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Differential *In Vivo* Gene Expression of Major *Leptospira* Proteins in Resistant or Susceptible Animal Models

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Transcripts of *Leptospira* 16S rRNA, FlaB, LigB, LipL21, LipL32, LipL36, LipL41, and OmpL37 were quantified in the blood of susceptible (hamsters) and resistant (mice) animal models of leptospirosis. We first validated adequate reference genes and then evaluated expression patterns *in vivo* compared to *in vitro* cultures. LipL32 expression was downregulated *in vivo* and differentially regulated in resistant and susceptible animals. FlaB expression was also repressed in mice but not in hamsters. In contrast, LigB and OmpL37 were upregulated *in vivo*. Thus, we demonstrated that a virulent strain of *Leptospira* differentially adapts its gene expression in the blood of infected animals.

Leptospirosis is a neglected tropical zoonosis caused by pathogenic spirochetes of the genus *Leptospira*, with a high incidence in developing countries (11). Infection occurs by direct contact with reservoir animals or exposure to an environment contaminated with their urine (10). The survival of leptospires upon transition into host conditions and leptospirosis pathogenesis include the modulation of bacterial gene expression (1). Microarray studies evidenced that shifts from environmental to mammalian host-simulating conditions (temperature or osmolarity) lead to major transcriptional modifications in *L. interrogans* (12, 14), especially for genes involved in chemotaxis, motility, or signal transduction. Exposure to guinea pig serum or mammalian macrophages also influenced genome-wide *Leptospira* gene expression (17, 21). It was notable that expression of several outer membrane proteins (OMPs) was downregulated. These results emphasized the transcriptomic regulation of pathogenic *Leptospira* upon a shift to host-simulating physiological conditions. However, the regulation of the leptospiral transcriptome has been evaluated *in vitro* only with host-simulating parameters that poorly reflected real *in vivo* conditions. Thus, its regulation in infected animals remains to be analyzed.

In a previous study (13), we found significant differences in the expression of major immune mediators between a susceptible hamster model and a resistant mouse model of leptospirosis. These animals presented distinct profiles of dissemination of bacteria in the blood. Indeed, the early clearance of *Leptospira* was evidenced in mice whereas a continuous and dramatic increase of the leptospiremia was observed in hamsters (13). Because the onset of infectious diseases involves a complex interplay between the host and the pathogen, we here evaluated the *in vivo* gene expression of several OMPs in the blood of these contrasting animal models infected with the virulent *L. interrogans* serovar *icterohaemorrhagiae* strain Verdun. Using the same blood specimens collected at early time points (13), we quantified the transcripts of OMPs and extracellular matrix (ECM)-interacting proteins but also the 16S rRNA and *flaB* genes by the use of reverse transcription-quantitative PCR (RT-qPCR).

Outbred OF1 mice and golden Syrian hamsters initially obtained from Charles River Laboratories (Charles Rivers Wiga GmbH, Sulzfeld, Germany) were infected with *L. interrogans* serovar *icterohaemorrhagiae* strain Verdun and studied as previously described (13). Animal experiments were conducted according to the appropriate ethical guidelines as mentioned before (13), following the guidelines of the Animal Care and Use Committees of the Institut Pasteur and European Recommendation 2007/526/EC. Briefly, animals were injected intraperitoneally with 2×10^8 bacteria. Blood was collected by cardiac puncture within the first 24 h after infection (3, 6, and 24 h) and conserved in PAXgene blood RNA tubes (PreAnalytix; Qiagen, Australia). Total RNA was extracted using a PAXgene blood RNA system from PreAnalytiX (Qiagen, Australia). *Leptospira* from *in vitro* cultures (4×10^8 bacteria) were also processed after overnight incubation at 30°C or 37°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. Bacterial total RNA was extracted using a High Pure RNA Isolation kit (Roche Applied Science, New Zealand) following the manufacturer's recommendations. RNA samples were treated with DNase (Turbo DNA-Free kit; Ambion, Applied Biosystems). Then, 1 μg of total RNA was reverse transcribed using a Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, New Zealand) on a GeneAmp PCR system 9700 instrument (Applied Biosystems) with the following program: 10 min at 25°C; 30 min at 55°C; and 5 min at 85°C.

After cDNA synthesis, qPCR assays were performed using primers (Table 1) purchased from Eurogentec (Seraing, Belgium) specific for the gene coding for the 16S rRNA, *flaB*, the gene coding for an Ig/cadherin-like protein (locus LIC10501), *ligB*, *lipL21*, *lipL32*, *lipL36*, *lipL41*, *lp95*, *lsa24*, *ompL37*, and the gene coding for a thermolysin-like protein (locus LIC13321). Amplifications were carried out on a LightCycler 480 II instrument using LightCycler 480 software (v. 1.5.0) and a LightCycler 480 SYBR green I master kit (Roche Applied Science, New Zealand) according to the provided instructions. The amplification programs are detailed in Table 1. Results were validated only when threshold cycle (C_T) values were under the limit value of 40 cycles and with an acceptable reproducibility between qPCR replicates (less than 5% of variation). Gene expression studies using RT-qPCR require the

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TABLE 1 Details and sequence of primers used for qPCR assays

Gene name	Locus tag[a]	Sequence (5′–3′)[b]	Efficiency[c]	Size (bp)[d]	T_m[e]	Prog.[f]
16S rRNA[g]		(F)GGCGGCGCGTCTTAAACATG (R)CTTAACTGCTGCCTCCCGTA	1.932 ± 0.010	309	86.2	A
flaB[h]		(F)AGCGAGACAACTTCTTCCGCCATA (R)ATGAAGCAGAGAGCGGATATGGGA	1.948 ± 0.027	139	80.8	A
Ig/Cad-like	LIC10501	(F)TACGGATCGTATGTTGGG (R)GTAACGGTCGGTGCATTT	1.907 ± 0.009	154	85.6	B
ligB	LIC10464	(F)ATCCGAAGTGGCATAACTCTCC (R)ATCGCCGGTTAGAGTCG	1.886 ± 0.014	263	81.3	A
lipL21	LIC10011	(F)TGGTGAAGCTACTGCATCT (R)CACCTGGAAATTTTGCG	1.903 ± 0.051	164	80.0	B
lipL32	LIC11352	(F)CGGACGGTTTAGTCGATG (R)GCATAATCGCCGACATTC	1.850 ± 0.009	228	84.0	B
lipL36	LIC13060	(F)GGTTCAAATTGCGCTGTAG (R)GCATAAACGGTTTTTCCGAG	1.806 ± 0.017	188	80.8	A
lipL41[h]		(F)TTTACCAGTTGCCATAGAAGCGGC (R)GGAAATCTGATTGGAGCCGAAGCA	1.982 ± 0.035	140	80.9	A
lp95	LIC12690	(F)GCCTTGTTTAGGTTGTCC (R)TCAGTCTTGCACCTTCG	1.883 ± 0.044	209	81.6	A
lsa24	LIC12906	(F)CTCCCGTCGTTACTTCTTG (R)TTGGACCGTCGACAATCA	1.921 ± 0.016	177	81.0	A
ompL37	LIC12263	(F)GGCGACTCACGAACAATA (R)CGGTTTATCCTCGGTTGG	1.865 ± 0.011	283	78.2	A
Thermolysin-like	LIC13321	(F)CCTAAGCTATCCGAGTCA (R)TTGGTGGATCTATCGAGGC	1.832 ± 0.020	238	77.5	A

[a] Locus tag of corresponding gene sequence from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 referenced in GenBank (NCBI) under accession number NC_005823.1 and used for primer design.
[b] (F) and (R), forward and reverse primer sequences, respectively.
[c] Efficiency for PCR was calculated as previously described by Matsui et al. (13).
[d] PCR product size (in base pairs).
[e] T_m, PCR product melting temperature (°C).
[f] qPCR amplification program (Prog.). A, 50 cycles of 5 s at 95°C, 5 s at 62°C, and 8 s at 72°C; B, 50 cycles of 5 s at 95°C, 5 s at 60°C, and 8 s at 72°C.
[g] As mentioned by Matsui et al. (13).
[h] As described by Carrillo-Casas et al. (3).

selection of internal controls for normalization, and potential reference genes (also known as housekeeping genes or HKG) were evaluated using the commonly employed software, geNorm and NormFinder (2, 20). We analyzed the expression stability of the genes (Table 2) in 33 samples (3 replicate cultures *in vitro* at both 30°C and 37°C; 11 and 16 blood samples of hamsters and mice, respectively). After the selection of the best reference genes, the normalization of gene expression was processed using qbase^{PLUS} software (Biogazelle, Belgium). The relative normalized expression ratio of target gene was then calculated as the ratio of the expression level *in vivo* to the expression level *in vitro* at 30°C, used as a calibrator.

TABLE 2 Expression stability values of *Leptospira* genes determined using geNorm and NormFinder software[a]

Gene name[b]	geNorm		NormFinder	
	M value	Rank position	NF value	Rank position
*lipL41**	1.382	1	0.242	1
*lipL36**	1.434	2	0.717	3
*lipL21**	1.513	3	0.617	2
flaB	1.651	4	0.830	4
16S rRNA	1.742	5	1.203	5
lipL32	1.811	6	1.280	6
ligB	2.230	7	1.753	7
ompL37	2.569	8	1.941	8

[a] Average expression stability values were calculated using geNorm (M value) and NormFinder (NF value) software after RT-qPCR assays were performed on 33 samples as described in the text. The rank positions of the expression stability values are given for each gene according to the software used.
[b] Gene names are given as used in the literature. See Table 1 for details. The most stable genes (*) were selected for the normalization step prior to relative quantification.

To our knowledge, only one paper has previously analyzed the stability of potential HKG in *Leptospira* (3) and evaluated the effect of the culture temperature on the expression of three genes frequently employed for normalization: *lipL41*, coding for an abundantly expressed OMP; *flaB*, coding for the flagellin subunit B; and the highly conserved 16S rRNA gene that was found to be the most stable gene for normalization in this study. In our *in vivo* study, however, the 16S rRNA gene was not a suitable HKG, as evidenced by its stability rank position (5th; Table 2) and high geNorm M and NormFinder stability values (1.742 and 1.203, respectively). The two algorithms evidenced *lipL21*, *lipL36*, and *lipL41* as the most stable of the genes studied under our experimental conditions. Previous studies evidenced that regulation of *lipL36* and *lipL41* *in vitro* was dependent on culture conditions (8, 21). Our contrasting results highlight the importance of evaluating expression stability under the experimental conditions of each study. As the minimal use of the three most stable genes is recommended for normalization (20), *lipL21*, *lipL36*, and *lipL41* were selected as the best set of internal controls to normalize gene expression using qbase^{PLUS} software (Biogazelle, Belgium). How-

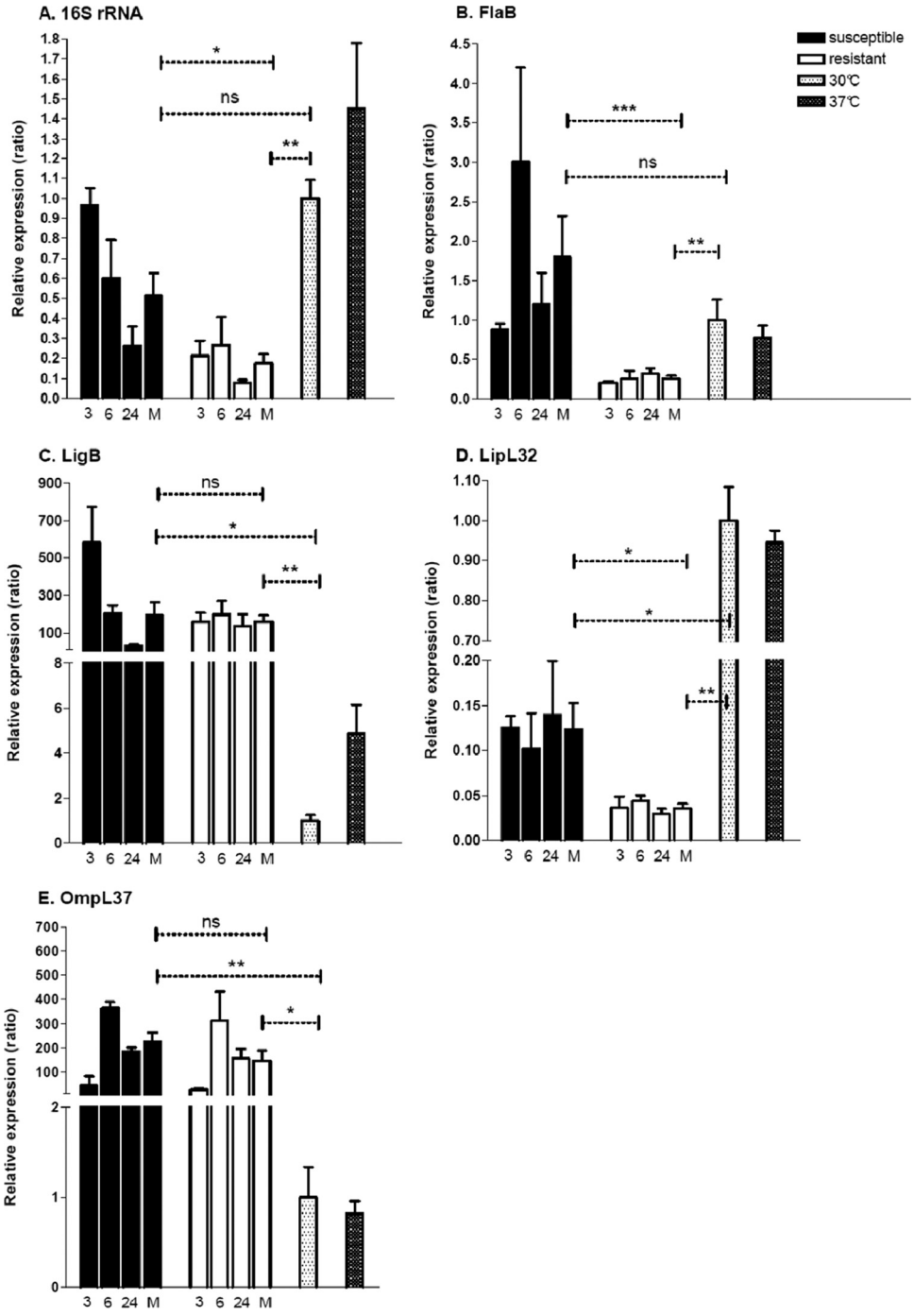

FIG 1 *In vivo* regulation of *Leptospira* gene expression. Relative expression of the target genes was studied as previously described by Matsui et al. (13) by quantifying transcripts from the blood of hamsters (susceptible; $n = 11$ animals) or mice (resistant; $n = 16$ animals) infected with *L. interrogans* serovar *icterohaemorrhagiae* strain Verdun. The *in vivo* results represent the expression levels in samples collected at 3, 6, and 24 h postinfection and the combination of all time points (M) as indicated. Transcripts from *in vitro* cultures were obtained after incubation at 30°C (30; $n = 3$) or 37°C (37; $n = 3$). Values represent means ± standard errors of the means (SEMs). Significant differences in gene expression between conditions (M and 30°C) were evaluated using a Mann-Whitney's nonparametric *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant.

ever, our set of internal-control genes has stability values that are higher than optimal and thus, selection and validation of HKG for *in vivo* expression quantification could still be improved.

Our results revealed that 16S rRNA gene expression is not stable but rather is downregulated in mouse blood (expression ratio of 0.17) compared to *in vitro* cultures (Fig. 1A). We previously quantified the 16S rRNA gene transcript level to assess the bacterial burden in tissues from infected animals and showed that this level presented an upward kinetic (13). However, because we were studying a bacterial burden, the normalization was based on host

HKG. Here we aimed to evaluate the transcriptomic function in the pathogen by quantifying the regulation of the 16S rRNA gene expression by calculating the ratio to that of formerly validated bacterial HKG. The decrease of the 16S rRNA gene expression *in vivo* is concordant with previous results showing that this gene, as with those encoding other ribosomal proteins, was downregulated in *Leptospira* cocultured with mammalian macrophages (21). In contrast, the mean expression ratio calculated during the first day postinfection was not different in the hamster blood compared to cultures. However, the expression ratio of the 16S rRNA gene at 24 h presented a significant downregulation compared to cultures ($P = 0.024$; data not shown). The decrease in 16S rRNA gene expression in mouse and hamster blood at 24 h cannot be explained but might reflect different phenomena in these two models: it can be speculated that it reflects a loss of fitness of the leptospires paralleling their rapid clearance in mouse blood (13) and, rather, a physiological and transcriptional shift in hamster blood, as hypothesized in a previous guinea pig serum study (17).

Colonization of host tissues might involve the motility of *Leptospira* bacteria that possess periplasmic flagellar filaments (5). Interestingly, we found that the expression of *flaB*, a major flagellin subunit, was significantly downregulated in mouse but not in hamster blood compared to *in vitro* culture (Fig. 1B). Although *flaB* expression is not regulated *in vitro* upon temperature changes (Fig. 1B) (16), it was downregulated upon interaction with murine but not human macrophages (21). Moreover, the amounts of flagellin decreased in leptospires excreted in urine from resistant reservoir hosts, suggesting a motility loss after tissue colonization (16). The differential regulation of *flaB* expression between mouse and hamster may reflect a disparity in invasiveness, partly accounting for the fatal issue in hamsters (13).

Direct adhesion to host cells or ECM proteins might also contribute to the dissemination of leptospires through the infected organism. Unfortunately, the transcripts of *lsa24*, *lp95*, LIC13321, and LIC10501 either were not detected or were inconsistently detected, most probably reflecting a very low expression level. The leptospiral immunoglobulin-like protein LigB is known to attach to host cells by binding several ECM proteins (6). Its expression is highly upregulated in the blood of infected animals of both species, with relative expression ratios higher than 150 compared to the results seen with *in vitro*-cultured bacteria (Fig. 1C). However, no significant differences in *ligB* expression between our animal models were observed. An upregulation of *ligB* was also observed *in vitro* upon temperature or osmolarity changes from environmental to host conditions (6, 12).

LipL32 is a major leptospiral OMP highly conserved among pathogenic *Leptospira* species (7) and was defined as an ECM-interacting protein (4). We show that *lipL32* expression was dramatically downregulated *in vivo* compared to culture conditions (Fig. 1D), correlating with microarray results produced upon interaction with mammalian macrophages (21). An interesting finding was differential gene expression of *lipL32* between our two animal models, with an expression ratio of 0.03 in mouse compared to 0.12 in hamster. It was evidenced that LipL32 was not required for the development of acute or nonlethal infection using a mutant *L. interrogans* strain (15). Because LipL32 also increases the permeability and apoptosis of human umbilical vein endothelial cells (19), it can be hypothesized that LipL32 is required for invasion only in the early stages of the infection through endothelial cells but is not required and therefore is repressed once *Leptospira* bacteria disseminate into the systemic circulation. Moreover, LipL32 was shown to interact with Toll-like receptor 2 (TLR-2), leading to the induced expression of inflammatory chemokine interleukin-8 (IL-8) (9). Thus, a decrease in *lipL32* expression might reflect a dissimulation strategy to avoid recognition by the host immune system.

OmpL37 was identified as another ECM-interacting OMP, exhibiting strong and dose-dependent binding activity to elastin (18). Quantification of the expression levels revealed that *ompL37* is highly upregulated *in vivo*, with expression ratios of up to 225.2 and 147.5 compared to *in vitro* results for hamster and mouse samples, respectively (Fig. 1E). However, it was not regulated in *Leptospira* cocultured with mammalian macrophages (21), and we found that it is also not influenced by culture temperature (Fig. 1E). As OmpL37 has a high affinity for skin and vascular elastin, it might play a role in the early invasion process by mediating attachment to skin and vascular tissues (18).

Surface-associated proteins like OMPs and ECM-interacting proteins, as a consequence of their location, are likely to be important in host-pathogen interactions: hence their potential to promote several activities, including motility, adhesion, or an effective immune response from the host. Our results highlight the need for global *in vivo* evaluation of *Leptospira* gene expression in both susceptible and resistant models to better understand leptospirosis pathophysiology.

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