenzyme found in extracellular fluid and plasma that can be converted to the enzyme plasmin by proteases such as urokinase plasminogen activator (uPA). Active plasmin may degrade numerous substrates, including fibrin clots, ECM proteins such as fibronectin and laminin, and immunoglobulins. Many pathogens bind to plasminogen, which is activated by endogenous or host proteases to produce the active protease plasmin. Surface-bound plasmin is involved in pathogenesis through degradation of ECM, complement components and antibodies, and the activation of matrix metalloproteases (Lähteenmäki et al. 2001) and plays an important role in the pathogenesis of bacteria such as streptococci (Li et al. 1999; Sanderson-Smith et al. 2008; Svensson et al. 2002).

Leptospires bind plasminogen in vitro, and bound plasminogen can be converted to plasmin in the presence of uPA (Verma et al. 2010a; Vieira et al. 2009). In vitro, *Leptospira*-bound plasmin degrades ECM components such as fibronectin (Vieira et al. 2009) and human fibrinogen (Oliveira et al. 2013), and may activate host matrix metalloproteases which in turn could contribute to tissue degradation. Plasmin-coated leptospires also crossed human umbilical vein epithelial cell monolayers more efficiently than normal leptospires, although the precise mechanism

was not investigated (Vieira et al. 2013). Taken together these data suggest that surface-bound plasmin may facilitate crossing of ECM and tissue barriers and degradation of fibrin clots by leptospires, resulting in dissemination throughout the host. As found in other pathogens (Lähteenmäki et al. 2001), leptospires may up regulate activators of plasminogen in host cells (Vieira et al. 2013). However, one study reported that saprophytic *L. biflexa* acquired more plasmin activity in vitro than some pathogenic strains, tempering these observations and complicating extrapolation to a host infection scenario (Vieira et al. 2009).

In vitro, leptospiral surface-bound plasmin also interferes with the deposition of C3b and immunoglobulin on the cell surface (Vieira et al. 2011). The elongation factor Tu (EF-Tu), involved in protein synthesis but also found to moonlight as a surface protein, binds plasminogen which may be activated to cleave C3b (Wolff et al. 2013). Reduced binding to C3b may diminish opsonization for phagocytosis and decrease the activation of the complement cascade at the cell surface by both the classical and alternative pathways. Plasmin-coated *L. interrogans* serovar Pomona displayed enhanced serum survival (Vieira et al. 2011).

Numerous leptospiral receptors for plasminogen have been identified. LenA binds plasminogen and it can be converted to plasmin to degrade fibronectin (Verma et al. 2010a). Enolase, a recognized plasminogen-binding protein of other bacterial pathogens, also binds plasminogen in *Leptospira* (Nogueira et al. 2013). Interestingly, leptospiral enolase is secreted and then associates with the leptospiral surface. More than a dozen additional receptors, including LipL32, have been reported to bind plasminogen and allow conversion to active plasmin in the presence of uPA, although evidence of surface localization for many of these proteins was inconclusive (Table 4). The significance of plasmin binding in vivo by these receptors is yet to be demonstrated.

3.5 Notable Proteins with Multiple Binding Affinities

Some leptospiral proteins bind to a remarkable number of diverse host proteins, potentially playing a role in varied aspects of pathogenesis. There are precedents for such proteins in the spirochetes; for example, the *Treponema denticola* protein OppA binds to plasminogen and fibronectin (Fenno et al. 2000), while Msp binds to fibronectin, keratin, laminin, collagen type I, fibrinogen, hyaluronic acid, and heparin (Edwards et al. 2005). Outside the spirochetes examples include Emp of *Staphylococcus aureus*, which interacts with fibronectin, fibrinogen, collagen, and vitronectin (Hussain et al. 2001).

3.5.1 Lig Proteins

The leptospiral Lig proteins are a group of three proteins (LigABC) that belong to a family of bacterial immunoglobulin-like proteins (Bigs) containing 12–13

immunoglobulin-like repeats (Matsunaga et al. 2003; Palaniappan et al. 2002). Bigs such as *E. coli* intimin and *Yersinia pseudotuberculosis* invasin mediate adhesion and invasion of host cells (Hamburger et al. 1999; Luo et al. 2000). The *ligA* gene is found in *L. interrogans*, *L. kirschneri*, and *L. santarosai*, although it is not yet clear whether it is universally present in all serovars and strains. LigA is one of the few promising vaccine candidate molecules for which statistically significant protection against acute leptospirosis has been demonstrated (see the chapter by B. Adler). *ligA* appears to have evolved from a partial gene duplication of *ligB* (McBride et al. 2009), and LigA is released from cells for an unknown purpose (Matsunaga et al. 2005). LigB is widely distributed in pathogenic leptospires. Many strains only have *ligB*, suggesting that it may be sufficient for pathogenesis. LigC has a wider distribution than LigA but is a pseudogene in multiple strains that retain virulence, indicating that it is unnecessary in these strains for disease pathogenesis (Cerqueira et al. 2009; McBride et al. 2009). LigC has not been functionally characterized.

Many factors indicate that Lig proteins are virulence factors. Lig proteins are significantly induced under conditions of increased osmolarity, emulating the transition from an environmental source to the host (Choy et al. 2007). Prolonged in vitro culture of leptospires leads to loss of Lig expression, correlating with a loss of virulence (Matsunaga et al. 2003). LigA and LigB bind to numerous host proteins and may play a role in disease by binding host ECM molecules at different stages (Choy et al. 2007, Table 3). Lig proteins also bind complement regulatory proteins (Table 4) and may play a role in potentiating tissue damage through binding fibrinogen and matrix components associated with wound healing (Choy et al. 2011). When *ligA* or *ligB* were expressed from a plasmid in the saprophyte strain *L. biflexa*, the resulting strain exhibited enhanced binding to some ECM components (fibronectin and laminin), but not others (Figueira et al. 2011). Lig proteins contribute to binding to host cells (Figueira et al. 2011; Lin et al. 2010; Toma et al. 2014). Lig proteins bind to complement regulatory proteins factor H and C4-binding protein (Castiblanco-Valencia et al. 2012), and LigB appears to contribute to serum resistance by inhibiting the alternative pathway of complement activation (Choy 2012).

However, evidence of an essential role in disease, or otherwise, for Lig proteins is inconclusive as a strain lacking all three Lig proteins has not been assessed in vivo. *L. interrogans* serovar Lai lacks *ligA* but retains *ligB* and *ligC* and is virulent (Ristow et al. 2007). A *ligB* mutant in *L. interrogans* serovar Copenhageni also retained virulence, but this strain still possessed *ligA* (*ligC* is a pseudogene in this strain) (Croda et al. 2008). Likewise, an *L. interrogans* serovar Manilae *ligC* mutant caused disease, but this strain retains *ligA* and *ligB* (Murray et al. 2009a). Given the extensive number of host substrates with which Lig proteins interact, in pathways including host matrix adhesion, complement resistance, and blood coagulation pathways, it seems likely that at least one Lig protein is required to cause disease.

3.5.2 The Len Proteins

This group of six proteins with similarity to human endostatins is found in pathogenic leptospires. It may have arisen through a process of gene duplication and recombination events, resulting in some overlap and some unique functions in the group (Stevenson et al. 2007). All of the Len proteins bind fibronectin and laminin. LenA (also known as LfhA/Lsa24) also binds plasminogen, factor H, and factor H-related protein, while LenB binds factor H (Verma et al. 2006, 2010a). Mutants in *lenB* and *lenE* retained virulence, but this is understandable given the functional redundancy of these proteins (Murray et al. 2009a).

3.5.3 LipL32

LipL32, also known as Hap1, is a dominant lipoprotein of the leptospiral outer membrane (Haake et al. 2000). It is the most abundant protein in *Leptospira* with an estimated 38,000 copies per cell (Malmstrom et al. 2009). The prominence of LipL32 combined with a high degree of conservation in pathogens and leptospires of intermediate pathogenicity, absence in saprophytic leptospires, and demonstrated expression in vivo make this protein a likely virulence factor (Murray 2013). An earlier indication that LipL32 may be associated with hemolysis has not been confirmed (Lee et al. 2000). Studies using recombinant LipL32 have identified binding substrates, including laminin, collagen I and V (Hoke et al. 2008), collagen IV and plasma fibronectin (Hauk et al. 2008). Notably, these studies had conflicting findings regarding laminin and collagen I binding by LipL32. Although reported binding strengths were moderate, the sheer number of LipL32 molecules on the surface may markedly increase the avidity of interaction (Vivian et al. 2009). LipL32 has also been reported to bind to plasminogen (Vieira et al. 2010a).

Despite all the indications of a role in virulence, a *lipl32* mutant remained virulent in both the hamster acute and rat colonization models of infection (Murray et al. 2009c). Notably, hamsters were challenged by both intraperitoneal and mucosal infection routes. The lack of attenuation of the *lipL32* mutant may be a result of functional redundancy, as many proteins share substrate specificity with LipL32 (Tables 3 and 4). Interestingly, while LipL32 is found in leptospires that are pathogenic or of intermediate pathogenicity, there are LipL32 orthologs in environmental organisms outside *Leptospira* (Murray 2013) including the marine organism *Pseudoalteromonas tunicata* (Hoke et al. 2008); perhaps this indicates a role for this LipL32 in transmission and environmental survival, two factors not assessed in current animal models. Furthermore, recent evidence using immunofluorescence and surface proteolysis suggests that LipL32 may not in fact be exposed on the surface of the cell (Pinne and Haake 2013). This may explain the general lack of protection conferred by immunization with LipL32 and naturally acquired immunity to LipL32 (Murray 2013, Chap. 10). As a result of this finding, it is advisable not to use LipL32 as a surface marker control when performing immunofluorescence and surface proteolysis experiments (see the chapter by D.A. Haake and W.R. Zückert, this volume).

4 Persistence

4.1 Evasion of Host Immunity

Phagocytes help to control the early stages of leptospiral infection, while protective acquired immunity is humoral in the vast majority of animal species and can be transferred passively by serum (Jost et al. 1986; Masuzawa et al. 1990; Schoone et al. 1989). Protective immunity is usually directed against lipopolysaccharides, and so is restricted to related serovars (Adler and de la Peña Moctezuma 2010) (see the chapter by R.L. Zuerner, this volume). Numerous interesting interactions have been characterized between leptospires and the immune system, which may increase the disease-causing potential of *Leptospira*.

4.1.1 Interaction with the Complement Cascade

During the initial stages of leptospirosis, bacteria are found in the blood for up to 2 weeks (Faine et al. 1999), necessitating a high degree of resistance to serum complement. Complement resistance distinguishes pathogenic leptospires from the highly susceptible saprophytes (Cinco and Banfi 1983). The difference between pathogen and saprophytes appears to be at the level of C3 deposition and this correlates with pathogen binding of host complement regulatory proteins factor H (Meri et al. 2005) and C4-binding protein (C4BP) (Barbosa et al. 2009). The consequence of inhibition of the complement cascade is not only reduced bacterial cell lysis, but also potentially diminished recruitment and activation of phagocytes (through reduced release of anaphylotoxins C3a and C5a) and reduced opsonophagocytosis (via phagocyte C3b receptors) (Blom et al. 2009).

Numerous proteins bind to the soluble host regulators of serum resistance factor H (and related proteins) and C4BP (Table 4). As most findings have only identified binding affinities using recombinant proteins, further work is required to elucidate the role of these proteins in serum resistance; for example, can binding to respective regulatory proteins be inhibited (by antibodies or ligand peptides), thereby enhancing complement sensitivity, or do mutants in these factors have enhanced serum sensitivity? Only *ligB* has been demonstrated to partially contribute to serum resistance when expressed in *L. biflexa* (Choy 2012). As is the case for ECM-binding proteins, a definitive in vivo role for complement pathway-interacting proteins in Table 4 is yet to be established. Mutants in genes encoding LenB (binds to factor H) and LigB (binds to factor H and C4BP) retained virulence, indicating that these proteins are not essential for disease (Croda et al. 2008; Murray et al. 2009a).

In alternative strategies for serum resistance, leptospires may inactivate bound complement proteins by proteases; plasmin-mediated reduction in C3b deposition and enhanced serum survival has been reported (Vieira et al. 2011), while secreted leptospiral proteases appear to degrade complement components (Fraga et al. 2014).

There is also evidence that leptospires can synthesize sialic acid and related non-ulosonic acids that may be added to surface proteins to promote serum resistance (Ricaldi et al. 2012), though further investigation is necessary.

4.1.2 Interaction with Phagocytic Immune Cells

Leptospires are not classical intracellular pathogens. However, recent discoveries suggest that intracellular phases may play a role in pathogenesis. In vitro studies have found leptospires maybe transiently intracellular when passing through cell layers (Barocchi et al. 2002; Thomas and Higbie 1990), and appear to persist in macrophages (Li et al. 2010; Toma et al. 2011).

In the murine macrophage/monocyte-like cell line J774A.1, internalization of leptospires occurred by receptor-mediated endocytosis rather than phagocytosis (Merien et al. 1997), suggesting entry into phagocytic cells by a non-phagocytic mechanism is beneficial to leptospires. A possible mechanism for this is via a mammalian cell entry (Mce) protein. Mce proteins are a group of proteins identified in *Mycobacterium tuberculosis* that mediate attachment and entry into host cells (Arruda et al. 1993). Pathogenic leptospires have an *mce*-like gene; when disrupted leptospiral adhesion and entry into macrophage-like cells was significantly reduced, and these capacities were restored upon complementation (Zhang et al. 2012). Adhesin and cell entry properties were conferred to *L. biflexa* upon complementation with the Mce protein and to Mce-coated latex beads. RGD protein motifs bind to integrins (Ruoslahti 1996) and this may be exploited by bacterial pathogens for cell adhesion and entry (Hauck et al. 2006). Binding of Mce to integrins α5β1 and $\alpha_V\beta3$ was demonstrated, and when the RGD motif of Mce was modified, binding of *L. biflexa* to host cells was lost (Zhang et al. 2012). The *mce* mutant had a modest attenuation upon infection of hamsters compared to the parent strain and complemented mutant (50-fold increase in LD_{50}) (Table 1) (Zhang et al. 2012), suggesting this entry mechanism is somewhat important for virulence. Leptospiral proteins LMB216 and LigB also contribute to the uptake of leptospires by phagocytic cells, as shown through analysis of *L. interrogans* mutants and by heterologous protein expression in *L. biflexa* (Toma et al. 2014).

Phagocytosis is an important immune control mechanism during leptospirosis (Faine 1957, 1964), therefore subversion of phagocytic outcomes may be an important mechanism of immune evasion. The production of reactive oxygen species is an important microbicidal mechanism for phagocytes. Catalase (KatE) found in pathogenic leptospires is required for resistance to hydrogen peroxide (Eshghi et al. 2012). While the role of KatE in survival in macrophages has not been directly tested, hamsters infected with *katE* mutants of *L. interrogans* serovars Pomona or Manilae survived challenge without signs of disease, indicating that oxidative stress resistance is essential for virulence (Table 1). Another mediator of resistance to oxidative stress is the molecular chaperone ClpB; this protein is also required for growth under nutrient restriction and heat stress (Lourdault et al. 2011). A *clpB* mutant was also highly attenuated; gerbils receiving a very high dose

survived infection with no clinical signs of leptospirosis and no macroscopic lesions normally associated with disease (Table 1). Restoration of growth under oxidative, heat, and nutrient stress conditions was achieved by complementation with an intact copy of *clpB*, along with partial restoration of virulence (Lourdault et al. 2011). The precise cause of attenuation of the *clpB* mutant is unknown, but may be due to altered expression of virulence factors, in vivo growth deficiency, or increased susceptibility to stress conditions including oxidative stress (Lourdault et al. 2011). Interestingly, a second chaperone, HtpG, has been shown to be required for virulence, but the mechanism of attenuation is yet to be determined (Tables 1 and 2).

Apoptosis is another potential mechanism for pathogens to escape killing in phagocytes, but paradoxically may also be a host mechanism to contain infection. Numerous potential mechanisms have been suggested for apoptosis observed in vitro (Jin et al. 2009; Hu et al. 2013), including the involvement of sphingomyelinase 2 in a human liver cell line (Zhang et al. 2008) and by calcium ion flux initiated by leptospiral phospholipase C (LB361) in human and murine macrophage cell lines (Zhao et al. 2013). However, it remains unclear what role macrophage apoptosis plays in leptospirosis as other studies have reported no evidence of apoptosis in vitro (Toma et al. 2011). Additionally, evidence of apoptosis in animal infection is limited, being reported in hepatocytes of laboratory infected guinea pigs (Merien et al. 1998).

4.2 Nutrient Acquisition

The nutritional requirements of *Leptospira* are relatively simple, comprising a source of B vitamins, iron, ammonium, and long chain fatty acids as an energy source for β-oxidation (see the chapter by C.E. Cameron, this volume). Leptospires have approximately 12 predicted TonB-dependent receptors that may be responsible for active nutrient import (Nascimento et al. 2004; Ren et al. 2003). However, little is known about the substrates for these receptors.

Fatty acids for β-oxidation may be obtained through degradation of host cells membranes by phospholipases or sphingomyelinases (Kasărov 1970; Narayanavari et al. 2012). Sphingomyelinases catalyze the hydrolysis of sphingomyelin into ceramide and phosphorylcholine and may be responsible for hemolysis and damage to host tissues. *L. interrogans* has five predicted sphingomyelinases (Sph1, Sph2, Sph3, Sph4, SphH) while *L. borgpetersenii* has three (SphA, SphB, Sph4), although only Sph2 and SphA are predicted to have complete catalytic sites (Narayanavari et al. 2012). The sphingomyelinase activities of SphA and Sph2 have been demonstrated (del Real et al. 1989; Segers et al. 1992), and Sph2 has cytotoxic effects on cultured cells (Artiushin et al. 2004), while the activities of the remaining enzymes are not fully resolved (Narayanavari et al. 2012). As noted in Table 3, Sph2 may be an adhesin binding to fibronectin (Pinne et al. 2012) and may initiate signaling that leads to cellular apoptosis (Zhang et al. 2008). It is hypothesized that the sphingomyelinases lacking key amino acid residues in catalytic sites may still

bind to sphingomyelin of the host cell followed by another effector function (Narayanavari et al. 2012). Sphingomyelinases may contribute directly to endothelial damage leading to hemorrhage, but this remains speculative. Sublytic properties of sphingomyelinases may also be important in disease; the generation of excess ceramide in the host cell membrane may lead to perturbations in cell biology in different tissues leading to different pathologies (Narayanavari et al. 2012).

Leptospires require iron for growth (Faine 1959). In vivo, free iron is scarce due to the rapid formation of oxidized forms under physiological conditions, and due to host sequestration of iron by iron-binding proteins, especially as a defense against pathogens during infection (Wooldridge and Williams 1993). The majority of iron in the mammalian host (74 %) is in the form of heme in the protein hemoglobin (Wooldridge and Williams 1993). Heme and hemoglobin are sufficient to support leptospiral growth as sole iron sources (Guégan et al. 2003). In vivo, hemoglobin may be obtained by lysis of erythrocytes by sphingomyelinases. There are orthologs of the *tlyABC* hemolysins of *Brachyspira hyodysenteriae* encoded on the leptospiral genome, but TlyB and TlyC do not appear to have hemolytic activity (Carvalho et al. 2009). Efficient use of hemoglobin requires heme oxygenase to liberate ferrous iron from the tetrapyrrole ring for use by the leptospires (Murray et al. 2008). A heme oxygenase mutant was moderately attenuated for virulence in the acute model of disease, confirming that heme is an important iron source in vivo (Murray et al. 2009b, Table 1).

Leptospira interrogans has one characterized heme import mechanism. HbpA is a TonB-dependent receptor that binds heme (Asuthkar et al. 2007). A mutant in *hbpA* was unable to colonize mice but was still virulent in the hamster model of infection (Marcsisin et al. 2013). LipL41 was also reported to bind to heme but there are conflicting findings regarding this potential function (Asuthkar et al. 2007; King et al. 2013; Lin et al. 2013).

5 Mechanisms of Damage to Host Tissues

Leptospirosis is characterized by various symptoms, including vasculitis, acute renal failure, jaundice, thrombocytopenia, pulmonary hemorrhage, myocarditis, conjunctival suffusion, and uveitis (Levett 2001, Chap. 5). The mechanisms by which damage occurs are not conclusively known. Injury to the endothelium of small blood vessels may contribute to ischemia and dysfunction of multiple organs, while circulating toxic cellular components or undefined toxins may contribute to tissue damage (Adler and de la Peña Moctezuma 2010). Disruption of tissue integrity may occur by activity of leptospiral sphingomyelinases and phospholipase D. Leptospires also encode multiple proteases that may damage host tissues (collagenase, metalloproteases, and multiple thermolysins) (Nascimento et al. 2004). While the virulence properties of most of these remain to be fully characterized, a collagenase mutant was recently reported to have modestly reduced virulence in the hamster model of infection (25-fold increase in LD_{50}), though it should be noted

that the challenge strain had an extremely high LD_{50} of around 10^6 leptospires (Table 1) (Kassegne et al. 2014). Decreased tissue distribution and reduced tissue pathology were also reported in animals infected with the mutant (Kassegne et al. 2014), though it is unclear if the reduced pathology was a direct result of the loss of collagenase activity. Activation of host proteases such as plasminogen and matrix metalloproteases may also contribute to host tissue destruction and bacterial dissemination. Hemorrhage may be a result from a combination of tissue damage, disruption of hemostatic mechanisms, and interference of wound repair.

Fever is a key feature of leptospirosis, and many pathologies associated with leptospirosis may result from inflammation. Inflammation may be a consequence of tissue damage rather than be directly mediated by bacterial factors (Faine et al. 1999), as leptospiral LPS has remarkably low pyrogenicity compared to the LPS of other bacteria; LPS extracts injected into rabbits were non-pyrogenic in doses up to 5 μg/kg, had reduced activity in *Limulus* lysate assay (Vinh et al. 1986), and 500-fold less acute lethality in mice and 20-fold less mitogenicity when compared to *Salmonella typhimurium* LPS (Shimizu et al. 1987). In contrast to LPS, glycolipoprotein extracts (containing polysaccharides, lipids and proteins) had cytotoxic activity (Vinh et al. 1986). Low LPS toxicity may contribute to the ability of leptospires to achieve high numbers in vivo, and may be a consequence of an unusual lipid A structure (Que-Gewirth et al. 2004). It should be noted that biological properties of leptospiral LPS have been elucidated from in vitro-grown bacteria and it is possible that LPS is modified in vivo (Nally et al. 2005), conferring different pyrogenic properties. Leptospiral LPS also signals via TLR2 (rather than the normal TLR4) in human macrophages, while signaling via TLR2 and TLR4 in murine cells (Nahori et al. 2005), which may also contribute to different outcomes in disease depending on host species.

Renal pathology during leptospirosis is associated with interstitial nephritis and cellular infiltrates containing neutrophils and monocytes, suggesting an inflammatory mechanism. Leptospiral membrane protein extracts induced inflammatory response in cultured murine proximal tubule cells (Yang et al. 2002) signaling through TLR2 (Yang et al. 2006); this activity may play a role in interstitial nephritis. It was found that LipL32 plays a role in this stimulation via TLR2 (Hsu et al. 2010; Yang et al. 2006). However, these experiments were performed using extracted proteins with cell lines in vitro; an in vivo role for LipL32 signaling via TLR2 is less clear as LipL32 in intact organisms may not be surface-exposed (Pinne and Haake 2013), and hamsters infected with a LipL32 mutant had the same renal pathology as hamsters infected with wild-type bacteria, indicating a role for other processes in renal pathology (Murray et al. 2009c). The leptospiral outer membrane protein Loa22 has also been implicated in causing necrosis of a rat proximal tubule cell line and inducing an inflammatory response (Zhang et al. 2010). An alternative inflammatory mechanism has recently been described, by down regulation of the Na/K-ATPase pump by leptospiral glycolipoprotein, thereby activating the NLRP3 inflammasome (Lacroix-Lamande et al. 2012). Inhibition of the Na/K-ATPase pump may also contribute to loss of lung integrity and kidney dysfunction, leading to hypokalemia (Goncalves-de-Albuquerque et al. 2012).

Uveitis is another complication of leptospirosis that may occur weeks to years after initial infection. The condition may result from a breakdown in the immune privilege status of the eye with a combination of inflammation and autoimmunity (Verma and Stevenson 2012). Self-reactive antibodies have been found in uveitic eyes, including antibodies to leptospiral proteins LruA that cross-react with lens proteins α-crystallin B and vimentin, and antibodies to LruB that cross-react to retinal protein β-crystallin B2 (Verma et al. 2010b).

6 Virulence-Associated Factors Without a Defined Function

The role in pathogenesis has not been determined for a significant number of virulence factors identified by mutagenesis and in vivo screening.

6.1 Loa22

Loa22 is a probable lipoprotein with an OmpA domain and peptidoglycan-binding domain, indicating that it could be both surface exposed and interact with the peptidoglycan layer. Loa22 was the first virulence factor identified by mutagenesis and testing in vivo (Ristow et al. 2007). A *loa22* mutant was attenuated in guinea pigs and hamsters; virulence was partially restored upon complementation. Guinea pigs infected with the *loa22* mutant showed little or no tissue pathology, but bacteremia was detected on day 3 and renal colonization was detected upon termination of the experiment at day 21. The level of attenuation for the *loa22* mutant was moderate given that at doses of approximately 10^8 leptospires not all animals in the control group died, while some animals challenged with the *loa22* mutant died; this may be due to the use of strain *L. interrogans* serovar Lai strain Lai 56601 which has reduced virulence.

The function of Loa22 remains unknown. Given that Loa22 is the second most abundant protein of the cell envelope of *L. interrogans* after LipL32 (Malmstrom et al. 2009), it may play an essential structural or other role in the cell not directly related to virulence (reviewed in Confer and Ayalew 2013). Surface exposure raises the possibility that it may interact directly with host proteins/structures (Ristow et al. 2007); in many bacterial species, OmpA proteins have been identified as adhesins for host cells and extracellular proteins (Confer and Ayalew 2013) and moderate binding to collagen type I, collagen type IV, and plasma fibronectin has been reported for Loa22 (Barbosa et al. 2006). OmpA domains are a pathogen-associated molecular pattern, thereby recognized by pattern recognition molecules such as TLR2, and OmpA proteins activate dendritic cells (Torres et al. 2006). Recombinant Loa22 was cytotoxic to a rat proximal tubule cell line and induced an

inflammatory response via TLR2 (Zhang et al. 2010), even in the absence of protein lipidation. OmpA family proteins have a diverse array of other virulence properties, including promoting invasion, intracellular survival, and evasion of host immune defenses such as complement (by binding fluid phase complement regulatory proteins) and antimicrobial peptides (Confer and Ayalew 2013).

6.2 *LruA*

LruA is a lipoprotein that is conserved across the different classes of leptospires and contains a LysM domain, suggesting that it binds to peptidoglycan. While LruA is at least partly surface exposed (Zhang et al. 2013) the majority remains cell-associated after TritonX 114 extraction (Verma et al. 2005), suggesting that the protein either has an unusual membrane topology or multiple subcellular locations; although unusual, lipoproteins with multiple subcellular locations have been described (Michel et al. 2013). LruA is a probable inducer of autoimmunity that causes reactive uveitis; antibodies to this protein cross-react with α-crystallin B and vimentin of the ocular lens (Verma et al. 2010b). Independent of this property, a recent study also identified this protein as essential for virulence (Zhang et al. 2013). Mutation of *lruA* led to a moderate attenuation; infection with a dose 100 times the estimated LD_{50} for serovar Manilae led to the death of 10 % of hamsters across two experiments. Interestingly, a second mutant with a minor truncation of LruA (Δ525-556) retained virulence, suggesting that the functional domains are not present at the carboxy terminus of the protein.

The mechanism of attenuation of the *lruA* mutant is unknown, but may be related to the interaction with host serum protein apolipoprotein A-I (Apo A-I). Apo A-I is involved in lipid transport, but can also play a role in LPS detoxification and inflammation during sepsis (Guo et al. 2013) and has been implicated in the killing of *Yersinia enterocolitica* by serum complement (Biedzka-Sarek et al. 2011). The LruA mutant bound considerably more Apo A-I than WT bacteria, but the significance of this is yet to be determined, and it did not increase susceptibility to killing by serum complement (Zhang et al. 2013). Given that LruA is a lipoprotein and therefore membrane bound, and binds to peptidoglycan, it may have a structural role not directly related to virulence.

6.3 *Lipopolysaccharide (LPS)*

Leptospires have unusually large LPS synthesis loci of approximately 100 genes, all encoded on the same DNA strand (Bulach et al. 2006; Nascimento et al. 2004; Ren et al. 2003). The structure of LPS is unknown, as are the roles of individual proteins in LPS synthesis. During a mutagenesis study of *L. interrogans*, relatively

few mutants disrupted in the LPS locus were identified, implying an essential role in the biology of *Leptospira* (Murray et al. 2009a).

In many pathogenic bacteria, LPS is essential for virulence. Two leptospiral mutants with modified LPS were highly attenuated in the acute model of infection even at very high dose (10^7 leptospires, more than 10^6 times estimated LD_{50}). No disease pathology and no symptoms of infection or tissue pathology were observed (Murray et al. 2010; Srikram et al. 2011). The first LPS mutant (M895) had a mutation in a gene of unknown function resulting in truncated LPS. The second mutant had no obvious change in molecular mass but different reaction with antibodies to LPS; subsequent bioinformatics analysis suggests that this gene may encode a methyltransferase of the LIC12133 family (NCBI Conserved Domain Database), which may explain the lack of a detectable mass difference by SDS PAGE. Both of these strains with modified LPS also failed to colonize the mouse maintenance host model (Marcsisin et al. 2013). The precise mechanism of attenuation is unknown, but was not due to increased susceptibility to complement-killing (Murray et al. 2010). It is predicted that the LPS locus contains long transcripts, raising the possibility that the mutations may affect the transcription of downstream LPS synthesis genes.

6.4 Bacterial Chaperone HtpG

Bacterial chaperone HtpG is a homolog of the eukaryotic Hsp90. It has been attributed variable roles in different bacteria, including resistance to heat and oxidative stress and survival in macrophages (Dang et al. 2011; Weiss et al. 2007). Attenuated *htpG* mutants have been described for *Edwardsiella tarda* (Dang et al. 2011) and *Francisella tularensis* (Weiss et al. 2007). A leptospiral *htpG* (*lb058*/*lic20044*) mutant was highly attenuated in hamsters, with animals surviving a dose of $>10^6$ times LD_{50} and lower bacterial burdens detected in tissues (King et al. 2014), although the *htpG* mutant colonized hamster kidneys. Additionally, microscopic and macroscopic pathology was observed in hamsters infected with the mutant. Virulence of the *htpG* mutant was fully restored upon complementation.

The mechanism of attenuation of the leptospiral *htpG* mutant is unknown, as bacteria displayed no increase in susceptibility to physical and chemical stresses (heat, osmolarity, and oxidative) and exhibited essential virulence phenotypes (LPS expression, motility, expression of Loa22, survival in macrophages) (King et al. 2014). The ClpB chaperone contributes to resistance to oxidative and heat stress (Lourdault et al. 2011) and there is a second *htpG* paralog (LA1231/LIC12469) encoded in the leptospiral genome that may also account for some of these properties, although a mutant in this gene retained virulence (King et al. 2014). It is difficult to predict what role HtpG plays in virulence as the substrates of this bacterial chaperone are poorly defined (Buchner 2010). Disruption of *htpG* may result in modulation of the expression of virulence factors and further characterization of this mutant may identify novel virulence processes.

6.5 Other Attenuated Mutants

Mutants in genes encoding *lb194*, *la2786*, and *la0589* (all of unknown function) were colonization-deficient in the mouse colonization model (Marcsisin et al. 2013). LB194 may be involved in iron utilization; it is up regulated under low iron conditions and is located in a preserved locus with HbpA (hemin-binding TonB-dependent receptor) in both pathogenic and saprophytic species of *Leptospira*. LA2786 is also marginally up regulated under low iron conditions (Lo et al. 2010).

LA0589 is one of around 12 highly similar paralogous proteins encoded in the *L. interrogans* genome. Of note, these genes are highly up regulated in vivo and point mutations in paralogs *la3490 la3388* were identified in a culture-attenuated leptospiral strain (Lehmann et al. 2013), further suggesting a role in virulence for this gene family. Interestingly, there are similar sets of paralogous proteins in *Bartonella* spp. Further work is required to elucidate the role of these proteins in host colonization.

7 The Renal Carrier State

Depending on the host and infecting serovar, leptospiral infection may cause a spectrum of syndromes from an asymptomatic carriage to a fulminant, acute disease. A carrier host, also termed maintenance, reservoir or chronically infected host, may be defined as a host in which infection is endemic, disease is mild or asymptomatic, and transmission occurs back to the same host species (Blackmore and Hathaway 1979). Carrier hosts are often rodents. Hosts that suffer acute disease such as humans are incidental hosts that are unlikely to serve as a source of transmission, constituting a dead end infection.

Upon infection, bacteria disseminate throughout the carrier host and are most likely cleared by the immune system from all tissues except the kidney. In the renal tubules, bacteria continue to multiply and are excreted in the urine at concentrations of as high as 10^7 leptospires/ml (Faine 1962; Monahan et al. 2008). While carrier hosts may become lifetime shedders of *Leptospira*, acute hosts become temporary urinary shedders, in the case of humans for 2 weeks to 1 month (Faine et al. 1999). The carrier host has a long-term evolutionary association with leptospires where equilibrium has been reached between virulence and host response, making the organism almost commensal. The contrast with an acute host can be remarkable; *L. interrogans* serovar Manilae has an LD_{50} of <10 bacteria in hamsters but a dose of 10^8 does not cause any overt signs of disease in rats apart from renal colonization (Murray et al. 2009c). Experimentally infected rats also display no tissue pathology apart from possible interstitial nephritis (Monahan et al. 2008; Tucunduva de Faria et al. 2007). It is feasible that incidental hosts become maintenance hosts over time, concomitant with a reduction in leptospiral virulence for the particular host. For example, in an area of high transmission rates in the Peruvian Amazon, around 5 %

of people may become long-term renal shedders of *Leptospira* suggesting host adaptation, although direct human to human transmission is yet to be demonstrated (Ganoza et al. 2010).

While the carrier state is required for leptospiral transmission cycle, and carrier animals are the exclusive reservoir for human infection, very little is known about the molecular basis for development of the carrier state. Specific mechanism may be required to cross into the lumen of proximal renal tubules, adhere to renal epithelial cells, evade antibodies in the filtrate, and to acquire nutrients. Some studies have analyzed antigenic properties relating to the carrier state. When compared to bacteria from an acute guinea pig model, leptospires derived from rat urine had comparatively more LPS present, although the significance of this is unknown (Nally et al. 2005). Furthermore, urine-derived bacteria exhibit reduced reactivity to host-derived antibodies (Faine 1962). This may avoid the activity of antibodies that leak into the tubule as a result of renal injury during infection (Lane and Faine 1963), possibly in part due to down regulation of proteins to which the host has mounted an immune response (Monahan et al. 2008).

Analysis of defined mutants may give insights into the molecular basis for the carrier state. Only a handful of mutants has been studied in carrier models of disease. LipL32 and LigB were found to be unnecessary for renal colonization of rats (Croda et al. 2008; Murray et al. 2009c). A third study analyzed 28 mutants for virulence in a mouse carrier host (Marcsisin et al. 2013). Two virulence factors required to cause disease in the acute model, LPS and HtpG, were also required to colonize mouse kidneys. An additional five mutants were unable to colonize the carrier host but still caused disease in the acute host (Marcsisin et al. 2013). These colonization-deficient leptospires had mutations in genes encoding several proteins of unknown function and with two proteins with potential roles in iron transport (Table 2), thus identifying the first colonization-specific virulence factors.

While bacterial factors may contribute to the outcome of infection, there are likely to be host factors that are also important, such as immune recognition of leptospires. Leptospiral LPS signals via TLR2 in human macrophages (rather than the more usual LPS receptor, TLR4), while signaling via TLR2 and TLR4 in murine cells (Nahori et al. 2005). Recognition of leptospires through TLR4 is important for the resistance of mice to acute leptospirosis, as mice defective in TLR4 are susceptible to acute leptospirosis (Chassin et al. 2009; Viriyakosol et al. 2006, Chap. 9). This point of difference between human and murine recognition of *Leptospira* may contribute to the contrasting disease outcomes (Werts 2010).

8 Future Directions

In the past decade, significant advances have been made in the understanding of the pathogenesis of leptospirosis. However, the molecular basis of disease remains poorly elucidated. For example, the molecular basis for the pathology of leptospirosis is largely unknown. Additionally, the functions of numerous essential virulence

factors (Tables 1 and 2) are either uncharacterized or poorly defined. The secretome also remains to be fully explored. It is likely that many more virulence factors remain to be discovered and considering the overrepresentation of hypothetical genes in those genes exclusively found in pathogenic leptospires, it is likely that these will be novel. Hence, unbiased screening experiments for attenuated mutants will be very useful (Marcsisin et al. 2013). Exclusively in vitro findings also require consolidation with the understanding in animal models; the plethora of leptospiral proteins with an in vitro-characterized function needs to be translated into the host, and any in vitro artefactual findings need to be identified and discarded. Finally, the elucidation of colonization mechanisms in the carrier host will be important in understanding human disease and may lead to methods of disease prevention.

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