Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily

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Summary

Proteins with bacterial immunoglobulin-like (Big) domains, such as the *Yersinia pseudotuberculosis* invasin and *Escherichia coli* intimin, are surface-expressed proteins that mediate host mammalian cell invasion or attachment. Here, we report the identification and characterization of a new family of Big domain proteins, referred to as Lig (leptospiral Ig-like) proteins, in pathogenic *Leptospira*. Screening of *L. interrogans* and *L. kirschneri* expression libraries with sera from leptospirosis patients identified 13 lambda phage clones that encode tandem repeats of the 90 amino acid Big domain. Two *lig* genes, designated *ligA* and *ligB*, and one pseudo-gene, *ligC*, were identified. The *ligA* and *ligB* genes encode amino-terminal lipoprotein signal peptides followed by 10 or 11 Big domain repeats and, in the case of *ligB*, a unique carboxy-terminal non-repeat domain. The organization of *ligC* is similar to that of *ligB* but contains mutations that disrupt the reading frame. The *lig* sequences are present in pathogenic but not saprophytic *Leptospira* species. LigA and LigB are expressed by a variety of virulent leptospiral strains. Loss of Lig protein and RNA transcript expression is correlated with the observed loss of virulence during culture attenuation of pathogenic strains. High-pressure freeze substitution followed by immunocytochemical electron microscopy confirmed that the Lig proteins were localized to the bacterial surface. Immunoblot studies with patient sera found that the Lig proteins are a major antigen recognized during the acute host infection. These observations demonstrate that the Lig proteins are a newly identified surface protein of pathogenic *Leptospira*, which by analogy to other bacterial immunoglobulin superfamily virulence factors, may play a role in host cell attachment and invasion during leptospiral pathogenesis.

Introduction

Leptospirosis is the most widespread zoonosis in the world and has emerged as an important public health problem in large urban centres of developing countries (Ko *et al*., 1999; Levett, 2001). Its severe disease form, known as Weil's syndrome, is an acute febrile illness associated

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with multiorgan system complications including jaundice, renal failure, meningitis and pulmonary haemorrhage, with a mortality rate that may exceed 15% (Faine *et al*., 1999; Marotto *et al*., 1999). Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*, which includes pathogenic and saprophytic species. Pathogenic *Leptospira* are highly motile and invasive organisms (Merien *et al*., 1997; Barocchi *et al*., 2002) which rapidly disseminate to target organs after entering the host, usually through abrasions in the skin or mucous membranes (Arean, 1962; Faine *et al*., 1999). These organisms may be cleared by the humoral immune response, but in carriers they have the ability to colonize and persist in the kidney tubules. The rat (*Rattus norvegicus*) is the principal reservoir associated with urban epidemics of leptospirosis (Ko *et al*., 1999). Urine excreted by infected animals contains viable *Leptospira*, which can survive for days to weeks in soil or water (Chang *et al*., 1948; Hellstrom and Marshall, 1978). Flooding following heavy rainfall is frequently associated with large outbreaks of leptospirosis (Trevejo *et al*., 1998; Ko *et al*., 1999).

To date, few leptospiral factors that contribute to the pathogenesis of disease have been identified. The periplasmic endoflagella involved in propeller movement of the bacterium is an important factor in tissue penetration and motility through highly viscous fluids (Greenberg and Canale-Parola, 1977; Trueba *et al*., 1992). Moreover, chemotaxis for haemoglobin has been demonstrated, which indicates that *Leptospira* may be attracted to abraded skin surfaces (Yuri *et al*., 1993). Many pathogenic *Leptospira* secrete sphingomyelinase C (SphA) and pore-forming haemolysins (SphH), possibly associated with the haemolytic anaemia observed in leptospirosis patients (Trowbridge *et al*., 1981; Segers *et al*., 1992; Lee *et al*., 2002). Leptospiral lipopolysaccharide (LPS) is a major outer membrane component recognized by Toll-like receptor 2 (TLR2) on macrophages (Werts *et al*., 2001). The acquired immune response to the carbohydrate component of leptospiral LPS confers serovar-specific immunity and underlies the serological classification of the genus *Leptospira* into >200 serovars (Jost *et al*., 1989; de la Peña-Moctezuma *et al*., 2001).

The ability of pathogenic leptospires to penetrate, disseminate and persist in mammalian host tissues appears to rely on the ability of these organisms to attach to eukaryotic cells and extracellular matrix proteins (Tsuchimoto *et al*., 1984; Vinh *et al*., 1984; Ballard *et al*., 1986; Thomas and Higbie, 1990). The variety of host cells recognized by *Leptospira* suggests the presence of several adhesins. However, the only putative leptospiral adhesin identified to date is a virulence-associated leptospiral surface protein that binds purified fibronectin (Merien *et al*., 2000). In a recent report, pathogenic leptospires were distinguished from saprophytic organisms by their ability to rapidly translocate through a polarized MDCK monolayer without disrupting tight junctions (Barocchi *et al*., 2002). Rapid translocation across tissue barriers may be a mechanism used by this pathogen to invade host organs.

Surface-exposed moieties or structures are presumably the factors that mediate adherence of the leptospiral pathogen to mammalian host cells. Like other spirochetes, leptospires have a large repertoire of lipoproteins, a subgroup of which may be involved in mediating host cell interactions, as in the case of *Borrelia* lipoproteins (Haake, 2000). A leptospiral integral membrane protein, OmpL1, lipoproteins, LipL41 and LipL32, and a peripheral membrane protein $P31_{LipL45}$ have been identified, some of which have been shown to be surface-exposed and expressed during host infection (Haake *et al*., 1993; 2000; Shang *et al*., 1996; Barnett *et al*., 1999; Matsunaga *et al*., 2002). More recently, a *L. interrogans* protein, LigA, was identified which has tandem repeats of the bacterial immunoglobulin-like (Big) domain (Palaniappan *et al*., 2002). This repeat motif is found in bacterial virulence factors that mediate mammalian host cell adherence and invasion, such as *Escherichia coli* intimin (Luo *et al*., 2000) and *Yersinia pseudotuberculosis* invasin (Hamburger *et al*., 1999). However, the sequence of LigA consists entirely of tandem Big repeats and does not have a C-terminal non-repeat domain that, by analogy to the structure of intimin and invasin, would serve as the binding domain to host

cell-associated receptors. In addition, the surface expression of LigA and its association with virulence were not characterized (Palaniappan *et al*., 2002).

Analysis of leptospiral antigens targeted by the humoral immune response to naturally acquired infection is an effective approach to identify proteins expressed during infection. Antibodies to the infection-associated proteins OmpL1, LipL41, LipL32, P31$_{LipL45}$ (Qlp42), and LigA are generated during human or animal infection (Flannery *et al*., 2001; Guerreiro *et al*., 2001; Nally *et al*., 2001; Palaniappan *et al*., 2002). In this report, we used sera from leptospirosis patients as a tool to identify DNA sequences from *Leptospira* genomic libraries which encode for proteins expressed during host infection. This approach allowed us to determine that there are in fact two intact leptospiral genes, *ligA* and *ligB*, and one disrupted gene, *ligC*, that encode domains belonging to the bacterial Ig-like superfamily. The *ligB* and *ligC* genes encode a C-terminal non-repeat domain following the tandem Big domain repeats, an organization which is similar to that of intimin and invasin. Furthermore, we found that LigA and LigB were surface-exposed lipoproteins whose expression correlated with the virulence of *Leptospira* strains. Together, these findings indicate that these newly identified surface lipoproteins may play an analogous role to intimin and invasin in mediating host cell interactions during leptospiral pathogenesis.

Results

Identification of leptospiral ligA, ligB and ligC genes

Pooled sera from leptospirosis patients were used to identify DNA sequences that encode host-expressed leptospiral proteins from λ genomic expression libraries of two virulent strains, *L. kirschneri* serovar grippotyphosa, strain RM52 and *L. interrogans* serovar copenhageni, strain Fiocruz L1-130. Antibody screening yielded 98 and 27 reactive clones from *L. kirschneri* and *L. interrogans* libraries, respectively. Triage of λ clones with antisera against characterized leptospiral proteins and sequencing of DNA inserts identified 70 clones with inserts encoding heat shock proteins GroEL and DnaK (Ballard *et al*., 1993), three clones containing the gene encoding the lipoprotein LipL41 (Shang *et al*., 1996), one clone with an insert encoding the putative inner membrane lipoprotein LipL31 (Haake and Matsunaga, 2002), and one encoding a putative 28 kDa lipoprotein. Thirteen clones had inserts that encoded imperfect tandem repeat sequences. A query of the repeat sequences against the Pfam 7.7b database (Bateman *et al*., 2002) identified each of these 90 residue repeats as Big2 (PF02368), a family of *b*acterial *i*mmuno*g*lobulin-like (Big) domains. In addition, the cloned insert sequences encoded polypeptides that were homologous to the recently described LigA protein from *L. interrogans* serovar pomona type kennewicki (Palaniappan *et al*., 2002).

Assembly of overlapping cloned sequences identified two novel leptospiral *big* genes, designated *ligB* and *ligC*, distinct from *ligA*. Of the recombinant clones, two *L. kirschneri* and three *L. interrogans* clones encoded LigA, six *L. kirschneri* clones encoded LigC, whereas one *L. interrogans* clone encoded LigB. Because none of the cloned sequences contained a complete open reading frame, inverse PCR of flanking DNA was performed to obtain the full-length nucleotide sequence. Primers derived from the *L. interrogans ligB* and *L. kirschneri ligC* sequences allowed for PCR amplification of the corresponding genes in *L. kirschneri and L. interrogans* respectively.

Sequence features of the ligA, ligB, and ligC genes

From the assembled sequences, *ligA (*3675 bp in both *L. interrogans* and *L. kirschneri)* and *ligB (*5673 bp in *L. interrogans* and 5661 bp in *L. kirschneri)* have open reading frames predicted to encode polypeptides of 128 and 212 kDa respectively. The *ligC* gene (5871 bp in *L. interrogans* and 5865 bp in *L. kirschneri*) appears to be a pseudogene in both *L.*

interrogans and *L. kirschneri*. In *L. interrogans,* the *ligC* open reading frame is interrupted at codon 499 by a TAA stop codon. Alignment of the *L. interrogans* and *L. kirschneri ligC* nucleotide sequences reveals an extra thymine at nucleotide 1008 (codon 336) in the *L. kirschneri* gene. This frameshift mutation results in a TAG stop codon downstream at codon 347. The predicted size of LigC, correcting for the stop and frame-shift mutations, is 210 kDa, similar to that predicted for LigB. LigA, LigB and LigC orthologues from *L. kirschneri* and *L. interrogans* had 90.7–94.9% amino acid sequence identity whereas the *L. kirschneri* and *L. interrogans* LigA sequences were 80.5% and 84.5% identical to that of *L. interrogans* type kennewicki LigA respectively (Palaniappan *et al.*, 2002). Horizontal transfer of DNA or convergent evolution may account for the closer relatedness of the *L. interrogans* strain Fiocruz L1-130 *ligA* gene to the *L. kirschneri* strain RM52 *ligA* gene than to that of *L. interrogans* type kennewicki.

The *ligA* and *ligB* genes are predicted to encode lipoproteins based on the identification of a 17 amino acid N-terminal signal peptide and lipoprotein signal peptidase cleavage site that conforms to the spirochetal lipobox (Haake, 2000). Following the signal peptide cleavage sites, the Lig proteins contain 10–11 repeats (Fig. 1D). Analysis of the primary amino acid sequences with the motif discovery tool MEME v3.0 (Bailey and Elkan, 1994) revealed two types of motifs within the 90 residue Big2 repeat sequences. Motif 1 is comprised of 52 residues, whereas Motif 2 is 24 residues in length (Fig. 1A and B). The repetitive nature of these motifs, along with their symmetrical placement within the Big domain, correlates with the predicted three-dimensional β-immunoglobulin sandwich structure of the Lig proteins (Fig. 1C).

Like the Big superfamily proteins intimin and invasin, *ligB* and *ligC* encode a C-terminal, non-repeat domain (771 and 789 residues, respectively) following the Big tandem repeat sequences. In contrast, LigA is a smaller truncated form consisting entirely of Big repeat domains. Whereas the amino acid sequence identity between C-terminal domains encoded by *ligB* and *ligC* is 51%, these domains do not have significant homology with proteins deposited in the NCBI database.

Inverse PCR with primers annealing to the 3′ end of the *ligA* gene (Table 2) demonstrated that the *ligB* gene is located <1.5 kb downstream of the truncated *ligA* gene. Both genes are transcribed in the same orientation (Fig. 1D). Open reading frames larger than 100 nucleotides are absent between *ligA* and *ligB*. Sequencing and Southern blot analyses indicate that *ligC* is not linked to the *ligA-ligB* locus (data not shown). Although *L. kirschneri* and *L. interrogans* sequences of *ligA* and *ligB* are homologous with >91% DNA sequence identity, there is considerable divergence between the *L. kirschneri* and *L. interrogans ligA-ligB* intergenic regions (943 bp and 1347 bp in length, respectively).

Interestingly, the 5′ portion of the *ligA* sequence is identical to the 5′ portion of the *ligB* sequence (Fig. 1D). This region of sequence identity begins 266 bp (*L. interrogans*) or 538 bp (*L. kirschneri*) upstream from the start codon of *ligA* and *ligB* and extends 1890 bp into the open reading frame sequence of both genes. Consequently, the first 630 amino acid residues of LigA and LigB, which includes the first four and part of the fifth Big2 repeat domain, are identical. In addition, there are four tandem imperfect 38-nucleotide repeats within the duplicated region upstream of the *L. kirschneri ligA* and *ligB* genes. The second and third repeats are identical. The first and fourth repeats differ from the other two repeats by two and six nucleotides respectively. The corresponding regions of the *ligA* and *ligB* genes in *L. interrogans* contain a single copy of a similar 39-nucleotide sequence that differs from the fourth repeat in *L. kirschneri* by two nucleotides. These small repeat sequences were not found in the 3′ flanking regions of either *ligA* or *ligB*. Several homopolymeric tracts were found in the *lig* genes. In *L. kirschneri*, *ligA* has a run of 8As, *ligB* has a run of 8As and a run of 9As, whereas *ligC* has a run of 8Ts. In *L. interrogans*, *ligB* has a run of 9As, whereas *ligC* has two runs of 8Ts and a

run of 6 Gs. However, changes in the length of homopolymeric tracts would not correct the frameshift and stop codon mutations of the *ligC* genes of *L. kirschneri* and *L. interrogans* respectively.

Distribution of lig genes in the genus Leptospira

Southern blot studies with *ligA*, *ligB*, and *ligC*-specific probes confirmed that *L. interrogans* Fiocruz L1-130 has a single copy of each *lig* gene (Fig. 2A, middle lanes). Further analysis found that specific *lig* sequences were not detected in the saprophytic, non-pathogenic *L. biflexa* Patoc 1 (Fig. 2A, right lanes) whereas the *L. kirschneri* (data not shown) and *L. interrogans* genomes contained the three genes encoding the Lig repetitive domain.

To examine the distribution of *lig* genes in a larger collection of pathogenic and saprophytic strains of *Leptospira* spp., a pair of degenerate PCR primers was designed that annealed to all six *lig* genes in *L. interrogans* Fiocruz L1-130 and *L. kirschneri* RM52. These primers amplified *lig* sequences in *L. kirschneri* RM52 and *L. interrogans* Fiocruz L1-130 (Fig. 2B, lanes 2 and 3, respectively) and in 12 strains belonging to six pathogenic *Leptospira* species (Fig. 2B, lane 4; Fig. 2C, lanes 2–13). *Leptospira fainei* and *L. inadai* are considered 'intermediate' pathogens and are phylogenetically distinct from typical pathogenic species (Perolat *et al.*, 1998). Polymerase chain reaction analysis yielded a product of the expected 500 bp size for a *L. fainei* strain, but not for a *L. inadai* strain (Fig. 2C, lanes 14 and 15). Polymerase chain reaction amplification of the *L. fainei* and *L. weilii* DNA yielded higher molecular weight products that may represent primers annealing to related sequences within the *lig* genes. Furthermore, *lig* sequences appear to be restricted to pathogenic strains since they were not detected in the two saprophytic strains that were evaluated (Fig. 2C, lanes 16–17).

Expression of Lig proteins in virulent and culture-attenuated Leptospira strains

Leptospires lose their virulence phenotype with sequential *in vitro* culture passages (Haake *et al.*, 1991). Therefore, expression of virulence factors may be downregulated during culture attenuation. In an earlier report, antisera to LigA of *L. interrogans* type kennewicki failed to react with antigens obtained from *in vitro* cultured leptospires (Palaniappan *et al.*, 2002). However, it was not determined whether the lack of antibody reactivity was due to the absence of detectable LigA expression in virulent strains or due to prolonged culture and virulence attenuation in the study strains.

To examine this issue, we performed immunoblot analysis with polyclonal antibodies raised against unique portions of recombinant LigA, LigB and LigC fragments and extracts from virulent, low-passage and culture-attenuated, high-passage (>200 passages) isolates of *L. kirschneri* strain RM52. Loss of virulence in high-passage isolates was documented by the failure of large ($\geq 10^7$) inoculum doses to produce a lethal infection in the hamster model (Haake *et al.*, 1991). In experiments with low-passage isolates, antibody against the last three Big2 repeats of LigA (amino acid residues 936–1224) reacted with a protein band with similar Mr (~130 kDa) to the predicted molecular weight for LigA (128 kDa) (Fig. 3A, lane1). Likewise, antibody against the C-terminal segment of LigB (residues 1113–1886) reacted with a >200 kDa protein band, which is close to the predicted molecular weights for LigB (212 kDa) and LigC, corrected for mutations (210 kDa) (Fig. 3B, lane 1). Anti-LigC antibodies (Table 1) did not react with a protein band >50 kDa in low-passage isolate extracts (data not shown), indicating that a high molecular weight *ligC* product is not expressed and that LigB is the >200 kDa protein detected in immunoblots with anti-LigB antibody. To confirm the expression of LigA and LigB, we raised antisera against residues 342 through 1224 of LigA, which includes a portion (residues 342–630) of the region of sequence identity with LigB. This antiserum reacted with ~130 and >200 kDa protein bands as expected (Fig. 3C, lane 1).

Loss of LigA and LigB expression correlates with loss of virulence during culture attenuation since antibodies to Lig proteins did not react with a protein band in extracts of high-passage *L. kirschneri* (Fig. 3A–C, lanes 2). This phenomenon appears to be specific for LigA and LigB, as expression of most leptospiral proteins, including LipL41 (Fig. 3A–C), is conserved in both low and high-passage isolates (Haake *et al*., 1991). DNA sequencing of the promoter regions upstream of *ligA* and *ligB* revealed no differences between virulent and attenuated forms of *L. kirschneri* strain RM52, ruling out changes in these regions as an explanation for the observed loss of LigA and LigB expression.

Immunoblot analysis was performed with strain Fiocruz L1-130 to determine whether these observations could be extended to *L. interrogans*. Figure 3D shows that a virulent, low-passage but not attenuated, high-passage (45 passages) isolates of this strain expressed LigA and LigB. The attenuated virulence of the high-passage Fiocruz L1-130 isolate was demonstrated when a large inoculum of 10^8 leptospires failed to produce a lethal infection. Additional low- and high-passage *L. kirschneri* and *L. interrogans* isolates were examined in immunoblots shown in Fig. 3E. GroEL was detected by anti-GroEL antibodies in low and high-passage strains whereas significant levels of LigA and LigB were found only in low-passage isolates. Although a small amount of LigB was detected in high passage *L. kirschneri* strain Moskva V, the quantity detected was markedly less than that in the low-passage isolate. The additional ~110 kDa band observed in the *L. interrogans* strains appears to be a proteolytic breakdown product produced when the sample is boiled repeatedly rather than expression of an additional Lig protein. Neither LigA nor LigB was detected by immunoblot analysis in the saprophytic strain, *L. biflexa* Patoc 1 (data not shown).

Reverse transcriptase-polymerase chain reaction analyses indicate that variation in *lig* transcript levels in low-and high-passage strains may account for the loss of LigA and LigB expression during culture attenuation (Fig. 4). For assays with RNA extracts of *L. kirschneri* strain RM52, PCR primers were used to amplify sequences corresponding to a ~0.5 kb region near the 3′ ends of the transcripts. Amplified products were not observed in control reactions lacking reverse transcriptase, indicating that extracts were not contaminated with DNA. The *ligA* and *ligB* transcripts were detected in low-passage strains while these transcripts were absent or detected at reduced levels in high-passage strains (Fig. 4). The *lipl45* gene expresses the $P31_{LipL45}$ protein, whose levels are unaffected by *in vitro* passaging and culture attenuation (Matsunaga *et al*., 2002). Differences in *ligA* and *ligB* transcript levels do not appear to be due to differential extraction of leptospiral RNA since equivalent levels of *lipl45* transcripts were detected in high and low-passage strains (Fig. 4, lanes 14 and 16). The *ligC* transcript was not observed in either low- or high-passage *L. kirschneri*, supporting the conclusion that *ligC* is not expressed as a gene product.

Early recognition of the Lig repeats by the immune system

At least one Lig protein is expressed during human infection since patient sera were used to identify *lig* clones from the λ genomic expression library. This finding was confirmed in immunoblot studies demonstrating that pooled patient sera specifically reacted with purified recombinant proteins corresponding to repeats one through five of LigB (Fig. 5, lane A3), repeats two through seven of LigB (data not shown), and repeats five through 10 of LigB (data not shown). In contrast, serum from healthy human volunteers did not react with any recombinant LigB proteins (Fig. 5, lane B3, and data not shown). These data suggest that LigB is expressed during infection; however, we can not exclude the possibility that the positive reaction in these blots is due to cross-reactivity to an antigenically related protein, such as LigA. Anti-recombinant LigB IgM antibody was detected in more than 95% of individual leptospirosis patients during their acute illness, suggesting that Lig proteins are expressed early in the course of infection (data not shown). In addition to human leptospirosis, Lig proteins

are expressed during natural infection of animal reservoirs: anti-LigB antibodies were found in sera from captured domestic rat (*Rattus norvegicus*) reservoirs (Fig. 5, lane C3), but not in sera from captured culture-negative rats (lane D3). In contrast to the findings observed with natural infection, multiple immunizations of laboratory rats with extracts of *in vitro* cultured low-passage strain Fiocruz L1-130 did not produce detectable serum antibodies against recombinant LigB (Fig. 5, lane E3). This immunization protocol produced strong antibody responses to more than 20 other leptospiral proteins found in whole extracts (Fig. 5, lane E1), as well as to recombinant LipL32 (Fig. 5, lane E2), a protein previously found to be expressed *in vitro* and during host infection (Haake *et al.*, 2000).

Surface localization of Lig proteins in immunocytochemical electron microscopy and cell fractionation

Immunoelectron microcopy (IEM) studies demonstrated that the C-terminal non-repeat (Fig. 6E and F) and Big repeat (data not shown) domains of Lig proteins are present on the surface of virulent, low passage leptospires. In whole-cell IEM assays (Fig. 6A–C), live *L. kirschneri* RM52 were settled onto EM grids, incubated with antibody, and labelled with gold-conjugated anti-rabbit secondary antibody. Gold particles were present on leptospires incubated with antibodies against the LigB C-terminal domain (32 ± 9 particles per bacterium, mean ± standard deviation) (Fig. 6E). Staining was similar to that observed when leptospires were incubated with antibodies against the leptospiral surface moiety, LPS (64 ± 6 particles per bacterium) (Fig. 6A). Assay conditions appeared to preserve the integrity of the leptospiral membranes as bacteria incubated with antibodies against the cytoplasmic heat shock protein, GroEL, showed low levels of background staining (5 ± 2 particles per bacterium) (Fig. 6C). Furthermore, IEM of thin-sectioned *L. kirschneri* strain RM52 demonstrated that antibodies against the LigB C-terminal non-repeat domain (Fig. 6F) and LPS (Fig. 6B) labelled the external surface of the outer membrane. Labelling with anti-GroEL antibodies was restricted to the cytoplasm of leptospires. Similar results were obtained for whole-cell and thin-section IEMs with *L. interrogans* Fiocruz L1-130 (data not shown). Indirect immunofluorescence assays were performed with live, intact leptospires as a third method to confirm the surface localization of Lig proteins. Polyclonal antibodies raised against the first to fifth Big repeat domains of LigA and LigB bound to the surface of the virulent, low passage *L. interrogans* Fiocruz L1-130 as did antibodies to the outer membrane lipoprotein LipL41 (data not shown). Surface labelling was not detected in control assays with antibodies against the inner membrane lipoprotein LipL31 (Haake and Matsunaga, 2002), and GroEL. Anti-LigA/B antibodies did not label the surface of culture attenuated, high passage *L. interrogans* Fiocruz L1-130. The loss of LigA/B surface expression during culture attenuation of virulence appeared to be specific as anti-LipL41 antibodies strongly labelled these leptospires.

The unique cellular architecture of spirochetes, in which the peptidoglycan is associated with the inner instead of the outer membrane, permits solubilization of the outer membrane with the non-ionic detergents Triton X-114 and Triton X-100 (Haake *et al.*, 1991; 1998; Zuerner *et al.*, 1991). The periplasmic cylinder (PC) and the Triton X-100-soluble fractions of virulent *L. kirschneri* were analysed by immunoblotting (Fig. 7). The fractions were probed with antibody to the inner membrane protein LipL31, which was found in the PC fraction, as expected (Haake and Matsunaga, 2002). Both LigA and LigB were partially solubilized by Triton X-100, consistent with the surface exposure demonstrated by immunogold electron microscopy. Incomplete solubilization of LigA and LigB suggests that the Lig proteins may be distributed between the inner and outer membranes.

Discussion

In this study, we characterized a new family of leptospiral surface-exposed proteins we refer to as Lig (leptospiral Ig-like), which may play a role in the pathogenesis of leptospirosis. Our results are consistent with LigA and LigB being expressed in the infected host (Fig. 5) and demonstrate that the *lig* genes are found only in pathogenic *Leptospira* species (Fig. 2). More importantly, LigA and LigB expression is correlated with virulence. The loss of the ability to produce lethal infection in hamsters by high-passage *L. interrogans* strain Fiocruz L1-130 (this study) and *L. kirschneri* strain RM52 (Haake *et al.*, 1991) is associated with loss of LigA and LigB expression (Fig. 3). Whereas the vast majority of leptospiral proteins expressed in virulent *L. kirschneri* RM52 is also expressed in the culture-attenuated form of the same strain (Haake *et al.*, 1991), LigA and LigB expression is specifically lost during culture attenuation, suggesting that the latter proteins are closely tied to the pathogenesis of leptospirosis. The molecular basis for the loss of Lig protein expression with culture passage is unknown. Changes in the number of the 38-nucleotide repeat in the promoter region may affect transcription. However, sequence analysis of the promoter regions, including the four 38-nucleotide tandem repeats, and the first 380 nucleotides of *ligA* and *ligB* of both virulent and culture-attenuated *L. kirschneri* revealed no sequence differences. Alternatively, several sites consisting of runs of at least six identical nucleotides are present within the *lig* genes, raising the possibility of the loss of *lig* expression by slipped-strand mispairing.

Our studies suggest that Lig expression is upregulated during infection of the mammalian host. Rats immunized with killed virulent *L. interrogans* Fiocruz L1-130, which expresses LigA and LigB *in vitro* (Fig. 3D), failed to produce detectable antibody against recombinant Lig protein (Fig. 5E). In contrast, anti-Lig antibody was detected in rats infected with live *L. interrogans* (Fig. 5C). Similarly, *lig* sequences were not found among 104 clones obtained from a leptospiral expression library probed against serum from rabbits immunized with killed virulent *L. kirschneri* RM52 (Matsunaga *et al.*, 2002), whereas in this study, 10% (13 of 125) of the clones isolated from expression libraries which reacted with leptospirosis patient sera had *lig* gene sequences. Upregulation of Lig expression during infection could also explain the inability of Palaniappan *et al.* (2002) to detect LigA in cultivated *L. interrogans* subtype kennewicki by immunoblot analysis while detecting expression of this moiety in leptospires from infected hamster kidneys by immunohistochemical methods.

The Lig proteins are new members of the bacterial immunoglobulin-like (Big) superfamily, some of which serve as adhesins and invasion-mediating determinants in other bacterial pathogens. Invasin of *Yersinia pseudotuberculosis* and intimin of enteropathogenic *E. coli* are two examples of virulence factors in this family. The structural organization and localization of Lig proteins share common themes to those observed for intimin and invasin. Lig proteins are presumed to be anchored to the outer membrane with the same N-terminal orientation as observed for intimin and invasin. However, LigA and LigB are predicted lipoproteins, which are anchored to the outer membrane via fatty acids attached to the amino-terminal cysteine residue rather than a transmembrane domain. The sequence of the amino termini of LigA and LigB largely conforms to the spirochetal lipobox, as previously defined (Haake, 2000). As with intimin and invasin, Lig proteins have tandem Big repeats that follow the membrane anchor. Computer modelling of Big2 domains from Lig proteins found significant sequence identity with Big domains from intimin and invasin, indicating that Lig Big2 domains adopt a folding structure resembling those observed for Big1 and 2 domains in these proteins (Fig. 1C).

The role of Big repeat domains in Lig proteins is unclear but they may serve a similar function as observed in intimin and invasion. Big domain repeats in these virulence factors are believed to act as linkers that project the receptor binding C-terminal domain from away from the bacterial surface therefore providing higher accessibility for interaction (Hamburger *et al.*,

1999; Luo *et al*., 2000). In addition, one of the four Big domains of *Y. pseudotuberculosis* invasin mediates efficient invasion by promoting homotypic interaction of invasin subunits (Dersch and Isberg, 2000). Finally, LigB has a large C-terminal domains (771 residues) following the Big domain repeats that, by analogy with invasin and intimin (106 and 99 residue C-terminal domains, respectively), may have a host cell binding function. LigA lacks the C-terminal domain found in LigB. Interestingly, *ligA* is located <2 kb upstream of *ligB*, and the finding that *ligA* and *ligB* have identical sequences in the 5′ regions raises the question whether these genes arose from a duplication event. Together, these findings suggest that LigB may be a more plausible candidate among the Lig family to serve as an adhesin. As a caveat, the possibility cannot be excluded that Big2 domains in LigA play a role in host cell receptor binding.

The sequences of the *L. kirschneri* and *L. interrogans ligC* genes have frameshift and stop codons, respectively, which interrupt the open reading frame. Introduction of early stop codons in bacterial mRNA often destabilize the entire message (Nilsson *et al*., 1987; Grunberg-Manago, 1999). Reverse transcriptase PCR and immunoblot studies could not detect expression of *ligC* at the RNA and protein level, providing additional evidence that *ligC* is a pseudogene. Nevertheless, the possibility that a truncated ~50 kDa or ~35 kDa protein is expressed from the open reading frames at the 5′ end of the *L. kirschneri* or *L. interrogans ligC* transcript, respectively, cannot be excluded.

Adherence of leptospires to host mammalian cells plays an essential role in the pathogenesis of leptospirosis. Host cell entry may play a role in the rapid dissemination of leptospires as *L. interrogans* has been shown to rapidly translocate across polarized cell monolayers (Thomas and Higbie, 1990; Barocchi *et al*., 2002). Electron microscopy studies found that leptospires tightly adhere to host cell plasma membrane during the translocation process, suggesting that this type of association may be a required early step for translocation (Barocchi *et al*., 2002). In addition, leptospires attach tightly to the luminal surface of the renal epithelium in order to establish persistent carriage in animal reservoirs (Marshall, 1976).

Proteins that mediate these host cell interactions are expected to be exposed on the leptospiral cell surface. In this study, imunoelectron microscopy and immunofluorescence microscopy of *L. kirschenri* and *L. interrogans* demonstrated that the Lig proteins are surface-exposed (Fig. 6, data not shown). Furthermore, surface expression of LigB proteins, as detected by immunofluorescent microscopy, disappeared as virulent strains were culture attenuated (data not shown). Previous studies have shown that virulent leptospires, in contrast to culture-attenuated forms of the same strains, attach more readily to tissue culture cells and extracellular matrix proteins (Ballard *et al*., 1986;Ito and Yanagawa, 1987). Merien *et al*. (1997) observed the same phenomenon with respect to the ability of virulent and culture attenuated leptospires to invade Vero cells *in vitro*. A 36 kDa surface-exposed protein potentially involved in fibronectin binding has been identified in virulent *L. interrogans* but is absent in the culture-attenuated form of that strain (Merien *et al*., 2000). The correlation of LigA and LigB expression and leptospiral adhesion with virulence and their structural similarity with invasin and intimin suggests that LigA and/or LigB may be another leptospiral adhesin.

In conclusion, we have identified a new family of Big proteins in *Leptospira* that may play a role in pathogenesis. The evidence that LigA and B proteins are virulence factors is that (i) they are found only in pathogenic *Leptospira* species; (ii) they are detected in virulent but not in culture-attenuated strains of *L. kirschneri* and *L. interrogans*; and (iii) like the Big proteins intimin and invasin of known enteric bacterial pathogens, LigB, and perhaps LigA, is surface exposed. Further studies are needed to determine the role of LigB and the other members of the Lig family in the attachment and invasion of *Leptospira* spp. to host tissues.

Experimental procedures

Bacterial strains and media

All leptospiral strains were cultivated at 30°C in EMJH medium supplemented with 1% rabbit serum (Johnson and Harris, 1967) (Faine, 1982). *Leptospira interrogans* serovar copenhageni, strain Fiocruz L1-130 was isolated from a patient during an outbreak of leptospirosis in Salvador, Brazil (Ko *et al*., 1999; Barocchi *et al*., 2001). The low passage clinical isolate Fiocruz L1-130 had an LD_{50} of 10^4 in the hamster model of virulence (Barocchi *et al*., 2002). Forty-five *in vitro* passages were performed to obtain a culture-attenuated strain for which an inoculum of 10^8 organisms of this high passage strain did not induce death or clinical disease in hamsters. All other leptospiral strains, including virulent and culture-attenuated *L. kirschneri* serovar grippotyphosa, strain RM52, were obtained from the National Leptospirosis Reference Center (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa) and are described in a recent study (Matsunaga *et al*., 2002). Experimental protocols involving hamsters were approved by the VA Greater Los Angeles Healthcare System Animal Research Committee.

Escherichia coli strains were grown in LB supplemented with 100 μg ml^{-1} ampicillin (Sigma) or 100 μg ml^{-1} carbenicillin (ICN) where appropriate. λ bacteriophage expression libraries were screened in *Escherichia coli* XL1-Blue MRF' (Stratagene) and *E. coli* XL1-Blue (Clontech). *Escherichia coli* SOLR and *E. coli* BM25.8 (Stratagene) allowed for *in vivo* excision of plasmid sequences from the λ expression vectors. Expression of His6-tagged and MBP-tagged recombinant proteins were done in *E. coli* BLR(DE3)/pLysS (Novagen) and *E. coli* TB1 (New England Biolabs) respectively.

Isolation of genes encoding leptospiral protein antigens

Genomic DNA prepared from low-passage *L. interrogans* serovar copenhageni, strain Fiocruz L1-130 and *L. kirschneri* serovar grippotyphosa, strain RM52 were partially digested with *Tsp*509I (New England Biolabs) and ligated to λZap II (Stratagene) and _TriplEx arms (Clontech) respectively. Recombinant phage DNA was packaged into λ heads following the instructions provided in the Gigapack III Gold Packaging Extract (Stratagene).

Sera from 10 to 20 leptospirosis patients identified during an urban outbreak in Brazil were pooled and adsorbed three times with lysates of *E. coli* XL1-Blue to remove antibodies to *E. coli* antigens (Gruber and Zingales, 1995). A titre of 1:100 to 1:500 of the adsorbed sera was used to probe the genomic expression libraries as described (Matsunaga *et al*., 2002). Reactive phage clones were purified by a round of plaque purification. The plasmid DNA was excised from the λ bacteriophage sequence by *in vivo* recombination in *E. coli* SOLR (Stratagene) or *E. coli* BM25.8 (Clontech) as instructed.

Gel electrophoresis and immunoblots

Electrophoresis and Western blots were performed as described (Guerreiro *et al*., 2001). Serum titres are indicated in the figure legends.

Oligodeoxynucleotides and DNA sequencing

The sequence of the first and last 500–700 nucleotides of each insert was obtained with vector-specific primers that anneal adjacent to the insert. The sequence of the upstream primer is 5′-TCCGAGATCTGGACGAGC-3′ (Clontech), and the sequence of the downstream primer is 5′-TAATACGACT CACTATAGGG-3′ (T7 promoter). The sequence of the remainder of each insert was obtained from plasmid clones deleted of restriction fragments extending from inside the insert into the multicloning sites or from intact plasmid clones with custom-designed gene-specific primers. Oligodeoxynucleotides were synthesized by Invitrogen. The sequencing

reactions were performed by the Yale/Keck Core DNA Sequencing Facility, the UC Berkeley Core DNA Sequencing Facility, and Instituto Butantan. The *lig* sequences were assembled with Sequencer 4.0.5 for Windows (Genecodes).

Polymerase chain reaction

The termini of the *lig* genes and their flanking regions were amplified by inverse PCR prior to sequencing. The 5′ and 3′ends of each *lig* sequence were subjected to separate inverse PCR reactions (Table 2). Leptospiral DNA was digested with an appropriate restriction enzyme (Table 2), treated with T4 DNA ligase, and amplified with *Taq* DNA polymerase (Qiagen). The primers listed in Table 2 (except for the *ligA* upstream primer) were designed to anneal near the ends of the *lig* sequences assembled from the clones obtained from the *L. kirschneri* expression library. After the duplicated segment was revealed by comparing the sequence of the 5′-most *ligA* clone with the inverse PCR product generated by the *ligA* 3′ end primers, the 5′ end of *ligA* and its promoter region were amplified with the sense primer 5′-ATTGTAATTTTTCTGATGGTCGTCAAAC-3′, which based on the sequence upstream of *ligB* was predicted to hybridize 265–238 nucleotides upstream of *ligA*, and the anti-sense primer 5′-CGGTAGCGGTCAGTTGTAGTGTAAGA-3′(positions 3175–3150 within *ligA*). Sequence upstream of the duplicated region of *ligA* was obtained by inverse PCR with the primers shown in Table 2 (*ligA* 5′ end).

To design PCR primers for the detection of *lig* sequences (Fig. 2B and C), the nucleotide sequences of all six *lig* genes in *L. interrogans* and *L. kirschneri* were aligned with MegAlign (DNAStar). Segments of near sequence identity were used to design the degenerate primers 5′-(G/C)AAAGTTG(T/C) (A/G)(T/C)G(T/G)CTTGGCC-3′ and 5′-(G/C)(A/T)ACC (A/G)TC (C/T)GAAAA(A/G)AT(A/T)CC-3′. About fifty nano-grams of leptospiral genomic DNA was amplified with *Taq* DNA polymerase (Qiagen) with an initial denaturation at 94°C for 1 min, then 35 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s, and a final 1 min extension at 72°C. The reactions were separated in a 1.4% agarose gel in TAE buffer (Fisher Biotech).

Plasmid constructions

Standard recombinant DNA techniques were used to construct all expression plasmids. The His6 expression vectors pRSETA and pRSETC were purchased from Invitrogen, and pMAL-c2x was purchased from New England Biolabs. pAE expression vector, a hybrid of pET and pRSET expression vectors, was kindly provided as a gift from Paulo Lee Ho (Instituto Butantan). The 2.8 kb *Kpn*I-*Hin*dIII fragment of plasmid clone 204 harbouring codons 342 through 1224 of *L. kirschneri ligA* was ligated to pRSETC to create p204orf1. All other expression plasmids were constructed by PCR with *lig*-specific primers with restriction sites introduced at their 5′ends (Table 1). Polymerase chain reactions were performed with *Pfu* Turbo DNA polymerase (Stratagene) during 30 cycles of amplification with *L. kirschneri* genomic DNA or 15 cycles of amplification with plasmid DNA. Polymerase chain reaction products were purified with the QIAquick PCR Purification Kit (Qiagen), subsequently gel-purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research), digested with the appropriate restriction enzymes, and ligated to a His6 expression vector with T4 DNA ligase (New England Biolabs).

Expression and purification of recombinant Lig

Expression plasmids were transformed into *E. coli* BLR(DE3)/pLysS. Transformed bacteria were grown at 37°C in 500 ml LB containing 100 μg ml^{-1} of carbenicillin to an OD_{600} of 0.4. IPTG was then added to a final concentration of 0.5 mM, and induction was continued at 37°C for 2.5–3 h. Except for p204orf1B (Table 1), all plasmids expressed insoluble His6-tagged recombinant protein. Ni^{2+}-nitrilotriacetic acid (NTA) affinity columns were used to purify His6-tagged recombinant proteins according to the instructions of the manufacturer (Qiagen).

The C-terminal domain of LigB fused to the maltose binding protein was expressed with pJIM520 (Table 1) transformed into *E. coli* TB1. Transformed bacteria were grown at 30°C in 500 ml containing 2 g litre^{-1} glucose and 100 μg ml^{-1} ampicillin to an OD_{600} of 0.5. Expression of the recombinant protein was then induced for 2.5 h with 0.5 mM IPTG. Bacteria were harvested, and the recombinant protein was purified by amylose resin as described in the instruction manual (New England Biolabs).

Animal sera

Purified recombinant His6-tagged Lig proteins were introduced into preparative 10% or 12% SDS-PAGE. The full-length recombinant protein was excised from the gel and used to immunize New Zealand White rabbits (Harlan Sprague Dawley) as previously described (Matsunaga *et al*., 2002). The MBP-LigB fusion protein was dialysed against PBS with a Float-A-Lyzer (Spectra/Por) and concentrated with the Centricon YM-10 spin column with a molecular weight cut-off of 10 000. Immunization protocols were approved by the VA Greater Los Angeles Healthcare System Animal Research Committee. Rabbit antibody to LipL41 and GroEL were prepared as previously described (Matsunaga *et al*., 2002; Shang *et al*., 1996). Domestic rats (*Rattus norvegicus*) were captured from urban communities from outbreaks in Brazil (Barocchi *et al*., 2001). Western blot analysis for Fig. 5C was done with sera from culture-positive rats identified as reservoirs for *L. interrogans*.

Reverse transcriptase PCR

Leptospira kirschneri strain RM52 was grown to late exponential phase. Total RNA was extracted from 1×10^{10} leptospiral cells by the hot-phenol method and resuspended in water following ethanol precipitation (Case *et al*., 1990). DNA was digested with the DNA-free kit from Ambion. RT-PCR was performed with ~100 ng leptospiral RNA and Omniscript RT as described (Qiagen). Transcripts were hybridized with the following primers:

ligA, 5′-CGCAGAAATTTTAGAGGAACCTACAG-3′;

ligC, 5′-TTTGACTCCAAGACGCAGAGGATGAT-3′;

ligB, 5′-ATTTTCAAGATTTGTTCTCCAGATTT-3′;

lipl45, 5′-ATTACTTCTTGAACATCTGCTTGAT-3′.

Following reverse transcription, the following primers were added to the reactions for DNA PCR:

ligA, 5′-CTGCTACGCTTGTTGACATAGAAGTA-3′;

ligC, 5′-TAGAACCAACACGAAATGGCACAACA-3′;

ligB, 5′-ATCCGAAGTGGCATAACTCTCCTCAT-3′;

lipl45, 5′-TGAAAAGAACATTACCAGCGTTGTA-3′.

Assembly of the reaction mixtures was completed in 10× PCR Buffer, dNTPs, and *Taq* DNA polymerase, as instructed by the manufacturer (Qiagen). Polymerase chain reaction was performed in a Techne Progene thermocycler. An initial denaturation step of 95°C for 1 min was followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. A final 72°C incubation for 30 s was then performed. Polymerase chain reaction products of 500 bp for *ligA*, 479 bp for *ligC*, 440 bp for *ligB*, and 438 bp for *lipl45* were expected.

Southern blot analysis

Genomic DNA was extracted from leptospiral strains with the Blood and Cell Culture kit (Qiagen) from 500 ml of 7-day cultures. Approximately 3 µg of DNA was digested with 5–20 units of *Nsi*I (NEB) overnight in a final volume of 50 µl. DNA was then purified with phenol:chloroform: isoamyl and precipitated with 100% cold ethanol and 3 M sodium acetate pH 5.2 and washed with 70% ethanol. Purified DNA was then re-digested with 5–20 units of *Pac*I overnight in a final volume of 25 µl. The double digested DNA was separated in a 0.8% agarose gel at 20 V overnight. The gel was then incubated twice for 30 min in denaturing buffer (1.5 M NaCl, 0.5 N NaOH), and twice for 30 min in neutralization buffer (1 M Tris 1.5 M NaCl, pH 7.4). Genomic DNA was transferred onto a positively charged nylon membrane (Roche Molecular Biochemicals) according to the method described by Southern (Southern, 1975).

Probes were synthesized with the PCR Dig-labelling kit (Roche). Reactions were assembled according to the manufacturer in a final volume of 50 µl. Temperature cycles for the amplification were 94°C for 5 min, 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with a final extension time of 7 min after 35 cycles of amplification. Primers were designed against the *L. interrogans* Fiocruz L1-130 *lig* gene sequences and are as follows: for the LigB repetitive domain 4–6 LigB_395 GATTTTAAAGTTACACAAGC, LigB_573 AAACCG GACTACTTACCTTTCC, for the C terminal specific probes: LigA.2078p TTACGGCTACAGGTATTTTTACG, LigA.2691p ATTGGAAGATTTCCAAGTAACC, LigB.5071p CATAACTC TCCTCATAACA, LigB.5548p TATGTAGAGATAAGATCC, LigC.5121p TATCTACGCTGCAAATGG, LigC.5865p TTGTT GGCGATACGTCCG.

The UV cross-linked membrane was subjected to prehybridization at 42°C for 1 h in Dig Easy Hybridization solution (Roche). Before hybridization, the Dig labelled probes were boiled for 10 min and rapidly transferred to ice for 5 min. The denatured probes were mixed with hybridization solution and incubated overnight with the membrane at 42°C. Following hybridization, the membranes were washed twice for 5 min at room temperature with 2× SSC (0.3 M NaCl, 30 mM sodium citrate), 0.1% SDS. The membranes were then washed twice for 30 min at 42°C with 0.1× SSC, 0.1% SDS. Membranes were exposed for 1–3 min to Biomax ML film (Eastman Kodak) for the detection of chemiluminescent products.

Computational analysis of bacterial-Ig like repeats and structural modelling

Lig sequences from both *L. interrogans* and *L. kirschneri* were used to search the nr NCBI database (http://www.ncbi.nlm.nih.gov), and the Pfam 7.7b database (http://pfam.wustl.edu). Schematic representation of the LigB protein was drawn with DSGene (Accelrys). Detection of motifs was based on the MEME algorithm (http://meme.sdsc.edu/meme/website/ intro.html), whereas structural modelling was performed by a web based iterative search method 3-D PSSM (http://www.sbg.bio.ic.ac.uk/~3dpssm). Briefly, PSI-BLAST generated a weighted position specific scoring matrix (PSSM), which then searched a structural database for remote homologues. This method combined secondary structure and solvation potentials to recognize structural relationships.

Detergent fractionation

Leptospira kirschneri strain RM52 was fractionated by solubilization with Triton X-100. 0.5–1×10^9 leptospiral cells were washed once in 0.5 ml 1× PBS-5 mM $MgCl_2$ and extracted in 1% Triton X-100 (Calbiochem), 20 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM EDTA, and 0.5% protease inhibitor cocktail (cat. # P8849 Sigma) for 30 min at 4°C. The insoluble protoplasmic cylinder was removed by centrifugation at 10 000 *g* for 10 min The soluble phase was precipitated with acetone before electrophoresis.

Immunoelectron microscopy

Leptospira interrogans (low-passage 4) and *L. kirschneri* (low-passage 6) were grown to log phase and centrifuged at 3000 r.p.m. for 5 min. Bacteria were resuspended in 0.1% gluteraldehyde in $1 \times$ PBS and processed by High Pressure Freezing (Bal-Tec HPM010) followed by Freeze substitution (Leica EMAFS Automatic Freeze Substitution System). Samples were embedded in LRWhite and thin sections were placed on carbon coated nickel grids. Thin sections were incubated with 1:50 or 1:100 primary antibody in blocking buffer ($1\times$ PBS 0.1% Fish gelatin, and Tween 20), and washed with $1\times$ PBS-Tween 20 rinse. Secondary gold conjugated goat anti-rabbit (IgG H + l) or goat anti-mouse (IgG H + l) were used at a dilution of 1:25. Grids were rinsed in $1\times$ PBS, fixed with 0.5% gluteraldehyde, rinsed five times with distilled water, and stained in 2% Methanolic Uranyl Acetate and Lead Citrate. Sections were examined with the FEI Tec-nai 12 Electron microscope.

Immunofluorescence-labelling assays

To detect surface labelling of live, intact leptospires, cultures were centrifuged at 5000 r.p.m. for 5 min and resuspended in EMJH media. After a second washing step, 20 μl of a suspension of 2×10^9 organisms per ml were placed on poly L-lysine (Sigma) coated slides and incubated with antisera (1:50 to 1:100 dilution) to leptospiral recombinant proteins for 60 min in a humidified chamber. The slides were gently washed with EMJH media, air-dried, fixed with methanol for 10 min and then washed with $1\times$ PBS with 10% goat serum. Antibody-labelled leptospires were incubated for 30 min with goat anti-rat Ig-FITC conjugated antibodies (Sigma), diluted 1:200. Air-dried slides were washed and mounted with a $1\times$ PBS-glycerol solution with *p*-phenylene-diamine (Sigma). Labelled organisms were visualized by fluorescence microscopy.

Accession numbers

The nucleotide sequence of *L. kirschneri* RM52 *ligA* and *ligB* was deposited in GenBank under the accession number AY190126. The *ligC* sequence was deposited under accession number AY190127. The nucleotide sequence of *L. interrogans* Fiocruz L1-130 *ligA* was deposited under accession number AY221109.

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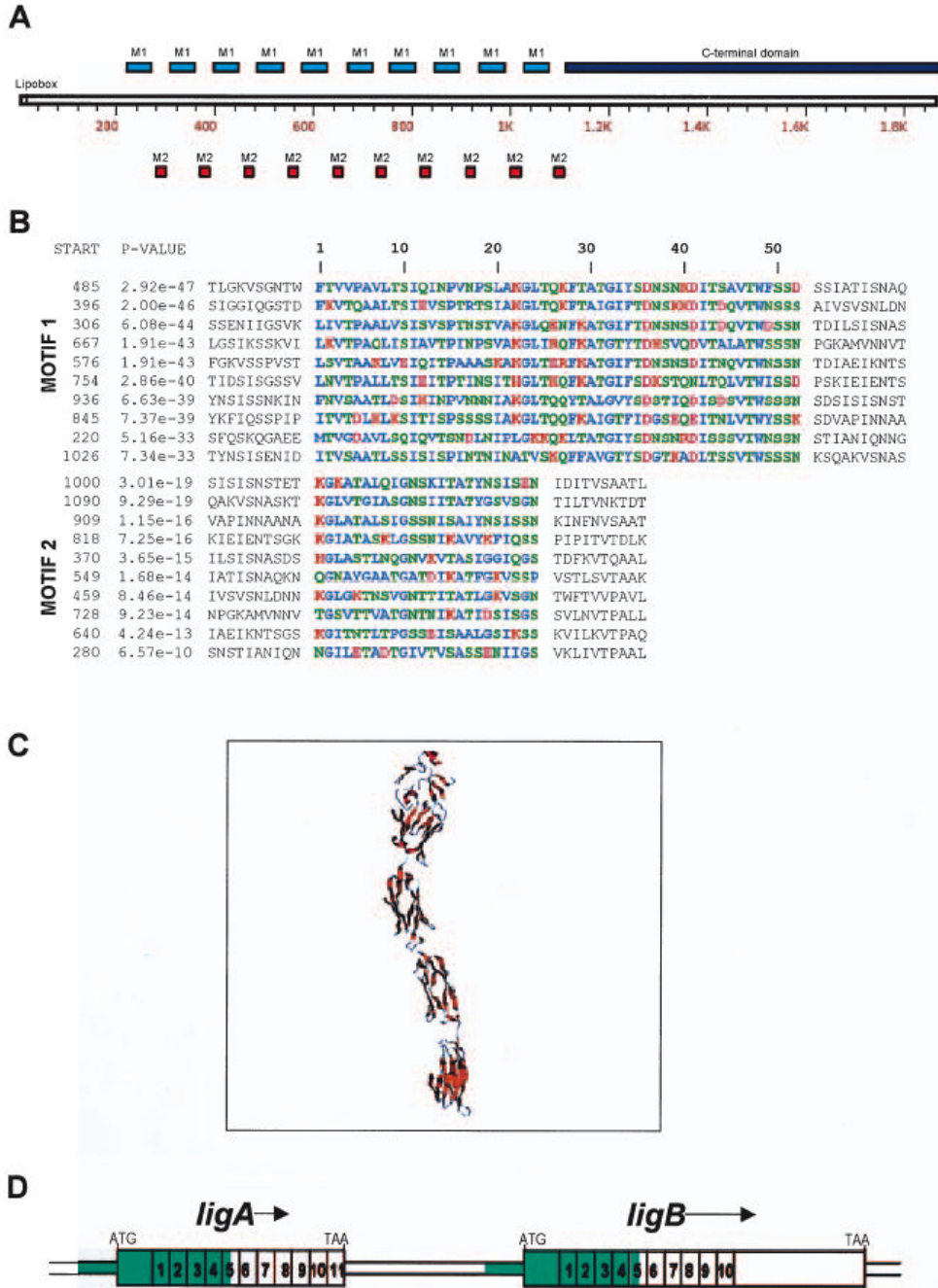
Fig. 1.
A. Schematic representation of LigB. Motif 1 (blue box) and Motif 2 (red box) comprise the 10 Big2 repetitive domains from residue 220 through 1109, whereas the C-terminus (dark blue box) is located between 1111 and 1890.

B. MEME Motif Discovery analysis of the Big2 repetitive domains of LigB. *L. interrogans* Lig proteins contain ~90mer repeats composed of two motifs of length 52 residues (M1) and 24 residues (M2), respectively. Residues are coloured based on their chemical properties: hydrophobic, basic, acidic and polar uncharged. The sites are listed in order of decreasing statistical significance (*P*-value). The *P*-values and start sites of the motifs are indicated. The *P*-value of a site is computed from the match score of the site with the position specific scoring

matrix for the motif and represents the probability of a random string (generated from the background letter frequencies) having the same match score or higher.

C. Comparative modelling of *L. interrogans* LigB repetitive domains (residues 493–917).

D. Genetic structure of the *ligAB* locus. The 11 and 10 Big2 domains encoded by *ligA* and *ligB*, respectively, are numbered. Two additional N-terminal Big2 domains with poor *E*-values ($>10^{-10}$) are not shown. The >2 kb segment of DNA sequence identity between the 5′ portions of *ligA* and *ligB* is indicated in green.
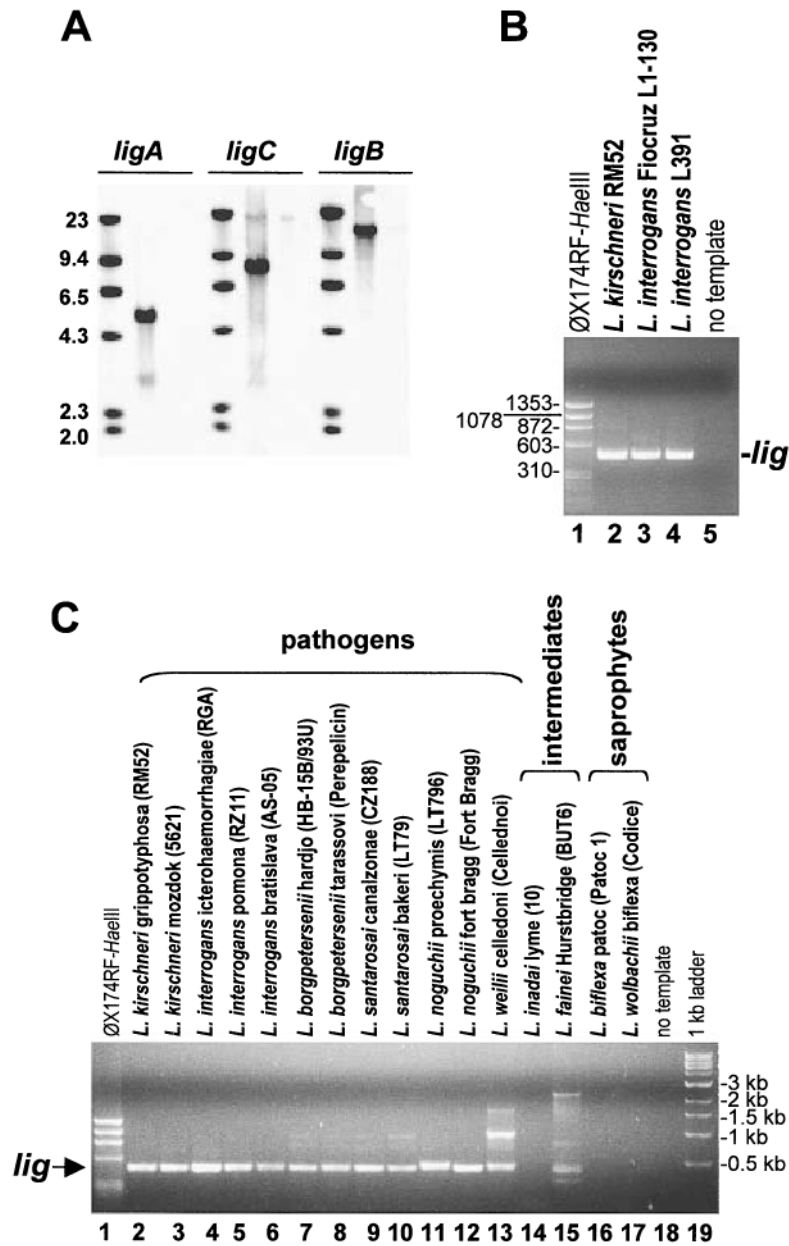
Fig. 2.
Detection of *lig* sequences in *Leptospira* species.

A. Southern blot analysis. Genomic DNA from *L. interrogans* Fiocruz L1-130 (*middle lane*) and *L. biflexa* Patoc 1 (*right lane*) were digested with *Nsi*I and *Pac*I and probed in Southern blots with *ligA*, *ligB* and *ligC* specific probes.

B and C. PCR analysis. Leptospiral DNA was subjected to PCR analysis by degenerate primers designed to anneal to all *lig* genes in *L. kirschneri* RM52 and *L. interrogans* Fiocruz L1-130. The expected size of the PCR product is −0.5 kb.
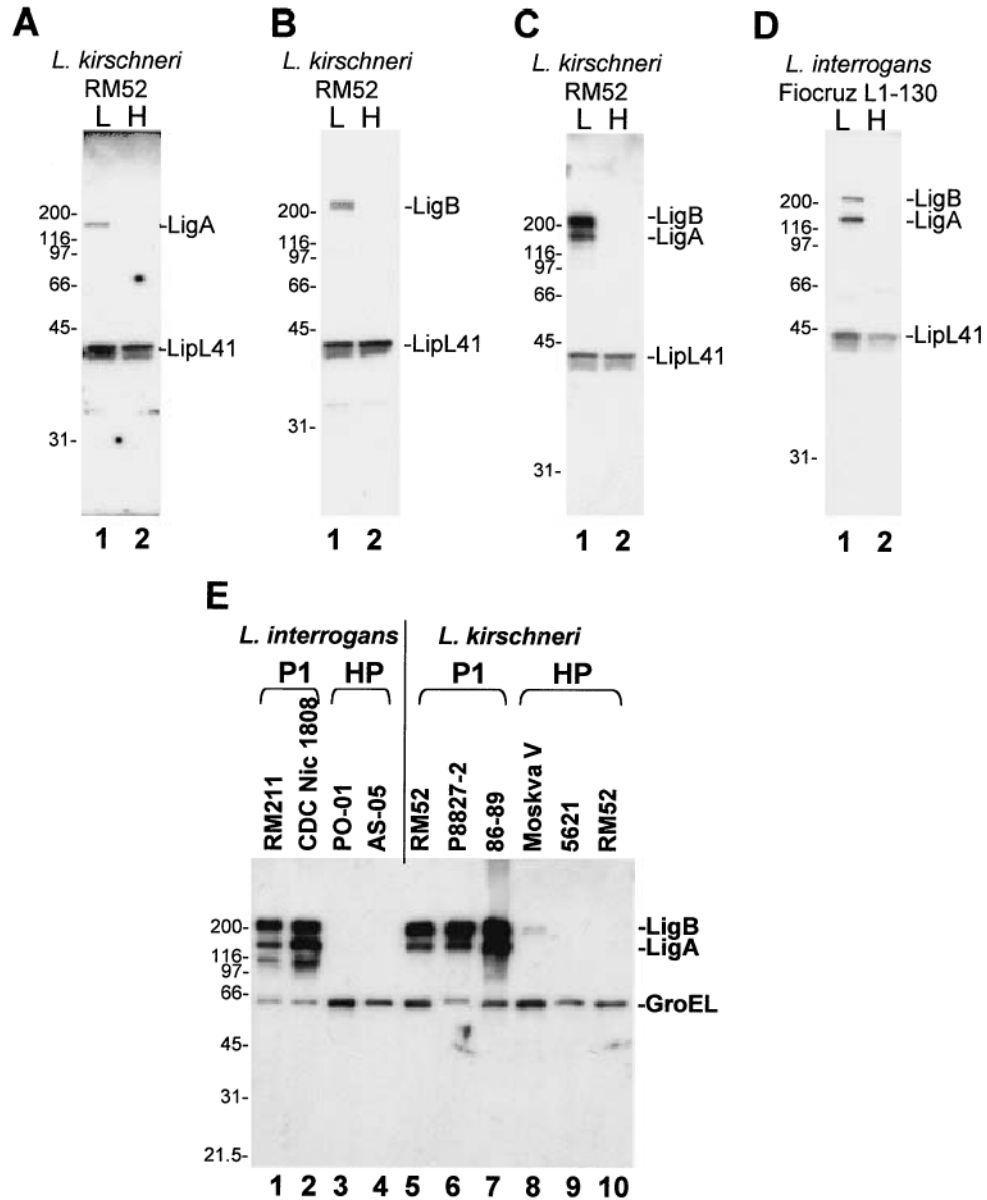
Fig. 3.
Western blot analysis of LigA and LigB expression in low- and high-passage *Leptospira*.
A and B. Immunoblots of low (9 passages) and high-passage (>200 passages) isolates of *L. kirschneri* RM52 were probed with LipL41 antiserum (1:10 000), and either LigA antiserum (A, 1:2000) or LigB antiserum (B, 1:2000). L, low passage; H, high passage.
C and D. Immunoblots of *L. kirschneri* RM52 or *L. interrogans* Fiocruz L1-130 (passage 25 and passage 69) were probed with LipL41 (1:10 000) and LigA/B repeat antisera (1:5000).
E. An immunoblot of *L. interrogans* and *L. kirschneri* isolates was probed with LigA/B repeat antisera (1:5000) and GroEL antisera (1:30 000). Strain names are shown above each lane. P1, passage 1; HP, high passage. The relative mobility (kDa) of molecular mass standards (Bio-Rad) is shown on the left of each panel.
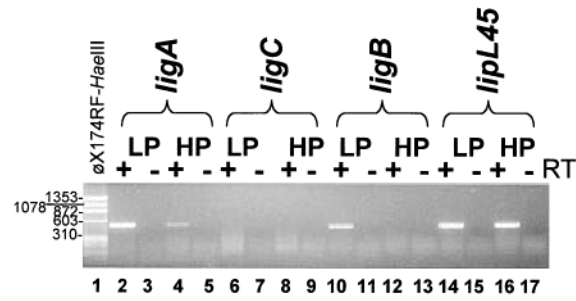
Fig. 4.
RT-PCR analysis of *lig* transcripts in low- and high-passage *L. kirschneri* RM52. Total RNA from low- (passage 4) and high-passage *L. kirschneri* RM52 was analysed by RT-PCR with primers specific for each *lig* gene. +, reverse transcriptase present in the reaction; −, reverse transcriptase omitted.
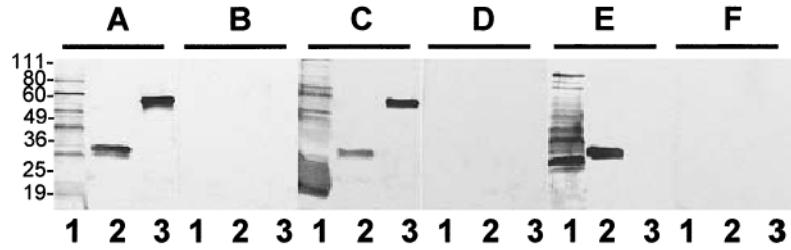
Fig. 5.
Reactivity of sera from leptospirosis patients and *Rattus norvegicus* reservoirs with recombinant LigB. Membranes were prepared from SDS-PAGE of whole *L. interrogans* extract (10^8 organisms per lane, lanes 1); recombinant *L. interrogans* lipoprotein, LipL32 (1 μg per lane, lanes 2) and recombinant protein containing repeat domains 1–5 of *L. interrogans* LigB (1 μg per lane, lanes 3). Membranes were probed with sera from leptospirosis patients (A); healthy individuals (B); captured *R. norvegicus* from which *L. interrogans* was isolated (C); captured culture-negative *R. norvegicus* (D); laboratory rats immunized with whole-cell preparations of *in vitro* cultured low-passage *L. interrogans* (E); and laboratory rats obtained prior to immunization (F). The relative mobility (kDa) of molecular mass standards (Invitrogen) is shown on the left.
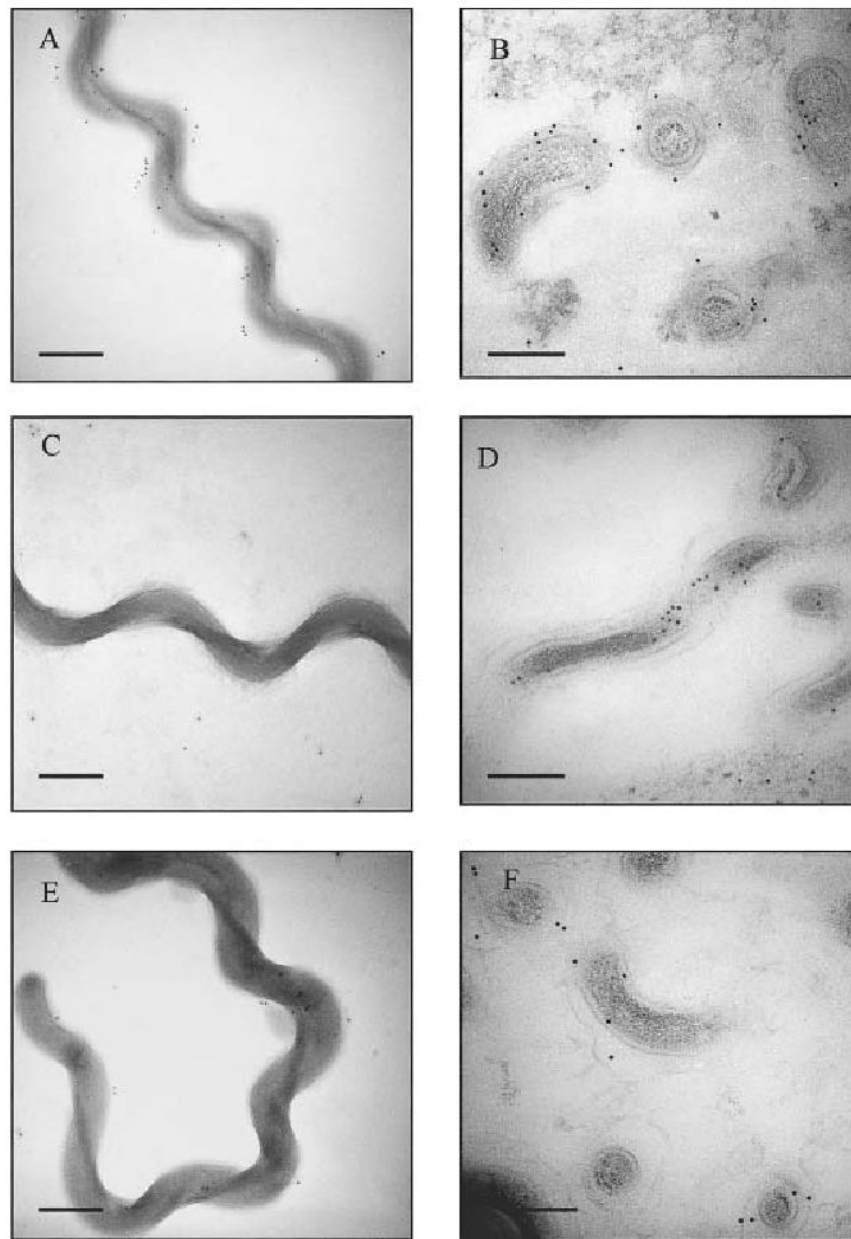
Fig. 6.
Immunoelectron microscopy of whole-cell (A, C and E) and thin-section (B, D, F) preparations of *L. kirschneri* strain RM52. Preparations were incubated with anti-LPS (A, B), anti-GroEL (C, D), and anti-LigB antibody (E, F), followed by anti-rabbit secondary antibody conjugated to 10 nm gold particles. Bars represent 100 nm.
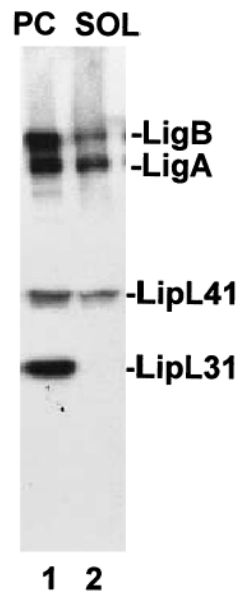
Fig. 7.
Triton X-100 extraction of Lig proteins from *L. kirschneri* RM52. Leptospires were incubated for 2 h in MEM (ATCC) at 37°C. Bacteria were then washed once in $1\times$ PBS with 5 mM $MgCl_2$ and extracted with 1% Triton X-100. The insoluble protoplasmic cylinder (PC) was pelleted by centrifugation. The protoplasmic cylinder and the Triton-soluble (SOL) fractions were probed in a Western blot using LigA/B repeat (1:5000), LipL41 (1:10 000), and LipL31 (1:8000) antisera. LipL31 is a marker for the leptospiral inner membrane (Haake and Matsunaga, 2002).

Table 1

Plasmids used to express recombinant Lig proteins in *E. coli.*

Protein	Source	Tag	Amino acid co-ordinates	Plasmid name
LigA	RM52	His6	936–1224	p204orf1B
LigA	RM52	His6	342–1224	p204orf1
LigB	RM52	MBP	20–581	pROY1
LigB	RM52	MBP	579–1126	pROY2
LigB	RM52	MBP	296–847	pROY3
LigB	RM52	MBP	1113–1886	pJIM520
LigB	RM52	His6	1113–1886	pJIM518
LigC	RM52	His6	621–1060	p249orf1
LigB	L1-130	His6	131–649	pAE-ligBrep

Table 2

Nucleotide sequence of inverse PCR primers.

Gene	Primer sequence 5′ to 3′	Co-ordinates[a]	Restriction enzyme[b]
ligA 5′ end	CAGATATTCTTACCGTTTCCAATACA	1883–1908	*Nsi*I
	ATCCGATCAGATTTTTGCATCAAGTC	−209–(−234)	
ligA 3′ end	CCGCTTTCTGTAGGTTCCTCTAAAAT	3562–3587	*Hinc*II
	CGGTAGCGGTCAGTTGTAGTGTAAGA	3175–3150	
ligB 3′ end	ATATCCGGAATGAATTTTGGTGTAAA	5569–5594	*Eco*RI
	ATTTTCAAGATTTGTTCTCCAGATTT	5487–5462	
LigC 5′ end	TCCTGCAAATCCAAGCGTAGCCAATG	1054–1079	*Xho*I
	GTAAGAACTGCGGAAGTGACGTTTACTA	1038–1011	
LigC 3′ end	ACCGGACTCTTGTTCTAACTGGGTGG	5209–5234	*Bgl*II
	ATCCGATTCCGTCGTTGCTTGACTTT	4868–4843	

[a]Relative to A in ATG start codon.

[b]Leptospiral DNA digested with restriction enzyme before treatment with T4 ligase and PCR.