Use of a high-throughput screen to identify Leptospira mutants unable to colonize the carrier host or cause disease in the acute model of infection

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The molecular basis for leptospirosis infection and colonization remains poorly understood, with no efficient methods available for screening libraries of mutants for attenuation. We analysed the attenuation of leptospiral transposon mutants in vivo using a high-throughput method by infecting animals with pooled sets of transposon mutants. A total of 95 mutants was analysed by this method in the hamster model of acute infection, and one mutant was identified as attenuated (M1233, lb058 mutant). All virulence factors identified in Leptospira to date have been characterized in the acute model of infection, neglecting the carrier host. To address this, a BALB/c mouse colonization model was established. The lb058 mutant and two mutants defective in LPS synthesis were colonization deficient in the mouse model. By applying the high-throughput screening method, a further five colonization-deficient mutants were identified for the mouse model; these included two mutants in genes encoding proteins with a predicted role in iron uptake (LB191/HbpA and LB194). Two attenuated mutants had transposon insertions in either la0589 or la2786 (encoding proteins of unknown function). The final attenuated mutant had an unexpected deletion of genes la0969–la0975 at the point of transposon insertion. This is the first description of defined, colonization-deficient mutants in a carrier host for Leptospira. These mutants were either not attenuated or only weakly attenuated in the hamster model of acute leptospirosis, thus illustrating that different factors that may be required in the carrier and acute models of leptospiral infection. High-throughput screening can reduce the number of animals used in virulence studies and increase the capacity to screen mutants for attenuation, thereby enhancing the likelihood of detecting unique virulence factors. A comparison of virulence factors required in the carrier and acute models of infection will help to unravel colonization and dissemination mechanisms of leptospirosis.

INTRODUCTION

Spirochaetes of the genus Leptospira are responsible for leptospirosis. Depending on the host and infecting serovar, infection may result in disease ranging between an asymptomatic carrier state to a severe, acute disease (Adler & de la
Peña Moctezuma, 2010). In carrier hosts (such as rodents, cattle, pigs and dogs), leptospires colonize the renal tubules and are excreted in urine. In some carrier hosts, leptospiral carriage is asymptomatic, an association that is indicative of the long evolutionary association between host and Leptospira. Acute disease is characterized by symptoms ranging from influenza-like headaches and fever to hepatic and renal failure, lung haemorrhage and death. Clearly, the mechanisms determining disease symptoms between the acute and carrier states differ, yet the vast majority of research on leptospirosis has focused on the acute disease, such as that seen in humans. Accordingly, very little is known about the molecular basis for colonization of carrier hosts.

The development of mutagenesis techniques has permitted the construction of defined L. interrogans mutants (Bourhy et al., 2005; Croda et al., 2008). These methods have helped identify a number of L. interrogans factors required for acute infection (Ristow et al., 2007; Liao et al., 2009; Murray et al., 2009a, 2010; Lourdault et al., 2011; Eshghi et al., 2012; Lambert et al., 2012; Zhang et al., 2012). However, some proteins that bear the hallmarks of virulence factors (such as abundance, absence in saprophytic Leptospira spp., cellular location, expression profiles, predicted function and in vitro characterized functions) are not required for virulence (reviewed by Adler et al., 2011). These include the extracellular matrix binding proteins LipL32 and LigB (Croda et al., 2008; Murray et al., 2009c) and the abundant surface lipoprotein LipL41 (King et al., 2013). These findings highlight the existing lack of understanding of leptospiral virulence and demonstrate the importance of improving the molecular characterization of the more than 40% of leptospiral proteins for which no function has been determined (Adler et al., 2011). Notably, no leptospiral virulence factors have been defined in the colonization host (Adler et al., 2011).

Transposon mutagenesis in L. interrogans is inefficient, making it unsuitable for signature-tagged mutagenesis that relies on the construction of large libraries of mutants. However, a library of almost 1000 partially characterized mutants (in which the identity of the inactivated gene is known) was reported previously (Murray et al., 2009a). Additional mutants were constructed as described previously (Bourhy et al., 2005), with the location of transposon insertion determined by direct sequencing from genomic DNA (Murray et al., 2008). Leptospires were cultured in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco) at 30 °C. The in vitro growth rate of all attenuated mutants was the same as WT unless indicated otherwise.

**Hamster model of acute disease.** L. interrogans mutants in the exponential phase of growth were enumerated by dark-field microscopy with a Petroff–Hauser counting chamber, then mixed in pools and administered intraperitoneally at the equivalent to 10⁹ of each mutant into 45 g golden hamsters of either sex (Fig. 1). Hamsters were monitored for 5 days before blood was collected, animals euthanized and kidneys removed for culture as described previously (Murray et al., 2009b). For testing the virulence of individual mutants, 10⁹ leptospires were administered intraperitoneally to groups of 10–11 hamsters. Animals were monitored for up to 21 days. Moribund animals were euthanized in accordance with animal ethics requirements and kidney cultures were taken.

**Mouse carrier host model.** For infection with strains in pools, bacteria were enumerated as described above, pooled and a dose equivalent to 2 × 10⁶ of each mutant was injected intraperitoneally into 6–8-week-old female BALB/c mice. Mice were monitored for 2 weeks before animals were euthanized and kidneys were removed for culture and PCR. Biopsies were taken from the kidney cortex using a glass Pasteur pipette and inoculated into 5 ml EMJH medium.

**METHODS**

**Bacterial strains and culture conditions.** The majority of mutant strains of L. interrogans serovar Manilae used in this study were constructed previously using the himar1-based transposon TnSC189 (Murray et al., 2009a). Additional mutants were constructed as described previously (Bourhy et al., 2005), with the location of transposon insertion determined by direct sequencing from genomic DNA (Murray et al., 2008). Leptospires were cultured in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco) at 30 °C. The in vitro growth rate of all attenuated mutants was the same as WT unless indicated otherwise.

![Fig. 1. High-throughput assay for screening of mutants for virulence.](image)

(a) Schematic representation of transposon TnSC189 insertion in the hypothetical gene ‘orfA’. The binding locations of transposon-specific primers (BAP3855 and BAP3453) and gene-specific primer are indicated. Elements of the transposon (in black) include inverted repeats (IR) and the kanamycin resistance gene (KanR).

(b) Flowchart outlining the experimental protocol.
containing 100 μg 5-fluorouracil ml⁻¹. For infection with individual leptospiral strains, bacteria were enumerated and inoculated intra-peritoneally at a dose of 10⁷ in EMJH medium. After 2 weeks, mice were euthanized and kidney and/or blood from cardiac puncture were removed for culture, where appropriate. Cultures were monitored for growth by dark-field microscopy for up to 6 weeks.

**PCR analysis of blood and kidney output pools.** DNA was prepared from bacterial cultures using a genomic DNA isolation kit (RBC Real Genomics). PCR was performed following a standard protocol, as described by the manufacturer (Roche), using Taq DNA polymerase (Roche), deoxynucleotide mix (New England Biolabs) and oligonucleotide primers. Reactions were set up for input/output pool DNA template for each mutant using a primer specific to each individual mutated gene and common oligonucleotides priming in opposite directions out of the transposon (BAP3453: 5’-CGTCAT-GGTCTTTGTAGTCTATGG-3’, and BAP3855: 5’-TTCTTGACG-AGTTCTTCTGA-3’) (Fig. 1a). For negative results in which a mutant was not detected, reactions were repeated for confirmation.

**PCR and histological analysis of mouse kidneys.** DNA was extracted from mouse kidneys with a DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer’s protocol with modifications. Samples of kidney (15–25 mg) were homogenized with 360 μl lysis buffer in a Whirl-Pak bag (Nasco), 40 μl proteinase K (20 mg ml⁻¹) was added and the homogenates were incubated at 56 °C for 2.5 h. Finally, 800 μl AL buffer and ethanol (1:1) were added and the samples were added to spin columns. DNA was eluted in 100 μl elution buffer passed through the column twice.

To detect kidney colonization for individual mutants, extracted DNA was amplified with Taq DNA polymerase and Leptospira-specific rrs (165) primers (forward 5’-GCGGCCGGCTCTTTACACATG-3’, reverse 5’-AAATCTTGCTCTAATGGGGGAA-3’ (Mérien et al., 1992). The following conditions were used: one cycle of 3 min at 94 °C, 1.5 min at 63 °C and 2 min at 72 °C, and 29 cycles of 1 min at 94 °C, 1.5 min at 63 °C and 2 min at 72 °C, and a final extension at 72 °C for 10 min.

For histological analysis of mouse kidneys, full cross-sections taken about mid-kidney were fixed in 10 % buffered formalin (pH 7.2); sections were then stained with haematoxylin and eosin.

**Genome sequences of attenuated mutants.** The genome sequence of each of the attenuated mutants was determined using an approach based on the whole genome shotgun DNA sequencing method (Staden, 1979). Libraries were generated from sheared genomic DNA and analysed on an Illumina HiSeq 2000. Approximately 24 million read-pairs (100 base reads) were generated for each mutant yielding 100x genome coverage. Read mapping was used to evaluate genomic differences (short indels, single base variations, and insertions and deletions) between each of the attenuated mutants and the parental L. interrogans serovar Manilae strain M001 (GenBank accession no. AHPR00000000.1). Reads were mapped using SHRIMP (David et al., 2011) as implemented in NESONI (http://www.vichioinformatics.com/software/nesonii.shtml).

**Statistical analysis.** Comparisons of survival rates were assessed with Fisher’s exact test. Statistical analysis for delay to death was calculated in Prism GraphPad version 5.03 with a Mantel–Cox test.

**RESULTS**

**Analysis of mutants by high-throughput screen in the hamster acute model of infection**

It is not feasible to test hundreds of mutants individually for attenuation in animal models of infection. Hence, they were tested simultaneously in pools of up to ten mutants by intraperitoneal injection into groups of five animals. One of two known avirulent mutants was included in each pool as a negative control (either M895 or M1352, both defective in lipopolysaccharide biosynthesis; Murray et al., 2010). L. interrogans serovar Manilae causes hamster death at approximately day 7 post-infection (Murray et al., 2009c); hence, hamsters were euthanized at day 5 and kidney and blood cultures taken. DNA was prepared from the input pool and output pools and compared by PCR, with a reaction set up for each mutant from each output source (Fig. 1a). The assay is detailed in the flow chart in Fig. 1(b).

Ninety-five mutants were selected for analysis from a library constructed previously (Murray et al., 2009a). Eighty-two mutants were detected in input or output pools by PCR (Table S1, available in JMM Online). The 13 mutants that were not detected in either pool were excluded from further analysis (data not shown). As expected, where the avirulent controls were detected in the input they were not detected in outputs (with the exception of M895 detected in one kidney sample). Some pools had low PCR success rate, i.e. amplification was unsuccessful for the majority of samples; this was likely a result of low yield for the corresponding DNA preparations and may give a false-positive result for attenuation. Seventeen mutants were detected in 20 % or fewer of all output samples (either blood or kidney tissue) and were considered potentially attenuated.

Seven potentially attenuated mutants were selected for further analysis by individual testing in groups of 11 hamsters (Table 1). Hamsters infected with mutant M1233 (mutation in lb058, encoding the bacterial heat-shock protein HtpG) showed 100 % survival (P<0.00001 compared with hamsters infected with WT). Although there was no significant difference in final survival for the hamsters infected with mutants M967 (mutant in gene of unknown function) and M708 (mutant in signal peptide peptidase) compared with those infected with WT, there was a significant delay in time to death (P<0.0001 and P<0.0022, respectively). The remaining mutants were indistinguishable from WT (P>0.05). All animals had macroscopic lung haemorrhages. Unexpectedly, all animals were kidney-culture positive for leptospires when infected with single mutants; these isolates were confirmed by PCR to be mutants and not WT revertants.

**Establishment of a mouse carrier model of leptospirosis and testing of mutants for attenuation**

Mice are natural renal carrier hosts of leptospirosis (Adler & de la Peña Moctezuma, 2010). A murine carrier host model was established to investigate virulence factors required for renal colonization. To determine an appropriate infection dose, mice were injected intraperitoneally with doses of L. interrogans serovar Manilae ranging from
10^3 up to 10^8 bacteria. After 2 weeks, mice were checked for colonization by culture (kidney and blood) and PCR. A dose of 10^5 leptospires was found to be optimal; at this dose all mice survived infection, showed no clinical signs of disease, exhibited renal colonization and did not have detectable leptospires in the blood. The kidneys of colonized mice were histologically normal (data not shown). At doses of 10^7 bacteria and above, mice exhibited signs of an acute infection and were euthanized.

A number of attenuated mutants have been described in the acute model of infection (Adler et al., 2011). Two available mutants were tested for colonization of the mouse host by individually infecting groups of five mice with 10^5 leptospires. No colonization occurred in mice infected with M895 or M1352 (mutants defective in LPS biosynthesis) (Murray et al., 2010), indicating that complete LPS is required to colonize the host. The mutant identified as attenuated in the hamster above, M1233 (mutant in \( \text{lb058} \), encoding \( \text{htpG} \)), did not colonize the mouse (Table 2).

### Analysis of mutants by high-throughput screen using the carrier model of infection

The high throughput screening method described above was applied in the mouse carrier model using 25 mutants. Five mutants were pooled and inoculated into groups of five mice. All 25 mutants were detected in the input pools. Mutants detected in less than two output samples were considered potentially colonization deficient. Nine of the 25 mutants were found not to colonize mouse kidneys (Table 2). From these, five mutants were retested in individual infections in groups of five mice; no colonization occurred in mice inoculated with M601 (mutant in putative TonB dependent receptor), M607, M620 or M655 (mutants in genes of unknown functions) (Table 2). Mutant M602 was also attenuated. Genome sequencing of each of the attenuated mutant strains was conducted to eliminate the possibility that other mutations were contributing to the observed phenotype. None of the attenuated mutants had any additional mutations (data not shown) except for M602, which had an ~4.5 kb deletion next to the transposon insertion point. This deletion removed part or all of genes \( \text{la0969} - \text{la0975} \) encoding the following putative products: an ATP-binding protein and permease of ABC transporter complex, a short-chain dehydrogenase and two hypothetical proteins.

The mutants found to be attenuated in mice were tested in the hamster in pools (see above) and had 20–40 % reisolation rate (Table S1). These mutants were therefore tested individually in the hamster model of infection to determine whether the mutation was specific for attenuation in one or both models (Table 2). None was significantly attenuated when comparing survival from infection. However, animals infected with M602, M607 and M655 had a significant delay in time to death (\( P = 0.0001 \) and M708 (\( P = 0.0028 \)), P = 0.0015 and P < 0.0014, respectively), suggesting marginal attenuation in these strains. Bacteria were recovered from hamster kidneys.

### DISCUSSION

This study used an increased-throughput method, similar to signature-tagged mutagenesis, for testing virulence of...
leptospiral mutants in vivo. This method overcomes some limitations of testing leptospiral mutants individually, thus providing a feasible means to screen the virulence of existing libraries of mutants. Analysis of 95 mutants for virulence in the acute (hamster) model by this method identified seven potentially attenuated mutants. Of these, three were confirmed to have varying degrees of attenuation. Only the mutation in lb058 led to a significant increase in hamster survival rates (the other two mutations led to an increased delay in time to death). The heat-shock protein encoded by lb058 (bacterial heat-shock protein 90 or HtpG) is likely to have a role in adaptation to the stress of infection; interestingly, a clpB chaperone mutant of L. interrogans was also attenuated (Lourdault et al., 2011). Previous studies on htpG mutants in other bacterial species have indicated that the protein has a role in the virulence of Edwardsiella tarda (Dang et al., 2011) but not Porphyromonas gingivalis (Sweier et al., 2003). The precise mechanisms of attenuation for the leptospiral htpG mutant M1233 are under investigation.

It is also of interest to note the genes that were not essential for hamster infection, including many genes encoding

Table 2. Mouse colonization analysis by high throughput assay, individual infections, and comparison with the acute hamster model

<table>
<thead>
<tr>
<th>Strain</th>
<th>Disrupted gene (serovar Lai)</th>
<th>Putative function or features of gene product(s)*</th>
<th>Detection in high throughput assay†</th>
<th>Mice positive for colonization (individual infection)‡</th>
<th>Hamster survival (individual infection)§</th>
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<tbody>
<tr>
<td>WT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5/5</td>
<td>0/10</td>
</tr>
<tr>
<td>EMJH</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/5</td>
<td>10/10</td>
</tr>
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<td>LPS biosynthesis</td>
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<td>lman1406</td>
<td>LPS biosynthesis</td>
<td>–</td>
<td>0/5</td>
<td>8/8</td>
</tr>
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<td>M1233</td>
<td>lb058</td>
<td>Molecular chaperone HtpG</td>
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<td>0/5</td>
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<td>Hypothetical</td>
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<td>M602</td>
<td>lb0969–la0975</td>
<td>ATP-binding protein and permease of ABC transporter complex, short chain dehydrogenase, hypothetical, hypothetical</td>
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<td>la2186</td>
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<td>la3869</td>
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<td>la3996</td>
<td>Sensor and regulator, TCSR</td>
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<td>M601</td>
<td>lb191</td>
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<td>M655</td>
<td>lb194</td>
<td>Hypothetical</td>
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*TCRR, two-component response-regulator; TCSR, two-component sensor and regulator. M602 had a deletion of five genes adjacent to the transposon insertion point.
†Detection in output pools from kidneys from five animals.
‡All results were significantly different from the WT control (P<0.01, Fisher’s exact test).
§The result for M1233 is reproduced from Table 1. Results for M895 and M1352 are included for reference and are reproduced from a previous study (Murray et al., 2010). Survival levels for animals infected with M601, M602, M607, M620 and M655 were not significantly different from those infected with WT (P>0.05, Fisher’s exact test).
proteins involved in signal transduction. These pathways may be required in environmental survival. Alternatively they may play a role in virulence in a different host or be functionally redundant, as discussed previously (Adler et al., 2011). Variations could be made to our high-throughput assay to enable more detailed analysis of infection. Sampling from multiple locations in the infected host may identify mutants that are defective in different aspects of virulence; for example, urine could be sampled to evaluate transmission potential. Moreover, infection routes could be varied, particularly via the conjunctiva, a natural route of transmission for *Leptospira*. As it stands, the current form of the animal models used is biased towards identification of highly attenuated leptospiral mutants; subtle attenuation could be gauged by analysing colonization levels by quantitative PCR or next generation sequencing.

When the virulence of some potentially attenuated mutants was tested individually in the acute model, they were found to colonize the hamster kidney, seemingly inconsistent with the findings of the screening experiments. However, the high-throughput method assesses colonization competitively, and mutants administered in pools may have been outcompeted (e.g. in colonization of a limited niche or access to restricted nutrients). Co-infection may be a more stringent assessment of virulence, as other studies have identified mutants that are attenuated when administered in competition with WT strains, but show a markedly higher level of virulence when administered individually. These include mutants in *Yersinia pseudotuberculosis* (defective in systemic spread), *Helicobacter pylori* (stomach colonization), *Bordetella pertussis* (respiratory tract colonization), *Mycobacterium tuberculosis* (systemic spread) and *Staphylococcus epidermidis* and *Staphylococcus aureus* (biofilm formation on implanted devices) (Logsdon & Mecsas, 2003; Fluckiger et al., 2005; Terry et al., 2005; Vergara-Irigaray et al., 2005; Joshi et al., 2006). Another factor that may have affected the outcome of infection is the different total bacterial inocula used for each type of experiment (individual: 10³ leptospires; pooled: 10⁴ leptospires). As with any screening assay, subsequent analysis of individual mutants remains important. It should be noted that previous studies have found that some virulence-attenuated *Leptospira* mutants still colonize the kidneys of acute host animals (Ristow et al., 2007; Murray et al., 2009b; Lambert et al., 2012; Zhang et al., 2012).

While a great deal of work has been carried out into the molecular basis of acute infection, study of the colonization host has largely been neglected. Some studies have identified changes in the proteome of leptospires within the kidney or urine (Monahan et al., 2008; Nally et al., 2011), although the mechanisms for localization and long-term survival in the renal tubules remain unknown. To investigate this aspect of the transmission cycle, a carrier host model was established using BALB/c mice. There has been limited work on mice as carrier hosts for leptospirosis. Carrier host infections have been established in Swiss white mice (Faine, 1962a, b), with mouse weight (equating to age) and challenge dose decisive in determining the outcome of infection. We found that 8–10-week-old BALB/c mice with a dose of 10⁵ leptospires were 100% colonized with no signs of disease after 2 weeks. This dose contrasts with the ID₅₀ of <10 bacteria of serovar Manilae seen in the acute hamster model of infection (Murray et al., 2009c).

A recent study analysed acute infection in mice and determined that mouse strains A, CBA, BALB/c and C57BL/6 were not susceptible to lethal leptospiral infection by strain *L. interrogans* serogroup Icterohaemorrhagiae strain Cop (Santos et al., 2010). The BALB/c mice were colonized, but did not develop the renal pathology associated with acute leptospirosis that was observed in all of the other mouse strains, suggesting that BALB/c mice are a more appropriate carrier model. In the current study, *L. interrogans* serovar Manna colonization likewise did not produce renal pathology such as inflammation or morphological alterations in the kidney. A similar lack of pathology is found in carrier rats (Nally et al., 2005; Tucunduva de Faria et al., 2007).

Through the use of transposon mutants, this study has defined the first factors required for colonization of the carrier host. Mouse-attenuated mutants included two LPS mutants, described previously (Murray et al., 2010), which are also attenuated in the acute hamster model, and the htpG mutant M1233. For each of these strains there may be a common method of attenuation in both the carrier and acute host. A further five mutants identified through the high-throughput assay were confirmed to be attenuated for mouse colonization. Interestingly, mutants M601 and M620 were not attenuated in the acute model, while mutants M602, M607 and M655 were only marginally attenuated (significant delay in time to death), thus highlighting different factors required for infection of different host species.

M601 had a mutation in *lb191* encoding a TonB-dependent outer-membrane receptor HbpA. HbpA is a haem-binding protein that is expressed *in vitro* under low-iron conditions (Asuthkar et al., 2007; Lo et al., 2010). These findings suggest that haem uptake and utilization are required in the carrier host, as in the acute host (Murray et al., 2009b). Mutant M655 had a mutation in *lb194* which is also iron regulated (Lo et al., 2010). The genes *lb191* and *lb194* are present in a locus conserved across *Leptospira*, suggesting a role in iron transport *in vivo*. M620 (*la2786* mutant) had a transposon insertion in a gene encoding a predicted hypothetical protein that is likely to be non-cytoplasmic. *la2786* was marginally upregulated under low-iron conditions (Lo et al., 2010).

Mutant M607 had an insertion in *la0589*. LA0589 (639 amino acids) is a hypothetical protein encoded within the published *L. interrogans* genomes (Ren et al., 2003; Nascimento et al., 2004). Intriguingly, there are another
ten paralogous proteins encoded by the serovar Lai genome (ranging from 69 to 81% identity and from 627 to 641 aa in length) and 11 paralogues in serovar Copenhageni. A BLASTP search reveals a similar set of 14 paralogous proteins of unknown function in Bartonella bacilliformis.

M602 had a deletion of five genes, three of which are in an operon structure associated with transport. It is not possible to say which of these genes is associated with virulence, but whole genome sequencing eliminated the possibility of other mutations being responsible. It is not known how this deletion occurred immediately adjacent to the transposon insertion point; we have not observed such a deletion before in mutants constructed with the Himar1 mariner transposon TnSCI89 (Murray et al., 2009a).

Only two other studies have tested defined mutants in the colonization model; these studies found that LigB and LipL32 were unnecessary for rat kidney colonization (Croda et al., 2008; Murray et al., 2009c). None of the factors identified as necessary for renal colonization in this study was highlighted in proteomic studies of rat urine-derived leptospires (Monahan et al., 2008; Nally et al., 2011). However, the combination of mutagenesis and proteomic approaches will build a picture of the molecular mechanisms for renal colonization and persistence. Comparing pathogenic mechanisms in the carrier and acute hosts may identify bacterial factors that contribute to different disease outcomes. Such knowledge will inform strategies for disease control, such as vaccine design.

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