Molecular Microbiology (2010) 78(3), 701–709 ■
doi:10.1111/j.1365-2958.2010.07360.x
First published online 14 September 2010

Mutations affecting *Leptospira interrogans* lipopolysaccharide attenuate virulence

Gerald L. Murray,[1] Amporn Srikram,[2†] Rebekah Henry,[1,3] Rudy A. Hartskeerl,[4] Rasana W. Sermswan[5] and Ben Adler[1,3]*

[1]*Department of Microbiology and* [3]*Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, Vic., Australia.*

[2]*Melioidosis Research Center and* [5]*Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand.*

[4]*Royal Tropical Institute (KIT), KIT Biomedical Research, Amsterdam, the Netherlands.*

Summary

***Leptospira interrogans* is the causative agent of leptospirosis. Lipopolysaccharide (LPS) is the major outer membrane component of *L. interrogans.* It is the dominant antigen recognized during infection and the basis for serological classification. The structure of LPS and its role in pathogenesis are unknown. We describe two defined mutants of *L. interrogans* serovar Manilae with transposon insertions in the LPS locus. Mutant M895 was disrupted in gene *la1641* encoding a protein with no known homologues. M1352 was disrupted in a gene unique to serovar Manilae also encoding a protein of unknown function. M895 produced truncated LPS while M1352 showed little or no change in LPS molecular mass. Both mutants showed altered agglutination titres against rabbit antiserum and against a panel of LPS-specific monoclonal antibodies. The mutants were severely attenuated in virulence via the intraperitoneal route of infection, and were cleared from the host animal by 3 days after infection. M895 was also highly attenuated via the mucosal infection route. Resistance to complement in human serum was unaltered for both mutants. While complementation of mutants was not possible, the attenuation of two independently derived LPS mutants demonstrates for the first time that LPS plays an essential role leptospiral virulence.**

Accepted 19 August, 2010. *For correspondence. E-mail ben.adler@med.monash.edu.au; Tel. (+61) 3 9902 9177; Fax (+61) 3 9902 9222. †Present address: Section of Food Technology, Faculty of Natural Resources and Agro-Industry, Chalermphrakiate Sakhonnakorn Province Campus, Kasetsart University, Sakhonnakorn, 47000, Thailand.

© 2010 Blackwell Publishing Ltd

Introduction

The spirochete *Leptospira interrogans* is a widespread zoonotic pathogen transmitted via the urine of carrier animals such as cattle, swine, dogs and rodents. Human infection occurs upon contact with urine or contaminated soil or water. Bacteria invade via skin abrasions or mucous membranes and cause a systemic illness that varies greatly in severity from a mild flu-like illness to one with multiple organ failure and death (Adler and de la Pena Moctezuma, 2010).

Lipopolysaccharide (LPS) is the main lipid component of the outer leaflet of the outer membrane of Gram-negative bacteria. LPS is generally composed of an endotoxic lipid A anchor, a conserved core oligosaccharide, and an oligosaccharide or polysaccharide known as the O antigen (Raetz and Whitfield, 2002). Leptospiral lipid A contains some unusual structural features and unexpectedly signals via TLR2 rather than TLR4 (Que-Gewirth *et al.*, 2004; Nahori *et al.*, 2005), at least in humans. Apart from a preliminary knowledge of sugar content, the polysaccharide structure of the LPS is unknown (Faine *et al.*, 1999).

LPS is responsible for the antigenic diversity of pathogenic *Leptospira*, and remains the primary identifier for diagnosis and classification. Currently, 24 serogroups containing more than 250 serovars of pathogenic *Leptospira* are recognized (Faine *et al.*, 1999). Immunity to *Leptospira* is largely serotype specific and antibodies directed against LPS are sufficient for protection from infection in most animal species (Jost *et al.*, 1986). This restricted degree of cross immunity has principally hindered attempts at generating a cross protective vaccine.

Genomic data for *L. interrogans* serovars (sv.) Lai and Copenhageni indicate that synthesis of LPS is encoded in large loci of approximately 100 kb all on one strand of the large chromosome (Ren *et al.*, 2003; Nascimento *et al.*, 2004). The LPS loci of these two very closely related serovars show a high level of similarity with few gene insertions or deletions. LPS loci have also been partially characterized for some other *L. interrogans* serovars (de

la Pena-Moctezuma *et al.*, 2001). However, without a structure for LPS the genetic basis for biosynthesis and antigenicity remains unknown.

The molecular mechanisms involved in the pathogenesis of leptospirosis are poorly defined, mainly due to a lack of genetic techniques that can be applied in the study of *L. interrogans*. The development of a transposon mutagenesis system has allowed production of defined mutants (Bourhy *et al.*, 2005). Application of mutagenesis has shown that Loa22 and HemO are required for full virulence (Ristow *et al.*, 2007; Murray *et al.*, 2008; 2009b), and found that the outer membranes proteins LipL32 and LigB are not essential for *Leptospira* to cause either acute disease or renal colonization (Croda *et al.*, 2008; Murray *et al.*, 2009a,c).

In this study, two defined transposon mutants with altered LPS are described. The mutants were avirulent in the hamster model of infection, demonstrating for the first time that *L. interrogans* LPS is essential for virulence.

Results

Construction of mutants defective in LPS synthesis

Transposon mutagenesis was conducted in *L. interrogans* sv. Manilae strain L495 as described previously (Bourhy *et al.*, 2005; Murray *et al.*, 2008; 2009a). In mutant M895 the transposon had inserted into the LPS synthesis locus within a gene named Lman_1456, homologous to *la1641* of *L. interrogans* sv. Lai (Fig. 1). This gene encodes a protein of unknown function with 11 predicted transmembrane helices and a predicted inner membrane location. The encoded protein has no significant sequence similarity to other proteins. A second mutant (M1352) was obtained within the LPS biosynthesis locus in a chromosomal region unique to sv. Manilae at the 3′ end of a gene encoding a protein of unknown function. This gene, named Lman_1408, is located downstream of a putative sugar pyridoxal-phosphate-dependent aminotransferase and 208 bp upstream of a putative *rmlC* (dTDP-4-dehydrorhamnose 3,5-epimerase). Both mutants showed normal motility and morphology when observed under dark-field microscopy, and showed normal *in vitro* growth rates (data not shown).

Mutants M895 and M1352 have altered LPS

The LPS profile of M895 was examined by silver-stained SDS-PAGE analysis (Fig. 2). As seen previously for *Leptospira*, the LPS formed a number of bands with the uppermost band predicted to be the mature LPS (Cinco *et al.*, 1986). In M895, the upper band was considerably reduced in molecular mass compared with that in the wild-type (WT) strain (Fig. 2). By Western blotting, M895 retained reactivity with anti-Manilae serum (Fig. 2). M895 showed a 75% reduction in MAT titre with antiserum against *L. interrogans* sv. Manilae (Table 1) and serum absorbed with M895 retained reactivity against WT LPS, but not against M895 (Fig. 2), consistent with the loss of some antigenic epitopes in the M895 mutant. In contrast, M1352 showed no apparent decrease in LPS molecular mass. M1352 also had a 75% reduced MAT titre with antiserum against *L. interrogans* sv. Manilae. Both mutants showed an increase in some titres when analysed by MAT against a panel of monoclonal antibodies against sero-

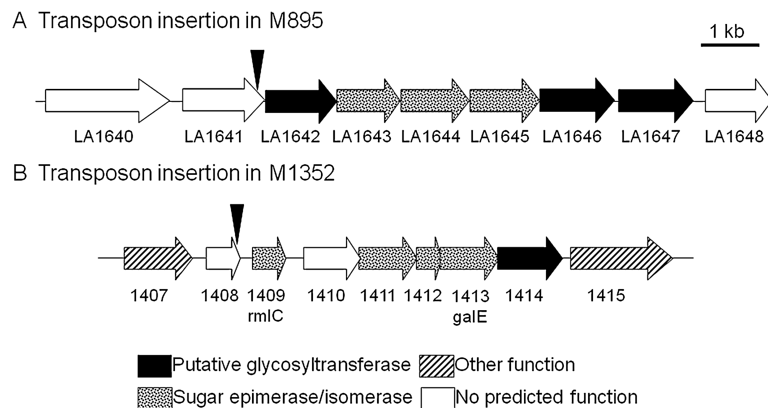

Fig. 1. The locations of transposon insertions in *L. interrogans* sv. Manilae. Insertion points are indicated by a vertical arrow head.
A. Transposon insertion in mutant M895. The map is derived from the sequence data of *L. interrogans* sv. Lai (Ren *et al.*, 2003). Locus tags are given based on the sv. Lai genome annotation. Predicted function of encoded proteins: LA1642, glycosyltransferase; LA1643, *N*-acetyl glucosamine/*N*-acetyl galactosamine epimerase; LA1644, NDP-sugar dehydratase or epimerase; LA1645, UDP-*N*-acetylglucosamine 2-epimerase; LA1646, glycosyltransferase; LA1647, undecaprenol-galactosyl transferase.
B. Transposon insertion in M1352. Open reading frames predicted to encode: 1407, sugar pyridoxal-phosphate-dependent aminotransferase; 1408, hypothetical; 1409, RmlC, dTDP-4-dehydrorhamnose 3,5-epimerase; 1410, hypothetical protein; 1411, nucleoside-diphosphate-sugar epimerase; 1412, sugar isomerase; 1413, GalE, UDP-glucose 4-epimerase; 1414, glycosyltransferase; 1415, ATP-binding protein of an ABC transporter complex.

© 2010 Blackwell Publishing Ltd, *Molecular Microbiology*, **78**, 701–709

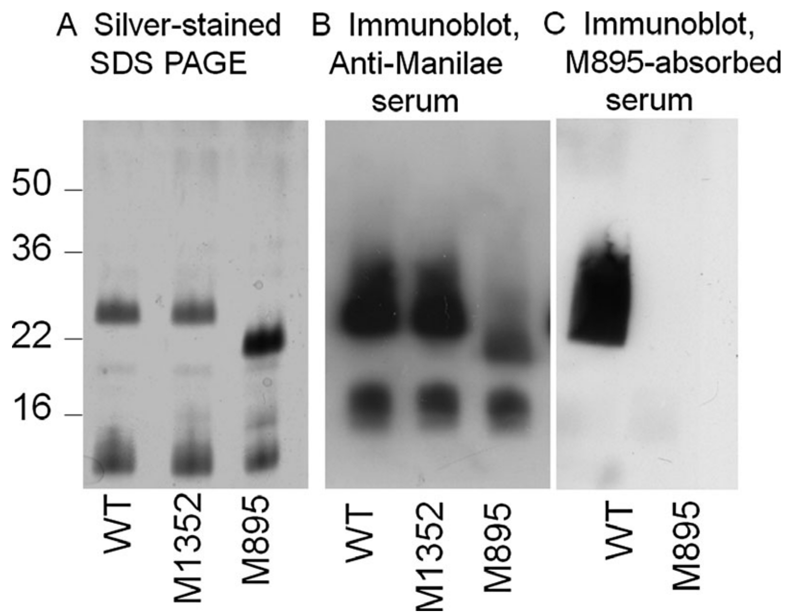

Fig. 2. Analysis of leptospiral LPS by SDS-PAGE and immunoblotting. Proteinase K-treated whole-cell lysates of wild-type L495 (WT), and mutants M895 or M1352 were separated on 12.5% polyacrylamide gels and either silver stained (A) or transferred to PVDF membrane and probed with a rabbit anti-Manilae serum (B) or antiserum pre-absorbed with M895 (C). The positions of standard molecular mass markers (kDa) are shown on the left.

group Pyrogenes LPS (Table 1), a finding consistent with LPS alterations resulting in greater exposure of the monoclonal-reactive epitopes.

Table 1. Microagglutination test (MAT) titres for mutant strains using various antibodies against *Leptospira* and *Leptospira* LPS.

			MAT titre for antibodies against strain[c]		
Antibody	Type[a]	Specificity[b]	L495 WT	M1352	M895
SIN1110	P	Manilae	102 400	25 600	25 600
F134C2	M	Pyrogenes	20	20	160
F134C5	M	Pyrogenes	20	40	160
F134C6	M	Pyrogenes	20	80	320

a. P, polyclonal; M, monoclonal.
b. Serovar or serogroup to which antibodies were raised. No reactivity was found against serogroup Canicola or Icterohaemorrhagiae monoclonal antibodies.
c. Results for unreactive antibodies are not shown.

Complementation of the mutant M895 was attempted by cloning the sv. Manilae gene equivalent of *la1641* incorporating upstream sequence into a modified transposon encoding spectinomycin resistance. Six transformants each contained the replacement intact gene and retained the original transposon mutation. However, the complemented strains did not exhibit a restored LPS phenotype.

Analysis of the Manilae LPS biosynthesis locus

A draft sequence of the *L. interrogans* sv. Manilae LPS locus was compared with other LPS loci. The region of *la1641* (mutated in M895) was similar between sv. Lai and Manilae (minimum of 97% protein identity), with the exception of the sv. Manilae gene at the location equivalent to *la1642* (encoding a predicted glycosyltransferase in both genomes, 48% identity, 66% similarity at the protein level) (Table 2). The genes downstream of LA1642 in sv. Lai (*la1643–la1647*, encoding three predicted sugar

Table 2. Comparison of proteins encoded by genetic loci surrounding the point of mutation in M895.

				% Identity to sv. Manilae protein (% similarity)[a]			
Strain	Serovar	Serogroup	Accession	LA1640	LA1641	LA1642	LA1643
L495	Manilae	Pyrogenes	–	100	100	100	100
Lai	Lai	Icterohaemorrhagiae	NC_004342	99	99	48 (66)	97
Hond Utrecht IV	Canicola	Canicola	AF316567	99	99	99	96
Naam	Naam	Icterohaemorrhagiae	AF316570	99	99	99	97
Salinem	Pyrogenes	Pyrogenes	AF316571	99	99	99	97

a. Encoded proteins are named according to the sv. Lai systematic nomenclature. Percentage similarity is given where identity was low. Putative function of encoded genes from original annotation: LA1640, hypothetical protein; LA1641, hypothetical protein; LA1642, glycosyltransferase; LA1643, *N*-acetyl glucosamine/*N*-acetyl galactosamine epimerase.

epimerases and two predicted sugar transferases) were also present in sv. Manilae and were highly similar to those in sv. Lai.

The locus encompassing *la1640–la1643* of sv. Manilae was more similar to the partial sequences of *L. interrogans* Hond Utrecht IV isolate CAN10, strain Salinem isolate PYR10, strain Naam isolate NAA10, and strain Salinem (Table 2). In the absence of LPS structural data for these different serovars, speculation on the role of these genes is not possible.

For mutant M1352, the region of insertion was unique to Manilae. The three genes immediately upstream of the insertion were predicted to encode a sugar pyridoxal-phosphate-dependent aminotransferase and two hypothetical proteins. The five downstream genes encoded (in order) a predicted dTDP-4-dehydrorhamnose 3,5-epimerase, hypothetical protein, nucleoside-diphosphate-sugar epimerase, sugar isomerase and UDP-glucose 4-epimerase (Fig. 1).

The LPS mutants are attenuated for virulence

The mutant M895 was inoculated intraperitoneally into hamsters at various doses. As reported previously (Murray *et al.*, 2009b), hamsters injected with the parent strain at 10^1, 10^2 or 10^3 leptospires per animal succumbed to infection from day 8 to day 10 (Table 3). Three animals (37.5%) survived in the 10^1 dose group, whereas no animals survived in the other groups. In contrast, the mutant M895 failed to produce any signs of illness in animals when injected at doses of up to 10^7 leptospires. Bacteria were readily cultured from animals infected with parent strain L495, but could not be cultured from the blood, urine or kidneys of M895-infected animals (cultures monitored for 27 days). The mutant M895 also failed to cause disease when administered by the ocular route of infection at a dose of 10^6 leptospires (Table 3), while 50% of animals infected by parent sv. Manilae via this route died over a 21-day period (Murray *et al.*, 2009c).

Histopathological examination was performed on animals infected by the intraperitoneal route. Tissues from animals infected with M895 were normal. In contrast, the lungs of hamsters infected with WT L495 showed mild to moderate multifocal alveolar haemorrhages, livers showed diffuse dissociation and single cell necrosis of hepatocytes with abundant leptospires in the sinusoids, and kidneys exhibited multifocal tubular necrosis, mild tubular proteinuria and rare haemoglobin casts with numerous leptospires in affected areas.

The mutant M1352 was also confirmed to be attenuated; when injected intraperitoneally into hamsters at a dose of 10^3 ($n = 8$) or 10^7 ($n = 10$) leptospires, M1352 failed to produce any symptoms of leptospirosis, with all animals surviving 14 days post infection and no kidney colonization detected. In contrast, hamsters infected with the same dose of the parent strain all died with all hamster kidney culture positive. These data indicate that LPS is essential for virulence in the hamster model of infection.

The LPS mutants have altered infection kinetics

During the normal course of infection of hamsters with *L. interrogans*, bacteria travel rapidly to the bloodstream via the lymphatics. They then invade the tissues and are later found in all organs and tissues prior to death of the animal (Faine *et al.*, 1999). To examine how the kinetics of infection were affected in the LPS mutant, bacteria were injected into hamsters intraperitoneally at a dose of 10^3 leptospires, blood samples were taken periodically for culture, and cultures were monitored by dark-field microscopy. Hamsters infected with WT Manilae succumbed to infection before day 7 and bacteria were recovered from kidneys. Hamsters infected with M895 or M1352 did not show any signs of disease for the duration of the experiment, and bacteria could not be recovered from kidneys. The parent sv. Manilae was cultured in the 1 h sample for some hamsters and appeared in the blood of all hamsters by day 3 (Table 4). In the case of the

Table 3. Survival of hamsters[a] infected with parent or mutant M895 strains of *L. interrogans* sv. Manilae.

Strain	Route of infection	Dose	Survival (%)	Statistical significance[b]
Manilae WT	Intraperitoneal	10^1	3/8 (37.5)	–
	Intraperitoneal	10^2	0/8 (0)	–
	Intraperitoneal	10^3	0/8 (0)	–
M895	Intraperitoneal	10^3	2/2 (100)	0.022
	Intraperitoneal	10^5	8/8 (100)	< 0.001
	Intraperitoneal	10^6	8/8 (100)	< 0.001
	Intraperitoneal	10^7	8/8 (100)	< 0.001
Manilae WT	Conjunctival	10^6	5/10 (50)	–
M895	Conjunctival	10^6	10/10 (100)	0.032

a. Some of these data also appeared previously in Murray *et al.* (2009c) due to concurrent use of control animals.
b. Statistical significance (Fisher's exact test) was calculated comparing the groups infected with equivalent doses of L495, or the most similar dose where infection doses differed.

Table 4. Bacterial infection kinetics measured by blood culture after intraperitoneal infection.

Challenge strain	Dose	No. Hamsters	Blood culture-positive hamsters at various time points (%)[a]						
			1 h	6 h	12 h	1 day	2 days	3 days	7 days
Manilae WT	10^3	5	60	0	0	0	20	100	ND[b]
M895	10^3	5	60	0	0	0	0	0	0
M1352	10^3	5	40	0	0	0	0	0	0

a. Cultures were monitored out to 36 days post inoculation. There was one positive culture for M895 (sample at day 3, identified 36 days after inoculation); however, it was not kanamycin resistant and was disregarded as a probable contaminant introduced through sampling error.
b. ND, not determined because animals did not survive to the indicated time point.

mutants M895 and M1352, leptospires were detected at 1 h, but not after this point.

Considering the possibility that infecting bacteria were cleared very quickly, an immunoblot using sera from infected hamsters was conducted to determine if an antibody response was mounted against the LPS mutants (Fig. 3). For each hamster examined, there was a number of strongly reactive bands in a *L. interrogans* sv. Manilae whole-cell lysate, indicating a specific antibody response against the LPS mutants. Unsurprisingly, there was variability in the proteins recognized by individual hamsters. Strongly reactive bands were not observed in the prebleed (prior to challenge), nor in hamsters mock infected with EMJH.

The LPS mutations do not lead to increased complement sensitivity

In other Gram-negative bacteria, complete LPS is clearly required for complement resistance (Murray *et al.*, 2006). The leptospiral mutants were tested for complement resistance using 90% pooled normal human serum, with counts performed immediately after serum addition and again after 60 min incubation at 37°C. A control of *Leptospira biflexa* sv. Patoc strain L41 was included as a complement-sensitive control. After 60 min incubation there were no detectable viable L41 organisms. In contrast, the parent sv. Manilae showed 92% survival (SD = 32), M895 78% survival (SD = 20) and M1352 97% survival (SD = 18). There was no significant difference in survival of either mutant (Student's *t*-test).

Analysis of surface exposed proteins

In other bacterial infection models, truncation of LPS can result in reduced expression, exposure or activity of specific surface virulence factors (Morona and Van Den Bosch, 2003; Bengoechea *et al.*, 2004). The surface exposure of proteins in the outer membrane was examined by biotinylation of intact, viable leptospires; there was no difference in profile between the WT strain and mutants (Fig. 4).

Discussion

Most genera of spirochetes, including *Borrelia* and *Treponema*, have an outer membrane devoid of LPS, instead containing a large proportion of lipoproteins. The LPS-

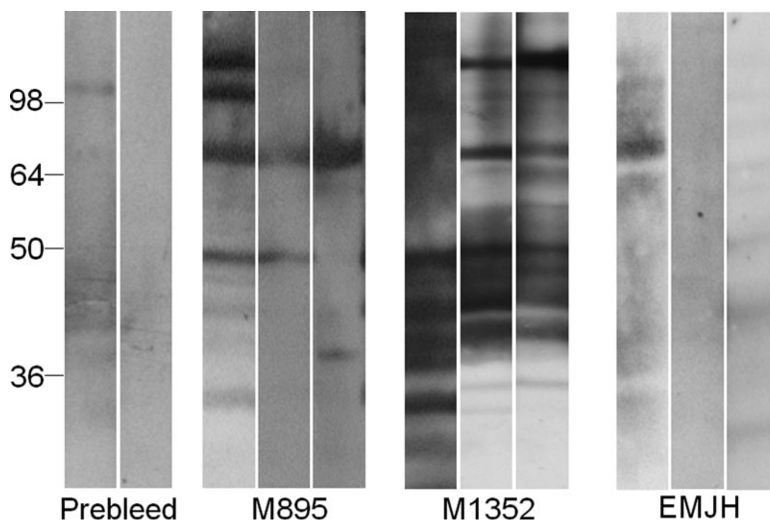

Fig. 3. Analysis of the antibody response to infection with M895 or M1352 as indicated. Sera were collected from infected hamsters at day 14 post infection and used to probe whole-cell lysates of *L. interrogans* serovar Manilae. Representative immunoblots are shown for individual hamsters. Pre-bleed: sera taken prior to infection. EMJH: sera from hamsters mock infected with EMJH medium. The positions of standard molecular mass markers (kDa) are shown on the left.

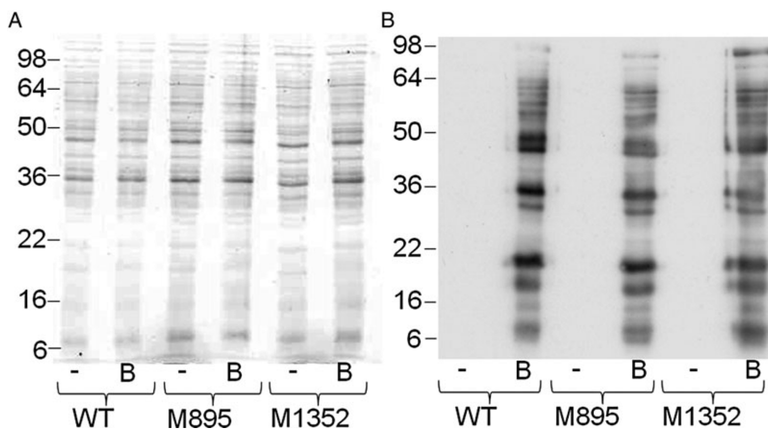

Fig. 4. Analysis of the leptospiral surfaceome. Wild-type (WT) or mutant (M895, M1352) bacteria were surface labelled with biotin, then lysates separated by SDS-PAGE.
A. Coomassie-stained SDS-PAGE gel.
B. Samples were transferred to a membrane for detection with horseradish peroxidase-conjugated streptavidin.
'B' indicates biotin labelling in the respective samples. The positions of standard molecular mass markers (kDa) are shown on the left.

containing outer membrane structure of *Leptospira* is therefore unusual amongst the pathogenic spirochetes. In this study we show that LPS plays an essential role in virulence of *L. interrogans*.

The *L. interrogans* genetic locus encoding the biosynthesis of LPS is very large, spanning approximately 100 kb. A recent large-scale mutagenesis study showed that this genetic region had an unusually low frequency of transposon insertion (Murray *et al*., 2009a), suggesting that most of the genes contained therein are essential for the viability of *L. interrogans*. Previously a mutant in *L. interrogans* sv. Pomona was identified in which an IS element had inserted into the LPS biosynthesis locus, resulting in a truncated LPS structure (Zuerner and Trueba, 2005). In the current study two mutants of *L. interrogans* sv. Manilae with changes in LPS was characterized. Both have mutations in a gene encoding a protein of unknown function, with no similarity to other known proteins. In both cases it is possible that modifications of LPS may not be due to mutation of particular genes *per se*, but may result from downstream polar effects on other LPS biosynthesis genes. Nevertheless, for M895 gel electrophoresis data clearly showed an altered LPS phenotype, while MAT and immunoblotting with absorbed serum indicated changes in antigenicity or loss of at least one antigenic epitope.

While M1352 LPS did not appear to have altered molecular mass, the mutant showed a diminished MAT titre to rabbit antiserum against the WT strain, but increased reactivity against LPS-specific monoclonal antibodies. This finding is consistent with the loss of epitope(s) recognized by polyclonal antiserum, but not by these monoclonal antibodies. The increase in reactivity with monoclonal antibodies suggests LPS alterations resulting in increased exposure and access to the monoclonal antibodies. Similar findings have been observed previously in *Pasteurella multocida* and *Neisseria meningitidis*, where removal of a phosphoethanolamine residue resulted in an imperceptible change in molecular mass of LPS, but increased reactivity to a monoclonal antibody (Mackinnon *et al*., 2002; St Michael *et al*., 2009).

The LPS mutants were avirulent in hamsters, demonstrating an increase in ID_{50} of $> 10^6$-fold. M895 was also avirulent when inoculated via a mucosal route of infection. Infection kinetics suggested that the mutant bacteria were cleared between 1 h and 3 days post infection. However, the mounting of a specific antibody response suggests a measure of persistence *in vivo*, raising the potential use of LPS mutant strains as live, attenuated vaccines. The attenuation of the leptospiral LPS mutants is consistent with results from other bacterial pathogens including *Salmonella typhimurium* and *Yersinia pseudotuburculosis*. In these species mutants with LPS O antigen truncations remain viable *in vitro* but are severely attenuated *in vivo* (Ohno *et al*., 1995; Murray *et al*., 2003; Bengoechea *et al*., 2004). The *Leptospira* LPS synthesis loci encode very long transcripts of at least 25 kb in length (de la Pena Moctezuma, 2002). Complementation was not possible in this study, probably because the mutation may also influence the expression of downstream genes. Indeed, complementation of these very long transcripts may well be impossible. In the absence of mutant complementation, the attenuation of two independently derived LPS mutants provides strong evidence that LPS is essential for infection in the hamster model. Notably, the presence of the transposon *per se* does not attenuate strains, as shown by the previous analysis of 29 mutants, of which only two showed signs of attenuation (Murray *et al*., 2009a).

The specific mechanisms of attenuation of these mutants are not known. LPS may provide protection against complement mediated killing (Murray *et al*., 2006) and host antimicrobial peptides (Boyce *et al*., 2009). The mutants in this study were not more susceptible to complement killing. However, this was not surprising, since *L. interrogans* can bind the host complement regulators factor H through proteins LenA and LenB (Verma *et al*., 2006; Stevenson *et al*., 2007), and C4b-binding

protein (C4 BP) through LcpA (Barbosa *et al.*, 2010). Similar to many other potential leptospiral virulence factors (Ko *et al.*, 2009; Murray *et al.*, 2009a), the role of leptospiral LPS in protecting against complement-killing may be functionally redundant. In *S. typhimurium* control of the molecular weight of O antigen is essential for virulence (Murray *et al.*, 2003; 2006) and O antigen chain length may be changed under the influence of *in vivo* conditions (Murray *et al.*, 2005). Likewise, the quantity of leptospiral LPS changes during acute infection, but not during kidney colonization of carrier hosts (Nally *et al.*, 2005). The mutations in M1352 and M895 may also interfere with *in vivo* variation in expression or structure, which would not be detected when examining *in vitro* grown cultures.

In *Yersinia enterocolitica* removal of the O antigen structure of LPS impacts upon the expression or function of several virulence factors (Bengoechea *et al.*, 2004; Perez-Gutierrez *et al.*, 2007), while shortening LPS has an impact on the surface exposure and activity of *Shigella flexneri* virulence factor *icsA* (Rajakumar *et al.*, 1994; Morona and Van Den Bosch, 2003; Morona *et al.*, 2003). However, no obvious differences in surface exposed proteins were found for the *L. interrogans* LPS mutants in this study.

In conclusion, LPS is only the fourth virulence factor to be defined in *L. interrogans*, after description of mutants affecting proteins Loa22 (Ristow *et al.*, 2007), haem oxygenase (Murray *et al.*, 2008) and motility (Liao *et al.*, 2009). Further work is needed to define the roles that LPS plays in leptospiral pathogenesis. As LPS is a major factor in immunity to *L. interrogans*, increasing knowledge of LPS will improve our understanding of immunity and may enhance vaccine development. Further study of LPS mutants combined with the elucidation of LPS structure will provide insights into the mechanisms of antigenic diversity of LPS.

Experimental procedures

Bacterial strains and culture conditions

Leptospira interrogans sv. Manilae strain L495 was kindly provided by N. Koizumi, National Institute of Infectious Diseases, Tokyo, Japan. *L. biflexa* sv. Patoc L41 was obtained from the laboratory collection of the Monash *Leptospira* laboratory. Bacteria were grown in EMJH medium (Becton Dickinson, Sparks, MD, USA) at 30°C without aeration. Plates were made by solidification of medium with 1.5% agar. Kanamycin (25 $\mu g\ ml^{-1}$) or spectinomycin (50 $\mu g\ ml^{-1}$) was added where appropriate.

Construction of mutants

Leptospires were made electrocompetent by washing log-phase bacteria in purified water, then transformed with Tn*SC189* (Murray *et al.*, 2009a) as described previously (Bourhy *et al.*, 2005; Murray *et al.*, 2009a). The location of chromosomal insertion was determined by sequencing directly from the genomic DNA as described previously (Murray *et al.*, 2008). Complementation was attempted as described previously by cloning genes onto a spectinomycin resistant plasmid for transfer to the leptospiral chromosome (Ristow *et al.*, 2007; Murray *et al.*, 2010). The transposon Tn*SC189* was modified, replacing most of the kanamycin resistance gene with a spectinomycin resistance gene driven by the *Borrelia burgdorferi flgB* promoter (a kind gift from Mathieu Picardeau, Pasteur Institute, Paris) producing the plasmid pSC189Spc^R. A PCR product using primers 5′-TTAGGTACCATAATACCCGAGCTTCAAG-3′ and 5′-TTTCCATGGGCCTAATTGAGAGAAGTTTC-3′ was cloned into pSC189Spc^R using KpnI and NcoI. This construct was used to transform the mutant as described above, with spectinomycin selection (50 $\mu g\ ml^{-1}$).

Sequencing of the L. interrogans *sv. Manilae LPS synthesis locus*

A draft sequence of the *L. interrogans* sv. Manilae genome was performed by Micromon (Monash University, Australia) with an Illumina Genome Analyser using 36-cycle single-read chemistry, yielding 50 times coverage. An automated annotation was performed using the *L. interrogans* sv. Lai genome (Accession No. NC_004342) as a scaffold. Relevant sequences have been submitted to the GenBank database under Accession Nos HQ127382 (Lman_1408) and HQ127383 (Lman_1456).

Analysis of LPS

SDS-PAGE analysis was conducted on log-phase cultures. Bacteria were enumerated by counting under dark-field microscopy, centrifuged to pellet cells and resuspended in 60 μl of SDS-PAGE sample buffer (1.25% SDS, 10% glycerol, 0.05% bromophenol blue, 0.1 M Tris, pH 6.8) final concentration of approximately 5×10^6 cells μl^{-1}, incubated at 100°C for 10 min, then proteinase K added to 30 $\mu g\ ml^{-1}$. Samples were digested for 1 h at 60°C. Between 1 and 5 μl (standardized by concentration) were loaded onto a 12.5% polyacrylamide SDS-PAGE gel. Silver staining was conducted using the SilverSnap stain kit II (Thermo Scientific, Rochford, IL, USA). Western blotting was performed with rabbit antiserum (R762) against *L. interrogans* sv. Manilae (a kind gift from Lee Smythe, WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia) detected with horse radish peroxidise-conjugated goat anti-rabbit immunoglobulin (Millipore, Temecula, CA, USA). To pre-absorb antiserum, it was first diluted (1/2000), then underwent two rounds of mixing with a suspension of approximately 2×10^9 leptospiral cells (4°C, 2 h) and centrifuged (9000 *g*, 10 min). The microscopic agglutination test (MAT) was conducted with early log-phase cultures as described previously (Terpstra *et al.*, 1985) with either a rabbit polyclonal serum R762 or a panel of Pyrogenes, Canicola and Icterohaemorrhagiae group monoclonal antibodies.

Biotin labelling of surface proteins

Surface biotinylation was conducted as described previously (Cullen *et al*., 2005). In brief, PBS-washed cultures were incubated with 0.5 mg ml^{-1} sulpho-succinimidyl-6-(bitoinamido) hexanoate (Sulfo-NHS-LC Biotin; Pierce) in PBS. Reactions were quenched by the addition of Tris-HCl (pH 7.2) to a final concentration of 50 mM and incubated at room temperature for 10 min. Cells were washed in PBS, lysed in SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to PVDF membrane and detected with horseradish peroxidase-conjugated streptavidin (GE Healthcare, Piscataway, NJ, USA).

Virulence in the hamster model of infection

Golden hamsters in groups were injected intraperitoneally with various doses of leptospires in 100 μl of EMJH and monitored for 14 days. Blood, urine and kidney tissue for leptospiral culture analysis was collected post mortem. For intraocular infection, bacteria at a concentration of 10^8 leptospires ml^{-1} were instilled in 10 μl onto one eye of each hamster in groups of 10 animals (dose of 10^6 leptospires), and animals were monitored for 21 days. After infection, kidneys were used for culture as described previously (Murray *et al*., 2009b) and tissues were fixed in 10% formalin for histopathology analysis (Murray *et al*., 2009c). Infection kinetics were measured by injecting hamsters intraperitoneally with 10^3 leptospires ($n = 5$ hamsters) and taking blood samples at 1, 6, 12 h, 1, 2, 3, 7 days for culture in EMJH. Blood cultures were monitored for leptospiral growth by dark-field microscopy for 36 days. Pre-bleeds for all hamsters were negative. In accordance with animal ethics requirements moribund animals were euthanized.

Analysis of serum resistance

Pooled normal human serum from healthy volunteers was mixed with cultures to a final concentration of 90% serum, 5×10^7 leptospires ml^{-1}. Viable cells were enumerated by counting under dark-field microscopy (t_0), incubated for 60 min at 37°C before counting again (t_{60}). Survival was calculated by t_{60} counts/t_0 counts. The experiment was performed four times.

Acknowledgements

This work was supported by the National Health and Medical Research Council and the Australian Research Council. Susan Thomas, Tim Witchell and Vicki Vallance are thanked for technical assistance. G.L.M. is supported by a NHMRC Peter Doherty Fellowship.

References

Adler, B., and de la Pena Moctezuma, A. (2010) *Leptospira* and leptospirosis. *Vet Microbiol* **140:** 287–296.

Barbosa, A.S., Monaris, D., Silva, L.B., Morais, Z.M., Vasconcellos, S.A., Cianciarullo, A.M., *et al*. (2010) Functional characterization of LcpA, a surface-exposed protein of *Leptospira* spp. that binds the human complement regulator C4 BP. *Infect Immun* **78:** 3207–3216.

Bengoechea, J.A., Najdenski, H., and Skurnik, M. (2004) Lipopolysaccharide O antigen status of *Yersinia enterocolitica* O:8 is essential for virulence and absence of O antigen affects the expression of other *Yersinia* virulence factors. *Mol Microbiol* **52:** 451–469.

Bourhy, P., Louvel, H., Saint Girons, I., and Picardeau, M. (2005) Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. *J Bacteriol* **187:** 3255–3258.

Boyce, J.D., Harper, M., St Michael, F., John, M., Aubry, A., Parnas, H., *et al*. (2009) Identification of novel glycosyltransferases required for assembly of the *Pasteurella multocida* A:1 lipopolysaccharide and their involvement in virulence. *Infect Immun* **77:** 1532–1542.

Cinco, M., Banfi, E., and Panfili, E. (1986) Heterogeneity of lipopolysaccharide banding patterns in *Leptospira* spp. *J Gen Microbiol* **132:** 1135–1138.

Croda, J., Figueira, C.P., Wunder, E.A., Jr, Santos, C.S., Reis, M.G., Ko, A.I., and Picardeau, M. (2008) Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. *Infect Immun* **76:** 5826–5833.

Cullen, P.A., Xu, X., Matsunaga, J., Sanchez, Y., Ko, A.I., Haake, D.A., and Adler, B. (2005) Surfaceome of *Leptospira* spp. *Infect Immun* **73:** 4853–4863.

Faine, S., Adler, B., Bolin, C., and Perolat, P. (1999) *Leptospira and Leptospirosis*. Australia: MediSci.

Jost, B.H., Adler, B., Vinh, T., and Faine, S. (1986) A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. *J Med Microbiol* **22:** 269–275.

Ko, A.I., Goarant, C., and Picardeau, M. (2009) *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat Rev Microbiol* **7:** 736–747.

Liao, S., Sun, A., Ojcius, D.M., Wu, S., Zhao, J., and Yan, J. (2009) Inactivation of the *fliY* gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. *BMC Microbiol* **9:** 253.

Mackinnon, F.G., Cox, A.D., Plested, J.S., Tang, C.M., Makepeace, K., Coull, P.A., *et al*. (2002) Identification of a gene (*lpt-3*) required for the addition of phosphoethanolamine to the lipopolysaccharide inner core of *Neisseria meningitidis* and its role in mediating susceptibility to bactericidal killing and opsonophagocytosis. *Mol Microbiol* **43:** 931–943.

Morona, R., and Van Den Bosch, L. (2003) Lipopolysaccharide O antigen chains mask IcsA (VirG) in *Shigella flexneri*. *FEMS Microbiol Lett* **221:** 173–180.

Morona, R., Daniels, C., and Van Den Bosch, L. (2003) Genetic modulation of *Shigella flexneri* 2a lipopolysaccharide O antigen modal chain length reveals that it has been optimized for virulence. *Microbiology* **149:** 925–939.

Murray, G.L., Attridge, S.R., and Morona, R. (2003) Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *Mol Microbiol* **47:** 1395–1406.

Murray, G.L., Attridge, S.R., and Morona, R. (2005) Inducible serum resistance in *Salmonella typhimurium* is dependent

© 2010 Blackwell Publishing Ltd,

on *wzz*(fepE)-regulated very long O antigen chains. *Microbes Infect* **7:** 1296–1304.
Murray, G.L., Attridge, S.R., and Morona, R. (2006) Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of *Salmonella enterica* serovar Typhimurium with macrophages and complement. *J Bacteriol* **188:** 2735–2739.
Murray, G.L., Ellis, K.M., Lo, M., and Adler, B. (2008) *Leptospira interrogans* requires a functional heme oxygenase to scavenge iron from hemoglobin. *Microbes Infect* **10:** 791–797.
Murray, G.L., Morel, V., Cerqueira, G.M., Croda, J., Srikram, A., Henry, R., *et al.* (2009a) Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. *Infect Immun* **77:** 810–816.
Murray, G.L., Srikram, A., Henry, R., Puapairoj, A., Sermswan, R.W., and Adler, B. (2009b) *Leptospira interrogans* requires heme oxygenase for disease pathogenesis. *Microbes Infect* **11:** 311–314.
Murray, G.L., Srikram, A., Hoke, D.E., Wunder, E.A., Jr, Henry, R., Lo, M., *et al.* (2009c) Major Surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. *Infect Immun* **77:** 952–958.
Murray, G.L., King, A.M., Srikram, A., Sermswan, R.W., and Adler, B. (2010) Use of luminescent *Leptospira interrogans* for enumeration in biological assays. *J Clin Microbiol* **48:** 2037–2042.
Nahori, M.A., Fournie-Amazouz, E., Que-Gewirth, N.S., Balloy, V., Chignard, M., Raetz, C.R., *et al.* (2005) Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. *J Immunol* **175:** 6022–6031.
Nally, J.E., Chow, E., Fishbein, M.C., Blanco, D.R., and Lovett, M.A. (2005) Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. *Infect Immun* **73:** 3251–3260.
Nascimento, A.L., Ko, A.I., Martins, E.A., Monteiro-Vitorello, C.B., Ho, P.L., Haake, D.A., *et al.* (2004) Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J Bacteriol* **186:** 2164–2172.
Ohno, A., Isii, Y., Tateda, K., Matumoto, T., Miyazaki, S., Yokota, S., and Yamaguchi, K. (1995) Role of LPS length in clearance rate of bacteria from the bloodstream in mice. *Microbiology* **141** (Part 10)**:** 2749–2756.
de la Pena Moctezuma, A. (2002) Studies on the genetic organisation of the *rfb* loci of *Leptospira interrogans* serovars. PhD thesis. Monash University, Melbourne, Australia.
de la Pena-Moctezuma, A., Bulach, D.M., and Adler, B. (2001) Genetic differences among the LPS biosynthetic loci of serovars of *Leptospira interrogans* and *Leptospira borgpetersenii*. *FEMS Immunol Med Microbiol* **31:** 73–81.
Perez-Gutierrez, C., Llompart, C.M., Skurnik, M., and Bengoechea, J.A. (2007) Expression of the *Yersinia enterocolitica* pYV-encoded type III secretion system is modulated by lipopolysaccharide O-antigen status. *Infect Immun* **75:** 1512–1516.
Que-Gewirth, N.L., Ribeiro, A.A., Kalb, S.R., Cotter, R.J., Bulach, D.M., Adler, B., *et al.* (2004) A methylated phosphate group and four amide-linked acyl chains in *Leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. *J Biol Chem* **279:** 25420–25429.
Raetz, C.R., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71:** 635–700.
Rajakumar, K., Jost, B.H., Sasakawa, C., Okada, N., Yoshikawa, M., and Adler, B. (1994) Nucleotide sequence of the rhamnose biosynthetic operon of *Shigella flexneri* 2a and role of lipopolysaccharide in virulence. *J Bacteriol* **176:** 2362–2373.
Ren, S.X., Fu, G., Jiang, X.G., Zeng, R., Miao, Y.G., Xu, H., *et al.* (2003) Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* **422:** 888–893.
Ristow, P., Bourhy, P., da Cruz McBride, F.W., Figueira, C.P., Huerre, M., Ave, P., *et al.* (2007) The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog* **3:** e97.
St Michael, F., Harper, M., Parnas, H., John, M., Stupak, J., Vinogradov, E., *et al.* (2009) Structural and genetic basis for the serological differentiation of *Pasteurella multocida* Heddleston serotypes 2 and 5. *J Bacteriol* **191:** 6950–6959.
Stevenson, B., Choy, H.A., Pinne, M., Rotondi, M.L., Miller, M.C., Demoll, E., *et al.* (2007) *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE* **2:** e1188.
Terpstra, W.J., Korver, H., van Leeuwen, J., Klatser, P.R., and Kolk, A.H. (1985) The classification of Sejroe group serovars of *Leptospira interrogans* with monoclonal antibodies. *Zentralbl Bakteriol Mikrobiol Hyg [A]* **259:** 498–506.
Verma, A., Hellwage, J., Artiushin, S., Zipfel, P.F., Kraiczy, P., Timoney, J.F., and Stevenson, B. (2006) LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect Immun* **74:** 2659–2666.
Zuerner, R.L., and Trueba, G.A. (2005) Characterization of IS1501 mutants of *Leptospira interrogans* serovar Pomona. *FEMS Microbiol Lett* **248:** 199–205.