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1 **Control of gene expression in *Leptospira* spp. by Transcription Activator-Like**
2 **Effectors (TALEs) demonstrates a potential role for LigA and LigB in virulence in *L.***
3 ***interrogans*.**

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27

28 **Abstract:**

29 Leptospirosis is a zoonotic disease that affects approximately one million people
30 annually with a mortality rate above ten percent. Currently, there is an absence of
31 effective genetic manipulation tools for targeted mutagenesis in pathogenic leptospire.
32 Transcription Activator-Like Effectors (TALEs) are a recently described group of
33 repressors which modify transcriptional activity in prokaryotic and eukaryotic cells by
34 directly binding to a targeted sequence within the host genome. To determine the
35 applicability of TALE within *Leptospira* spp., two TALE constructs were designed. First,
36 a constitutively expressed *tale* specific for the *lacO*-like region upstream of *bgaL* was
37 trans inserted in the saprophyte *Leptospira biflexa* (TALE_{*bgaL*}). RT-PCR analysis and
38 enzymatic assays demonstrated BgaL was not expressed in TALE_{*bgaL*}. Second, to study
39 the role of LigA and LigB in pathogenesis, a constitutively expressed *tale* with specificity
40 for the homologous promoter regions of *ligA* and *ligB* was cis inserted into the pathogen
41 *Leptospira interrogans* (TALE_{*lig*}). LigA and LigB expression were studied using three
42 independent clones: TALE_{*lig1*}, TALE_{*lig2*}, and TALE_{*lig3*}. Immunoblot analysis of osmotically
43 induced TALE_{*lig*} clones demonstrated a 2 to 9 fold reduction in expression of LigA and
44 LigB, with highest reductions noted in TALE_{*lig1*} and TALE_{*lig2*}, which were avirulent in
45 vivo and non-recoverable from animal tissues. This study reconfirms galactosidase
46 activity in the saprophyte, and suggests a role for LigA and LigB in pathogenesis.
47 Collectively, this study demonstrates that TALE is effective at reducing expression of
48 targeted genes within saprophytic and pathogenic strains of *Leptospira* spp., providing
49 an additional genetic manipulation tool for this genus.

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51

52 **Introduction**

53 Leptospirosis, a bacterial infection transmitted by animal reservoirs, has emerged to
54 become a major public health concern in much of the developing world. There are more
55 than one million cases of severe leptospirosis reported each year for which mortality is
56 greater than 10% (1). As a spirochete, *Leptospira* spp. which includes the causative
57 agent of leptospirosis, differ considerably from other members of Gram positive and
58 Gram negative bacteria. Progress in our understanding of the general biology and
59 virulence mechanisms of pathogenic *Leptospira* has been slow and difficult. This is
60 mainly due to the lack of adequate and efficient genetic tools (2). Genetic modification of
61 the pathogen are limited primarily to random transposon mutagenesis and there exist
62 only a few examples of mutants obtained by targeted mutagenesis (3). Thus, there is a
63 clear need for additional tools to develop genetic studies in *Leptospira* spp..

64 The transcription activator-like effector (TALE) family forms a subset of proteins made
65 by *Xanthomonas* bacterial species that are injected into plants to modulate host gene
66 expression, with each effector directly binding a specific DNA target (4, 5). TALEs are
67 composed of three domains: (i) a central repeat domain, with each repeat unit of 34
68 amino acids specifying one target base via two adjacent amino acids termed repeat-
69 variable diresidues, (ii) an N-terminal translocation signal, and (iii) a C-terminal region
70 containing a transcriptional activation domain as well as a nuclear localization signal.
71 These DNA binding proteins can be engineered to target novel DNA sequences (6).
72 Transcriptional repression by TALEs is a common approach to control gene expression
73 in higher organisms as diverse as plants, fruit flies, zebrafish, mice, and human cell lines.
74 More recently, the TALE genetic system has been imported in *Escherichia coli* (7). Here,
75 we imported this new genetic system based on TALE, to allow targeted repression of
76 gene expression in both the saprophyte *L. biflexa* and the pathogen *L. interrogans*.

77 **Materials and Methods**

78 **Strains and culture conditions**

79 The following *Leptospira* strains were used in this study: pathogen *Leptospira*
80 *interrogans* serovar Manilae strain L495, and saprophyte *Leptospira biflexa* serovar
81 Patoc strain Patoc1. *E. coli* XL-10 gold ultracompetent cells (Agilent, Santa Clara, CA)
82 were used for construction of *flgB-tale* constructs, and *E. coli* strain S17.1 (8) was used
83 for conjugation. Leptospirees were grown at 30°C on a rotary shaker in Ellinghausen-
84 McCullough-Johnson-Harris (EMJH) liquid media as previously described (9, 10).
85 *Leptospira* transformants were initially isolated on solid EMJH media with 40 µg/ ml
86 spectinomycin (Sigma-Aldrich Chimie, Saint-Quentin-Fallavier, France) as previously
87 described (11). *E. coli* cells were grown in Luria broth or Luria solid media
88 supplemented with ampicillin (100 µg / ml), kanamycin (50 µg /ml) and spectinomycin
89 (50 µg / ml) when required. For NaCl induced *Leptospira* cultures, strains were grown
90 at 30°C to late exponential phase in 50 ml of liquid EMJH with a final concentration of
91 120 mM NaCl.

92 **Bioinformatic analyses**

93 Bioinformatic analyses were completed by Microscope Microbial Genome Annotation &
94 Analysis Platform web based software (<https://www.genoscope.cns.fr/agc/mage>) (12).
95 LigA, LigB, and FlaA2 expression were quantitated using ImageJ software (Rasband, W.S.,
96 ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,
97 <http://imagej.nih.gov/ij/>, 1997-2014) which is made available by the United States
98 National Institutes of Health (Bethesda, MD).

99 **TALE construct design**

100 The custom TALE genes used in this study were synthesized by GeneArt (Life
101 Technologies, Grand Island, NY, USA). The genes (2523 bp), which include 5' NdeI and 3'

102 XbaI restriction sites, were codon-optimized for expression in *Leptospira* spp. The
103 availability of codon usage tables for the genomes of *L. interrogans* strains allowed us to
104 define preferred and rare codons. The TALE constructs were then manually codon-
105 optimized by using codons frequently used in *L. interrogans*.

106 Based on this methodology, a TALE specific for the *lacO*-like region of *L. biflexa*, named
107 *tale_{βgal}*, was designed to anneal at sequence 5'-AAGATAGATTCTGCAATCG-3'. The *tale_{βgal}*
108 gene was digested with NdeI and XbaI, purified, and inserted between the NdeI and SpeI
109 restriction sites of pCRPromFlgB (13) to generate a transcriptional fusion between the
110 *tale* gene and the *Borrelia burgdorferi flgB* promoter. The DNA fragment containing the
111 transcriptional fusions was released by PvuII digestion and cloned into the SmaI site of
112 the *E. coli-L. biflexa* shuttle vector pMAT (14) to generate the plasmid *ptale_{βgal}*, which
113 replicates in *L. biflexa*. Another TALE construct, named *tale_{lig}*, was designed to anneal to
114 the sequence 5'-TCCAATAAATCTTAAGAGA-3' which is located in homologous promoter
115 regions of *ligA* and *ligB* in *L. interrogans* 170 basepairs upstream of +1 (15). The *tale_{lig}*
116 gene was inserted downstream of the *B. burgdorferi flgB* promoter (as described above)
117 and the DNA fragment containing the fusion was released by ApaI and KpnI digestions
118 (Thermo Scientific, Waltham MA), gel purified (Qiagen, Venlo, Netherlands), and
119 subcloned into the corresponding sites of pAL614 (16). Plasmid constructs (*ptale_{lig}*)
120 were introduced into *Leptospira* strains by conjugation with *E. coli*, as described
121 previously (17). Spectinomycin-resistant clones were confirmed by PCR amplification of
122 *aadA* (primers spcA/spcB) and *tale_{lig}* (primers TA5F/TA5R) and *flgB-tale_{lig}*
123 (pFLGA/TA5R) (table1). The position of *tale_{lig}* cis insertion in *Leptospira interrogans*
124 transformants were determined as previously described (18) using primers
125 TnSp1/Deg1 or TnSp1/Deg2 (for initial amplification) followed by primers 3855/Tag

126 (for nested PCR) (table 1). DNA sequencing was completed by Eurofins Genomics
127 (Ebersberg, Germany).

128 **Reverse Transcriptase PCR**

129 For RT-PCR experiments, 8 mL of cells were grown at 30°C on a rotary shaker to late
130 logarithmic growth in EMJH liquid media. Cells were collected by centrifugation for 15
131 minutes at 4000 x g. Pelleted cells were re-suspended in 700 µl of TRIzol and RNA was
132 purified according to manufacturer's instructions (Invitrogen, Saint Aubin, Ile de France,
133 France). DNA was degraded using Turbo DNA-free kit according to manufacturer's
134 instructions (Invitrogen). 1 µg of DNase-treated RNA per sample was used to generate
135 cDNA with iScript cDNA synthesis kit as per manufacturer's instructions (Bio-Rad,
136 Marnes-la-Coquette, France). PCR amplifications were performed to determine the
137 presence of cDNA for *tale_{βgal}* using primers TAF5/TA5R (table 1), and *bgaL* using
138 primers LEPBla0024F/LEPBla0024R (table 1).

139 **β-galactosidase assay**

140 A β-galactosidase colorimetric assay adapted from Miller (19) was used for
141 determination of β-galactosidase activity in *L. biflexa*. The cell concentration of
142 leptospire cells in logarithmic growth was determined by Petroff-Hausser counting
143 chamber according to manufacturer's instructions (Hausser Scientific, Horsham PA). 3 x
144 10⁸ cells per sample were centrifuged at 6700 cfm for 10 minutes to pellet cells. The
145 supernatant of each sample was discarded, and cells were re-suspended in 1 ml of Z
146 buffer pH7 (130 mM monosodium phosphate, 40 mM disodium phosphate, 10 mM
147 potassium chloride, 2 mM magnesium sulfate, 40 mM β-mercaptoethanol). Optical
148 density of each culture was measured by spectrophotometry (OD₄₂₀). 20 µl of
149 chloroform (final volume 2% v/v) and 20 µl of 0.1% SDS (final volume 0.02% v/v) was
150 added to each re-suspended sample. The sample was vortexed for 10 seconds, and then

151 samples were placed at 30°C for 5 minutes. Following incubation, 200 µl of a 13.27 mM
152 solution of o-nitrophenyl-B-D-galactopyranoside (ONPG, 4 mg/ mL) re-suspended in Z
153 buffer was added to samples. Samples were gently rocked and observed until samples
154 turned yellow, or for 24 hours if no color change was observed. The reaction was
155 stopped by adding 248 µl of sample with 100 µl of 1M sodium carbonate to samples.
156 Color change was measured by spectrophotometric analyses at OD₄₂₀ and OD₅₅₀. To
157 calculate Miller units (mU), the following formula was used: $mU = 1000(OD_{420} - (1.75 \times$
158 $OD_{550})) / (\text{time in minutes} \times \text{volume of culture in mL} \times \text{initial } OD_{420} \text{ of culture}).$

159 **Blue-White Screening**

160 β-galactosidase activity was also determined by blue-white screening of *L. biflexa*
161 colonies. *L. biflexa* colonies were grown on 20 ml of solid EMJH media. After colony
162 formation, approximately 0.04 ml of 5-bromo-4-chloro-3-indolyl-β-D-galacto-
163 pyranoside in DMSO (X-gal, 40 mg/ml) was added to plates. Plates were incubated
164 overnight at 30°C before photographs were taken. Colonies of wild type (n=910), *βgal*-
165 (n=221) and TALE_{*βgal*} (n=557) were counted from at least 4 independent plates.
166 Percentage of blue versus white colonies was calculated, and the average and standard
167 error mean were reported. Statistical analysis was performed by one-way analysis of
168 variance.

169 **Immunoblot analysis**

170 Cells were grown to late logarithmic phase under physiological osmotic shock
171 conditions (120 mM NaCl in EMJH liquid medium). Cells were pelleted, washed in
172 phosphate-buffered saline (PBS), resuspended in 0.2 mL of lysis buffer (25 mM Tris-HCl,
173 2 mM EDTA, 150 mM KCl, 5mM DTT in ddH₂O, pH 7.5), and sonicated. For the
174 representative membrane shown, 3.5 µg of protein per sample was used for SDS-PAGE
175 and PVDF membrane transfer according to manufacturer's instructions (Bio-Rad).

176 Immunoblots were performed as previously described (20) with the following primary
177 antibodies: anti-LigA/B (1:1000 dilution), or anti-FlaA2 (1:2000 diluted in blocking
178 buffer). Membranes were probed with goat anti-rabbit IgG-HRP conjugated antibody
179 (1:100,000 dilution) according to manufacturer's instructions (Sigma-Aldrich Chimie).
180 Chemiluminescent detection was performed with Supersignal West Pico
181 Chemiluminescent substrate according to manufacturer's instructions (Life
182 Technologies). The mean and SEM of three independent replicates per transformant
183 and wild type were used to calculate the mean and SEM of LigA/LigB fold reduction in
184 TALE_{lig} transformants compared to wild type. Statistical analysis was performed by
185 one-way analysis of variance.

186 **Virulence assay**

187 Low passage leptospire were grown to mid-late logarithmic phase. Cell concentration
188 was quantitated by Petroff-Hausser counting chamber as described above. Cells were
189 diluted to 10⁶ cells/ mL in EMJH medium. Four week old male Golden Syrian Hamsters
190 (*Mesocricetus auratus*, RJHan:AURA) were purchased from Janvier Labs (Saint-Berthevin,
191 France). Four hamsters per group were inoculated via intraperitoneal injection with 1
192 mL of 10⁶ cells. The animals were observed daily for symptoms of leptospirosis. The
193 animals were euthanized if symptoms of leptospirosis were present, or at 21 days post
194 inoculation in absence of disease manifestation as previously described (19). Kidney
195 tissue and blood were collected from euthanized animals, homogenized under sterile
196 conditions in 5 mL of EMJH media, and incubated at 30°C for 4 weeks to recover viable
197 spirochetes. Statistical analysis of survival data was performed by the Log-rank (Mantel-
198 Cox) test. Protocols for animal experiments conformed to the guidelines of the Animal
199 Care and Use Committees of the Institut Pasteur (Paris, France).

200 **Results and Discussion**

201 ***tale_{βgal}* represses β-galactosidase activity in *L. biflexa*.**

202 A putative DNA-binding site for the lactose repressor, also called the lac operator or *lacO*,
203 which is homologous to the one characterized in *E. coli* (5'-AATTGTGAGCGCTCACAATT-
204 3') was identified downstream of the Shine-Dalgarno sequence within the promoter
205 region of *LEPBIa0024* (*bgaL*) encoding for a β-galactosidase (21). Accordingly, a *tale*
206 gene was designed for the promoter region 5'-AAGATAGATTCTGCAATCG-3' (NT 24090-
207 24107) which is located 14 basepairs upstream of the +1 site of *bgaL* and named *tale_{βgal}*.
208 *tale_{βgal}* was cloned into the replicative vector pMAT, then transferred into *L. biflexa* as
209 described above. The transformant was named TALE_{βgal}. RT-PCR verified that *tale_{βgal}* is
210 expressed by TALE_{βgal} in *L. biflexa* (fig. 1A). Amplification of *tale* from TALE_{βgal} also
211 serves as a positive control that RNA was purified, and that cDNA was generated from
212 TALE_{βgal}. Further, *tale_{βgal}* abrogated transcription of *bgaL* in TALE_{βgal} (fig. 1A). To
213 confirm that *bgaL* repression by *tale_{βgal}* results in abrogation of β-galactosidase activity
214 in the transformant TALE_{βgal}, enzymatic activity analysis was completed comparing β-
215 galactosidase activity in wild type to *βgal*⁻ (a spontaneous *L. biflexa* frameshift mutant at
216 NT24353), and TALE_{βgal}. First, strains were tested by blue-white colony screening to
217 determine if they were capable of cleaving X-gal. Colonies grown on EMJH solid media
218 were treated with 80 μg/ ml of X-gal and left for 16 hours at 30°C. Wild type was
219 capable of β-galactosidase activity, hydrolysing X-gal into galactose and 4-chloro-3-
220 brom-indigo, creating a characteristic blue colony in 97.6%±1.7 of colonies. All *βgal*⁻
221 (100%±0) and TALE_{βgal} (100%±0) colonies remained white after incubation with X-gal
222 (fig. 1B), which were statistically significant compared to wild type (P<0.01, and P<0.01,
223 respectively). Additionally, a modified Miller colorimetric assay (19) was used to
224 measure β-galactosidase activity as described above. Wild type exhibited 1.43 mU of

225 activity, while *βgal* and TALE_{*βgal*} both exhibited 0 mU of activity. Together, this data
226 demonstrates that TALE is an effective modulator of expression in the saprophyte *L.*
227 *biflexa*, and concurs with previous data that *bgal* has β-galactosidase activity (21).

228

229 **TALE_{*lig*} reduces expression of LigA and LigB in *L. interrogans***

230 A *tale* specific for the leptospiral immunoglobulin-like (*lig*) genes *ligA* and *ligB* was
231 designed to anneal to 5'- TCCAATAAATCTTAAGAGA -3'. This sequence corresponds to
232 the -10 promoter region, which is a homologous region 170 basepairs upstream of +1 of
233 *ligA* (*lmanv2_30028*) and *ligB* (*lmanv2_350013*) (15). Three biologically distinct TALE_{*lig*}
234 transformants were characterized: TALE_{*lig1*}, TALE_{*lig2*}, TALE_{*lig3*}. Since insertion is due to
235 random insertion by the transposon *Himar1*, sequence analysis by nested PCR using
236 degenerate primers was completed to determine *tale_{lig}* insertion position in each
237 transformant. *tale_{lig}* inserted in TALE_{*lig1*} at position NT818455 within the proximal
238 region of *lmanv2_90191* (NT 818032-819462, putative polysaccharide deacetylase),
239 *tale_{lig}* inserted in TALE_{*lig2*} at NT 1270962 within the proximal region of *lmanv2_150048*,
240 (NT 1270809-1271918, putative *pilF*), and *tale_{lig}* inserted in TALE_{*lig3*} at the distal region
241 of *lmanv2_340018* (NT 2894815-2896014, putative conserved membrane protein of
242 unknown function).

243 TALE_{*lig*} transformants were grown in physiological osmotic conditions in vitro, and
244 expression of LigA and LigB was determined by immunoblot analysis. This study
245 focused on immunoblot analysis, as these data are more informative of the ability of
246 *tale_{lig}* to repress expression at the protein level within Tale_{*lig*} transformants, which was a
247 pre-requisite for in vivo experimentation. Expression of Lig proteins were normalized
248 to expression of FlaA2 per sample. TALE_{*lig1*} had a 3.1±1.2 fold reduction of LigA
249 (P<0.05), and a 6.6±1.2 fold reduction of LigB (P<0.05) compared to wild type. TALE_{*lig2*},

250 had a 5.3 ± 1.3 fold reduction of LigA ($P < 0.05$), and a 9.1 ± 3.8 fold reduction of LigB
251 ($P < 0.05$) compared to wild type. TALE_{lig3} had a of 2.1 ± 0.4 fold reduction of LigA
252 ($P < 0.05$), and a 4.6 ± 1.6 fold reduction of LigB compared to wild type ($P < 0.05$) (fig. 2A).
253 The variability in *ligA* and *ligB* repression amongst transformants may suggest that
254 expression of *tale_{lig}* (and concordant repression of *ligA* and *ligB*) might be affected by
255 the chromosomal location of Tn-*tale_{lig}*. Since the TALE system currently relies on
256 random insertion by the Himar1 transposon system, it is suggested that multiple
257 transformants should be selected and screened when using the TALE system in the
258 pathogen.

259

260 **Repression of LigA and LigB may suggest a role of virulence of *L. interrogans***

261 Hamsters were inoculated intraperitoneally with 10^6 cells of wild type, TALE_{lig1}, TALE_{lig2},
262 or TALE_{lig3} transformants and observed for leptospirosis for 21 days. TALE_{lig3}
263 transformant had reduced virulence compared to wild type ($P = 0.09$), while TALE_{lig1} and
264 TALE_{lig2} transformants were avirulent compared to wild type ($P < 0.01$, $P < 0.01$,
265 respectively) (fig. 2B). Tissue samples from hamsters were harvested following
266 euthanasia to attempt recovery of viable spirochetes. Motile leptospire were recovered
267 from wild type and TALE_{lig3} transformant infected animals, however no spirochetes
268 were recovered nor observed from tissues harvested from animals infected by neither
269 TALE_{lig1} nor TALE_{lig2} transformants. LigA and LigB protein expression differences
270 between TALE_{lig} transformants may explain their different virulence profiles.
271 Alternatively, it remains possible that attenuation of virulence of TALE_{lig} transformants
272 is the result of chromosomal insertion of Tn-*tale_{lig}* and the subsequent inactivation of
273 these disrupted genes in the TALE_{lig} transformants that were studied. Therefore, future

274 work to determine the role of *lmanv2_90191*, *lmanv2_150048* (*pilF*), and *lmanv2_340018*
275 is warranted.

276 Previous research has shown that LigA and LigB are surface exposed proteins (22), and
277 that they play a role in adhesion of spirochetes to extracellular matrix proteins,
278 complement regulators, and animal cells (15, 23, 24). Further, single gene mutants of
279 either *ligA* in *L. interrogans* serovar Manilae strain L495 or *ligB* in *L. interrogans* serovar
280 Copenhageni strain Fiocruz L1-130 did not reduce virulence in standard animal models,
281 and remained recoverable from animal tissue (25, 26). The distribution and sequences
282 of *lig* genes, including *ligA*, *ligB*, and *ligC*, (27, 28) as well as the level of expression of Lig
283 proteins (22) may vary in each pathogenic serovar. Depending on the strain, inactivation
284 of only one *lig* gene may therefore be compensated by the expression of another Lig
285 protein(s). The status of the *lig* genes in the *L. interrogans* serovar Manilae remains to be
286 clarified.

287 Previous analyses have demonstrated homology in the N-terminal repeat domains of
288 LigA and LigB (encompassing 266 bp upstream of their start codon until 1890 bp into
289 *ligA* and *ligB*) with variation at the C-terminal region of LigB (22). As has been
290 previously suggested, LigA and LigB may work as a redundant system to ensure
291 spirochete adhesion and survival within a mammalian host (25, 29). As the current data
292 suggests, reduced expression of both LigA and LigB proteins within the TALE_{*lig*}
293 transformants conferred reduced virulence within hamsters, which may suggest a
294 cumulative role of LigA and LigB in pathogenesis.

295 In conclusion, the current study demonstrates that the TALE system is an efficient
296 system of targeted gene repression in saprophytic and pathogenic leptospires, and
297 suggests a role of Lig proteins in pathogenesis. TALEs act as DNA-binding repressors by
298 inhibiting promoter recognition by RNA polymerase or blocking transcription; however,

299 the promoter regions of *Leptospira* are poorly characterized at present. Insight into the
300 promoter motif of leptospires allows increased efficiency of the TALE system, as
301 demonstrated here by TALE_{*βgal*}. It is therefore suggested that future research focused on
302 determining these promoter motifs and transcriptional start sites will aid in designing
303 TALEs exhibiting tight repression of gene expression in *Leptospira* spp.

304

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308

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406 **Figure legends**

407

408 **Figure 1:** TALE_{*βgal*} represses β-galactosidase activity in *L. biflexa*.

409 **A.** RT-PCR of *L. biflexa* wild type (WT) and TALE_{*βgal*}. Left image shows that TALE_{*βgal*}
410 transcribes *tale_{βgal}*. Right image shows that WT transcribes *bgaL*, while TALE_{*βgal*} does
411 not.

412 **B.** Wild type, natural mutant Patoc *βgal*⁻, and TALE_{*βgal*} were grown on solid phase EMJH
413 and exposed to X-gal for 16 hours. Wild type was able to cleave X-gal to galactose and 5-
414 bromo-4-chloro-3-hydroxyindole, creating a blue colour. Natural mutant Patoc *βgal*⁻ and
415 TALE_{*βgal*} were unable to cleave X-gal; thus the colonies remained colourless.

416

417 **Figure 2:** TALE_{*lig*} represses expression of LigA and LigB in *L. interrogans*.

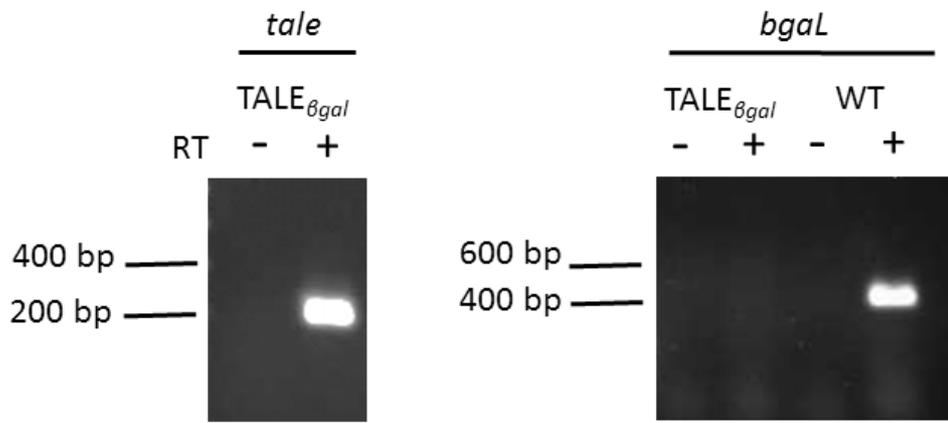
418 **A.** A representative immunoblot of *L. interrogans* wild type (WT) and 3 biologically
419 distinct TALE_{*lig*} transformants (TALE_{*lig1*}, TALE_{*lig2*}, TALE_{*lig3*}). 3.5 μg of protein extract/
420 sample was reacted with antisera specific for LigA/B (top images) or FlaA2 (bottom
421 images). Analysis revealed a 3.1±1.2 fold reduction of LigA (P<0.05), and a 6.6±1.2 fold
422 reduction of LigB (P<0.05) in TALE_{*lig1*} compared to WT; a 5.3±1.3 fold reduction of LigA
423 (P<0.05), and a 9.1±3.8 fold reduction of LigB (P<0.05) in TALE_{*lig2*} compared to WT; a
424 2.1±0.4 fold reduction of LigA (P<0.05), and a 4.6±1.6 fold reduction of LigB (P<0.05) in
425 TALE_{*lig3*} compared to WT.

426 **B.** Survival analysis of intraperitoneal inoculation of 10⁶ cells of WT, TALE_{*lig1*}, TALE_{*lig2*},
427 or TALE_{*lig3*} in hamsters showed reduced virulence of TALE_{*lig3*} compared to wild type
428 (P=0.09) and significant attenuation of virulence in TALE_{*lig1*} (P<0.01) and TALE_{*lig2*}
429 (P<0.01) compared to wild type.

Table 1: Primers used in this study

Primer Name	Sequence (5' → 3')	Expected Size	Target
TA5F	CATATGGTTGATCTTCGTAC	264 bp	amplification of proximal region of <i>tale</i>
TA5R	AAGTGCACGTGCACCTGACC		
LEPBIa0024F	AATATAAAACTCTCGATTGCGTTAACA	500 bp	amplify internal region of <i>L. biflexa bgal</i>
LEPBIa0024R	TCACAAATTGCTTGTGCAACC		
<i>spcA</i>	GGGGTGAATTTGAGAATGGA	246 bp	<i>aadA</i> cassette
<i>spcB</i>	GTCAGTGTGGCCACATTCC		
<i>tnsp1</i>	GAAACTTCTCAATTAGG	varies	<i>spc^R</i> transposon
Deg1	GGCCACGCTCGACTAGTACNNNNNNNNNNNGATAT		
Deg2	GGCCACGCTCGACTAGTACNNNNNNNNNNNTCTT	varies	Degenerate oligo with 5' tag Degenerate oligo with 5' tag nested primer
3855	TTCTTGACGAGTCTTCTGA		
tag	GGCCACGCTCGACTAGTAC	671 bp	binds to tag on Deg1/ Deg2 <i>flgB-tale</i>
pFLGA	TAATACCCGAGCTTCAAGGAAG		
TA5R	AAGTGCACGTGCACCTGACC		

A



B

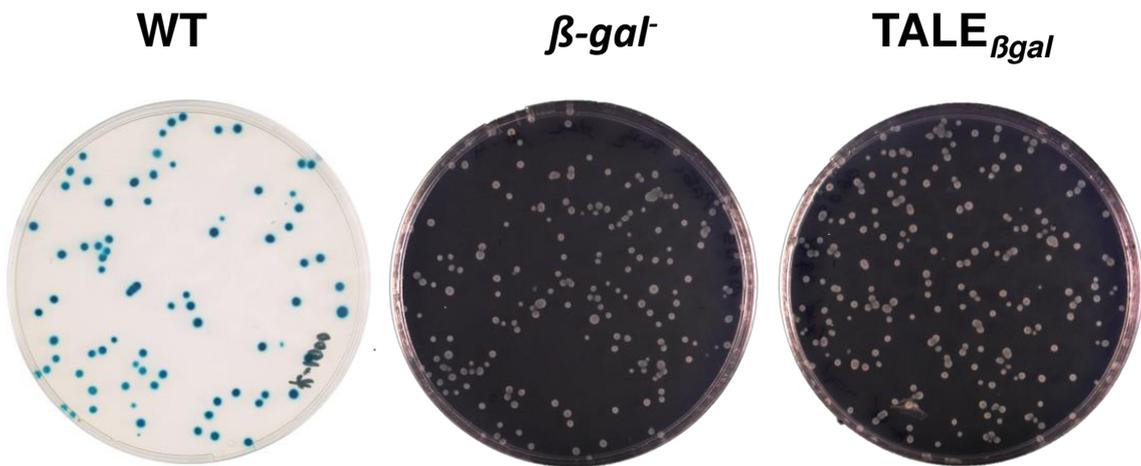
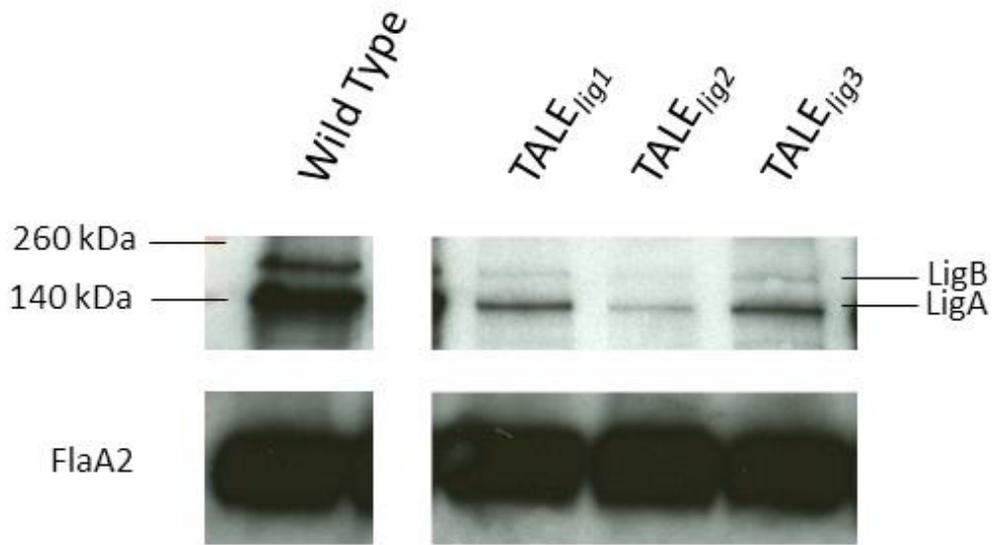
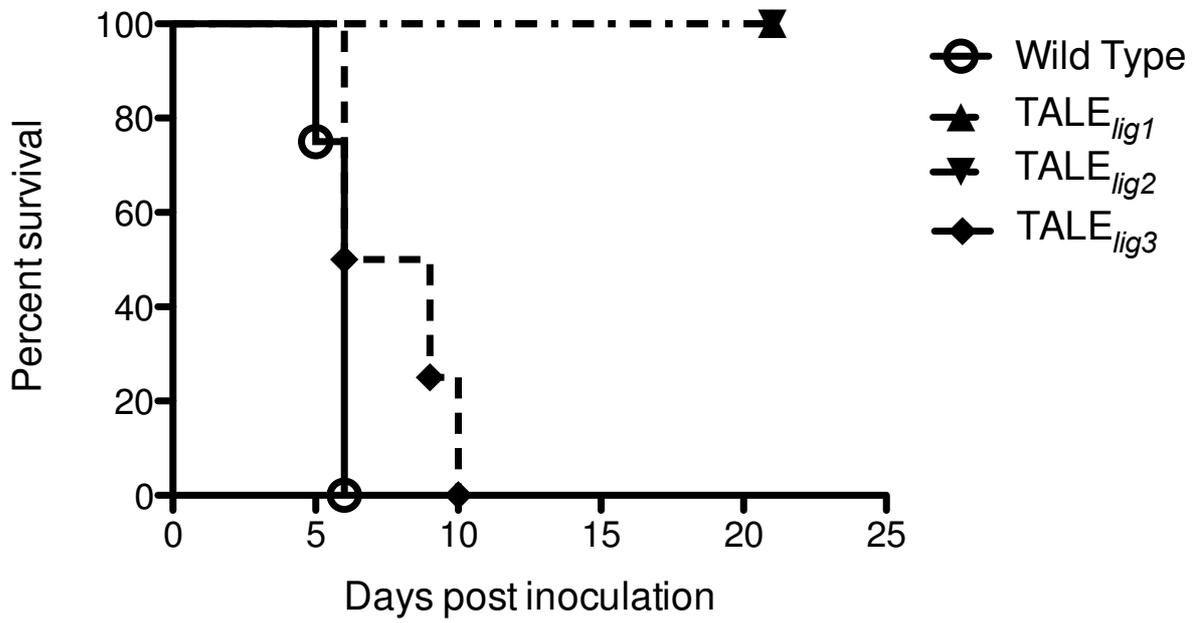


Figure 1

A**B****Figure 2**