

# The EbpA-RpoN regulatory pathway of the pathogen Leptospira interrogans is essential for survival in the environment.

Wei-Lin Hu, Christopher J Pappas, Jun-Jie Zhang, You-Yun Yang, Jie Yan, Mathieu Picardeau, X Frank Yang

▶ **To cite this version:**

Wei-Lin Hu, Christopher J Pappas, Jun-Jie Zhang, You-Yun Yang, Jie Yan, et al.. The EbpA-RpoN regulatory pathway of the pathogen Leptospira interrogans is essential for survival in the environment.. Applied and Environmental Microbiology, 2016, Epub. 10.1128/AEM.02377-16 . pasteur-01423062

**HAL Id: pasteur-01423062**

**https://pasteur.hal.science/pasteur-01423062**

Submitted on 28 Dec 2016

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

# The EbpA-RpoN regulatory pathway of the pathogen *Leptospira interrogans* is essential for survival in the environment

Wei-Lin Hu[1, 2*], Christopher J. Pappas[3, 4*], Jun-Jie Zhang[2*], You-Yun Yang[2], Jie Yan[1§], Mathieu Picardeau[3§], X. Frank Yang[2§]

*Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang, P.R. China[1]; Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana, USA[2]; Institut Pasteur, Unité Biologie des Spirochètes, Paris, France[3]; Manhattanville College, Department of Biology, Purchase, New York, USA[4]*

* WLH, CJP, and JJZ contributed equally to this work.

§ Address correspondence to:

Jie Yan, med_bp@zju.edu.cn

Mathieu Picardeau, mpicard@pasteur.fr

X. Frank Yang, xfyang@iupui.edu

Running title: EbpA-RpoN pathway in *Leptospira interrogans*

Key words: *Leptospira*, RpoN

## ABSTRACT

*Leptospira interrogans* is the agent of leptospirosis, a re-emerging zoonotic disease. Leptospirosis is transmitted to humans through environmental surface waters contaminated by the urine of mammals that are chronically infected by pathogenic strains that are able to survive in water for long periods. Little is known about the regulatory pathways underlying environmental sensing and host adaptation of *L. interrogans* during its enzootic cycle. This study identifies the EbpA-RpoN regulatory pathway in *L. interrogans*. In this pathway, EbpA, a $\sigma^{54}$ activator and putative prokaryotic enhancer-binding protein (EBP), and the alternative sigma factor RpoN ($\sigma^{54}$) control expression of at least three genes – encoding AmtB (an ammonium transport protein) and two proteins of unknown function. Electrophoresis mobility shift assay (EMSA) demonstrated that recombinant RpoN and EbpA bind to the promoter region and upstream of these three identified genes, respectively. Genetic disruption of *ebpA* in *L. interrogans* serovar Manilae virtually abolished expression of the three genes, including *amtB* in two independent *ebpA* mutants. Complementation of the *ebpA* mutant restored expression of these genes. Intra-peritoneal inoculation of gerbils with the *ebpA* mutant did not affect mortality. However, the *ebpA* mutant had decreased cell length *in vitro* and had a significantly lowered cell density at stationary phase when grown with L-alanine as the sole nitrogen source. Furthermore, the *ebpA* mutant has dramatically reduced long-term survival ability in water. Together, these studies identify a regulatory pathway, the EbpA-RpoN pathway, that plays an important role in the zoonotic cycle of *L. interrogans*.

## IMPORTANCE

Leptospirosis is a re-emerging disease with global importance. However, our understanding of gene regulation of the spirochetal pathogen *L. interrogans* is still in its infancy, largely due to the lack of robust tools for genetic manipulation of this spirochete. Little is known about how the pathogen achieves its long-term survival in the aquatic environment. By utilizing bioinformatic, genetic, and biochemical methods, we discovered a regulatory pathway in *L. interrogans*, the EbpA-RpoN pathway, and demonstrated that this pathway plays an important role in environmental survival of this pathogen.

## INTRODUCTION

Leptospires are a group of spirochetes that include both saprophytic and pathogenic species within the genus *Leptospira* (1). Pathogenic species including *L. interrogans,* are the causative agents of leptospirosis, the most widespread zoonotic infectious disease in the world. Saprophytic species including *L. biflexa*, are found primarily in aquatic and terrestrial environments and are considered non-pathogenic. Pathogenic leptospires also can survive in the terrestrial environment for a prolonged period, and humans are typically infected by direct or indirect contact with soil or water that is contaminated with the urine of infected animals.

*L. interrogans* must adapt to both aquatic and mammalian host conditions in order to be successfully maintained in its zoonotic cycle. Some progress has been made on how *L. interrogans* alters its gene expression to cope with environmental change. Whole-genome microarrays and high-throughput RNA sequencing analyses revealed global changes in transcript levels of *L. interrogans* in response to temperature (2), osmolality (3), iron depletion (4), serum exposure (5), interaction with phagocytic cells (6), and cultivation in a host-adapted model (the dialysis membrane chambers model) (7). Understanding how *L. interrogans* senses environmental and host signals and regulates its gene expression has just begun to be explored.

Little is known about the regulation of gene expression in *L. interrogans*. It was shown that the PerR homolog in *L. interrogans* acted as a regulator of the oxidative stress response (3, 4, 8). The KdpE sensor was shown to be an activator of the KdpABC potassium

transporter (9)., The LexA repressor bound to the promoter of *recA* is involved in DNA repair (10). HemR regulates the heme biosynthetic pathway (11, 12). Despite these advances, the regulatory pathways underlying environmental sensing and host adaptation of *L. interrogans* remain poorly understood, largely due to the lack of a robust system for genetic manipulation of *Leptospira*.

One common mechanism of regulating bacterial gene expression is the use of an alternative sigma factors (σ). In addition to the housekeeping sigma factor $\sigma^{70}$ (RpoD), *Leptospira* species have several alternative sigma factors, including $\sigma^{54}$ (RpoN), $\sigma^{F}$ (FliA,), $\sigma^{B}$, and 5 to 11 extracytoplasmic function (ECF) sigma factors ($\sigma^{E}$) (13-15). Among these alternative σ factors, $\sigma^{54}$ is phylogenetically different from the other σ factors; all other alternative σ factors belong to the $\sigma^{70}$ family. $\sigma^{54}$ recognizes a unique -24/-12 promoter sequence (instead of the -35/-10 promoter sequence for $\sigma^{70}$). In addition, transcriptional activation from a $\sigma^{54}$-type promoter always requires a bacterial enhancer binding protein (EBP) (16, 17). A typical EBP consists of an N-terminal allosteric effector binding domain for sensing the signal, an AAA+ NTPase domain for $\sigma^{54}$ activation, and a C terminal helix-turn-helix domain for DNA binding. The EBP binds to the enhancer region, where it curves toward and interacts with the $\sigma^{54}$ holoenzyme. Upon sensing a signal, the EBP is activated, which in turn activates $\sigma^{54}$ by AAA+-type ATPase activity. This activation energy promotes the closed complex of the holoenzyme and the formation of an open complex at the promoter, resulting in the transcriptional activation of $\sigma^{54}$-dependent genes.

92 $\sigma^{54}$ has been shown to modulate many cellular processes including nitrogen
93 assimilation, motility, virulence, and biofilm formation in bacteria (16-18). Previous
94 experiments have ascertained that *rpoN*, while typically associated with pathogenic
95 adaptation to host environment, has also been shown to play a role in ecological adaptation
96 and survival. Therefore, we attempted to characterize the role of an *rpoN* regulon in
97 pathogenic *Leptospira* (18-20). In this study, using a combination of bioinformatic,
98 biochemical, and genetic approaches, we report identification of the EbpA-RpoN regulatory
99 pathway in *L. interrogans*. This pathway constitutes a $\sigma^{54}$ activator (LMANv2_37011,
100 hereafter referred to as EbpA), $\sigma^{54}$ (LMANv2_590036, RpoN), and at least three downstream
101 targets. We found that this pathway is not essential for mammalian infection but plays an
102 important role in *L. interrogans* adaptation to diverse environmental conditions during the
103 enzootic cycle.
104

## MATERIALS AND METHODS

**Strains and culture conditions.** The pathogen *L. interrogans* serovar Manilae strain L495 and biologically distinct transposon mutant *LMANv2_37011*$^{-}$/ebpA$^{-}$ strains – ebpA1$^{-}$ (Man250) and ebpA2$^{-}$ (Man 1407) (21) – were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (22, 23) at 30°C. *Escherichia coli* strains XL-10, BL21 (DE3), and SM10 were grown in Luria-Bertani (LB) broth or agar with antibiotics as described below.

**Construction of *ebpA* mutants and the complemented strain.** *ebpA* was previously disrupted in wild type *L. interrogans* serovar Manilae strain L495 by the Himar1 random transposon system (21). Transposon insertion of *epbA* in ebpA1$^{-}$ occurred at NT 3149215 in the proximal region of *epbA*. Transposon insertion of *epbA* in ebpA2$^{-}$ occurred at NT 3148873 in the proximal region of *epbA*. The native *ebpA* allele along with its native promoter region (including 300 bp upstream of +1) from *L. interrogans* serovar Manilae strain L495 was PCR amplified with XhoI 5' and 3' overhangs using primers ebpAx1 & ebpAx2 (**Table 1**) and T-A ligated into pCRII-TOPO plasmid (Life Technologies, Waltham, MA). The resulting plasmid was transformed into XL-10 competent cells according to the manufacturer's instructions (Stratagene, La Jolla CA). This plasmid was re-named 'pCRII-TOPO-*ebpA*'. XL-10 cells containing pCRII-TOPO-*ebpA* were grown in 5 ml of LB broth with 50 μg/ml of kanamycin (Sigma) and 50 μg/ml ampicillin (Euromedex) to late log phase ($10^7$/ml), followed by plasmid extraction by QIAprep Spin Miniprep Kit (Qiagen). The

*ebpA* gene was released from pCRII-TOPO-*ebpA* by 1 unit of XhoI restriction endonuclease (Thermo Scientific, Waltham, MA) and gel extracted by QIAquick Gel Extraction Kit (Qiagen), following the respective manufacturer's instructions. and the gene was ligated with T4 ligase according to the manufacturer's instructions (New England Biolabs) into the dephosphorylated XhoI site of conjugative plasmid pAL614 carrying a modified Himar1 transposon element along with an *aadA* (spectinomycin resistance) cassette (gift from Ben Adler). The resulting plasmid was renamed pAL614-*ebpA*. It was transformed into BL21(DE3) *E. coli* and grown in LB broth with 0.3 mM diaminopimelic acid (DAP, Sigma) and 50 μg/ml of spectinomycin (Sigma) as previously described (24). The *ebpA* construct was then randomly *cis*-inserted into the genome of ebpA1$^{-}$ (Man 250) via conjugation between Man250 and *E. coli* strain SM10 carrying pAL614-*ebpA* as previously described (25). Sequence analysis was performed as previously described (25) and revealed the *ebpA*$^{-/+}$ clone used in this study was *cis* inserted into *LMANv2_590053*.

**RNA isolation and qRT-PCR.** For the initial qRT-PCR analysis of wild type and epbA1$^{-}$ samples by relative quantity, leptospires were grown in EMJH at 28°C and harvested when bacterial growth reached late logarithmic phase ($10^7$/ml). Total RNA was isolated using Trizol according to the manufacturer's instructions (Invitrogen) and genomic DNA was digested using RNase-free DNase I (Promega). RNA was then further purified using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript III reverse transcriptase with random primers (Invitrogen, Carlsbad, CA). Using the cDNAs as the templates, the mRNA was assessed by real-time fluorescence quantitative PCR (qPCR) using

148 a SYBR green PCR master mixture (ABI, Pleasanton, CA) in an ABI 7000 Real-Time PCR
149 System (ABI). The primers used in the qPCR are listed in **Table 1**. In the RT-qPCR, the 16S
150 rRNA gene of *L. interrogans* serovar Manilae strain L495 was used as the internal reference.
151 The RT-qPCR data were analyzed using the ΔΔCt model and randomization test in REST
152 2005 software. For subsequent qRT-PCR analysis of wild type, epbA1$^{-}$, epbA2$^{-}$ and epbA$^{-/+}$,
153 RNA was generated from strains grown in EMJH with 1% rabbit serum until late logarithmic
154 phase as described above. cDNA was generated by 1 μg of triple-DNase-treated RNA with
155 the iScript cDNA synthesis kit as per the manufacturer's instructions (Bio-Rad). 2 μl of 1:10
156 diluted cDNA was added to SsoFast Evagreen Supermix along with 250 nM of primers
157 according to the manufacturer's instructions (Bio-Rad). qPCR was performed using CFX 96
158 Real Time System/C1000 Thermal Cycler (Bio-Rad) with an amplification program of 1
159 cycle of 95°C for 3 minutes; followed by 40 cycles of 95°C for 10 seconds, 55°C for 30
160 seconds, a plate read; and then 1 cycle at 95°C for 10 seconds; and finally followed by
161 melting curve analysis of 65°C-95°C in 0.5°C increments for 5 seconds each. Bio-Rad CFX
162 Manager V3.1 was used to determine the Cq threshold, and absolute quantity (ΔCt) was
163 determined by comparing copy number of each gene to copy number of *16S*. Statistical
164 analysis was completed by one-way ANOVA and Tukey's HSD test, where significance was
165 defined as $P<0.05$.

166 Standard curves were generated for the *16S* rRNA gene (*LArRNA2417572R*), *ebpA*
167 *(LMANv2_370110)*, *LMANv2_200027*, *LMANv2_290065*, and *LMANv2_310003* (*amtB*)
168 using the primers described in **Table 1**. Each cassette was amplified from *L. interrogans*

serovar Manilae strain L495 by PCR as described above, T-A ligated into pCRII-TOPO plasmid (Life Technologies), and transformed into XL-10 Gold or XL-1 Blue competent cells (Stratagene) as per the manufacturer's instructions. Cells were subsequently grown in 2 ml of Luria-Bertani broth with 50 μg/ml of kanamycin and 50 μg/ml ampicillin until late logarithmic phase (107/ml) and plasmid extracted by QIAprep Spin Miniprep Kit as per the manufacturer's instructions (Qiagen). Plasmid concentration was calculated by spectrophotometric analysis ($A_{260/280}$) by NanoDrop Lite (Thermo Scientific). Total copy numbers of each plasmid were then calculated by http://cels.uri.edu/gsc/cndna.html, and a standard curve was generated.

**Recombinant protein expression and purification.** Recombinant RpoN was produced in *E. coli* using the aLICator LIC cloning and expression system (Thermo Scientific). Briefly, the full-length *rpoN* gene was amplified from *L. interrogans* serovar Manilae strain L495 using primers pLATE52-rpoN F/pLATE52-rpoN R (**Table 1**) and cloned into pLATE52 to generate the pLATE52$^{rpoN}$ plasmid, which was then transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) for sequencing. The plasmid containing the *rpoN* sequence was transformed into *E. coli* BL21 (DE3) (Novagen) to form *E. coli* BL21DE3$^{pLATE52\text{-}rpoN}$. All of the engineered bacteria were cultured in 100 μg/ml ampicillin-containing LB broth to express the recombinant proteins under induction by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma). The His6-tagged recombinant proteins were purified using the Ni-NTA column according to the manufacturer's instructions (Qiagen) and analyzed by SDS-PAGE. To obtain the recombinant protein EbpA, the

full-length *ebpA* gene was PCR amplified from *L. interrogans* strain L495 genomic DNA using the primers pGEX-4T-2-ebpA F/pGEX-4T-2-ebpA R (**Table 1**). The product was cloned into pMD18-T to form a recombinant pMD18-T$^{ebpA}$ plasmid for sequencing. The pMD18-T$^{ebpA}$ and pGEX-4T-2 vectors (Amersham Pharmacia Biotech) were digested with both BamHI and NotI endonucleases (New England Biolabs, NEB). The recovered *ebpA* gene segment was linked to the linearized pGEX-4T-2 using T4 DNA ligase (NEB) to form the pGEX-4T-2$^{ebpA}$ plasmid. pGEX-4T-2$^{ebpA}$ was transformed into *E. coli* BL21 (DE3) (Novagen) to generate *E. coli* BL21DE3$^{pGEX-4T-2-ebpA}$. The recombinant EbpA protein (rEbpA) was expressed by induction with 0.5 mM IPTG (Sigma), and the fusion protein with GST was extracted from the cell lysates after ultrasonication using Bio-Scale Mini Profinity GST Cartridges (Bio-Rad). The rEbpA protein was cleaved with thrombin (Amersham Pharmacia Biotech) and purified further with Amicon Ultra-4 centrifugal filter (Millipore, NMWL 50 kDa) to remove the GST and thrombin. The soluble recombinant protein was filtered by using a 0.22 μm pore size membrane filter, examined by SDS-PAGE and measured by using the BCA protein assay kit (Bio-Rad).

**Electrophoretic mobility shift assay (EMSA).** Primers used in EMSA are listed in **Table 1**. Synthesized or PCR-amplified DNA probes were end-labeled with biotin using biotin-labeled forward primers (Invitrogen, Shanghai, China). EMSA was performed by using the LightShift™ Chemiluminescent EMSA Kit (Thermo Scientific). Briefly, 200 nM labeled probe was mixed with various amounts of purified rRpoN or rEbpA in 20 μl of the gel shift binding buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM dithiothreitol (DTT), 5

211 mM $MgCl_2$ and 5% v/v glycerol. After being incubated at 37°C for 30 min, the samples were
212 analyzed by 10% non-denaturing polyacrylamide gel electrophoresis at 60 V for 3-6 h. Then,
213 DNA was transferred onto a positively charged nylon membrane (Roche Applied Science,
214 USA) by electroblotting (380 mA for 80 min). After UV cross-linking, blotting and washing,
215 the membrane was conjugated by stabilized streptavidin-horseradish peroxidase. Signals were
216 detected following the manufacturer's instructions after exposure using a camera equipped
217 with a CCD imager (FluorChem E, ProteinSimple).

218 **Growth curve, survival, and cell morphology.** *L. interrogans* strains, including the
219 *ebpA1* mutant, were first grown in standard EMJH medium to late log phase ($10^7$/ml). Then,
220 the bacteria-containing media was diluted at a ratio of 1:10 into EMJH or EMJH with 5 mM
221 alanine replacing the $NH_4Cl$. For the water survival test, *L. interrogans* strains were grown to
222 late logarithmic phase before they were harvested by centrifugation. The supernatants were
223 drained thoroughly, and the cells were re-suspended to the original volume in spring water
224 (pH 7.2) (Kirkland Signature). All cultures were incubated at 30°C, and the motile leptospires
225 were enumerated every 24 hours under dark-field microscopy by Petroff-Hausser counting
226 chamber according to the manufacturer's instructions (Hausser Scientific, PA). The viability
227 of the spirochetes was confirmed by growth in standard EMJH medium.

228 Cell morphology was measured in wild type, ebpA1$^-$, ebpA2$^-$, and ebpA$^{-/+}$ cells grown
229 in standard EMJH medium to late-logarithmic phase ($10^7$/ml) as described above. The lengths
230 of 100-200 cells of each strain were measured using an Olympus Bx53 microscope and
231 Olympus CellSens Dimension 1.7.1 software (Olympus, Hamburg, Germany). Fields were

randomly selected for measuring leptospire cell length. Average length and standard error of the mean were calculated by Graphpad software (Prism, San Diego, CA). Statistical analyses were completed by one-way ANOVA and Tukey's HSD test, where significance was defined as $P<0.05$.

**Determination of the contribution of EbpA to virulence in Mongolian gerbils.** Three-week-old male Mongolian gerbils (*Meriones unguiculatus*) were purchased from Janvier labs (Janvier, Le Geneste-Saint-Isle, France). Four gerbils per group were tested for survival against wild type, epbA1$^-$, or epbA2$^-$ strains in accordance with the guidelines of the Animal Care and Use Committees of the Institut Pasteur (authorization B-75-1132). Following intraperitoneal injection with $10^4$ or $10^6$ leptospires, gerbils were observed twice daily until day 21 post inoculation. Following euthanasia at day 21, approximately 300 µl of blood was collected along with kidneys by dissection. The outgrowth of spirochetes in these tissue cultures was assessed using dark-field microscopy.

## RESULTS

***In silico* prediction of RpoN ($\sigma^{54}$) regulatory network in *L. interrogans*.** Genome sequences reveal that all sequenced *Leptospira* species (including pathogenic, saprophytic, and intermediate strains) have the alternative sigma factor $\sigma^{54}$. To study the role of $\sigma^{54}$ in the enzootic cycle of *L. interrogans*, we first performed *in silico* analysis of the $\sigma^{54}$ network in *L. interrogans*. Since its activation always requires an activator, an EBP, which has a conserved domain for activation of $\sigma^{54}$-polymerase-dependent transcriptional initiation, we analyzed the presence of EBPs among *Leptospira* species. Our analyses revealed that all pathogenic *Leptospira* have two activators (herein named as *Leptospira* enhancer-binding protein A and B: EbpA [LMANv2_37011, fhlA-like (16, 18)] and EbpB [LMANv2_590040, NtrC-like (16, 18)]. These results suggest that within the $\sigma^{54}$ regulon, there are two pathways, EbpA-RpoN ($\sigma^{54}$) and EbpB-RpoN ($\sigma^{54}$), that activate two sets of genes that utilize a $\sigma^{54}$-type promoter (**Fig. 1**).

Because $\sigma^{54}$ recognizes a unique -24/-12 promoter sequence, $\sigma^{54}$-dependent genes can be predicted *in silico*. In this regard, Francke et al. performed a comparative genome analysis of over 300 genuses, based on the frequency representation of the $\sigma^{54}$ promoter (18). Based on their results, we searched the *L. interrogans* genomes using Promscan Analysis Tools (http://www.promscan.uklinux.net) and queried for genes containing a putative RpoN binding sequence in putative promoter regions. We identified 13 putative RpoN ($\sigma^{54}$)-type promoters for 22 genes in *L. interrogans* 56601 and L495 strains (where genes shared by the same

promoter were grouped) (**Table 2**). Twenty-one of these genes were located on chromosome I, and one gene was located on chromosome II. Five of these genes encode hypothetical proteins, and seventeen genes encode proteins with assigned functions such as nitrogen assimilation, glutamine synthesis, alanine racemization, flagellar synthesis, sodium bile acid symport, and lipid A biosynthesis. Given that *L. interrogans* has two EBPs, we predicted that one set of these twenty-two genes is controlled by the EbpA-RpoN pathway, and the other set is governed by EbpB-RpoN.

**Inactivation and complementation of the enhancer binding protein gene *ebpA*.** To investigate the function of the $\sigma^{54}$ regulon in *L. interrogans*, we initially attempted to inactivate *rpoN* (encoding $\sigma^{54}$) in *L. interrogans* strains. However, multiple attempts were unsuccessful. A random transposon mutant library in *L. interrogans* serovar Manilae strain L495 also has not yet yielded an *rpoN*-disrupted mutant (26). However, the library did yield two biologically distinct mutants with transposon insertion within the proximal region of *ebpA* (21) . These biologically distinct mutants were named *ebpA1* (transposon library ID: M250) and *ebpA2* (transposon library ID: M1407).

To study the function of *ebpA* and to fulfill molecular Koch's postulates, we first performed complementation for *ebpA* (named epbA$^{-/+}$) by randomly *cis*-insertion of a wild-type copy of *ebpA* (including its upstream native promoter) into the *ebpA1* mutant. Subsequent sequence analysis revealed that one of the ebpA$^{-/+}$ clones used in this study harbored an *epbA* insertion at the distal region of LMANv2_590053 (conserved protein of

288 unknown function) at NT 4079032. qRT-PCR analyses demonstrated that the two *epbA*
289 mutants did not express *ebpA*; whereas the complemented strain ebpA$^{-/+}$ successfully restored
290 *ebpA* expression (**Fig. 2**).
291
292 **Deletion of *ebpA* affects cell morphology in *L. interrogans.*** Qualitative examination of
293 the *ebpA* mutant by microscopy revealed no defect in motility. However, we observed that
294 the *ebpA* mutant appeared shorter in length than the wild type strain. Further quantitative
295 analyses of cell length showed that the two *ebpA* mutants were significantly shorter in cell
296 length (7.365 μm ± 0.1782 μm, P<0.01 and 7.566 μm ± 0.1631 μm, P<0.01) than the wild
297 type (10.99 μm ± 0.3118 μm) when grown to mid-to-late logarithmic phase.
298 Complementation of *ebpA1* restored the cell length in epbA$^{-/+}$ (10.47 μm ± 0.2129 μm,
299 P<0.01) (**Fig. 3**).
300
301 **Identification of EbpA-regulated genes by qRT-PCR.** To identify genes controlled by
302 the EbpA-RpoN pathway, initial qRT-PCR analysis by relative fold change between wild
303 type and the *ebpA* mutants was performed. Among the twenty-two predicted RpoN-controlled
304 genes, three genes – LMANv2_31003 (*amtB,* ammonia transporter gene), *LMANv2_200027*
305 (a conserved exported protein), and *LMANv2_290065* (a putative outer membrane protein) –
306 had significantly lower expression in the *ebpA* mutant than the wild-type strain (P<0.01) (**Fig.**
307 **4A**). Based on this information, a second analysis was performed by comparing the absolute

transcript levels of the above identified genes to copies of *16S rRNA,* which is constitutively expressed under standard *in vitro* growth conditions at 30˚C (27) in wild-type, the *ebpA* mutants and epbA$^{-/+}$. This study re-confirmed the above findings of significantly lower expression of *amtB, LMANv2_200027*, and *LMANv2_290065* in the *ebpA* mutant compared to wild type (5-10 fold change) (**Fig. 4B**). The complemented strain epbA$^{-/+}$ restored expression of *amtB, LMANv2_200027*, and *LMANv2_290065*) (**Fig. 4B**). These results suggest that EbpA-RpoN regulates expression of *amtB*, *LMANv2_200027*, and *LMANv2_290065*.

**RpoN and EbpA bind to the promoter region of *amtB, LMANv2_200027*, and *LMANv2_290065*.** To demonstrate that *amtB, LMANv2_200027,* and *LMANv2_290065* have an RpoN ($\sigma^{54}$) promoter, we purified recombinant RpoN of *L. interrogans* and performed EMSA assay. A 50 bp oligonucleotide encoding the predicted -24/-12 promoter region of *amtB, LMANv2_200027,* and *LMANv2_290065* was end-labeled with biotin and mixed with different amounts of purified recombinant RpoN in binding buffer. The results showed that *L. interrogans* RpoN bound to the predicted $\sigma^{54}$ promoter of *amtB, LMANv2_200027*, and *LMANv2_290065* in a dose-dependent manner (**Fig. 5A**). The bindings were specific, since unlabeled DNA fragments could compete for binding and no binding was observed when bovine serum albumin (BSA) was used instead of RpoN. Furthermore, mutated probes (MRN027: -25TGGCA>AAAAT; -14TTGC>AATT, MRN065: -25TGGCA>AAAAT; -14TTGC>AATT or MRN003: -25TGGTA>AAAAT; -14TTGC>AATT) in the -24/-12

329 region did not show binding (**Fig. 5B**). These data provide biochemical evidence that *amtB,*
330 *LMANv2_200027*, and *LMANv2_290065* have an RpoN ($\sigma^{54}$)-type promoter.

331 Since *L. interrogans* has two predicted EBPs, a gene with an RpoN ($\sigma^{54}$)-type
332 promoter can be activated either by EbpA or by EbpB. To gain biochemical evidence that
333 *amtB, LMANv2_200027*, and *LMANv2_290065* are under the control of EbpA, we further
334 performed EMSA using recombinant EbpA. Because EbpA is a putative enhancer-binding
335 protein that can bind at a site far from the promoter, 500 bp DNA fragments of the *amtB*,
336 LMANV_200027, or LMANV_290065 promoter regions (EA003, EA027, EA065, -450 bp
337 and +50 bp relative to the ATG start codon) were used for the EMSA assays. As shown in
338 **Fig. 6A**, rEbpA was capable of binding to the upstream region of *amtB, LMANv2_200027*,
339 and *LMANv2_290065*. Unlabeled EA003, EA027, or EA065 could compete for binding, but
340 sheared salmon sperm DNA did not. In addition, no DNA-BSA complex was observed (**Fig.**
341 **6B**). Furthermore, neither rRpoN nor rEbpA bound to the sigma70-type promoter region
342 (L41) of *lipL41*. On the other hand, rRpoN bound to the promoter region of *lipL41* when the
343 sigma70-type promoter was replaced with a sigma54-type promoter (RL41) (**Fig. 6C**).
344 However, rEbpA could not bind to RL41 despite that RL41 contained a sigma54-type
345 promoter, suggesting that rEbpA binding to *amtB, LMANv2_200027*, and *LMANv2_290065*
346 is specific. The results of these biochemical experiments, along with the genetic data above,
347 suggest that EbpA is the regulator that activates *amtB, LMANv2_200027*, and
348 *LMANv2_290065* by binding to the upstream binding regions near their promoters.

349

**The *ebpA* mutant had reduced growth and survival under various conditions.** *amtB,* predicted to encode an ammonia transporter protein (PSORT-B, TMhmm), is putatively co-transcribed with *glnK,* which encodes a nitrogen regulatory protein in *L. interrogans* (5, 29). We therefore examined the growth of the *ebpA* mutant in EMJH liquid media with alternative nitrogen sources. There was no significant difference in growth observed between wild-type and ebpA1$^-$ grown in standard EMJH media with 5 mM ammonium chloride (**Fig. 7A**) (22, 23). However, when 5 mM L-alanine, a poor nitrogen source, was supplemented in nitrogen-free EMJH media, the ebpA1$^-$ mutant had significantly reduced growth compared to the wild type (**Fig. 7B**).

To investigate the survival abilities of the *ebpA1* mutant in stress conditions, wild type, ebpA1$^-$, and *epbA1*$^{-/+}$ strains were cultivated in either 1:10 diluted EMJH or in spring water. The *ebpA* mutant had a lag phase at the initial stage in nutrient-limited media (**Fig. 7C**). Wild-type *L. interrogans* can survive in spring water for weeks, and the spirochetal numbers remained high till week 5, and the numbers began to drop at week 6 (**Fig. 7D**). At weeks 6, wild-type spirochetes remained alive and cultivable (data not shown). In contrast, the numbers of the *ebpA* mutant began dropped significantly at less than week 1 (**Fig. 7D**). At week 4, although some mutant spirochetes remained (**Fig. 7D**), but no live and cultivable mutant spirochetes could be detected (data not shown). These results suggest that EbpA is important for Leptospira to survive in water.

***ebpA* is dispensable for virulence.** To determine if *ebpA* plays a role in virulence, $10^4$ cells of either wild type or *epbA2* were inoculated into gerbils, and the gerbils were observed for 21 days. Data indicate that the abrogation of the *ebpA* gene does not reduce mortality in gerbils (**Fig. 8**). Similar results were found between wild type and *ebpA1* when $10^6$ cells were inoculated intraperitoneally (data not shown). Viable spirochetes for all of the strains inoculated were recovered from renal tissue and blood. These results suggest that EbpA is not essential for mammalian infection.

## DISCUSSION

Little is known about the signaling and regulatory pathways underlying the differential gene expression of *L. interrogans*. In this study, we provide bioinformatic, genetic, and biochemical evidence to demonstrate the presence of the EbpA-RpoN pathway in which the bacterial enhancer-binding protein EbpA and the sigma factor $\sigma^{54}$ work together to activate at least three genes from their $\sigma^{54}$-type promoters: *amtB* (encoding ammonia transporter), *LMANv2_200027* (encoding a conserved exported protein), and *LMANv2_290065* (encoding a putative outer membrane protein). This pathway appears not to be essential for mammalian infection, but plays an important role for *L. interrogans* to survive in the environment.

RpoN has been shown to be involved in regulation of many bacterial functions such as nitrogen metabolism, flagellar biosynthesis, osmotolerance, pH changes, biofilm formation, motility, colonization, lipoprotein biosynthesis and a Type III secretion system (18, 30-32). Bioinformatics analysis predicted 13 putative RpoN ($\sigma^{54}$)-type promoters controlling 22 genes in *L. interrogans*. Using the *ebpA* mutant and EMSA assay, we demonstrated that at least three of these genes *amtB*, *LMANv2_200027*, and *LMANv2_290065*, are under the control of EbpA. Other predicted RpoN-dependent genes could be controlled by EbpB. Interestingly, 3 of these genes have been demonstrated to be under the control of RpoN in other bacteria: *lpxC*, which is responsible for the first committed step in lipid A biosynthesis regulated by RpoN in *Aquifex aeolicus* (33); *pstA*, encoding phosphoenolpyruvate-protein phosphotransferase, which participates in regulation of the $\sigma^{54}$-dependent promoter in *Rhizobium etli* (34); and *glnA*, encoding glutamine synthetase, the RpoN-dependent gene in

399 *E. coli* (35). Other putative RpoN-dependent genes include ones encoding a sodium bile acid
400 symporter, a pyridoxamine-phosphate oxidase, an endoflagellar biogenesis protein, an alanine
401 racemase, and proteins with unknown function. Although they remain to be confirmed, these
402 observations suggest multi-functional roles of RpoN in *L. interrogans*.

403 We demonstrated that the *ebpA* mutant had impaired ability to use alanine as an
404 alternative nitrogen source, and more importantly, had reduced survival in water. Bacteria
405 assimilate a variety of inorganic nitrogen sources; such as ammonia, amino acids, peptides,
406 polyamines and related compounds, cytosine, nucleosides, and other compounds (36).
407 Ammonia supports the fastest growth rate and is therefore considered the preferred nitrogen
408 source among most bacteria. Ammonia and ammonium transport proteins belonging to the
409 Amt protein family are ubiquitous in prokaryotes. Nearly all prokaryotes encode at least one
410 Amt protein and in some cases two or three homologues within their respective genomes
411 (37). Based on analyses of the genomes of leptospires, three genes were predicted to be
412 involved in nitrogen assimilation. *amtB* (LIC10441) and *glnK* (LIC10440), co-transcribed as
413 an operon and encoding ammonia permease and nitrogen regulatory protein II (PII),
414 respectively (5), were dramatically down-regulated in both of the *ebpA* mutant strains in our
415 study. PII serves as a signal transduction protein for sensing external ammonium availability
416 and nitrogen status of the cell. Ammonia permease acts as a channel for ammonium transport.
417 In *E. coli* and other bacteria, *amtB-glnK* are also considered a signal transduction pathway. In
418 a nitrogen/ammonia-rich environment, GlnK is physically bound to AmtB, disrupting uptake
419 of free ammonia (38). However, in an ammonium/nitrogen-scarce environment, GlnK is

420 unbound from AmtB, allowing GlnK to interact intracellularly with other proteins, most of
421 which are unknown (39, 40). In turn, this suggests that AmtB moonlights as a sensor of
422 external nitrogen availability. Further, it has been previously suggested the AmtB-GlnK
423 complex is critical for sensing and responding to nitrogen scarcity (36). Our data suggest a
424 similar pattern in *Leptospira*: the reduced expression of *amtB* and *glnK* in *ebpA* mutants may
425 create an inadequate intracellular stress response to nitrogen scarcity. There is another gene,
426 *amt3* (LIC10589, LMANv2_130007), which encodes an alternative ammonium transporter
427 that does not appear having a RpoN-type promoter. The presence of the alternative
428 ammonium transporter *LMANv2_130007* may explain the lack of growth defect in the *ebpA*
429 mutant when growing in a nitrogen-rich medium. Activation of *LMANv2_130007* or another
430 as yet unidentified ammonium transporter may work redundantly to ensure sufficient uptake
431 of nitrogen under conditions when EbpA-RpoN is inactivated. Concordantly, previous studies
432 have shown that expression of the *glnK-amtB* operon is generally induced during growth
433 under limited ammonium conditions. Thus, loss of ammonia transport along with loss of
434 GlnK intracellular signaling may explain the observed growth phenotype of the *ebpA* mutant
435 when grown with a poor nitrogen source such as alanine and spring water (37). During
436 infection, leptospires are exposed to a nutrient-rich host environment. It has been reported
437 that *amtB* and *glnK* were down-regulated when exposed to serum (5). This may explain why
438 *ebpA* and *amtB* are dispensable for virulence during infection.

439 We found that the *ebpA* mutant has shortened cell length. This may be due to a variety of
440 global response changes within the bacterium upon disruption of the epbA-rpoN pathway.

441 One possibility is that the lack of a strong nitrogen source in the bacterium may partially
442 activate the stringent response pathway (*rel*) in leptospires, as *rel* activation in *Borrelia*
443 *burgdorferi* is important as an alarmone response that results in global changes within that
444 species 41). Likewise, *rel* has been associated with cell morphology changes in other bacteria
445 (41). This phenotype was also observed in a previously described *lpxD* mutant (25). In
446 addition to shortened length of the *ebpA* mutant, it remains unclear whether the mutant also
447 has abnormal cell width, helical pitch or flagellar length. Further study using cryo-electron
448 microscopy will likely yield interesting finding on the nature of the morphological phenotype
449 of the ebpA mutant.

450 We observed that the complemented strain produces higher levels of *ebpA* transcript
451 levels than the wild type (Fig. 2.), as well as for those EbpA-controlled genes (Fig. 4). One
452 possibility is the gene dosage. This is possible but unlikely since our random nested PCR
453 only revealed insertion into one gene (lmanv2_590053). Another possibility is that insertion
454 of *ebpA* into lmanv2_590053 has allowed increased expression of *ebpA*. Increased expression
455 of EbpA in turn increased expression of EbpA-dependent genes.

456 In summary, we demonstrate that the presence of the EbpA-RpoN regulatory pathway
457 is important for the environmental survival of *L. interrogans*. This pathway does not appear
458 to be essential for mammalian survival based on our findings. The non-essential nature of
459 EbpA-RpoN for virulence is somewhat not surprising, since the saprophytic strain *L. biflexa*
460 also contains EbpA and RpoN. Both species may face various hostile and nutrient-scarce
461 conditions when positioned in the natural environment (42, 43). Both species also encode

462 other factors (e.g., $\sigma^E$) to respond to environmental stresses (44, 45). On the other hand,
463 pathogenic *L. interrogans* encodes EbpB, which is lacking in saprophytic *L. biflexa,* where
464 EbpB is an NtrC-type of EBP. Interestingly, *B. burgdorferi*, another spirochetal pathogen, has
465 an NtrC-type EBP, Rrp2. Rrp2, working together with RpoN, controls expression of RpoS,
466 which in turn governs multiple factors required for mammalian infection (46-48). Previous
467 research on the *ntrC* locus of pathogenic *Leptospira* has demonstrated that this locus has
468 undergone several evolutionary rearrangements compared to other pathogenic spirochetes
469 (49). Therefore, it is tempting to postulate that for the two RpoN pathways of *L. interrogans*,
470 while EbpA-RpoN is important for environmental survival, EbpB-RpoN may play a role in
471 mammalian-phase adaptation.

472 **ACKNOWLEDGMENTS**

473 This work was supported in part by grants from the National Natural Science Foundation of
474 China (No. 81501713, WLH), China Postdoctoral Science Foundation (No. 2015M570516,
475 WLH), National Science Foundation grant IIA-1159099 (CJP) and the Institut Pasteur (MP).

476
477

478 **TABLE 1. Primers used in this study.**

479

| Gene | Primer name | sequence (5' -> 3')# | size | purpose |
|---|---|---|---|---|
| ***RpoN (LMANv2_590036)*** | pLATE52-rpoN F | GGTTGGGAATTGCAAATGAATCTAAGTCAGTCAC | 1419 | rpoN gene expression |
| | pLATE52-rpoN R | GGAGATGGGAAGTCATTATCTAGACTCCAAAGAC | | |
| ***16S (lArRNA3064233R)*** | plasmid-16s F | GCGGCGAACGGGTGAGTAACACGTGGG | 968 | Generation of plasmid for standard curve |
| | plasmid-16s R | CATCTCACGACACGAGCTGACGACAACC | | |
| | qRT-16sRNA F | GGCGGACATGTAAGTCAGGT | 179 | qRT-PCR primer set |
| | qRT-16sRNA R | GCCTAGCGTCAGTTTTAGG | | |
| ***ebpA (LMANv2_370110)*** | plasmid-ebpA F | ATGTCAGGATATGTGAAGCC | 1214 | Generation of plasmid for standard curve |
| | plasmid-ebpA R | GCAGAGGCAATCATTTCTTTACCGG | | |
| | qRT-ebpA F | TCCGATGATTGTTGAAGACG | 262 | qRT-PCR primer set |
| | qRT-ebpA R | GGATTTTTCCAAACGATCCA | | |
| | ebpA-x1 | CCGCTCGAGCATAGGTTTAGTGATC | 2673 | Generation of plasmid for complementation |
| | ebpA-x2 | CCGCTCGAGCGTTCACACAATCTTCTC | | |
| | pGEX-4T-2-ebpA F | CGCGGATCCATGTCAGGATATGTGAAG | 2097 | ebpA gene expression |
| | pGEX-4T-2-ebpA R | ATAAGAATGCGGCCGCATAATCGATTTT | | |
| ***LMANv2_200027*** | plasmid-200027 F | CAAGTTTGCGGCAATTGCTCTTTTTTTAAGC | 495 | Generation of plasmid for standard curve |
| | plasmid-200027 R | CGGGTCAAATCAGTTAGAACTAGAGATGGG | | |

| | | | | |
|---|---|---|---|---|
| | qRT-LMANv2_200027 F | AACCAATCCCTTCATGTTCG | 195 | qRT-PCR primer set |
| | qRT-LMANv2_200027 R | AGCTTCTTCTTCCGCATTGA | | |
| | RN027 F | GAATCTTTATAAATTT**TGGCACGCTGCTTGCA**TATATCTATACAAGAAC | 49 | EMSA probes for rRpoN, 3' biotin labeled |
| | RN027 R | TTCTTGTATAGATATA**TGCAAGCAGCGTGCCA**AAATTTATAAAGATTCT | | |
| | MRN027 F | GAATCTTTATAAATTT**aaaatCGCTGCaattA**TATATCTATACAAGAAC | 49 | EMSA probes for rRpoN, 3' biotin labeled |
| | MRN027 R | TTCTTGTATAGATATA**TaattGCAGCGatttt**AAATTTATAAAGATTCT | | |
| | FA027 F | TATCTTGCTTCGAAAGTTACC | 500 | EMSA probes for rEbpA, 5' biotin labeled |
| | FA027 R | AGTCCGGTAAAAATGCTTAAA | | |
| | LMANv2_290065 F | CGTCACAACTGCTCCTTTCA | 216 | Generation of plasmid for standard curve and qRT-PCR primer set |
| | LMANv2_290065 R | GCCATTGCTGCTTAGAAGG | | |
| ***LMANv2_290065*** | RN065 F | TCTAAATTTTAAACTT**TGGCATATATTTTGCA**TAGTCTGAGCATGGAGA | 49 | EMSA probes for rRpoN, 3' biotin labeled |
| | RN065 R | CTCCATGCTCAGACTA**TGCAAAATATATGCCA**AAGTTTAAAATTTAGAA | | |
| | MRN065 F | TCTAAATTTTAAACTT**aaaatTATATTaattA**TAGTCTGAGCATGGAGA | 49 | EMSA probes for rRpoN, 3' biotin labeled |
| | MRN065 R | CTCCATGCTCAGACTA**TaattAATATAattttt**AAGTTTAAAATTTAGAA | | |

| | | | | |
|---|---|---|---|---|
| | FA065 F | GGAACTCATACTTTTAGAAAA | 500 | EMSA probes for rEbpA, 5' biotin labeled |
| | FA065 R | ACCAGCAGACAAAAAACAGAA | | |
| | plasmid-310003 F | GGGACTTCTTGTAAGTCCGGTATGCTACGC | 538 | Generation of plasmid for standard curve |
| | plasmid 310003 R | CTTGGTATTCGTGGCCTCTGAAAACTTCCG | | |
| | qRT-LMANv2_310003 F | GTTAGGCGCGGTTCTTACAG | 219 | qRT-PCR primer set |
| | qRT-LMANv2_310003 R | TCTCCGTGAATTTCCGAATC | | |
| ***amtB (LMANv2_310003)*** | RN003 F | ATTATTCTTCAAT**TGGTACTAATCTTGCA**AACAAAGAATAAAAATTGAT | 49 | EMSA probes for rRpoN, 3' biotin labeled |
| | RN003 R | TCAATTTTTATTCTTTGTT**TGCAAGATTAGTACCA**ATTGAAGAATAATT | | |
| | MRN003 F | ATTATTCTTCAAT**aaaatCTAATCaattA**AACAAAGAATAAAAATTGAT | 49 | EMSA probes for rRpoN, 3' biotin labeled |
| | MRN003 R | TCAATTTTTATTCTTTGTT**TaattGATTAGattttA**TTGAAGAATAATT | | |
| | FA003 F | TATATTTTATAAGCATACTTT | 500 | EMSA probes for rEbpA, 5' biotin labeled |
| | FA003 R | GTGCTAGCTTCCTGCCCAAAT | | |
| ***lipL41*** | L41/RL41 F | TTGTAATTCAGTATCTTG | 221 | EMSA probes for rRpoN or rEbpA, 5' biotin labeled |
| | L41/RL41 R | AAGAACATAAGGAGAACT | | |
| ***raiA*** | q-LMANv2_200026 | CCGCTGAAAAATACGCAAAT | 193 | qRT-PCR |

| | | | | |
|---|---|---|---|---|
| | F | | | primer set |
| ***(LMANv2_200026)*** | q-LMANv2_200026 R | TCTTCCAGACCGTCGATACA | | |
| ***lpxC*** | q-LMANv2_430009 F | CGGCAGAACTCAGCAATGTA | 162 | qRT-PCR |
| ***(LMANv2_430009)*** | q-LMANv2_200026 R | CGTCCATGATCGGAACTTCT | | primer set |
| ***secE*** | q-LMANv2_260002 F | GCAGAATTGGAAAAGGTCCA | 189 | qRT-PCR |
| ***(LMANv2_260002 F)*** | q-LMANv2_260002 R | CTAACCAGCCACTTCCCAAA | | primer set |
| ***glnA*** | q-LMANv2_500019 F | TTGCGGATACCGCTTATTTC | 170 | qRT-PCR |
| ***(LMANv2_500019)*** | q-LMANv2_500019 R | AGTACCAGGACGGTGACCAG | | primer set |
| ***glnK*** | q-LMANv2_310004 F | AAGCGACGTTCAAGGTTACG | 249 | qRT-PCR |
| ***(LMANv2_310004)*** | q-LMANv2_310004 R | GCGGAACTTCCTCTTTCTCC | | primer set |
| ***nctP1*** | q-LMANv2_210009 F | TCCCGCAAATTTGAAAAGAC | 208 | qRT-PCR |
| ***(LMANv2_210009)*** | q-LMANv2_210009 R | TCGACGTTCTTTGTGGATGA | | primer set |
| ***pdxH*** | q-LMANv2_210047 F | GTTAGCCTCACCGCAAAGTC | 178 | qRT-PCR |
| ***(LMANv2_210047)*** | q-LMANv2_210047 R | ATCGTGTAAACGGCTGGAAC | | primer set |
| ***LMANv2_240139*** | q-LMANv2_240139 F | TTCCGGTCCAGAGACTTTTG | 250 | qRT-PCR |
| | q-LMANv2_240139 R | GATCGAAACGTTTTCGGAGA | | primer set |
| ***LMANv2_240051*** | q-LMANv2_240051 F | TTTGTTTGCCCGGATAAAAG | 151 | qRT-PCR |
| | q-LMANv2_240051 R | TTTTCCGAAATCGATCCAAG | | primer set |

| | | | | |
|---|---|---|---|---|
| ***LMANv2_300019*** | q-LMANv2_300019F | TTCGTGTTTGATCGTTTCCA | 248 | qRT-PCR primer set |
| | q-LMANv2_300019R | CCTTTCCGGCAAAAAGTAGA | | |
| ***fliO (LMANv2_150090)*** | q-LMANv2_150090F | GGAATCGCAGGGACTCTTCT | 217 | qRT-PCR primer set |
| | q-LMANv2_150090R | CATTGTCAGCAACTCCGAGA | | |
| ***LMANv2_90055*** | q-LMANv2_90055F | TGTATTGAAGCGGGGATTTC | 184 | qRT-PCR primer set |
| | q-LMANv2_90055R | TACGAGCTGTTTCCGGTCTT | | |

480

481 # Restriction endonuclease cut sites are underlined. The sequences in bold are the predicted binding

482 sites for RpoN. Lowercase nucleotides are the mutated sites.

**Table 2: *Leptospira* genes containing a perfect RpoN binding sequence in the putative promoter regions.**

| Gene | Function | Matching sequence |
|---|---|---|
| LMANv2_200027 | tetratricopeptide repeat protein, outermembrane protein | TGGCACGCTGCTTGCA |
| *raiA* (LMANv2_200026) | sigma 54 modulation protein, ribosomal subunit interface protein | TGGCACGCTGCTTGCA |
| *lpxC* (LMANv2_430009) | UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase | TGGCATAGAGTTTGCT |
| *ptsA* (LMANv2_430010) | phosphoenolpyruvate-protein phosphotransferase | TGGCATAGAGTTTGCT |
| *secE* (LMANv2_260002) | preprotein translocase subunit | TTCAAAAGCCGTGCCG |
| *nusG* (LMANv2_260003) | transcription antiterminator | TTCAAAAGCCGTGCCG |
| *rplK* (LMANv2_260004) | 50S ribosomal protein L11 | TTCAAAAGCCGTGCCG |
| *rplA* (LMANv2_260005) | 50S ribosomal protein L1 | TTCAAAAGCCGTGCCG |
| *rplJ* (LMANv2_260006) | 50S ribosomal proteinL10 | TTCAAAAGCCGTGCCG |
| *rplL* (LMANv2_260007) | 50S ribosomal protein L7/L12 | TTCAAAAGCCGTGCCG |
| LMANv2_290065 | outer membrane protein | TGCAAAATATATGCCA |
| *glnA* (LMANv2_500019) | glutamine synthetase | TGGTACAATATTTGCA |

| | | |
|---|---|---|
| *amtB* (LMANv2_310003) | ammonia permease | TGGTACTAATCTTGCA |
| *glnK* (LMANv2_310004) | nitrogen regulatory protein P-II, nitrogen assimilation regulatory protein for GlnL, GlnE, and AmtB | TGGTACTAATCTTGCA |
| *nctP1* (LMANv2_210009) | sodium bile acid symporter | AGCAAAAAGCGCGCCG |
| *pdxH* (LMANv2_210047) | pyridoxamine-phosphate oxidase | AGGCAAGCGTTTTGCT |
| LMANv2_240139 | putative membrane protein | ATCAAGTTGTATGCCA |
| LMANv2_240051 | HD family protein | AGCACTTTCGATGCCA |
| LMANv2_300019 | putative membrane protein | TGGCACTAAATTTGAT |
| *lepB* (LMANv2_300018) | signal peptidase I | TGGCACTAAATTTGAT |
| *fliO* (LMANv2_150090) | endoflagellar biogenesis protein | TGCAATCAGCTTGCCA |
| *alr* (LMANv2_90055) | alanine racemase | TGGCATGGACTCTGCA |

# Reference


1. **Adler B, de la Pena MA.** 2010. *Leptospira* and leptospirosis. Vet Microbiol **140:**287-96.

2. **Lo M, Bulach DM, Powell DR, Haake DA, Matsunaga J, Paustian ML, Zuerner RL, Adler B.** 2006. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. Infect Immun **74:**5848-59.

3. **Matsunaga J, Lo M, Bulach DM, Zuerner RL, Adler B, Haake DA.** 2007. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. Infect Immun **75:**2864-74.

4. **Lo M, Murray GL, Khoo CA, Haake DA, Zuerner RL, Adler B.** 2010. Transcriptional response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog. Infect Immun **78:**4850-9.

5. **Patarakul K, Lo M, Adler B.** 2010. Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. BMC Microbiol **10:**31.

6. **Xue F, Dong H, Wu J, Wu Z, Hu W, Sun A, Troxell B, Yang XF, Yan J.** 2010. Transcriptional responses of *Leptospira interrogans* to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. PLoS Negl Trop Dis **4:**e857.

7. **Caimano MJ, Sivasankaran SK, Allard A, Hurley D, Hokamp K, Grassmann AA, Hinton JC, Nally JE.** 2014. A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar Copenhageni. PLoS Pathog **10:**e1004004.

8. **Pappas CJ, Benaroudj N, Picardeau M.** 2015. A replicative plasmid vector allows efficient complementation of pathogenic *Leptospira* strains. Appl Environ Microbiol **81:**3176-81.

9. **Matsunaga J, Coutinho ML.** 2012. Positive regulation of *Leptospira interrogans kdp* expression by KdpE as demonstrated with a novel beta-galactosidase reporter in *Leptospira biflexa*. Appl Environ Microbiol **78:**5699-707.

10. **Cune J, Cullen P, Mazon G, Campoy S, Adler B, Barbe J.** 2005. The *Leptospira interrogans lexA* gene is not autoregulated. J Bacteriol **187:**5841-5.

11. **Louvel H, Betton JM, Picardeau M.** 2008. Heme rescues a two-component system *Leptospira biflexa* mutant. BMC Microbiol **8:**25.

12. **Morero NR, Botti H, Nitta KR, Carrion F, Obal G, Picardeau M, Buschiazzo A.** 2014. HemR is an OmpR/PhoB-like response regulator from *Leptospira*, which simultaneously effects transcriptional activation and repression of key haem metabolism genes. Mol Microbiol **94:**340-52.

13. **Fouts DE, Matthias MA, Adhikarla H, Adler B, Amorim-Santos L, Berg DE, Bulach D, Buschiazzo A, Chang YF, Galloway RL, Haake DA, Haft DH, Hartskeerl R, Ko AI, Levett PN, Matsunaga J, Mechaly AE, Monk JM, Nascimento AL, Nelson KE, Palsson B, Peacock SJ, Picardeau M, Ricaldi JN, Thaipandungpanit J, Wunder EA Jr, Yang XF, Zhang JJ, Vinetz JM.** 2016. What makes a bacterial species pathogenic?: comparative genomic analysis of the genus *Leptospira*. PLoS Negl Trop Dis **10:**e0004403.

14. **Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, Creno S, Kuczek ES, Bommezzadri S, Davis JC, McGrath A, Johnson MJ, Boursaux-Eude C, Seemann T, Rouy Z, Coppel RL, Rood JI, Lajus A, Davies JK, Medigue C, Adler B.** 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One **3:**e1607.

15. **Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, Zhang YX, Xiong H, Lu G, Lu LF, Jiang HQ, Jia J, Tu YF, Jiang JX, Gu WY, Zhang YQ, Cai Z, Sheng HH, Yin HF, Zhang Y, Zhu GF, Wan M, Huang HL, Qian Z, Wang SY, Ma W, Yao ZJ, Shen Y, Qiang BQ, Xia QC, Guo XK, Danchin A, Saint GI, Somerville RL, Wen YM, Shi MH, Chen Z, Xu JG, Zhao GP.** 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature **422:**888-93.

16. **Bush M, Dixon R.** 2012. The role of bacterial enhancer binding proteins as specialized activators of sigma54-dependent transcription. Microbiol Mol Biol Rev **76:**497-529.

17. **Wigneshweraraj S, Bose D, Burrows PC, Joly N, Schumacher J, Rappas M, Pape T, Zhang X, Stockley P, Severinov K, Buck M.** 2008. Modus operandi of the bacterial RNA polymerase containing the sigma54 promoter-specificity factor. Mol Microbiol **68:**538-46.

18. **Francke C, Groot KT, Hagemeijer Y, Overmars L, Sluijter V, Moezelaar R, Siezen RJ.** 2011. Comparative analyses imply that the enigmatic Sigma factor 54 is a central controller of the bacterial exterior. BMC Genomics **12:**385.

19. **Cytryn EJ, Sangurdekar DP, Streeter JG, Franck WL, Chang WS, Stacey G, Emerich DW, Joshi T, Xu D, Sadowsky MJ.** 2007. Transcriptional and physiological responses of

548 *Bradyrhizobium japonicum* to desiccation-induced stress. J Bacteriol **189:**6751-62.
549 20. **Hwang S, Jeon B, Yun J, Ryu S.** 2011. Roles of RpoN in the resistance of *Campylobacter*
550 *jejuni* under various stress conditions. BMC Microbiol **11:**207.
551 21. **Murray GL, Morel V, Cerqueira GM, Croda J, Srikram A, Henry R, Ko AI,**
552 **Dellagostin OA, Bulach DM, Sermswan RW, Adler B, Picardeau M.** 2009. Genome-wide
553 transposon mutagenesis in pathogenic *Leptospira* species. Infect Immun **77:**810-6.
554 22. **Johnson RC, Harris VG.** 1967. Differentiation of pathogenic and saprophytic letospires. I.
555 Growth at low temperatures. J Bacteriol **94:**27-31.
556 23. **Jr Ellinghausen HC, Mccullough WG.** 1965. Nutrition of *Leptospira pomona* and growth
557 of 13 other serotypes: fracionation of oleic albumin complex and a medium of bovine albumin
558 and polysorbate 80. Am J Vet Res **26:**45-51.
559 24. **Eshghi A, Becam J, Lambert A, Sismeiro O, Dillies MA, Jagla B, Wunder EA Jr, Ko**
560 **AI, Coppee JY, Goarant C, Picardeau M.** 2014. A putative regulatory genetic locus modulates
561 virulence in the pathogen *Leptospira interrogans*. Infect Immun **82:**2542-52.
562 25. **Eshghi A, Henderson J, Trent MS, Picardeau M.** 2015. *Leptospira interrogans lpxD*
563 homologue is required for thermal acclimatization and virulence. Infect Immun **83:**4314-21.
564 26. **Bourhy P, Louvel H, Saint GI, Picardeau M.** 2005. Random insertional mutagenesis of
565 *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. J Bacteriol
566 **187:**3255-8.
567 27. **Carrillo-Casas EM, Hernandez-Castro R, Suarez-Guemes F, de la Pena-Moctezuma**
568 **A.** 2008. Selection of the internal control gene for real-time quantitative rt-PCR assays in
569 temperature treated *Leptospira*. Curr Microbiol **56:**539-46.
570 28. **Shang ES, Summers TA, Haake DA.** 1996. Molecular cloning and sequence analysis of
571 the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species.
572 Infect Immun. **64:**2322-30.
573 29. **Heinrich A, Woyda K, Brauburger K, Meiss G, Detsch C, Stulke J, Forchhammer K.**
574 2006. Interaction of the membrane-bound GlnK-AmtB complex with the master regulator of
575 nitrogen metabolism TnrA in *Bacillus subtilis*. J Biol Chem **281:**34909-17.
576 30. **Fisher MA, Grimm D, Henion AK, Elias AF, Stewart PE, Rosa PA, Gherardini FC.**
577 2005. *Borrelia burgdorferi* sigma54 is required for mammalian infection and vector transmission
578 but not for tick colonization. Proc Natl Acad Sci U S A **102:**5162-7.

31. **Jovanovic M, James EH, Burrows PC, Rego FG, Buck M, Schumacher J.** 2011. Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity. Nat Commun **2:**177.

32. **Zhao K, Liu M, Burgess RR.** 2010. Promoter and regulon analysis of nitrogen assimilation factor, sigma54, reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis. Nucleic Acids Res **38:**1273-83.

33. **Vidangos NK, Heideker J, Lyubimov A, Lamers M, Huo Y, Pelton JG, Ton J, Gralla J, Berger J, Wemmer DE.** 2014. DNA recognition by a sigma(54) transcriptional activator from *Aquifex aeolicus*. J Mol Biol **426:**3553-68.

34. **Michiels J, Van Soom T, D'hooghe I, Dombrecht B, Benhassine T, de Wilde P, Vanderleyden J.** 1998. The *Rhizobium etli rpoN* locus: DNA sequence analysis and phenotypical characterization of *rpoN*, *ptsN*, and *ptsA* mutants. J Bacteriol **180:**1729-40.

35. **Reitzer L, Schneider BL.** 2001. Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev **65:**422-44, table of contents.

36. **Reitzer L.** 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. Annu Rev Microbiol **57:**155-76.

37. **Javelle A, Merrick M.** 2005. Complex formation between AmtB and GlnK: an ancestral role in prokaryotic nitrogen control. Biochem Soc Trans **33:**170-2.

38. **Conroy MJ, Durand A, Lupo D, Li XD, Bullough PA, Winkler FK, Merrick M.** 2007. The crystal structure of the *Escherichia coli* AmtB-GlnK complex reveals how GlnK regulates the ammonia channel. Proc Natl Acad Sci U S A **104:**1213-8.

39. **Jack R, De Zamaroczy M, Merrick M.** 1999. The signal transduction protein GlnK is required for NifL-dependent nitrogen control of *nif* gene expression in *Klebsiella pneumoniae*. J Bacteriol **181:**1156-62.

40. **Coutts G, Thomas G, Blakey D, Merrick M.** 2002. Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. EMBO J **21:**536-45.

41. **Drecktrah D, Lybecker M, Popitsch N, Rescheneder P, Hall LS, Samuels DS.** 2015. The *Borrelia burgdorferi* RelA/SpoT homolog and stringent response regulate survival in the tick vector and global gene expression during starvation. PLoS Pathog **11:**e1005160.

42. **Trueba G, Zapata S, Madrid K, Penafiel N.** 2002. Adaptation of *Leptospira interrograns*

(sensu stricto) to fresh water. Rev Cubana Med Trop **54:**11-4.

43. **Andre-Fontaine G, Aviat F, Thorin C.** 2015. Waterborne leptospirosis: survival and preservation of the virulence of pathogenic *Leptospira* spp. in fresh water. Curr Microbiol **71:**136-42.

44. **La Scola B, Bui LT, Baranton G, Khamis A, Raoult D.** 2006. Partial *rpoB* gene sequencing for identification of *Leptospira* species. FEMS Microbiol Lett **263:**142-7.

45. **Redfield AR, Price CW.** 1996. General stress transcription factor sigmaB of *Bacillus subtilis* is a stable protein. J Bacteriol **178:**3668-70.

46. **Boardman BK, He M, Ouyang Z, Xu H, Pang X, Yang XF.** 2008. Essential role of the response regulator Rrp2 in the infectious cycle of *Borrelia burgdorferi*. Infect Immun **76:**3844-53.

47. **Hubner A, Yang X, Nolen DM, Popova TG, Cabello FC, Norgard MV.** 2001. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. Proc Natl Acad Sci U S A **98:**12724-9.

48. **Yang XF, Alani SM, Norgard MV.** 2003. The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*. Proc Natl Acad Sci U S A **100:**11001-6.

49. **Zuerner RL, Huang WM.** 2002. Analysis of a *Leptospira interrogans* locus containing DNA replication genes and a new IS, IS*1502*. FEMS Microbiol Lett **215:**175-82.

## FIGURE LEGENDS

**Figure 1. Predicted RpoN regulatory network in *L. interrogans.*** The *L. interrogans* genome encodes a $\sigma^{54}$ alternative sigma factor, and two bacterial enhancer-binding proteins (EbpA, EbpB) that activate the genes with a $\sigma^{54}$-type promoter. Both EbpA and EbpB are predicted to have three functional domains: an N-terminal domain that receives a distinct signal, a central domain that activates $\sigma^{54}$, and a C-terminal domain that binds to DNA. EbpA contains two putative GAF domains in its receiver domain, whereas EbpB contains a receiver domain (REC) conserved for all the two-component system response regulator proteins. EbpA and EbpB respond to different signals, forms oligomer, interact with RpoN ($\sigma^{54}$), and each activates a specific set of $\sigma^{54}$-dependent genes. RNP: RNA polymerase.

**Figure 2. qRT-PCR analysis of *ebpA* expression.** RNAs were isolated from wild-type (WT), the *ebpA* mutants (*ebpA1*$^{-}$ , *ebpA2*$^{-}$), and the complemented strain (*ebpA*$^{-/+}$) and subjected to qRT-PCR analyses. Levels of *ebpA* expression were reported relative to the level of 16S rRNA in each sample.

**Figure 3. Cell morphology of the *ebpA* mutants and the complemented strain. A:** Morphology of wild type and ebpA1$^{-}$ grown in EMJH medium examined under a dark-field microscope. Images were taken at a magnification of 500X. **B:** Quantitation of cell length. A total of 200 randomly selected cells from each strain were measured. WT: 10.99 μm ± 0.3118,

651 ebpA1$^{-}$ : 7.365 μm ± 0.1782, ebpA2$^{-}$ 7.566 μm ± 0.1631, ebpA$^{-/+}$: 10.47 μm ± 0.2129. WT vs.
652 either ebpA1$^{-}$ or ebpA2$^{-}$ : $P < 0.01$; ebpA$^{-/+}$ vs. ebpA1$^{-}$: $P < 0.01$.

653

654 **Figure 4. Identification of genes regulated by EbpA. A:** qRT-PCR assessment of expression
655 of putative RpoN-regulated genes. **B:** qRT-PCR analyses to confirm EbpA-dependent expression
656 of *LMANv2_200027, LMANv2_290065* and *LMANv2_310003/amtB.* For expression of all three
657 genes: WT vs. ebpA1$^{-}$ or ebpA2$^{-}$: $P < 0.01$; ebpA$^{-/+}$ vs. ebpA1$^{-}$ : $P < 0.01$; WT vs. ebpA$^{-/+}$: $P <$
658 0.01.

659

660 **Figure 5. EMSA analysis for identification of RpoN (sigma$^{54}$)-type promoter. A**: RpoN
661 binding to the promoter region of LMANv2_200027, LMANv2_290065 or *amtB*. Various
662 concentrations of rRpoN (labeled on the top, in nM) were incubated with DNA fragments
663 containing the promoter region of LMANv2_200027, LMANv2_290065 or *amtB* (labeled at the
664 bottom). Probe names are indicated on the left at the position of unbound DNA, while bound
665 DNA is denoted by arrows. **B:** Specificity of rRpoN binding to the promoter. MRN027,
666 MRN065 and MRN003 are the probes with mutation at the predicted sigma54 sequence region
667 (labeled at the bottom, the far right lane in each gel). Exclusion (-) or inclusion of 10, 50, and
668 100-fold unlabeled competitor DNA (labeled as ×10, x50, and x100, respectively), or absence (-)
669 or presence of 60 nM (+) of BSA are indicated on top of each lane. Probe names are labeled
670 below the images. Bound DNA is denoted by arrows.

671

672

673 **Figure 6: EMSA analysis for identification of EbpA-regulated genes. A**: EbpA bindings to
674 the upstream region of *LMANv2_200027, LMANv2_290065* and *amtB.* Various concentrations of
675 rEbpA (labeled on the top, in nM) were incubated with DNA fragments from upstream region of
676 *LMANv2_200027, LMANv2_290065* or *amtB*. Probe names are indicated on the left at the
677 position of unbound DNA, while bound DNA is denoted by arrows. **B**: Specificity of rEbpA
678 binding to the upstream region of *LMANv2_200027, LMANv2_290065* and *amtB.* ). Exclusion (-)
679 or inclusion of 50 or 100-fold unlabeled competitor DNA (labeled as x50 and x100, respectively),
680 absence (-) or presence of 60 nM (+) of BSA, absence (-) or presence of 100-fold (+) of sheared
681 salmon sperm (SS) DNA, are indicated on top of each lane. **C**: Ability of rRpoN and rEbpA to
682 bind to the promoter region of *lipL41*. Probe L41 (top panel) contains a sigma70-type *lip41*
683 promoter. Probe RL41 (bottom panel) contains the *lipL41* promoter region that the promoter
684 sequence was replaced with the sigma54 promoter sequence.

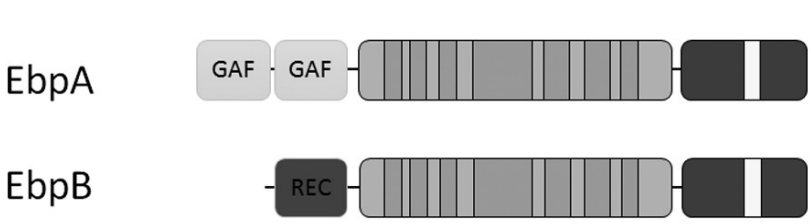
685

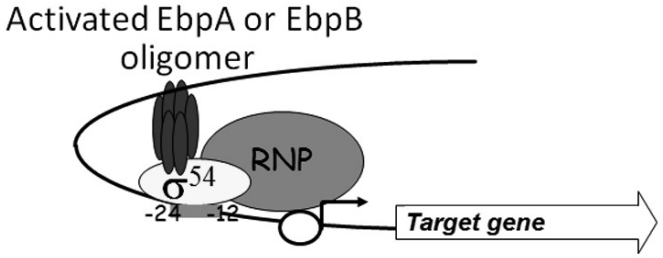
686 **Figure 7. Growth of the *ebpA* mutant in various conditions.** The wild type *L. interrogans*
687 strain, the *ebpA* mutant and its complementary strain were grown to late-log phase ($10^7$/ml) and
688 then subcultured into regular EMJH (**A**), EMJH with 5 mM alanine replacing $NH_4Cl$ (**B**), or 1:10
689 diluted regulated EMJH (**C**). For the water survival test (**D**), *L. interrogans* wild type, the *ebpA*
690 mutant and its complementary strains were harvested by centrifugation and re-suspended in
691 mineral water. The dotted line in D is the limit of detection. All cultures were incubated at 30°C,
692 and the motile leptospires were counted directly under dark-field microscopy. Error bars indicate
693 standard error from triplicate cultures. * represents statistical differences between the wild-type

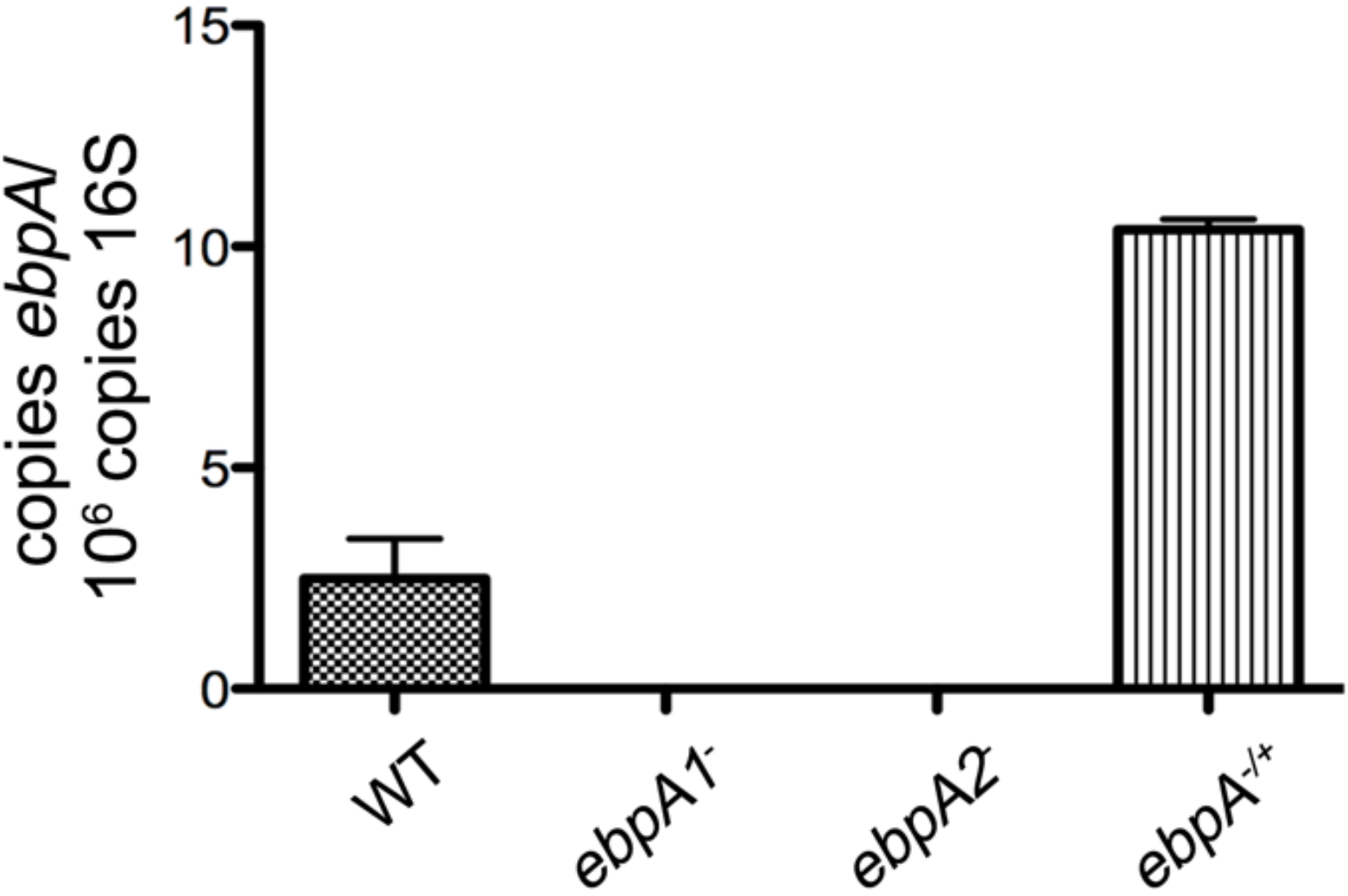
694 and the *ebpA* mutants with a $P < 0.001$ using the Student *t* test.

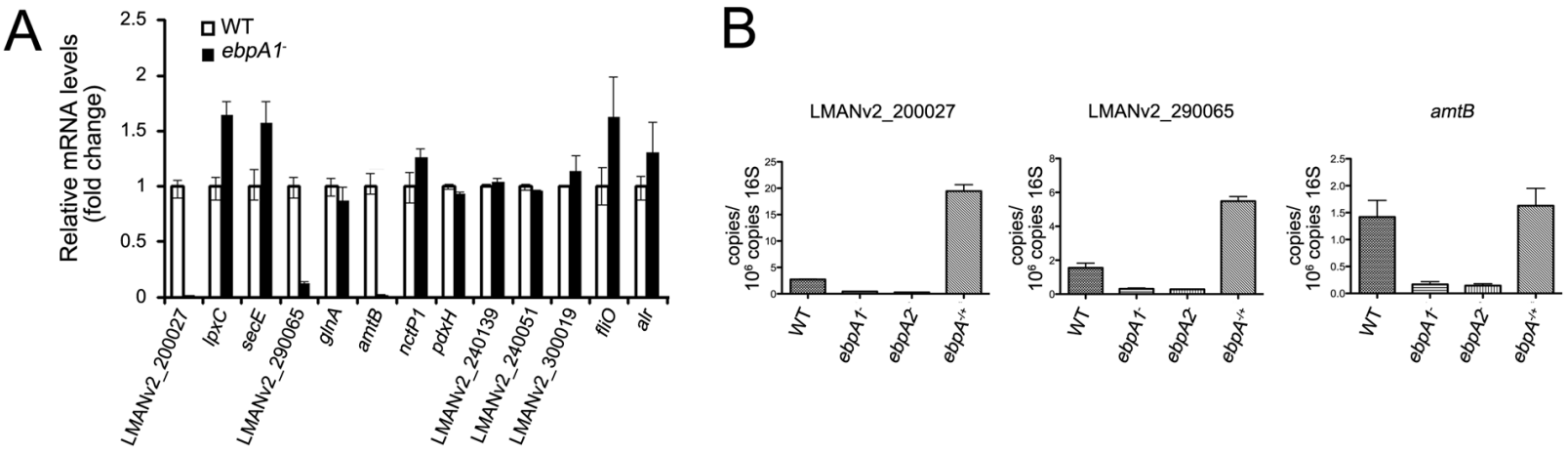
695

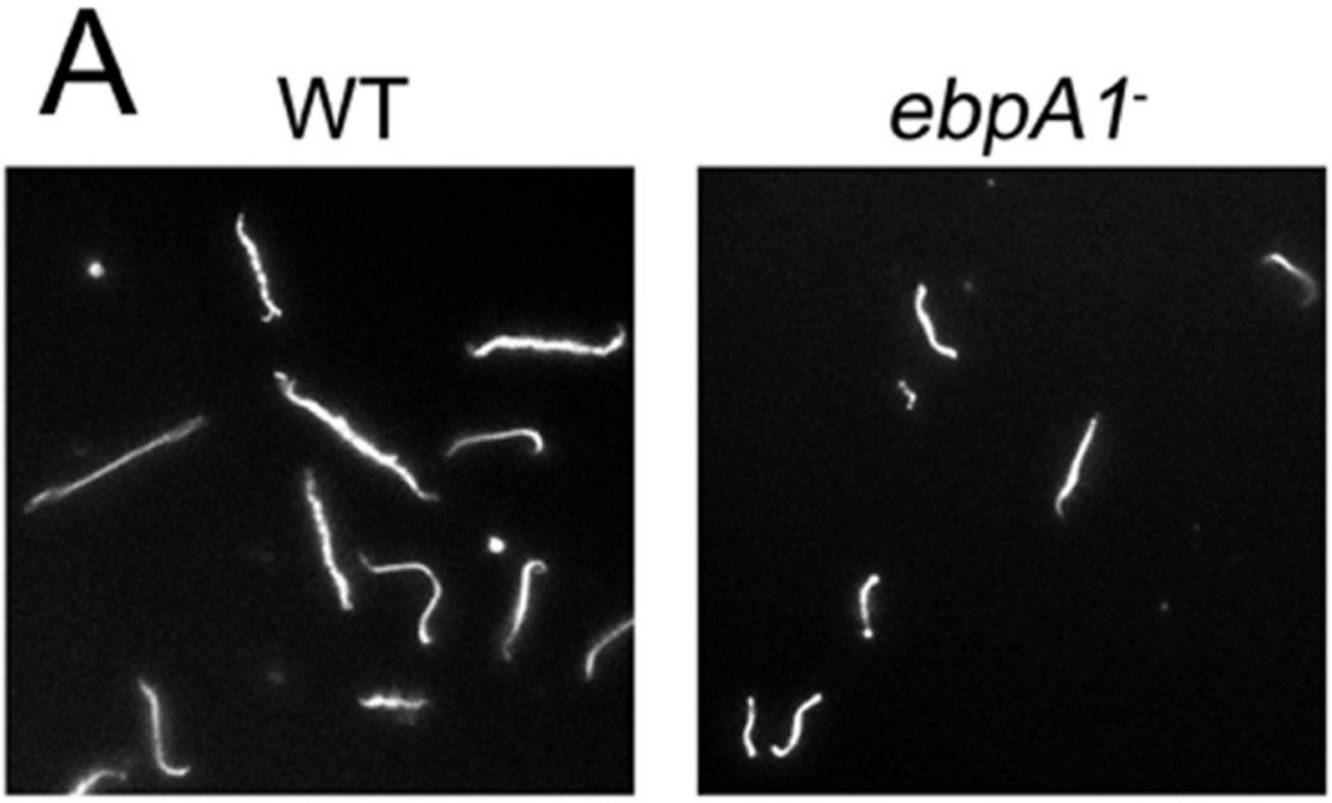
696 **Figure 8. EbpA is dispensable for mammalian infection.** Four 3-week-old male Mongolian
697 gerbils per group were intraperitoneally inoculated with $10^4$ cells of either WT or *ebpA2*$^-$. Each
698 group contained four gerbils, and the infectivity of each strain was reported as percentage of
699 animal survival.

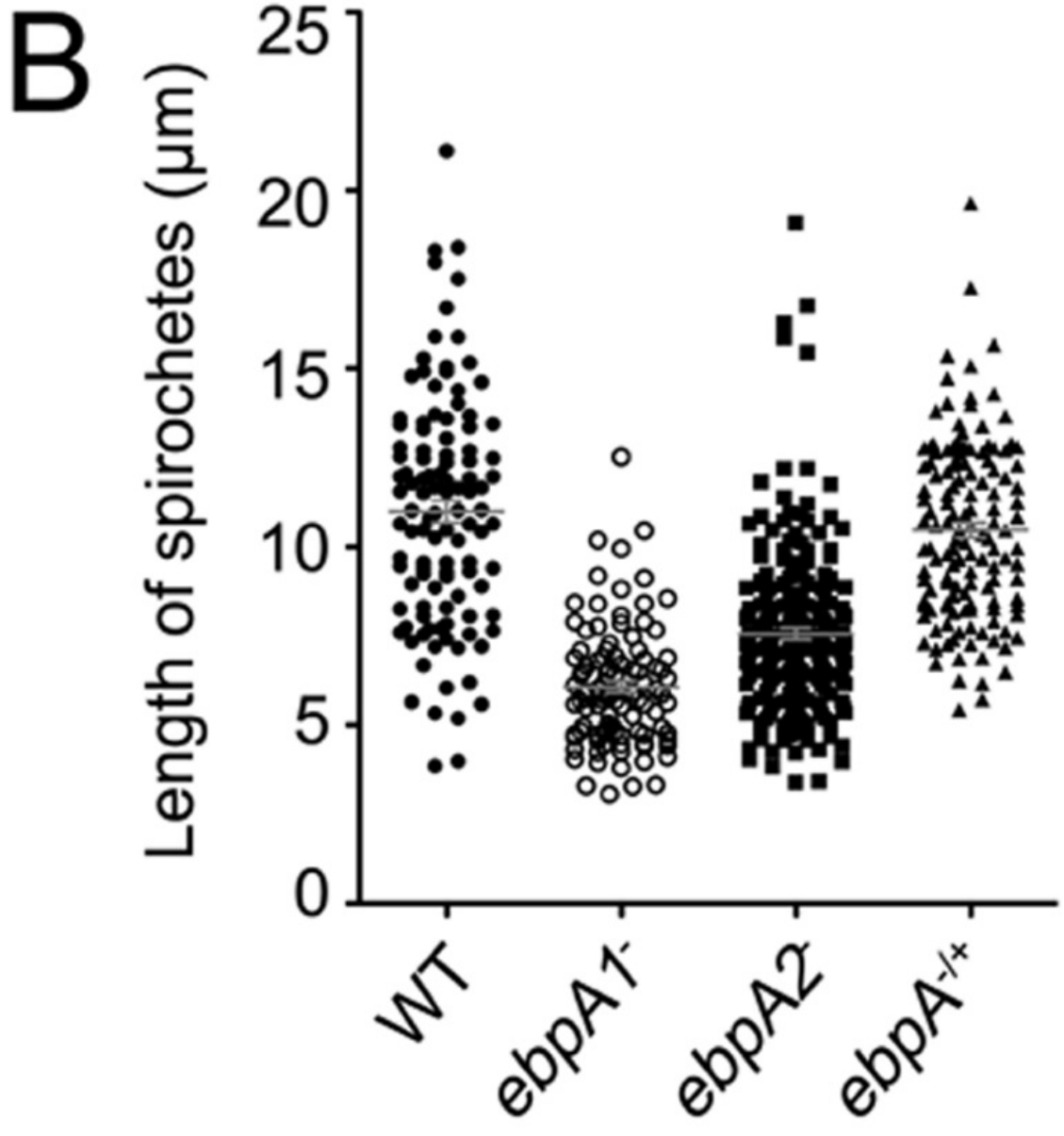

EbpA
GAF
GAF
EbpB
REC

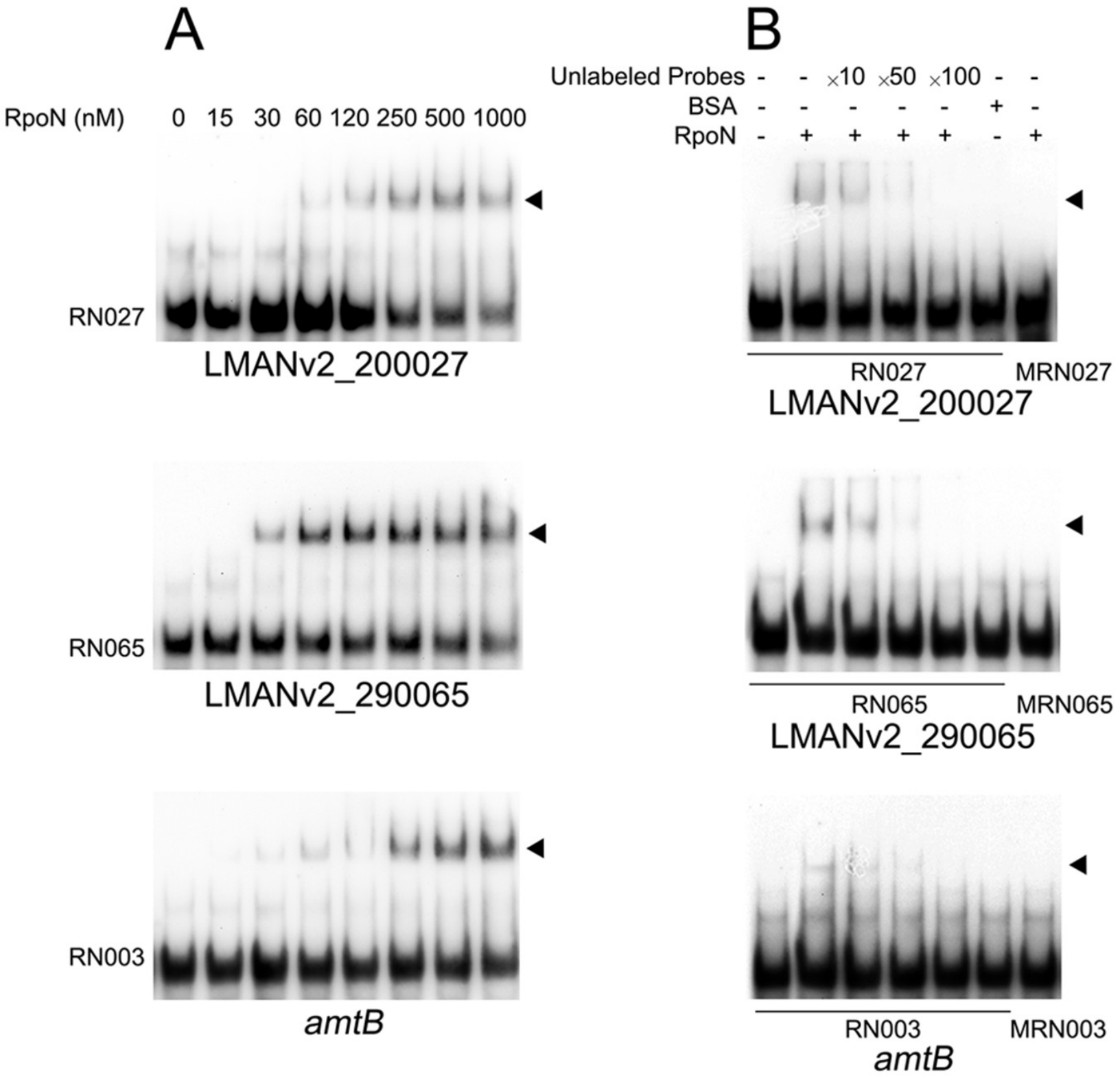

Activated EbpA or EbpB
oligomer
RNP
σ54
-24
-12
Target gene

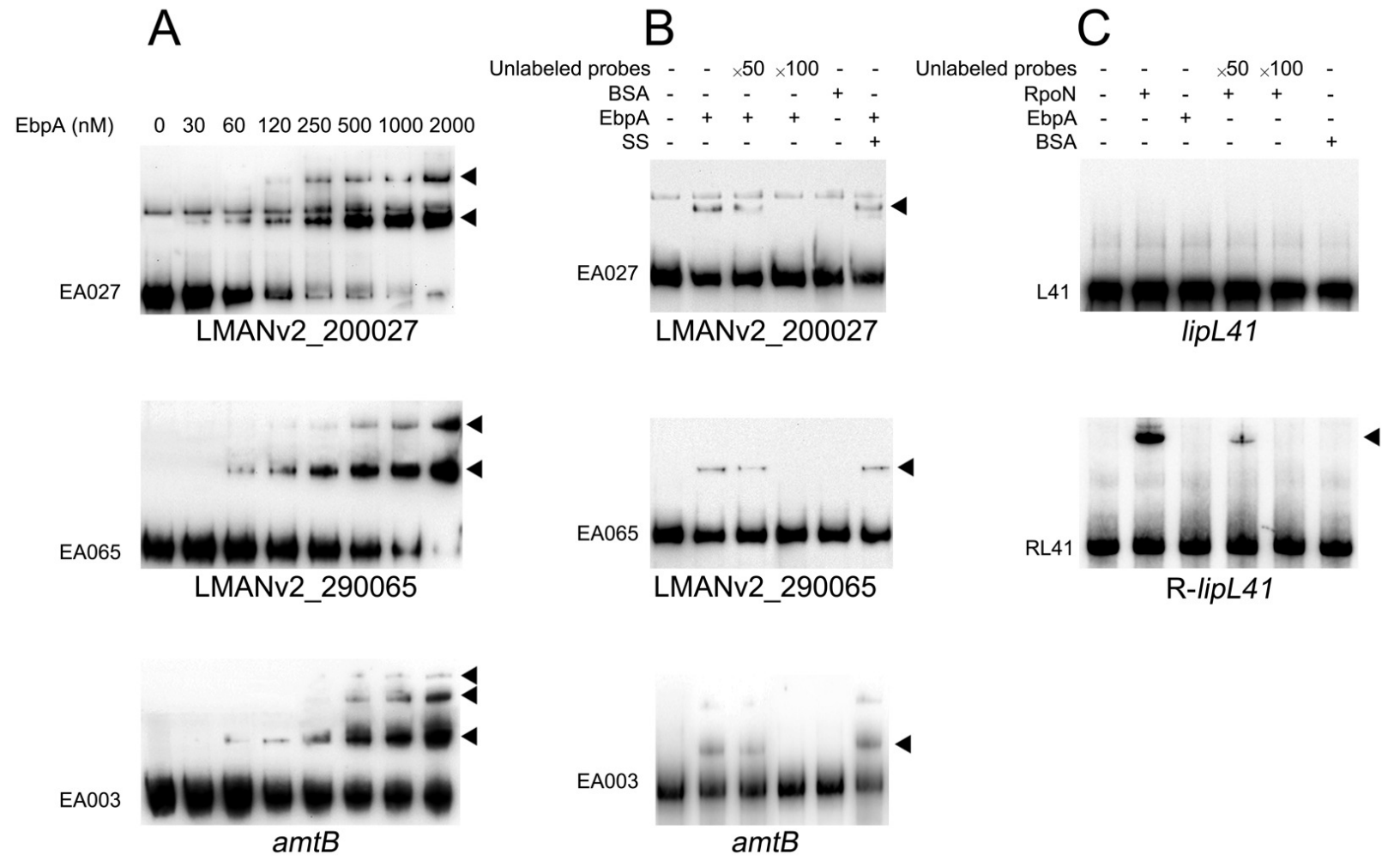

15
10
5
0
copies ebpA/
10^6 copies 16S
WT
ebpA1-
ebpA2-
ebpA-/+

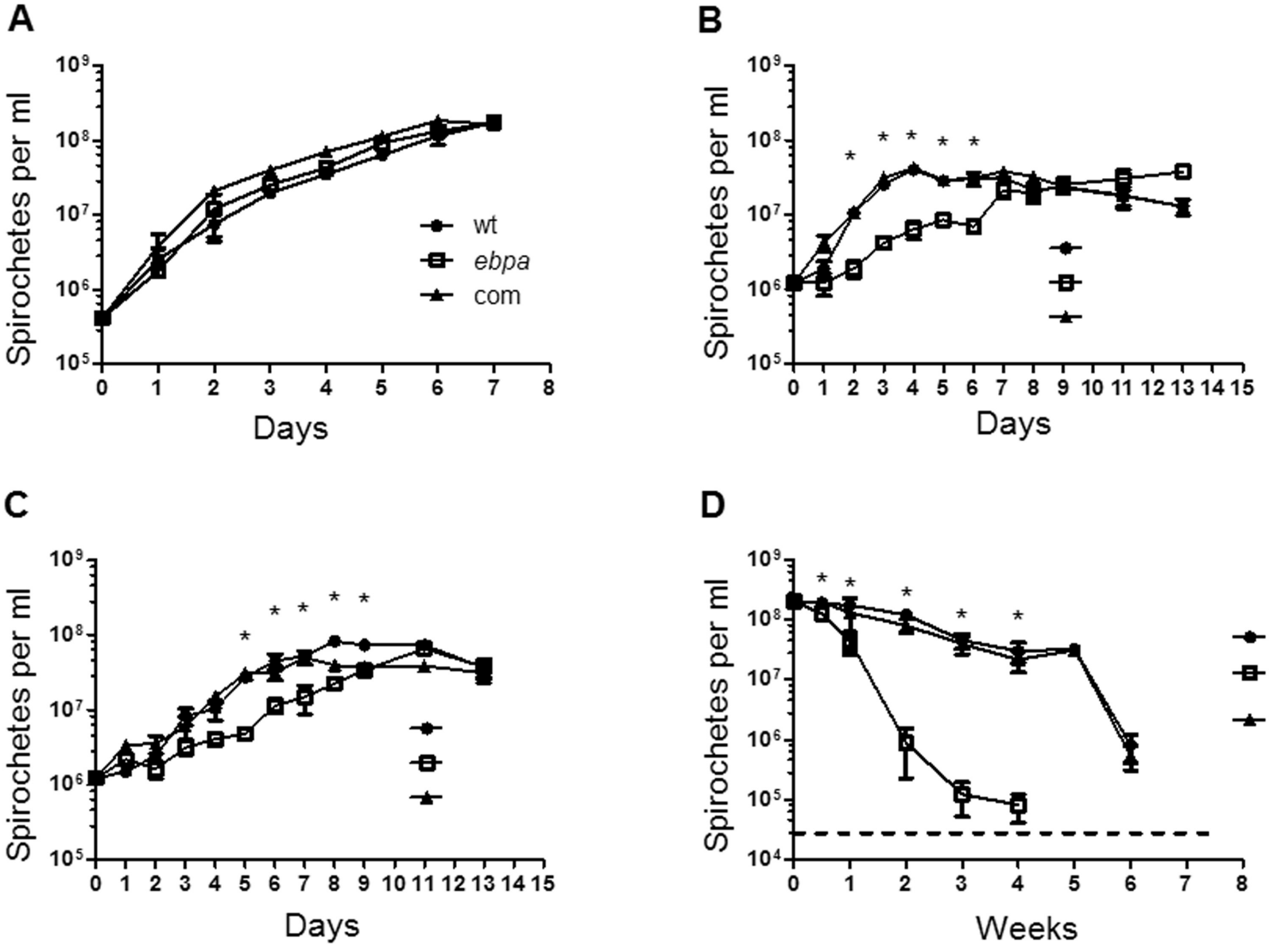

A
WT
ebpA1-
Relative mRNA levels
(fold change)
2.5
2
1.5
1
0.5
0
LMANv2_200027
lpxC
secE
LMANv2_290065
glnA
amtB
nctP1
pdxH
LMANv2_240139
LMANv2_240051
LMANv2_300019
fliO
alr
B
LMANv2_200027
LMANv2_290065
amtB
copies/
10^6 copies 16S
WT
ebpA1-
ebpA2
ebpA+/+

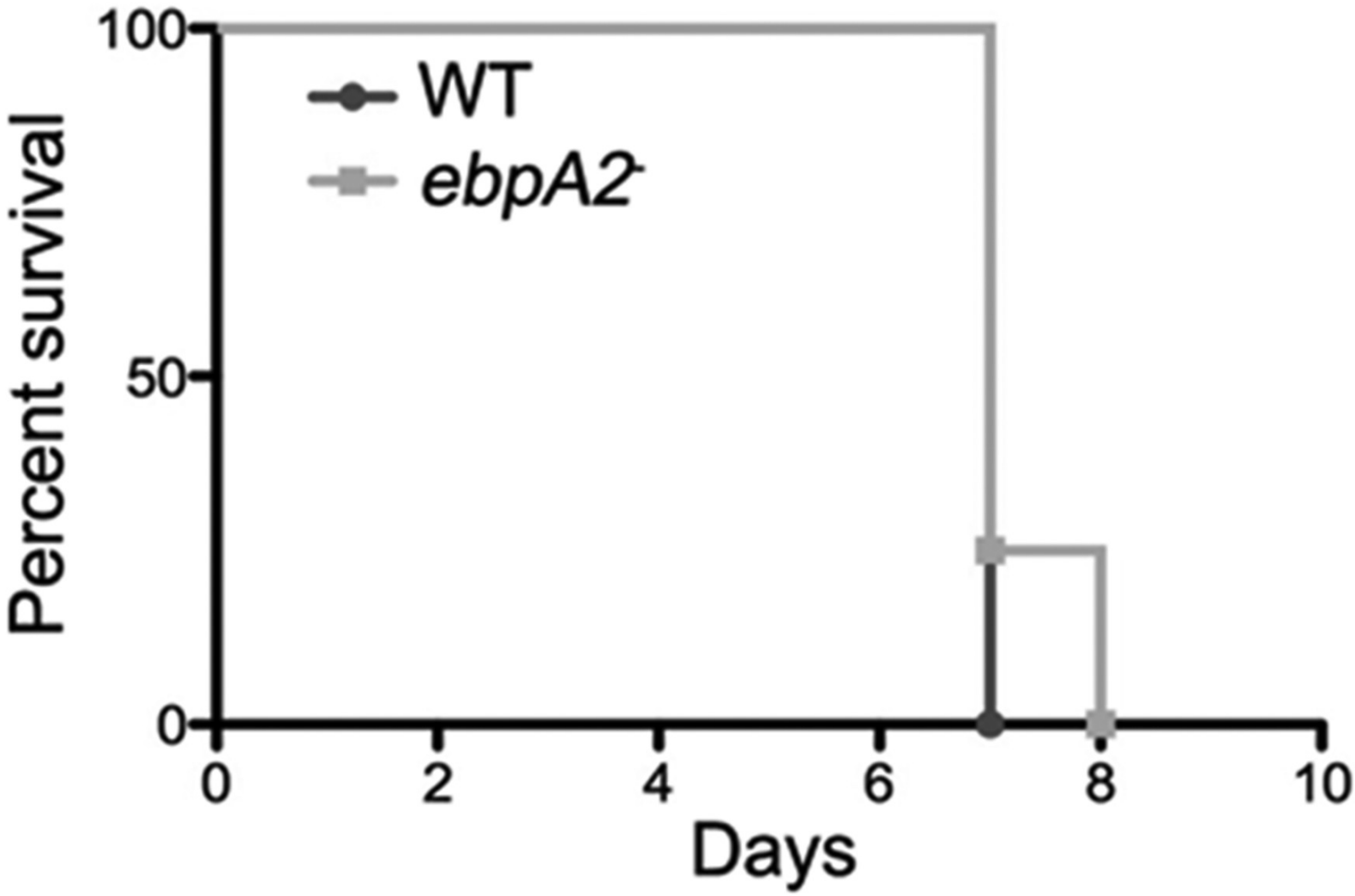

A
WT
ebpA1-


B
Length of spirochetes (μm)
25
20
15
10
5
0
WT
ebpA1-
ebpA2-
ebpA-/+


A
RpoN (nM) 0 15 30 60 120 250 500 1000
RN027
LMANv2_200027
RN065
LMANv2_290065
RN003
amtB
B
Unlabeled Probes - - ×10 ×50 ×100 - -
BSA - - - - - + -
RpoN - + + + + - +
RN027
MRN027
LMANv2_200027
RN065
MRN065
LMANv2_290065
RN003
MRN003
amtB


A
EbpA (nM) 0 30 60 120 250 500 1000 2000
EA027
LMANv2_200027
EA065
LMANv2_290065
EA003
amtB
B
Unlabeled probes - - ×50 ×100 - -
BSA - - - - + -
EbpA - + + + - +
SS - - - - - +
EA027
LMANv2_200027
EA065
LMANv2_290065
EA003
amtB
C
Unlabeled probes - - - ×50 ×100 -
RpoN - + - + + -
EbpA - - + - - -
BSA - - - - - +
L41
lipL41
RL41
R-lipL41


A
B
C
D
Spirochetes per ml
Days
Weeks
wt
ebpa
com


100
50
0
Percent survival
WT
ebpA2-
0
2
4
6
8
10
Days