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

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19

ABSTRACT

20

21 *Leptospira interrogans* is the agent of leptospirosis, a re-emerging zoonotic disease.

22 Leptospirosis is transmitted to humans through environmental surface waters contaminated

23 by the urine of mammals that are chronically infected by pathogenic strains that are able to

24 survive in water for long periods. Little is known about the regulatory pathways underlying

25 environmental sensing and host adaptation of *L. interrogans* during its enzootic cycle. This

26 study identifies the EbpA-RpoN regulatory pathway in *L. interrogans*. In this pathway,

27 EbpA, a σ^{54} activator and putative prokaryotic enhancer-binding protein (EBP), and the

28 alternative sigma factor RpoN (σ^{54}) control expression of at least three genes – encoding

29 AmtB (an ammonium transport protein) and two proteins of unknown function.

30 Electrophoresis mobility shift assay (EMSA) demonstrated that recombinant RpoN and EbpA

31 bind to the promoter region and upstream of these three identified genes, respectively.

32 Genetic disruption of *ebpA* in *L. interrogans* serovar Manilae virtually abolished expression

33 of the three genes, including *amtB* in two independent *ebpA* mutants. Complementation of the

34 *ebpA* mutant restored expression of these genes. Intra-peritoneal inoculation of gerbils with

35 the *ebpA* mutant did not affect mortality. However, the *ebpA* mutant had decreased cell length

36 *in vitro* and had a significantly lowered cell density at stationary phase when grown with

37 L-alanine as the sole nitrogen source. Furthermore, the *ebpA* mutant has dramatically reduced

38 long-term survival ability in water. Together, these studies identify a regulatory pathway, the

39 EbpA-RpoN pathway, that plays an important role in the zoonotic cycle of *L. interrogans*.

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IMPORTANCE

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

50

INTRODUCTION

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52

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

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

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

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

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

92 σ^{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 σ^{54} activator (LMANv2_37011,
100 hereafter referred to as EbpA), σ^{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.

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MATERIALS AND METHODS

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

114 **Construction of *ebpA* mutants and the complemented strain.** *ebpA* was previously
115 disrupted in wild type *L. interrogans* serovar Manilae strain L495 by the HimarI random
116 transposon system (21). Transposon insertion of *ebpA* in *ebpA1*⁻ occurred at NT 3149215 in
117 the proximal region of *ebpA*. Transposon insertion of *ebpA* in *ebpA2*⁻ occurred at NT
118 3148873 in the proximal region of *ebpA*. The native *ebpA* allele along with its native
119 promoter region (including 300 bp upstream of +1) from *L. interrogans* serovar Manilae
120 strain L495 was PCR amplified with XhoI 5' and 3' overhangs using primers *ebpAx1* &
121 *ebpAx2* (**Table 1**) and T-A ligated into pCRII-TOPO plasmid (Life Technologies, Waltham,
122 MA). The resulting plasmid was transformed into XL-10 competent cells according to the
123 manufacturer's instructions (Stratagene, La Jolla CA). This plasmid was re-named
124 'pCRII-TOPO-*ebpA*'. XL-10 cells containing pCRII-TOPO-*ebpA* were grown in 5 ml of LB
125 broth with 50 µg/ml of kanamycin (Sigma) and 50 µg/ml ampicillin (Euromedex) to late log
126 phase (10⁷/ml), followed by plasmid extraction by QIAprep Spin Miniprep Kit (Qiagen). The

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

140 **RNA isolation and qRT-PCR.** For the initial qRT-PCR analysis of wild type and *ebpA1*⁻
141 samples by relative quantity, leptospire were grown in EMJH at 28°C and harvested when
142 bacterial growth reached late logarithmic phase (10⁷/ml). Total RNA was isolated using
143 Trizol according to the manufacturer's instructions (Invitrogen) and genomic DNA was
144 digested using RNase-free DNase I (Promega). RNA was then further purified using RNeasy
145 Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript III reverse
146 transcriptase with random primers (Invitrogen, Carlsbad, CA). Using the cDNAs as the
147 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 $\Delta\Delta C_t$ 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 μ g of triple-DNase-treated RNA with
155 the iScript cDNA synthesis kit as per the manufacturer's instructions (Bio-Rad). 2 μ l 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 C_q threshold, and absolute quantity (ΔC_t) was
163 determined by comparing copy number of each gene to copy number of *I6S*. 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 *I6S* 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*

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

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

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

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

211 mM MgCl₂ 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 (10⁷/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 NH₄Cl. 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 leptospire
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 (10⁷/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

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

236 **Determination of the contribution of EbpA to virulence in Mongolian gerbils.**

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

245

RESULTS

246

247

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

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

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

274

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

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

288 unknown function) at NT 4079032. qRT-PCR analyses demonstrated that the two *ebpA*
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 ($7.365 \mu\text{m} \pm 0.1782 \mu\text{m}$, $P < 0.01$ and $7.566 \mu\text{m} \pm 0.1631 \mu\text{m}$, $P < 0.01$) than the wild
297 type ($10.99 \mu\text{m} \pm 0.3118 \mu\text{m}$) when grown to mid-to-late logarithmic phase.
298 Complementation of *ebpA1* restored the cell length in *ebpA*^{-/+} ($10.47 \mu\text{m} \pm 0.2129 \mu\text{m}$,
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

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

316

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

331 Since *L. interrogans* has two predicted EBPs, a gene with an RpoN (σ^{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 *LMANv2_200027*, or *LMANv2_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

350 **The *ebpA* mutant had reduced growth and survival under various conditions.** *amtB*,
351 predicted to encode an ammonia transporter protein (PSORT-B, TMhmm), is putatively
352 co-transcribed with *glnK*, which encodes a nitrogen regulatory protein in *L. interrogans* (5,
353 29). We therefore examined the growth of the *ebpA* mutant in EMJH liquid media with
354 alternative nitrogen sources. There was no significant difference in growth observed between
355 wild-type and *ebpA1*⁻ grown in standard EMJH media with 5 mM ammonium chloride (**Fig.**
356 **7A**) (22, 23). However, when 5 mM L-alanine, a poor nitrogen source, was supplemented in
357 nitrogen-free EMJH media, the *ebpA1*⁻ mutant had significantly reduced growth compared to
358 the wild type (**Fig. 7B**).

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

369

370 ***ebpA* is dispensable for virulence.** To determine if *ebpA* plays a role in virulence, 10^4 cells
371 of either wild type or *ebpA2* were inoculated into gerbils, and the gerbils were observed for
372 21 days. Data indicate that the abrogation of the *ebpA* gene does not reduce mortality in
373 gerbils (**Fig. 8**). Similar results were found between wild type and *ebpA1* when 10^6 cells were
374 inoculated intraperitoneally (data not shown). Viable spirochetes for all of the strains
375 inoculated were recovered from renal tissue and blood. These results suggest that EbpA is not
376 essential for mammalian infection.

377

378

DISCUSSION

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

387 RpoN has been shown to be involved in regulation of many bacterial functions such
388 as nitrogen metabolism, flagellar biosynthesis, osmotolerance, pH changes, biofilm
389 formation, motility, colonization, lipoprotein biosynthesis and a Type III secretion system (18,
390 30-32). Bioinformatics analysis predicted 13 putative RpoN (σ^{54})-type promoters controlling
391 22 genes in *L. interrogans*. Using the *ebpA* mutant and EMSA assay, we demonstrated that at
392 least three of these genes *amtB*, *LMANv2_200027*, and *LMANv2_290065*, are under the
393 control of EbpA. Other predicted RpoN-dependent genes could be controlled by EbpB.
394 Interestingly, 3 of these genes have been demonstrated to be under the control of RpoN in
395 other bacteria: *lpxC*, which is responsible for the first committed step in lipid A biosynthesis
396 regulated by RpoN in *Aquifex aeolicus* (33); *pstA*, encoding phosphoenolpyruvate-protein
397 phosphotransferase, which participates in regulation of the σ^{54} -dependent promoter in
398 *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 leptospire, 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, leptospire 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 *ebpA-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 leptospire, 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 findings 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., σ^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.

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476

477

478 **TABLE 1. Primers used in this study.**
 479

Gene	Primer name	sequence (5' -> 3')#	size	purpose
<i>RpoN</i> (<i>LMANv2_5</i> <i>90036</i>)	pLATE52-rpoN F	GGTTGGGAATTGCAAATGAATCTAAGT CAGTCAC	1419	rpoN gene expression
	pLATE52-rpoN R	GGAGATGGGAAGTCATTATCTAGACTC CAAAGAC		
<i>16S</i> (<i>IArRNA306</i> <i>4233R</i>)	plasmid-16s F	GCGGCGAACGGGTGAGTAACACGTGG G	968	Generation of plasmid for standard curve
	plasmid-16s R	CATCTCACGACACGAGCTGACGACAAC C		
	qRT-16sRNA F	GGCGGACATGTAAGTCAGGT	179	qRT-PCR primer set
	qRT-16sRNA R	GCCTAGCGTCAGTTTTAGG		
<i>ebpA</i> (<i>LMANv2_3</i> <i>70110</i>)	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 compleme ntation
	ebpA-x2	CCGCTCGAGCGTTCACACAATCTTCTC		
<i>LMANv2_20</i> <i>0027</i>	pGEX-4T-2-ebpA F	CGCGGATCCATGTCAGGATATGTGAAG ATAAGAATGCGGCCGCATAATCGATT T	2097	ebpA gene expression
	pGEX-4T-2-ebpA R			
	plasmid-200027 F	CAAGTTTGCGGCAATTGCTCTTTTTTTA AGC	495	Generation of plasmid for standard curve
	plasmid-200027 R	CGGGTCAAATCAGTTAGAAGTAGAGAT GGG		

	qRT-LMANv2_2000 27 F	AACCAATCCCTTCATGTTTCG		
	qRT-LMANv2_2000 27 R	AGCTTCTTCTTCCGCATTGA	195	qRT-PCR primer set
	RN027 F	GAATCTTTATAAAATTT TGGCACGCTGC TTGCATATATCTATAACAAGAAC		EMSA probes for
	RN027 R	TTCTTGATAGATATAT GCAAGCAGC GTGCCAAAATTTATAAAGATTCT	49	rRpoN, 3' biotin labeled
	MRN027 F	GAATCTTTATAAAATTT aaaatCGCTGCaat tATATATCTATAACAAGAAC		EMSA probes for
	MRN027 R	TTCTTGATAGATATAT aattGCAGCGatt ttAAAATTTATAAAGATTCT	49	rRpoN, 3' biotin labeled
	FA027 F	TATCTTGCTTCGAAAGTTACC		EMSA probes for
	FA027 R	AGTCCGGTAAAAATGCTTAAA	500	rEbpA, 5' biotin labeled
	LMANv2_290065 F	CGTCACAACCTGCTCCTTTCA		Generation of plasmid for
	LMANv2_290065 R	GCCATTGCTGCTTAGAAGG	216	standard curve and qRT-PCR primer set
LMANv2_29 0065	RN065 F	TCTAAATTTTAAACTTT TGGCATATATT TTGCATAGTCTGAGCATGGAGA		EMSA probes for
	RN065 R	CTCCATGCTCAGACTAT GCAAAATATA TGCCAAAGTTTAAAATTTAGAA	49	rRpoN, 3' biotin labeled
	MRN065 F	TCTAAATTTTAAACTT aaaatTATATTaatt ATAGTCTGAGCATGGAGA		EMSA probes for
	MRN065 R	CTCCATGCTCAGACTAT aattAATATAaatt ttAAGTTTAAAATTTAGAA	49	rRpoN, 3' biotin labeled

	FA065 F	GGAACTCATACTTTTAGAAAA		EMSA
	FA065 R	ACCAGCAGACAAAAACAGAA	500	probes for rEbpA, 5' biotin labeled
	plasmid-310003 F	GGGACTTCTTGTAAGTCCGGTATGCTA CGC		Generation of plasmid
	plasmid 310003 R	CTTGGTATTCGTGGCCTCTGAAAACCTT CCG	538	for standard curve
	qRT-LMANv2_3100 03 F	GTTAGGCGCGTTCTTACAG		qRT-PCR
	qRT-LMANv2_3100 03 R	TCTCCGTGAATTTCCGAATC	219	primer set
<i>amtB</i> (<i>LMANv2_3</i> <i>10003</i>)	RN003 F	ATTATTCTTCAATTGGTACTAATCTTG CAAACAAAGAATAAAAATTGAT		EMSA
	RN003 R	TCAATTTTTATTCTTTGTTTGCAAGATT AGTACCAATTGAAGAATAATT	49	probes for rRpoN, 3' biotin labeled
	MRN003 F	ATTATTCTTCAAT aaaatCTAATCaattAA ACAAAGAATAAAAATTGAT		EMSA
	MRN003 R	TCAATTTTTATTCTTTGTT TaattGATTA GattttATTGAAGAATAATT	49	probes for rRpoN, 3' biotin labeled
	FA003 F	TATATTTTATAAGCATACTTT		EMSA
	FA003 R	GTGCTAGCTTCTGCCCAAAT	500	probes for rEbpA, 5' biotin labeled
<i>lipL41</i>	L41/RL41 F	TTGTAATTCAGTATCTTG		EMSA
	L41/RL41 R	AAGAACATAAGGAGAACT	221	probes for rRpoN or rEbpA, 5' biotin labeled
<i>raiA</i>	q-LMANv2_200026	CCGCTGAAAAATACGCAAAT	193	qRT-PCR

	F			primer set
(<i>LMANv2_2</i> <i>00026</i>)	q-LMANv2_200026 R	TCTTCCAGACCGTCGATACA		
<i>lpxC</i>	q-LMANv2_430009 F	CGGCAGAACTCAGCAATGTA	162	qRT-PCR primer set
(<i>LMANv2_4</i> <i>30009</i>)	q-LMANv2_200026 R	CGTCCATGATCGGAACTTCT		
<i>secE</i>	q-LMANv2_260002 F	GCAGAAATTGAAAAGGTCCA	189	qRT-PCR primer set
(<i>LMANv2_2</i> <i>60002 F</i>)	q-LMANv2_260002 R	CTAACCAGCCACTTCCCAAA		
<i>glnA</i>	q-LMANv2_500019 F	TTGCGGATACCGCTTATTTTC	170	qRT-PCR primer set
(<i>LMANv2_5</i> <i>00019</i>)	q-LMANv2_500019 R	AGTACCAGGACGGTGACCAG		
<i>glnK</i>	q-LMANv2_310004 F	AAGCGACGTTCAAGGTTACG	249	qRT-PCR primer set
(<i>LMANv2_3</i> <i>10004</i>)	q-LMANv2_310004 R	GCGGAACTTCCTCTTTCTCC		
<i>nctP1</i>	q-LMANv2_210009 F	TCCCGCAAATTTGAAAAGAC	208	qRT-PCR primer set
(<i>LMANv2_2</i> <i>10009</i>)	q-LMANv2_210009 R	TCGACGTTCTTTGTGGATGA		
<i>pdxH</i>	q-LMANv2_210047 F	GTTAGCCTCACCGCAAAGTC	178	qRT-PCR primer set
(<i>LMANv2_2</i> <i>10047</i>)	q-LMANv2_210047 R	ATCGTGTAACGGCTGGAAC		
<i>LMANv2_24</i> <i>0139</i>	q-LMANv2_240139 F	TTCCGGTCCAGAGACTTTTG	250	qRT-PCR primer set
	q-LMANv2_240139 R	GATCGAAACGTTTTCCGAGA		
<i>LMANv2_24</i> <i>0051</i>	q-LMANv2_240051 F	TTTGTTTGCCCGGATAAAAG	151	qRT-PCR primer set
	q-LMANv2_240051 R	TTTTCCGAAATCGATCCAAG		

<i>LMANv2_30019</i>	q-LMANv2_300019	TTCGTGTTTGATCGTTTCCA	248	qRT-PCR primer set
	F			
<i>0019</i>	q-LMANv2_300019	CCTTTCCGGCAAAAAGTAGA		
	R			
<i>fliO</i>	q-LMANv2_150090	GGAATCGCAGGGACTCTTCT	217	qRT-PCR primer set
	F			
<i>(LMANv2_150090)</i>	q-LMANv2_150090	CATTGTCAGCAACTCCGAGA		
	R			
<i>LMANv2_90055</i>	q-LMANv2_90055	TGTATTGAAGCGGGGATTTC	184	qRT-PCR primer set
	F			
<i>055</i>	q-LMANv2_90055	TACGAGCTGTTCCGGTCTT		
	R			

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.

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

485
 486

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

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

487

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629

FIGURE LEGENDS

630

631

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

641

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

646

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

651 $ebpA1^-$: $7.365 \mu\text{m} \pm 0.1782$, $ebpA2^-$: $7.566 \mu\text{m} \pm 0.1631$, $ebpA^{-/+}$: $10.47 \mu\text{m} \pm 0.2129$. 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 (σ^{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 σ^{54} 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 $\times 10$, $\times 50$, and $\times 100$, 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 leptospire 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.

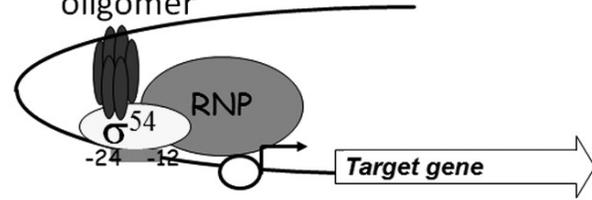
EbpA

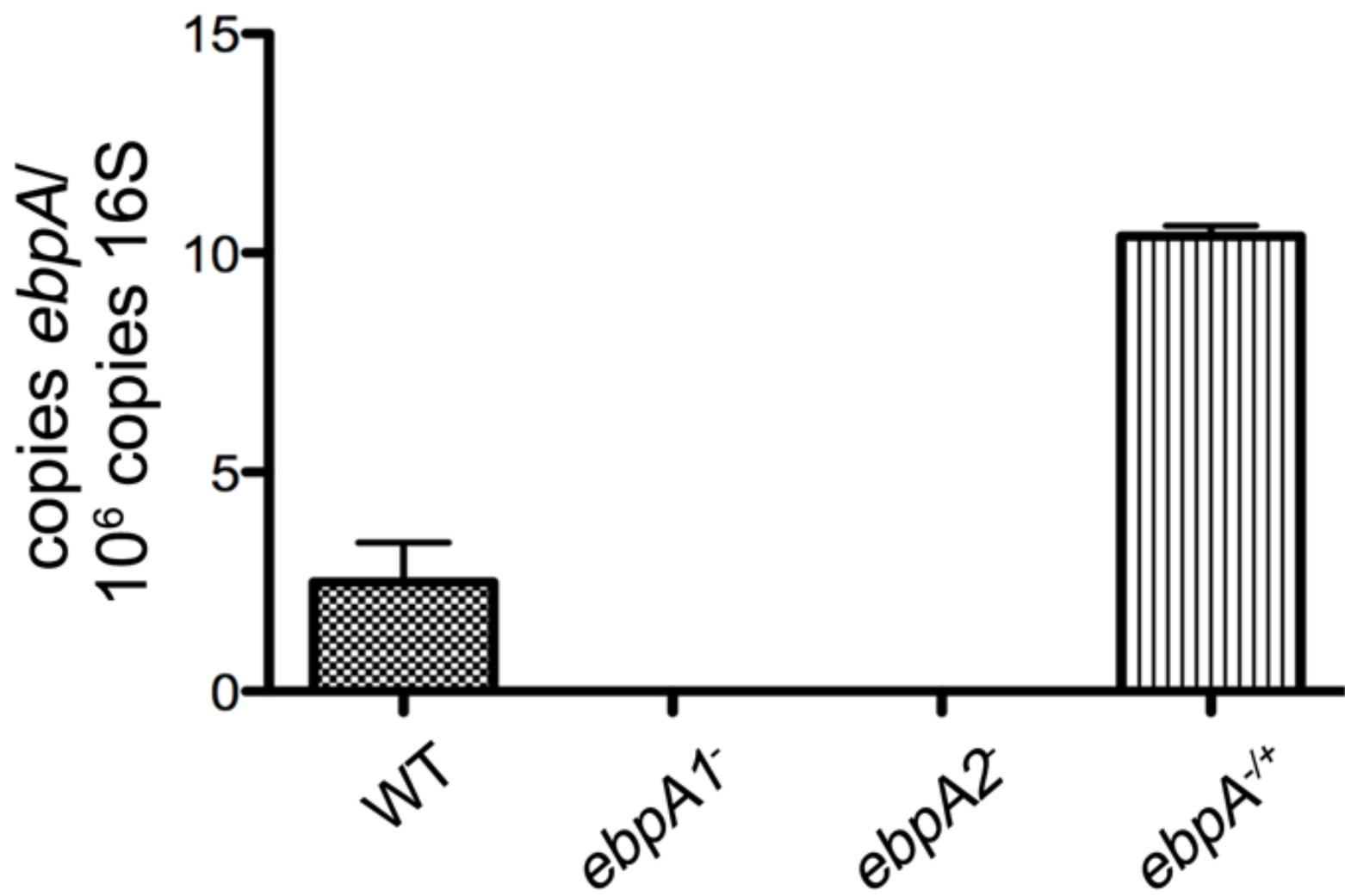


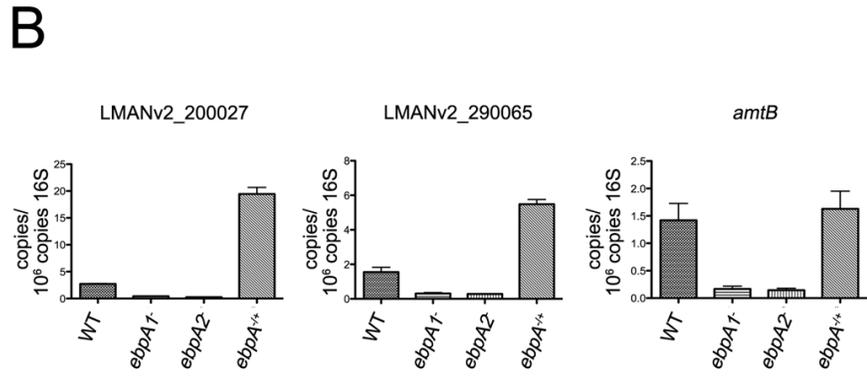
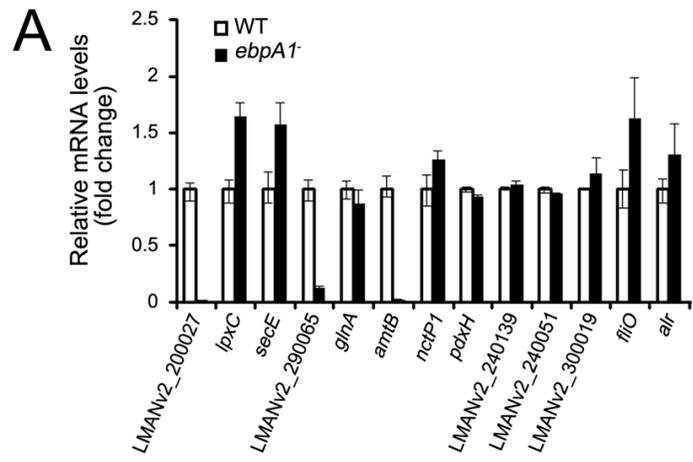
EbpB



Activated EbpA or EbpB oligomer

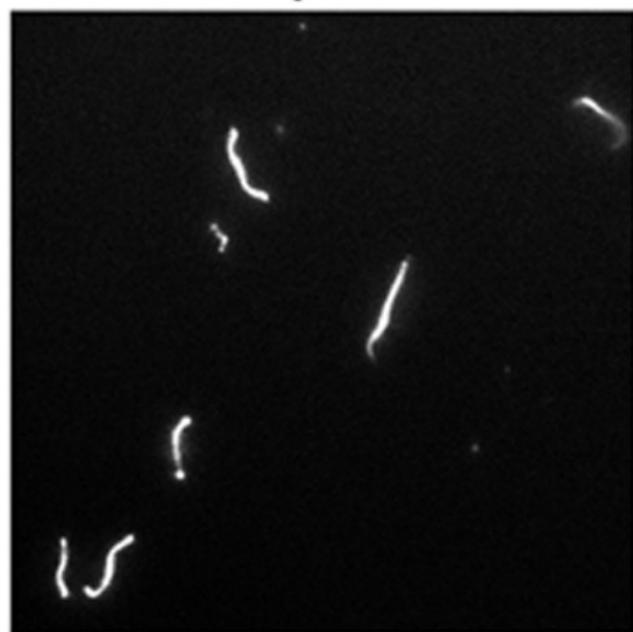
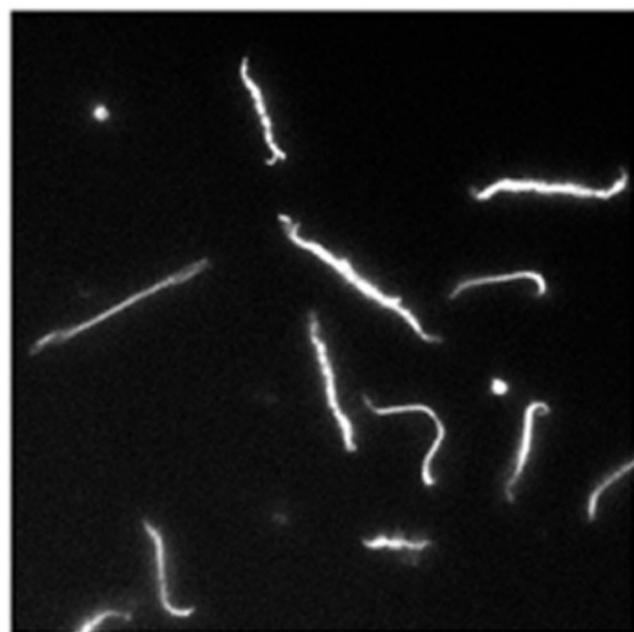
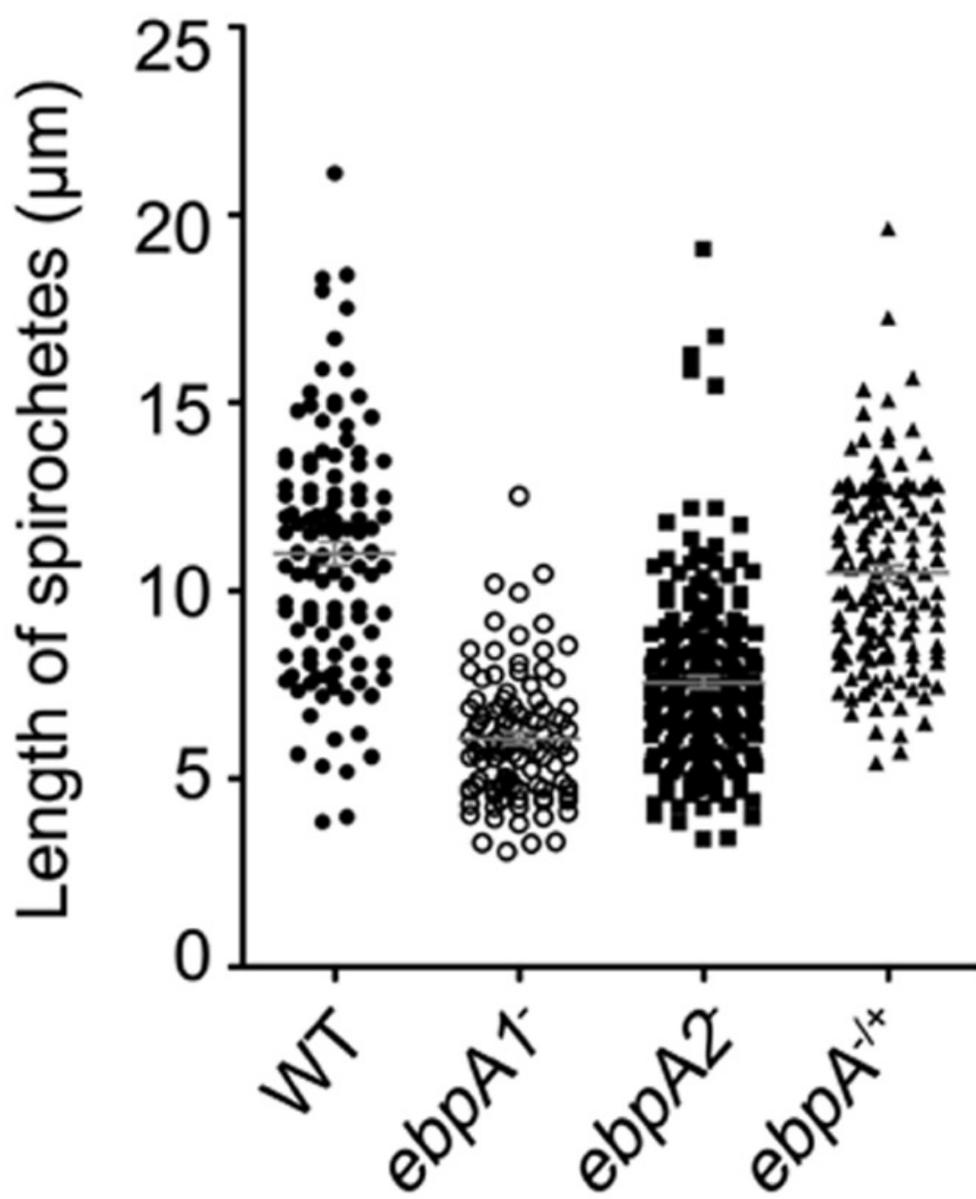






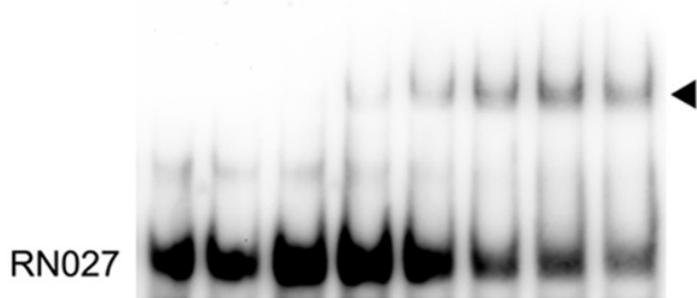
A

WT

ebpA1⁻**B**

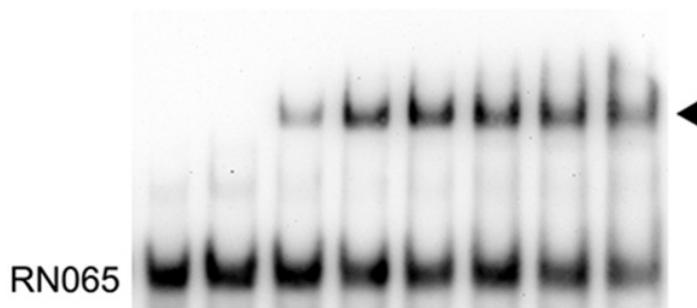
A

RpoN (nM) 0 15 30 60 120 250 500 1000



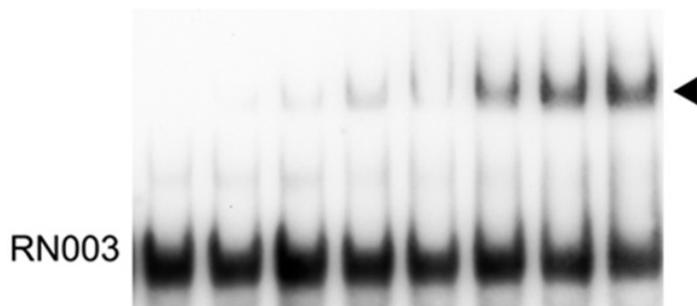
RN027

LMANv2_200027



RN065

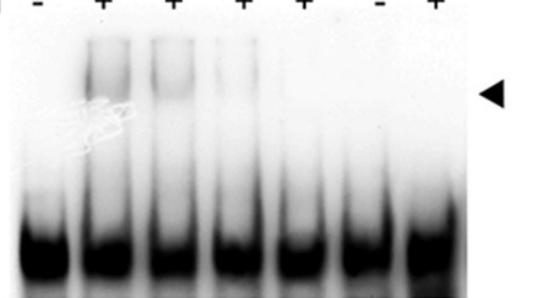
LMANv2_290065



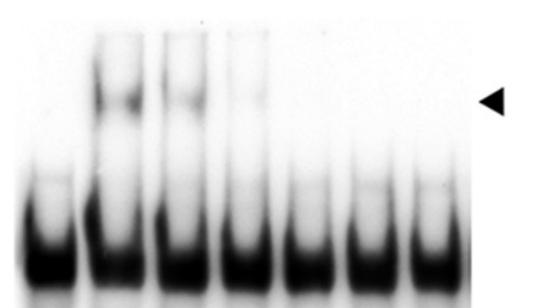
RN003

*amtB***B**

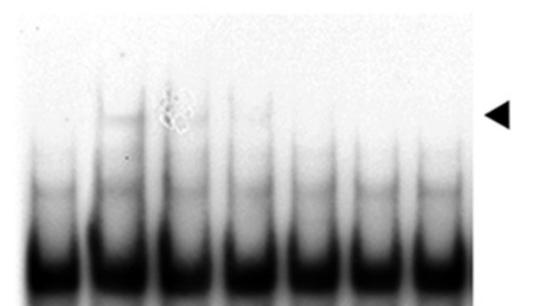
Unlabeled Probes	-	-	×10	×50	×100	-	-
BSA	-	-	-	-	-	+	-
RpoN	-	+	+	+	+	-	+



	RN027					MRN027	
	LMANv2_200027						



	RN065					MRN065	
	LMANv2_290065						



	RN003					MRN003	
	<i>amtB</i>						

