



HAL
open science

A Replicative Plasmid Vector Allows Efficient Complementation of Pathogenic *Leptospira* Strains

Christopher Pappas, Nadia Benaroudj, Mathieu Picardeau

► **To cite this version:**

Christopher Pappas, Nadia Benaroudj, Mathieu Picardeau. A Replicative Plasmid Vector Allows Efficient Complementation of Pathogenic *Leptospira* Strains. *Applied and Environmental Microbiology*, 2015, 81 (9), pp.3176-3181. 10.1128/AEM.00173-15 . pasteur-02536538

HAL Id: pasteur-02536538

<https://pasteur.hal.science/pasteur-02536538v1>

Submitted on 8 Apr 2020

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39

A replicative plasmid vector allows efficient complementation of pathogenic *Leptospira* strains

Christopher J. Pappas^{1,2}, Nadia Benaroudj¹, and Mathieu Picardeau^{1*}

¹ Institut Pasteur, Unité de Biologie des Spirochètes, Paris, France

² Manhattanville College, Department of Biology, Purchase, NY 10577

Key words: *Leptospira*, replicative vector, genetic tools, complementation, PerR

* Corresponding author
Mailing address: Mathieu Picardeau, Institut Pasteur, Unité de Biologie des Spirochètes, 28 rue du docteur Roux, 75724 Paris Cedex 15, France. Tel: 33 (1) 45 68 83 68. Fax: 33 (1) 40 61 30 01. E-mail: mathieu.picardeau@pasteur.fr

40 **ABSTRACT**

41 Leptospirosis, an emerging zoonotic disease, remains poorly understood due to a lack of
42 genetic manipulation tools available for pathogenic leptospires. Current genetic manipulation
43 techniques include insertion of DNA by random transposon mutagenesis and homologous
44 recombination via suicide vectors. The current study describes the construction of a shuttle
45 vector, pMaORI, which replicates within saprophytic, intermediate, and pathogenic
46 leptospires. The shuttle vector was constructed by insertion of a 2.9 kb DNA segment
47 including *parA*, *parB*, and *rep* genes into pMAT, a plasmid that cannot replicate in *Leptospira*
48 spp. that contains a backbone consisting of an *aadA* cassette, *ori* R6K, and *oriT* RK2/RP4.
49 The inserted DNA segment was isolated from a 52 kb region within *Leptospira mayottensis*
50 strain 200901116 that is not found in the closely related *L. mayottensis* strain 200901122.
51 Due to the size of this region and the presence of bacteriophage-like proteins it is possible
52 this region is a result of a phage-related genomic island. Stability of the pMaORI plasmid was
53 tested within pathogenic strains by passaging cultures 10 times without selection and
54 confirming the presence of pMaORI. Concordantly, we report the use of trans
55 complementation in the pathogen *Leptospira interrogans*: transformation of a pMaORI vector
56 carrying a functional copy of the *perR* gene in a null mutant background restores expression
57 of PerR and susceptibility to hydrogen peroxide comparable to that of wild-type cells. In
58 conclusion, we demonstrate replication of a stable plasmid vector in a large panel of
59 *Leptospira* strains, including pathogens. The described shuttle vector will expand our ability
60 to perform genetic manipulation of *Leptospira* spp.

61

62

63

64 **INTRODUCTION**

65 Leptospirosis, which is caused by one of the ten pathogenic *Leptospira* spp. described to
66 date, is a neglected zoonotic disease that has a worldwide distribution with a high incidence
67 in tropical countries. Our current understanding of the virulence mechanisms and more
68 generally the biology of pathogenic *Leptospira* remains largely unknown. This hindrance is
69 partly due to a lack of efficient genetic tools available for use in pathogenic *Leptospira* spp.
70 (8, 11). While genetic modification tools allow flexible manipulation of the genome of the
71 saprophyte *Leptospira biflexa*, including targeted mutagenesis and cis/ trans
72 complementation (11), genetic modification of the pathogen are limited primarily to random
73 transposon mutagenesis.

74 Previously, genetic analysis of *Leptospira* was impeded by the absence of methods for the
75 introduction of DNA into leptospiral cells. Currently, DNA can be introduced in *Leptospira*
76 spp. by electroporation (14) or conjugation between *E. coli* and *Leptospira* spp. using RP4
77 derivative conjugative plasmids (10). Transformed *Leptospira* can be visualized on solid
78 medium as subsurface colonies after one week for saprophytes and up to four weeks for
79 pathogens. Markers for the selection of transformants include kanamycin-, spectinomycin-,
80 and gentamicin- resistance cassettes (1, 13, 14). The replication origins of the *L. biflexa*
81 phage LE1 (14), *L. biflexa* plasmid p74 (12) and a phage-related genomic island from *L.*
82 *interrogans* (4) have previously been used to construct *L. biflexa*- *E. coli* plasmid shuttle
83 vectors. However, a shuttle vector construct has not been reported in intermediate nor
84 pathogenic *Leptospira* spp.

85 In this study, analysis of the genomes of two genetically related strains of a recently
86 discovered pathogenic *Leptospira* species (2) revealed a prophage-like region of
87 approximately 52 kb present in only one of the strains. Further analysis of this region allowed
88 the identification of a putative replication origin. Cloning of a DNA fragment containing this
89 replication origin into an *E. coli* conjugative plasmid allowed autonomous replication in the
90 saprophyte *L. biflexa* and several intermediate and pathogenic strains. Subsequent to
91 plasmid construction and analysis, we employed this plasmid for functional trans

92 complementation of a mutant in the pathogen *L. interrogans* serovar Manilae which carries
93 an inactivation in the peroxide stress regulator-encoding gene *perR*. The PerR transcriptional
94 regulator belongs to the Fur family and controls the expression of genes participating in the
95 cell's defense against oxidants. It has been previously shown that in a *Leptospira perR*
96 mutant a catalase (*katE*) and a putative cytochrome C peroxidase (*maugG-2*) had higher
97 expression compared to the wild type strain. Previous work has also shown that this *L.*
98 *interrogans perR* mutant has enhanced survival in the presence of hydrogen peroxide
99 compared to wild-type cells (9). We report here the successful production of PerR and
100 concomitant restoration of reduced resistance to hydrogen peroxide when PerR is expressed
101 in trans in the *perR* mutant using the described plasmid.

102 **MATERIALS AND METHODS**

103 **Bacterial strains and culture conditions.**

104 The following *Leptospira* spp. were used in this study: intermediate strains *L. fainei* serovar
105 Hurstbridge strain BUT 6 and *L. licerasiae* serovar Varillal strain VAR010; pathogenic strains
106 *L. interrogans* serovar Copenhageni strain Wijnberg, *L. interrogans* serovar Canicola strain
107 Hond Utrecht IV, *L. interrogans* serovar Icterohaemorrhagiae strain Verdun, *L. interrogans*
108 serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Manilae strain L495, *L.*
109 *interrogans* serovar Manilae *perR* mutant 'M776' (generous gift from Gerald Murray and Ben
110 Adler; Monash University, Melbourne, Australia) (9), *L. interrogans* serovar Lai strain 56601,
111 *L. mayottensis* strain 200901116; and the saprophyte *L. biflexa* serovar Patoc strain Patoc1.
112 Strains were grown at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid
113 medium (6, 7) with or without spectinomycin (40 µg/ ml, Sigma Aldrich, St. Louis, MO).
114 EMJH solid medium was prepared by adding 1% noble agar (w/v) to EMJH liquid medium
115 containing spectinomycin (40 µg/ml), and incubated at 30°C for 7 to 30 days.
116 *E. coli* strain П1 ($\Delta thyA$) cells were used for plasmid transformation. *E. coli* strain β 2163
117 ($\Delta dapA$) was used for transfer of pMaORI vector into *Leptospira* spp. via conjugation. *E. coli*
118 strains were grown in Luria-Bertani broth or agar containing spectinomycin (50 µg/ml). Strain
119 β 2163 was additionally cultured with 0.3 mM diaminopimelic acid (DAP, Sigma Aldrich),
120 while strain П1 was additionally cultured with 0.3 mM thymidine (dT, Sigma Aldrich) as
121 previously described (5).

122

123 **Plasmid construction and transformation of *Leptospira* spp.**

124 The nucleotide sequence of the replication region of *L. mayottensis* strain 200901116 was
125 amplified with primer pairs ori1 and ori2 (**Table 1**) and inserted into pCR2.1-TOPO by using
126 the TOPO TA cloning kit as per manufacturer's instructions (Life Technologies, Waltham,
127 MA). After BamHI- XbaI digestion and gel purification, the DNA fragment containing the
128 replication region was inserted into the corresponding restriction sites of the conjugative
129 spectinomycin-resistant plasmid pMAT, and designated pMaORI. Plasmid constructs were

130 introduced into *Leptospira* strains by conjugation as previously described (10) with *E. coli*
131 β 2163 containing the pMaORI plasmid construct. Transformed *Leptospira* strains were
132 recovered from solid media, grown in EMJH liquid media with spectinomycin selection (40
133 μ g/ml), and filtered with a 0.22 μ m filter into fresh EMJH liquid media with spectinomycin (40
134 μ g/ml). Once these samples had grown to mid logarithmic growth all samples were visually
135 verified to ensure the presence of motile leptospira cells without the presence of *E. coli* cells
136 or other contaminants. Once it was determined contaminants were not present, the sample
137 was tested for the shuttle vector pMaORI as described below.

138 Gene amplification of *aadA* and *adk* (an *E. coli* specific adenylate kinase gene) was
139 performed via PCR. Gene specific primers are listed in table 1.

140 Plasmid extraction of pMaORI from *Leptospira* spp. and *E. coli* strains was performed by
141 QiaPrep Spin Miniprep Kit as per manufacturer's instructions (Qiagen, Venlo, Netherlands).
142 The restriction endonuclease KpnI was used to determine pMaORI restriction profile isolated
143 from *E. coli* cells that were previously transformed with pMaORI from *Leptospira* species. 18
144 μ l of extracted plasmid was added to 2.1 μ l of 10x FastDigest buffer, and 1 μ l (unit) of
145 FastDigest KpnI (Thermo Scientific, Waltham, MA). Samples were digested for 30 minutes
146 at 37°C followed by gel electrophoresis.

147

148 **Passage experiment**

149 *Leptospira* strains were passaged ten times to determine if the pMaORI construct could be
150 conserved without selection. For each passage, a 100 μ l aliquot was passaged into 9 ml of
151 fresh EMJH liquid media without selection until it reached late logarithmic growth. Each
152 sample was then subsequently passaged, and the remaining 8.9 ml of sample in late
153 logarithmic growth was plasmid extracted (described above). Extracted plasmid was tested
154 by PCR for the *aadA* cassette, and subsequently transformed into Π 1 *E. coli* strain where
155 pMaORI was verified by KpnI restriction profile.

156

157 **Complementation of *L. interrogans* serovar Manilae *perR* mutant**

158 To complement the *L. interrogans perR* 'M776' mutant (9), the wild-type *perR* allele (including
159 its own promoter region, 310 nucleotides upstream of the *perR* start codon) was amplified
160 from *L. interrogans* serovar Manilae strain L495 using primers PerR5 and PerR3 (**Table 1**).
161 This PCR product was cloned into pCR2.1-TOPO (Invitrogen). The *perR* DNA fragment was
162 then released with EcoRI and inserted into the dephosphorylated EcoRI site of pMaORI to
163 generate plasmid pMaORI-*perR* (pNB138). The pMaORI-*perR* plasmid construct was
164 introduced by conjugation into the M776 mutant (*L. interrogans perR* mutant) as described
165 above and spectinomycin-resistant colonies were inoculated into liquid EMJH containing 40
166 µg/ml spectinomycin for further analysis.

167 PerR cellular content was measured by immunoblot analysis. Exponentially growing cells
168 were lysed by sonication in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, in the
169 presence of protease inhibitors (Roche, Basel, Switzerland). 15 µg of total cell extracts were
170 loaded on a 15% SDS-PAGE and proteins were transferred onto nitrocellulose. PerR protein
171 was detected by using a rabbit anti-PerR antibody at 1:2000 dilution as primary antibodies, a
172 goat anti-rabbit HRP-conjugated IgG at a 1:150000 dilution (Sigma Aldrich) as secondary
173 antibodies, and the SuperSignal West Pico reagent (Thermo Scientific) as HRP substrate.
174 Survival in the presence of H₂O₂ was assessed by incubating exponentially growing cells in
175 the presence or in the absence of 10 mM H₂O₂ for 30 min at 30°C. Twenty microliters of cells
176 were then incubated with the Alamar Blue cell viability reagent (Life Technologies) according
177 to the manufacturer's recommendations.

178

179 **Bioinformatic Analysis**

180 Bioinformatic analysis was completed using Microscope Microbial Genome Annotation &
181 Analysis Platform web based software (<https://www.genoscope.cns.fr/agc/mage>).

182

183 **Accession number**

184 The annotated sequence of pMaORI has been deposited in Genbank under accession
185 number KP784428.

186 **RESULTS AND DISCUSSION**

187

188 **Genome analysis**

189 All members of the *Leptospira* genus that have been analyzed carry at least two circular
190 replicons. The large circular (cl, >3.6 Mb) and the small circular (cII, 278 to 350 kb)
191 chromosomes encode genes involved in housekeeping and other functions (17, 18). More
192 recently, plasmids have also been identified by whole genome sequencing such as p74 in
193 the saprophyte *L. biflexa* (12), and pGui1, pGui2 in the pathogen *L. interrogans* (16). The
194 presence of small replicative plasmids within *Leptospira* spp. implied possible conservation
195 of small genetic elements for our current study.

196 We previously reported a group of *Leptospira* strains isolated from the blood of patients with
197 leptospirosis in Mayotte (Indian Ocean) which were identified as belonging to a novel
198 pathogenic species (2, 3). High-throughput genome sequencing of two representative
199 strains, 200901116 and 200901122, of this new species designated as *Leptospira*
200 *mayottensis* was performed at the J. Craig Venter Institute (<http://gsc.jcvi.org/>). The draft
201 genome sequences of strains 200901116 (4,135,276 bp, 84 contigs) and 200901122
202 (4,161,553 bp, 85 contigs) are deposited in GenBank under accession numbers
203 AKWB00000000 and AKWM00000000, respectively. The G+C content of these strains are
204 39.5 mol%, which is within the range 35-45 mol% reported for members of the genus
205 *Leptospira* (2). Comparative genome analysis was performed using the MaGe interface in
206 the SpiroScope database (<https://www.genoscope.cns.fr/agc/mage>) (15).

207 The genomes of strains 2009001116 and 200901122 are highly similar as demonstrated by
208 their high number of common genes: they share 3,501 CDS with an average pair-wise amino
209 acid identity higher than 99%. In comparison, using the same criteria, strain 200901116
210 shares only 34 CDS with *L. interrogans* strain Fiocruz L1-130 and 194 CDS with *L.*
211 *borgpetersenii* strain L550, with >99% amino acid identity. By analyzing genome disparity,
212 we found a unique region of approximately 52 kb carried by 200901116 but not 200901122
213 at approximate position NT440186-NT492268 (**Figure 1**). The majority of genes in this

214 region encode hypothetical proteins. Genes identified in this region include phage-related
215 gene homologues coding for Integrase, Late control protein D, Portal protein, GpA terminase,
216 Bacteriophage resistance factor (PF05565 family), and a toxin (RelE-like family)-antitoxin
217 (Xre-like family). Another gene (AKWB270011) in this region codes for a putative protein of
218 234 amino acids containing an N-terminal helix-turn-helix domain (25-76 amino acids), which
219 is typically associated with DNA binding. Upstream of this gene, two genes, one of which,
220 AKWB270009, exhibited similarities with homologs of the ParA partition protein. The other
221 gene (AKWB270010) could constitute the *parB* of the putative partition locus. This genetic
222 organization is similar to the *parA-parB* organizations identified to date in *L. biflexa* serovar
223 Patoc strain Patoc1 and *L. interrogans* serovar Lai strain 56601 (4, 12, 14) and includes
224 genes coding for a DNA-binding protein that could constitute a Rep protein for initiation of
225 plasmid replication and a partitioning system (**Figure 2a**). Interestingly, orthologues of the *L.*
226 *mayottensis* Rep-like protein were also found in *L. interrogans* pathogenic strains with a
227 sequence identity superior to 75% (**Figure 2b**). Together, this suggests the 52 kb genomic
228 island located in *L. mayottensis* strain 200901116 may represent a putative prophage. The
229 occurrence of the *L. mayottensis rep* gene in other sequenced *Leptospira* strains suggests a
230 widespread distribution of this prophage in *L. interrogans* strains.

231

232 **Design of a replicative vector**

233 To construct the pMaORI replicative vector, a 2.9-kb autonomously replicating sequence
234 (ARS) consisting of *parA* locus (AKWB270009), *parB* locus (AKWB270010), and *rep*
235 (AKWB270011) located within the sequence of *L. mayottensis* strain 200901116 was
236 amplified and inserted into the 2.2kb conjugative plasmid pMAT, derived from pSW29T (10).
237 The pMAT vector includes the *aadA* spectinomycin resistant-cassette, *ori* R6K for replication,
238 and *oriT* RK2/RP4 for conjugation. The engineered vector is 5035bp and was named
239 pMaORI (**Figure 3**).

240

241 **Determination of pMaORI replication in various *Leptospira* spp.**

242 The pMaORI construct was confirmed to be a viable shuttle vector in saprophytic,
243 intermediate, and pathogenic *Leptospira* strains. Compared with original conjugative vector
244 carrying the *Himar1* transposon (10), the number of transconjugates increased from 2 to 5
245 fold when using our replication vector pMaORI into *L. biflexa* serovar Patoc strain Patoc, *L.*
246 *interrogans* serovar Copenhageni strain Fiocruz LA-130, and *L. interrogans* serovar Manilae
247 strain L495. It was possible to transform the pMaORI construct into *E. coli* β 2163, conjugate
248 pMaORI into leptospire, recover the plasmid via plasmid extraction, and then re-transform
249 pMaORI into *E. coli* Π 1 (**Table 2**). Transformed *Leptospira* strains were initially confirmed
250 positive for pMaORI construct via *aadA* amplification. To ensure confirmation was not a
251 result of *E. coli* contamination, positive *Leptospira* strains were tested for *adk*, an *E. coli*
252 gene. Further, to ensure *aadA* confirmation was not a result of naked DNA contamination
253 within the sample, pMaORI that was extracted from *Leptospira* strains was transformed into
254 *E. coli* Π 1, plasmid extracted from *E. coli*, and pMaORI was confirmed by KpnI restriction
255 profile.

256 The pMaORI vector was introduced (via conjugation) into *L. mayottensis* strain 200901116 to
257 determine if the pMaORI plasmid could replicate in its parental strain. Analysis revealed this
258 strain was incapable of maintaining the pMaORI vector.

259 Following confirmation that pathogenic *Leptospira* strains were able to replicate and maintain
260 the pMaORI plasmid, strains were passaged ten times in EMJH media without spectinomycin
261 to determine if the pMaORI plasmid could be conserved in the absence of selection. It was
262 confirmed that pathogenic strains were indeed capable of maintaining this shuttle vector
263 through ten passages without selection (**Table 2**). This was confirmed via plasmid extraction
264 of pMaORI from *Leptospira* cultures, followed by PCR amplification of the *aadA* cassette,
265 ability to re-transform into *E. coli* Π 1, and subsequent plasmid restriction profile analysis. A
266 limitation in the method utilized for detecting plasmid stability is that it does not quantify the
267 percentage of the population that retain pMaORI. Future studies should focus on quantifying
268 the percentage of the bacterial population that retain pMaORI, and determining copy number
269 per cell.

270

271 **Plasmid-complementation of *perR* in *Leptospira interrogans***

272 A *perR* mutant was previously obtained by random transposon mutagenesis in *L. interrogans*
273 serovar Manilae strain L495 (9). This *perR* mutant was shown to have greater survival in the
274 presence of hydrogen peroxide compared to wild-type cells. However, repeated attempts to
275 complement the mutation were unsuccessful (9).

276 In this study, complementation of the *perR* mutant was performed by cloning the *perR* wild-
277 type allele with its native promoter into the pMaORI plasmid vector. Hundreds of
278 transconjugants were obtained, and complemented cells showed expression of PerR
279 (**Figure 4A**), indicating that *perR* ORF was expressed in trans from the pMaORI expression
280 vector. Next, the ability of the complemented *perR* mutant cells to resist lethal concentrations
281 of H₂O₂ were tested and compared to those of wild type and the *perR* mutant. Cell viability
282 was assessed via the Alamar Blue assay, which measures the ability of cells to carry out
283 redox reactions; reducing blue resazurin to pink resorufin. As shown in figure 4B, in the
284 presence of H₂O₂, *perR* mutant cells display increased viability compared to wild type cells
285 as shown by their ability to reduce resazurin, indicating as expected that the *perR* mutant
286 had greater survival in the presence of peroxide. *PerR* mutant cells that had been
287 transformed by the pMaORI vector containing the *perR* allele were no longer able to reduce
288 the resazurin, which indicated that they had a decreased viability in the presence of H₂O₂
289 (**Figure 4B**). This demonstrated that expressing the *perR* coding sequence from the pMaORI
290 vector could restore an H₂O₂-associated phenotype comparable to that of wild-type cells.
291 While replication of plasmid vectors in pathogens has not been previously described, these
292 results demonstrate for the that plasmid complementation is possible in pathogenic
293 *Leptospira* strains. The pMaORI vector will therefore be useful for the purpose of genetic
294 complementation of mutants obtained by random transposon mutagenesis or in the
295 generation of conditional mutants.

296

297 **ACKNOWLEDGMENTS**

298 Authors thank J. Vinetz and D. Fouts for their permission to use the draft genomes which are
299 part of a project which was funded with federal funds from the National Institute of Allergy
300 and Infectious Diseases, National Institutes of Health, Department of Health and Human
301 Services under contract number HHSN272200900007C. This work was supported in part by
302 National Science Foundation grant IIA-1159099 (CJP) and the Institut Pasteur (MP).

303

304 REFERENCES

- 305 1. **Bauby, H., I. Saint Girons, and M. Picardeau.** 2003. Construction and
306 complementation of the first auxotrophic mutant in the spirochaete *Leptospira meyeri*.
307 *Microbiology* **149**:689-693.
- 308 2. **Bourhy, P., L. Collet, S. Brisse, and M. Picardeau.** 2014. *Leptospira mayottensis*
309 sp. nov., a pathogenic *Leptospira* species isolated from humans. *Int. J. Syst. Evol.*
310 *Microbiol.* **64**:4061-4067.
- 311 3. **Bourhy, P., L. Collet, T. Lernout, F. Zinini, R. A. Hartskeerl, H. van der Linden,**
312 **J. M. Thiberge, L. Diancourt, S. Brisse, C. Giry, F. Pettinelli, and P. M.** 2012.
313 Human leptospira isolates circulating in Mayotte (Indian Ocean) have unique
314 serological and molecular feature. *J. Clin. Microbiol.* **50**:307-311.
- 315 4. **Bourhy, P., L. Salaün, A. Lajus, C. Médigue, C. Boursaux-Eude, and M.**
316 **Picardeau.** 2007. A genomic island of the pathogen *Leptospira interrogans* serovar
317 Lai can excise from its chromosome. *Infect. Immun.* **75**:677-683.
- 318 5. **Demarre, G., A. M. Guerout, C. Matsumoto-Mashimo, D. A. Rowe-Magnus, P.**
319 **Marliere, and D. Mazel.** 2005. A new family of mobilizable suicide plasmids based
320 on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative
321 machineries and their cognate *Escherichia coli* host strains. *Res. Microbiol.* **156**:245-
322 255.
- 323 6. **Ellinghausen, H. C., and W. G. McCullough.** 1965. Nutrition of *Leptospira pomona*
324 and growth of 13 other serotypes: fractionation of oleic albumin complex and a
325 medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.* **26**:45-51.
- 326 7. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and
327 saprophytic leptospires. *J. Bacteriol.* **94**:27-31.
- 328 8. **Ko, A. I., C. Goarant, and M. Picardeau.** 2009. *Leptospira*: the dawn of the
329 molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.*
330 **7**:736-747.
- 331 9. **Lo, M., G. L. Murray, C. A. Khoo, D. A. Haake, R. L. Zuerner, and B. Adler.**
332 2010. Transcriptional response of *Leptospira interrogans* to iron limitation and
333 characterization of a PerR homolog. *Infect. Immun.* **78**:4850-4859.
- 334 10. **Picardeau, M.** 2008. Conjugative transfer between *Escherichia coli* and *Leptospira*
335 spp. as a new genetic tool. *Appl. Environ. Microbiol.* **74**:319-322.
- 336 11. **Picardeau, M.** 2015. Genomics, Proteomics, and Genetics of *Leptospira*, *Leptospira*
337 and Leptospirosis. Springer-Verlag Berlin Heidelberg.
- 338 12. **Picardeau, M., D. M. Bulach, C. Bouchier, R. L. Zuerner, N. Zidane, P. J.**
339 **Wilson, S. Creno, E. S. Kuczek, S. Bommezzadri, J. C. Davis, A. McGrath, M. J.**
340 **Johnson, C. Boursaux-Eude, T. Seemann, Z. Rouy, R. L. Coppel, J. I. Rood, A.**
341 **Lajus, J. K. Davies, C. Médigue, and B. Adler.** 2008. Genome sequence of the

- 342 saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and
343 the pathogenesis of leptospirosis. PLoS ONE **3**:e1607.
- 344 13. **Poggi, D., P. Oliveira de Giuseppe, and M. Picardeau.** 2010. Antibiotic resistance
345 markers for genetic manipulations of *Leptospira* spp. Appl. Environ. Microbiol.
346 **76**:4882-4885.
- 347 14. **Saint Girons, I., P. Bourhy, C. Ottone, M. Picardeau, D. Yelton, R. W. Hendrix,**
348 **P. Glaser, and N. Charon.** 2000. The LE1 bacteriophage replicates as a plasmid
349 within *Leptospira biflexa*: construction of an *L. biflexa*-*Escherichia coli* shuttle vector.
350 J. Bacteriol. **182**:5700-5705.
- 351 15. **Vallenet, D., E. Belda, A. Calteau, S. Cruveiller, S. Engelen, A. Lajus, F. Le**
352 **Fèvre, C. Longin, D. Mornico, D. Roche, Z. Rouy, G. Salvignol, C. Scarpelli, A.**
353 **A. Thil Smith, M. Weiman, and C. Médigue.** 2013. MicroScope--an integrated
354 microbial resource for the curation and comparative analysis of genomic and
355 metabolic data. Nucleic Acids Res. **41**:D636-647.
- 356 16. **Zhu, W. N., L. L. Huang, L. B. Zeng, X. R. Zhuang, C. Y. Chen, Y. Z. Wang, J.**
357 **H. Qin, Y. Z. Zhu, and X. K. Guo.** 2014. Isolation and Characterization of Two
358 Novel Plasmids from Pathogenic *Leptospira interrogans* Serogroup Canicola Serovar
359 Canicola Strain Gui44. PLoS Negl Trop Dis. **8**:e3103.
- 360 17. **Zuerner, R. L.** 1991. Physical mapping of chromosomal and plasmid DNA
361 comprising the genome of *Leptospira interrogans*. Nucleic Acids Res. **19**:4857-4860.
- 362 18. **Zuerner, R. L., J. L. Herrmann, and I. Saint Girons.** 1993. Comparison of genetic
363 maps for two *Leptospira interrogans* serovars provides evidence for two
364 chromosomes and intraspecies heterogeneity. J. Bacteriol. **175**:5445-5451.
- 365
- 366

367

368 **Table 1:** Primers Used in this Study
 369

	Sequence (5' -> 3')^a	target
spcA	GGGGTGAATTTGAGAATGGA	<i>aadA</i>
spcB	GTCACTGTTTGCCACATTCC	cassette
ori1	CGCGGATCCTAATCAGCATACTGCAATCC	<i>ori</i>
ori2	CTAGTCTAGAAATCCGTATAGCATATTCC	<i>ori</i>
adkF	ATTCTGCTTGGCGCTCCGGG	<i>E. coli</i>
adkR	CCGTCAACTTTCGCGTATTT	<i>adK</i>
PerR5	CTCCTTAGAATGGACCGAAG	<i>perR</i>
PerR3	GGATTCTATGTAGGATCAGTAG	<i>perR</i>

370
 371
 372
 373

^a: BamHI and XbaI restriction sites are underlined

374
375
376
377
378
379

Table 2: *Leptospira* strains are capable of replication and conservation of pMaORI

type	species	serovar	strain	spcR colonies ^b	pMaORI conservation ^d
saprophyte	<i>L. biflexa</i>	Patoc	Patoc1	+++	ND
intermediate	<i>L. fainei</i>	Hurstbridge	BUT 6	++	ND
intermediate	<i>L. licerasiae</i>	Varillal	VAR010	+	ND
pathogen	<i>L. interrogans</i>	Canicola	Hond Utrecht IV	+++	positive ^c
pathogen	<i>L. interrogans</i>	Copenhageni	Fiocruz L1-130	++	positive
pathogen	<i>L. interrogans</i>	Copenhageni	Wijnberg	+	positive
pathogen	<i>L. interrogans</i>	Icterohaemorrhagiae	Verdun	+++	positive
pathogen	<i>L. interrogans</i>	Lai	56601	+++	positive
pathogen	<i>L. interrogans</i>	Manilae	L495	++	positive
pathogen	<i>L. mayottensis</i>	Mini ^a	200901116	-	NA

^a: serogroup

^b: - = no colony, +=1-40 colonies, ++= 41-100 colonies, +++ = >100 colonies.

^c: strain verified until passage 4

^d: pMaORI conserved after 10 passages without spectinomycin selection. Results verified by KpnI restriction profile of plasmid preps, and PCR of *aadA* cassette and the *E. coli* *adh* gene.

NA : not applicable

ND : not determined

380
381

382 **Figure legends**

383

384

385 **Figure 1: Comparative genome analysis between the draft genomes of strains**
386 **200901116 and 200901122.**

387 Comparative genome analysis was performed using the MaGe interface (15) in the
388 SpiroScope database (<https://www.genoscope.cns.fr/agc/mage>). The arrow indicates the
389 phage-like region present in strain 200901116, but not in strain 200901122.

390

391

392 **Figure 2: Genetic organization of replication origins of prophage and plasmids**
393 **identified in *Leptospira* spp.** A :Figure compares organization of *parA* and *parB* between

394 saprophytic and pathogenic *Leptospira* spp. Sequence analysis of *L. mayottensis* strain
395 200901116 reveals a putative replication origin constituted by genes encoding a DNA-
396 binding protein that could constitute a Rep protein for initiation of plasmid replication and
397 ParA/ParB partitioning system. This genetic organization is similar to previously identified
398 replication origins in *L. biflexa* (12, 14) and *L. interrogans* (4). B : Sequence alignment of
399 putative Rep proteins from *L. mayottensis* serovar Mini strain 200901116, *L. interrogans*
400 strain UI08452, *L. interrogans* serovar Medanensis strain L0448, *L. interrogans* strain L0996,
401 *L. interrogans* serovar Zanoni strain LT2156. Identical residues are shaded in black,
402 conserved residues are shaded in grey.

403

404

405 **Figure 3: Schematic representation of plasmid vector pMaORI.**

406 Plasmid pMaORI (5035 bp) was generated by cloning the replication origin of *L. mayottensis*
407 strain 200901116, which carries the *rep*, *parA*, and *parB* genes, into the XbaI-BamHI
408 restriction sites of conjugative plasmid pMAT, which contains the spectinomycin resistance
409 cassette (*aadA*/ *Spc*^r), *ori* R6K, and *oriT* RK2/RP4. The *aadA* gene is expressed from the *B.*
410 *burgdorferi* *flgB* promoter.

411

412

413 **Figure 4: Complementation of a *L. interrogans perR* mutant**

414 **A.** PerR production in the wild-type (WT), *perR* mutant (*perR*), and complemented *perR*
415 mutant (*perR* + pMaORI-*perR*) cells. Western blot analysis shows recovery of expression of
416 PerR in the *perR* mutant carrying the replicative plasmid pMaORI-*perR*.

417 **B.** Cell viability of the wild-type (WT), *perR* mutant (*perR*), and complemented *perR* mutant
418 (*perR* + pMaORI-*perR*) cells during peroxide stress. Exponentially growing wild-type, *perR*

419 mutant, and complemented *perR* mutant cells were exposed to H₂O₂ and cell viability was
420 assessed in the presence of resazurin. Cell viability is measured by the capacity of the cell to
421 carry out the redox reaction of reducing blue resazurin into bright-pink resorufin.
422

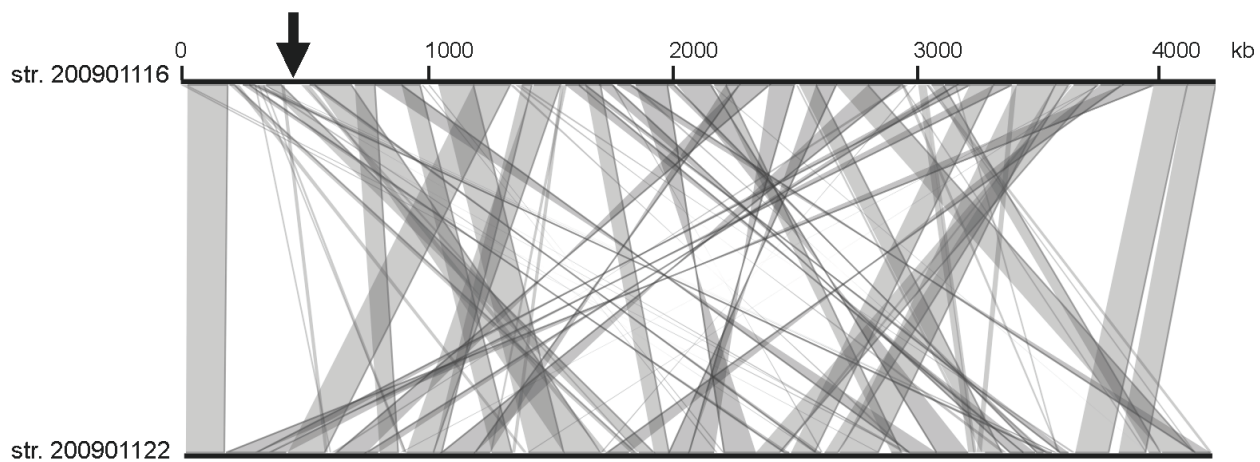


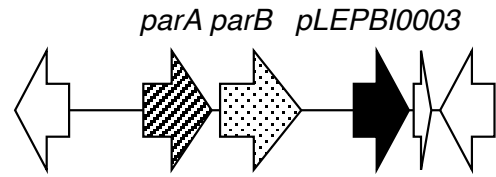
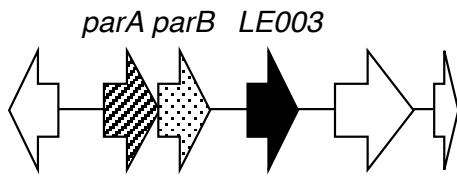
Figure 1

A

500 bp

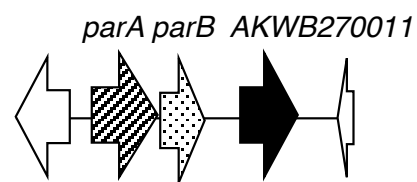
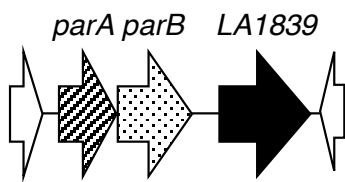
L. biflexa LE1

L. biflexa p74



L. interrogans Lai GI

L. mayottensis 200901116 GI



B

LINTstrL0448	1	MEENYVGEFIPRAVINTKLSRGLRDLLAKITLLDIAGRCEGRNGCYAGNEYLATCLGMAA
LINTstrL0996	1	MEENYVGEFIPRAVINTKLSRGLRDLLAKITLLDIAGRCEGRNGCYAGNEYLATCLGMAA
LINTstrUI08452	1	MEENYVGEFIPRAVINTKLSRGLRDLLAKITLLDIAGRCEGRNGCYAGNEYLATCLGMAA
LINTstrLT2156	1	MEENYVGEFIPRAVINTKLSRGLRDLLAKITLLDIAGRCEGKGCYAGNEYLATCLGTAAT
LMAostr200901116	1	MD E LLTYGEFIPRAVINTKLSRGTRDVLAKITLLDIAGRCEGRGCGCYAGNEYLATMLGMAT
LINTstrL0448	61	TTIAKYISRLRKAGYIEQVSFDGRVRVIRSTLHDAVVMERVQYKISKALANNPSQPRTN
LINTstrL0996	61	TTIAKYISRLRKAGYIEQVSFDGRVRVIRSTLHDAVVMERVQYKISKALANNPSQPRTN
LINTstrUI08452	61	TTIAKYISRLRKAGYIEQVSFDGRVRVIRSTLHDAVVMERVQYKISKALANNPSQPRTN
LINTstrLT2156	61	TTVAKYISRLRKAGYIEQVSFDGRVRVIRSTLHDAVVNERAQYKISKALANNPSQPRTN
LMAostr200901116	61	TTIAKYISRLRKAGYIEQISFDGRVRVIRSTLHDMVVMERAQYKISKASANNPSQTRTN
LINTstrL0448	121	GQDRGIQQGRAAWDNRSVAVRTKEESKKTLLNVSAEKIKSEPTWNGFLDWAERLTRSSND
LINTstrL0996	121	GQDRGIQQGRAAWDNRSVAVRTKEESKKTLLNVSAEKIKSEPTWNGFLDWAERLTRSSND
LINTstrUI08452	121	GQDRGIQQGRAAWDNRSVAVRTKEESKKTLLNVSAEKIKSEPTWNGFLDWAERLTRSSND
LINTstrLT2156	121	GQDRGVQQGRAAWDNRSVAVRTKEENKKTLLNVSAEKIKSEPTWNGFLDWAERLTRSSND
LMAostr200901116	121	GQGREVROGRAAEWDVRSASVRTKEESKKTLLNVDAAKNQ-KPTWIGFLDWANERLTRSSND
LINTstrL0448	181	VLKNLKIDFKVSELRLLEPVTNSLSMIILKYFTEEVKRPISVKFVEKTEQGAA-
LINTstrL0996	181	VLKNLKIDFKVSELRLLEPVTNSLSMIILKYFTEEVKRPISVKFVEKTEQGAA-
LINTstrUI08452	181	VLKNLKIDFKVSELRLLEPVTNSLSMIILKYFTEEVKRPISVKFVEKTEQGAA-
LINTstrLT2156	181	ILKNLKIDFKVNELRLEPVTNSLSMVIILKYFTEEVKNQFQ-----
LMAostr200901116	180	ILRNKIDFNGTELRLEPVSDSLGMVIQKYFTEETEKPIEVVFKNSDEQSRAA

Figure 2

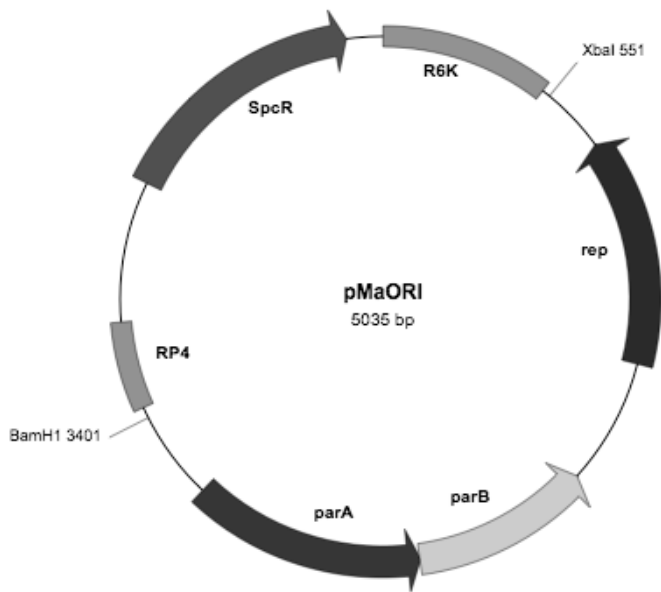


Figure 3

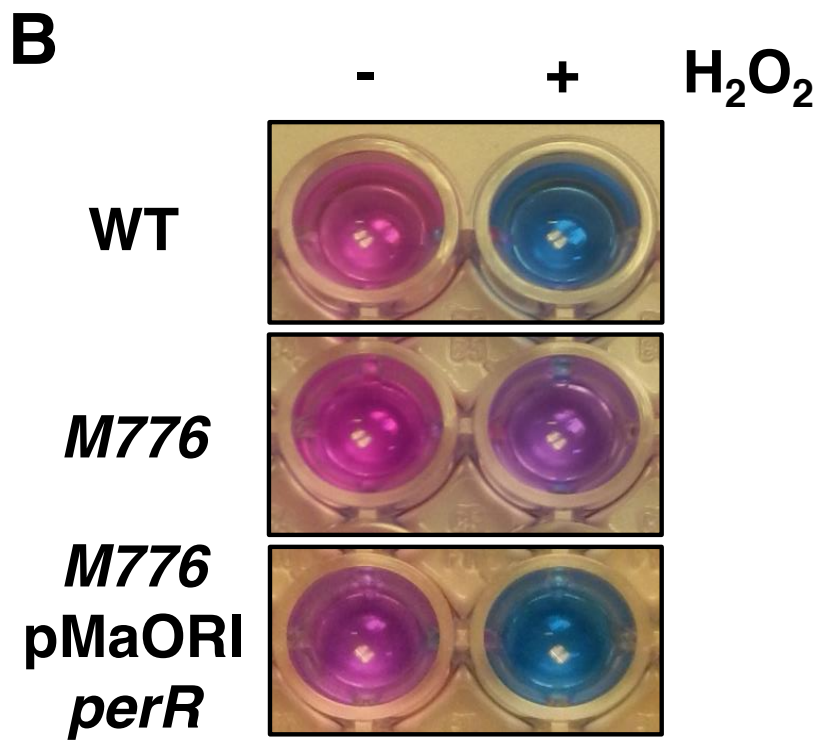
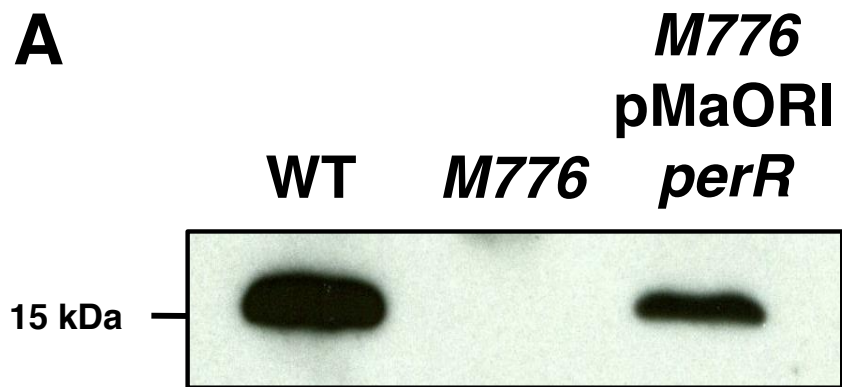


Figure 4