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1 **A novel flagellar sheath protein, FcpA, determines filament coiling, translational motility**
2 **and virulence for the *Leptospira* spirochete**

3

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23

24 **Summary**

25 *Leptospira* are unique among bacteria based on their helical cell morphology with hook-
26 shaped ends and the presence of periplasmic flagella (PF) with pronounced spontaneous
27 supercoiling. The factors that provoke such supercoiling, as well as the role that PF coiling plays
28 in generating the characteristic hook-end cell morphology and motility, have not been elucidated.
29 We have now identified an abundant protein from the pathogen *L. interrogans*, exposed on the
30 PF surface, and named it Flagellar-coiling protein A (FcpA). The gene encoding FcpA is highly
31 conserved among *Leptospira* and was not found in other bacteria. *fcpA*- mutants, obtained from
32 clinical isolates or by allelic exchange, had relatively straight, smaller-diameter PF, and were not
33 able to produce translational motility. These mutants lost their ability to cause disease in the
34 standard hamster model of leptospirosis. Complementation of *fcpA* restored the wild-type
35 morphology, motility and virulence phenotypes. In summary, we identified a novel *Leptospira*
36 36-kDa protein, the main component of the spirochete's PF sheath, and a key determinant of the
37 flagella's coiled structure. FcpA is essential for bacterial translational motility and to enable the
38 spirochete to penetrate the host, traverse tissue barriers, disseminate to cause systemic infection,
39 and reach target organs.

40 **Introduction**

41 Leptospirosis is the major zoonotic cause of mortality and morbidity worldwide (Costa *et*
42 *al.*, 2015). The disease can be caused by >200 serovars distributed among ten pathogenic species
43 that belong to the genus *Leptospira*, which also encompasses saprophytic and intermediate
44 species (Ko *et al.*, 2009). A key feature of pathogenic *Leptospira* is their ability to produce rapid
45 translational motility (Noguchi, 1917). Pathogenic *Leptospira* rapidly penetrate abraded skin
46 and mucous membranes, traverse tissue barriers and cause a systemic infection within minutes to
47 hours (McBride *et al.*, 2005, Ko *et al.*, 2009, Bharti *et al.*, 2003). Motile spirochetes rely on their
48 helical or flat wave morphology and asymmetrical rotation of periplasmic flagella (PF) attached
49 near each cell cylinder extremity to generate translational motility (Charon & Goldstein, 2002,
50 Charon *et al.*, 2012, Motaleb *et al.*, 2000). However, leptospiral morphology is markedly
51 different from what is observed for other spirochetes. Leptospires are unique as they have hook-
52 shaped cell ends when PF are not rotating and cells thus resemble to a question mark, a feature
53 initially observed by Stimson in 1907, who named the organism *Spirocheta* (now *Leptospira*)
54 *interrogans* (Stimson, 1907). During translational motility, as viewed from the center of the cell,
55 counterclockwise PF rotation produces spiral-shaped ends at the leading end, while
56 concomitantly clockwise PF rotation causes the gyrating cell to remain hook-shaped at the tail
57 end (Berg *et al.*, 1978, Kan & Wolgemuth, 2007, Goldstein & Charon, 1990, Nakamura *et al.*,
58 2014).

59 Spirochete PF are similar in structure and function to flagella of externally flagellated
60 bacteria, as each consists of a basal body complex or motor, a flexible hook and a flagellar
61 filament (Charon & Goldstein, 2002, Limberger, 2004). Whereas PF from non-*Leptospira*
62 spirochetes display curved forms when viewed by electron microscopy once purified (Charon *et*

63 *al.*, 1991, Li *et al.*, 2000a), leptospire PF are instead extensively supercoiled in the form of a
64 spring (Berg *et al.*, 1978, Bromley & Charon, 1979, Wolgemuth *et al.*, 2006, Kan & Wolgemuth,
65 2007). Furthermore, leptospire mutants that form uncoiled PF, or lacking PF altogether,
66 maintain their helical cell body shape but display straight cell axes, and are also unable to
67 generate translational motility (Bromley & Charon, 1979, Picardeau *et al.*, 2001). These
68 findings, taken together, suggest that the coiled phenotype of PF and their interaction with the
69 helical cell cylinder are key determinants in producing the peculiar cell morphology observed for
70 leptospire and their ability to produce translational motility.

71 The molecular factors that contribute to the coiled flagella phenotype in *Leptospira* have
72 not been fully unveiled. In contrast to the flagellar filaments of enterobacteria, which are
73 composed of a single flagellin protein (Macnab, 1996, Berg, 2003), spirochete PF are a multi-
74 protein complex that comprise of a core, composed of the FlaB family of flagellin-like proteins,
75 and sheath proteins (Charon & Goldstein, 2002, Wolgemuth *et al.*, 2006, Li *et al.*, 2000b).
76 Although FlaA, a protein family conserved across spirochetes (Wolgemuth *et al.*, 2006, Li *et al.*,
77 2000b, Charon & Goldstein, 2002, Liu *et al.*, 2010, Li *et al.*, 2008), was found to form a flagellar
78 sheath for *Brachyspira hyodysenteriae* (Li *et al.*, 2000a), *Treponema pallidum* (Cockayne *et al.*,
79 1987, Isaacs *et al.*, 1989) and *Spirochaeta aurantia* (Brahamsha & Greenberg, 1989) this
80 observation was not confirmed for *Borrelia burgdorferi* (Motaleb *et al.*, 2004) and *L. interrogans*
81 (Lambert *et al.*, 2012). Spirochetes appear thus to differ in the composition and organization of
82 their flagella. Elucidation of the complex structure of leptospire PF may reveal new mechanisms
83 for flagella-associated motility. Although recent advances have been made to genetically
84 manipulate *Leptospira* and address this question (Picardeau, 2015), the leptospire PF structure
85 remains poorly characterized.

86 Herein, we report our investigation of motility-deficient and motile strains from a clinical
87 isolate of *L. interrogans*, which in turn led to the identification of a novel and highly abundant
88 leptospire protein, Flagellar-coiling protein A (FcpA). Targeted mutagenesis and
89 complementation, together with immuno-electron microscopy, demonstrated that FcpA is a key
90 component of leptospiral PF sheath and is an essential requirement for the hook-shaped
91 morphology of the cell ends, coiled flagella phenotype and translational motility. We also
92 provide evidence that these features are essential for the process of bacterial penetration and
93 dissemination in host tissues.

94 **Results**

95

96 **Isolation of a motility-deficient clone in *L. interrogans***

97 *L. interrogans* serovar Copenhageni strain Fiocruz LV2756 was isolated from a Brazilian
98 patient with pulmonary hemorrhage syndrome due to leptospirosis (Gouveia *et al.*, 2008). Two
99 colony morphologies, large and small, were observed after plating the isolate onto solid EMJH
100 media. The phenotype was confirmed by measuring the ability of the cells to grow in motility
101 plate assays (Fig. 1A), confirming the previous observation. Sub-culturing of larger colonies
102 yielded leptospire, which had phenotypes similar to wild-type (WT) *L. interrogans* with respect
103 to the characteristic hook-end cell morphology (Figs. 1B and 1C) and translational motility
104 (Video S1). In contrast, sub-cultures of small colonies yielded leptospire that lacked the
105 terminal hook-ends (Figs. 1B and 1C), and did not produce translational motility (Video S2).
106 Leptospire from small colonies retained the characteristic corkscrew cell body morphology,
107 were able to gyrate their ends, forming spiral waveforms at their terminal ends. We also
108 observed that a proportion of these leptospire grew as long chains with incomplete division
109 planes (Video S2). Sub-cultures of large and small colonies were named motile and motility-
110 deficient strains, respectively.

111 Velocity measurements of both strains showed a statistically significant decrease of the
112 mean velocity for the motility-deficient strain ($2.77 \pm 1.7 \mu\text{m/s}$) when compared with the motile
113 strain cells ($11.4 \pm 4.47 \mu\text{m/s}$, $p < 0.0001$) (Fig. S2A). The path of individual cells shows that the
114 Fiocruz LV2756 motility-deficient strain lacks translational motility, indicating that the residual
115 velocity measured most-likely derives from gyration-related movement combined with Brownian
116 motion (Fig. S2B). Transmission electron microscopy of purified negatively stained PF revealed

117 that the motility-deficient strain had straightened PF, opposed to the extensively coiled PF
118 observed from the motile strain and WT strains (Fig. 1D). Furthermore, diameters of PF from
119 motility-deficient strain (mean 16.3 ± 2.9 nm) were significantly smaller than those from motile
120 strain (mean 21.5 ± 2.2 nm, $p < 0.0001$). However, there was no difference in the length of the
121 flagella between motile strain ($2.14 \pm 0.57 \mu\text{m}$) and motility-deficient strain ($2.17 \pm 0.52 \mu\text{m}$,
122 $p = 0.8870$). Taken together, these data suggest that the alteration of the morphology of the
123 flagellum is responsible for the loss of hook-shaped ends in the motility-deficient strain.

124

125 **Motility is required for host penetration and virulence**

126 Intraperitoneal inoculation of hamsters with motile strain uniformly induced a moribund
127 state between 6-8 days post-infection (Table 1). In contrast, hamsters infected with motility-
128 deficient strain had significantly ($p < 0.05$) reduced mortality and prolonged survival. Loss of the
129 *fcpA* gene was associated with a greater than seven log increase (4.64 to $\geq 10^8$ bacteria) in the
130 LD_{50} , indicating that motility is an essential determinant for virulence (Table S1).

131 To determine whether the loss of virulence observed for the motility-deficient mutant was
132 due to a defect in its ability to disseminate in the host, we performed quantitative PCR analysis
133 of tissues from animals that were infected intraperitoneally with motile and motility-deficient
134 strains and perfused prior to harvesting. One hour after inoculation, the motile strain was
135 detected at $> 10^3$ genome equivalents (GEq) per gram in tissues, including immune privileged
136 sites such as the eye (Fig. 2A). Four days after inoculation, bacterial loads reached $> 10^7$ GEq per
137 gram in blood, lung, liver and kidneys (Fig. 2B). Infection with the motility-deficient strain did
138 not yield detectable bacteria in tissue one hour after infection but did produce bacterial loads of
139 up to 10^4 GEq per gram in tissues obtained four days after challenge, indicating that the motility-

140 deficient strain was able to disseminate from the peritoneum, cause a systemic infection and
141 reach organs, although at a low burden when compared with the motile strain (Figs. 2A and 2B).
142 Bacteria were not detected in tissues 21 days after infection (data not shown), indicating that
143 infection with the motility-deficient strain was transient.

144 We then determined whether the motility-deficient strain was capable of penetrating
145 epithelial barriers and entering the host, the key initial step in infection. Inoculation of hamsters
146 with 10^8 bacteria of motile strain via conjunctival route produced bacteremia and bacterial loads
147 of $>10^5$ GEq per gram in tissues collected seven days post-infection and uniformly caused death
148 at 8-9 days post-infection (Fig. 2C). In contrast, inoculation with the motility-deficient strain did
149 not yield detectable bacteria in tissues or produce a lethal infection. Furthermore, we found that
150 motility-deficient strain was unable to translocate *in vitro* across polarized MDCK cell
151 monolayers, in contrast to WT strain (Fig. S1).

152

153 **Identification of a novel *Leptospira* flagellar-associated protein**

154 SDS-PAGE analysis identified a prominent band with a molecular weight of 36kDa in
155 whole cell lysates (data not shown) and purified PF (Fig. 3A, lane 1) of the motile strain, which
156 was absent in preparations of the motility-deficient strain (Fig. 3A, lane 2). Mass spectrometry
157 (MS) of this 36-kDa protein excised from the gel band identified 10 unique peptides, which were
158 associated with the motile strain and not the motility-deficient strain. Peptide sequences were
159 identical and covered 83% of the predicted hypothetical protein LIC13166 of *L. interrogans*
160 strain Fiocruz L1-130, a virulent strain (Ko *et al.*, 1999) whose genome was previously
161 sequenced (Nascimento *et al.*, 2004).

162 All of the *Leptospira* genomes present in public databases (>500 at the time of writing)
163 have orthologs of the gene *lic13166*. However, no orthologs were identified in the genome of
164 other spirochetes or any other bacterial species. The amino acid sequence identity between
165 LIC13166 orthologs of pathogenic, intermediate and saprophytic species of *Leptospira* was 95-
166 100%, 88% and 76-79%, respectively. LIC13166 is predicted to encode a protein of 306 amino
167 acids, of which the first 25 encode a signal peptide. A previous study reported that the LIC13166
168 gene product was the 13th most abundant among all cell proteins in *L. interrogans* (Malmstrom *et*
169 *al.*, 2009). Polyclonal antibodies to recombinant LIC13166 protein recognized a 36kDa protein
170 in whole-cell lysates (data not shown) and purified PF (Fig. 3B, lane 1) of the motile strain and
171 did not label moieties in western-blot of the motility-deficient strain (data not shown) or its
172 purified PF (Fig. 3B, lane 2). Since the LIC13166 protein was specifically associated with coiled
173 PF and not straight PF, we named the protein, Flagellar-coiling protein 1 (FcpA).

174 Further analysis of SDS-PAGE (Fig. 3A) also revealed the presence of a band below
175 FlaB1 in the motile strain (Fig. 3A line 1), which is absent on the motility-deficient strain (Fig.
176 3A line 2). This uncharacterized protein could correspond to a FcpA-associated protein of the
177 flagellum. Furthermore, quantitative immunoblotting showed that there was a reduction of
178 expression of both FlaA1 (26% \pm 2.9) and FlaA2 (57.5% \pm 3.3) proteins in the motility-deficient
179 strain, but no significant reduction of FlaB1 (98.2% \pm 2.4). Together, these observations indicate
180 that the lack of FcpA protein modify the composition of proteins in the flagellum.

181 The *fcpA* gene from the motility-deficient strain had an insertion of a deoxythymidine at
182 base pair position 855, which introduced a frame shift at amino acid position 286 and resulted in
183 a premature stop codon at amino acid position 294 (Fig. 4). In contrast, the *fcpA* gene sequence
184 from the motile strain was identical to WT strain. Whole genome sequencing of motile (NCBI

185 accession number PRJNA63737) and motility-deficient (NCBI accession number PRJNA65079)
186 strains found 10 single nucleotide polymorphisms and 5 indels between the genomes. Among
187 these, only the insertional mutation in *fcpA* (*L. interrogans* strain Fiocruz L1-130 genome
188 position 3876852) was predicted to disrupt a gene product, indicating that a single spontaneous
189 mutation abolished FcpA protein expression in the motility-deficient strain.

190

191 **Allelic exchange and genomic complementation confirm that FcpA is a flagellar protein**

192 To confirm that inactivation of the *fcpA* gene specifically causes loss of translational
193 motility, we used a homologous recombination approach (Fig. 4) to generate a *fcpA*- mutant of *L.*
194 *interrogans* strain Fiocruz L1-130 (Video S3). The resultant mutant, Fiocruz L1-130 *fcpA*-, not
195 only lacked the expression of FcpA (Fig. 3 line 4) when compared with the wild-type (Fig. 3 line
196 5), but also exhibited identical phenotypes as observed previously for the motility-deficient strain
197 (Figs. 1 and 3), including loss of translational motility (Video S4).

198 Complementation of WT *fcpA* gene into the motility-deficient and *fcpA*- strains restored
199 the hook-end morphology of cells (Figs. 1B and C), the expression of FcpA (Fig. 3 lines 3 and 6)
200 and translational motility (Videos S5 and S6). PF purified from complemented strains were
201 coiled (Fig. 1D). SDS-PAGE and immunoblotting analysis demonstrated that complementation
202 of the *fcpA* gene rescued FcpA and other related flagellar proteins expression and confirmed the
203 presence of this protein in the flagella structure (Fig. 3). Altogether, these findings indicate that
204 the lack of FcpA expression resulted in the disappearance of the hook-shaped end of the cell and
205 loss of the coiled morphology of the flagella when purified, ultimately resulting in cells without
206 the ability to produce translational motility.

207 Generation of *fcpA*- mutants and complemented strains provided the opportunity to
208 confirm the role of motility in leptospiral pathogenesis. Loss of FcpA in the knock-out strain
209 was also associated with an attenuated phenotype (Table 1) and a statistically significant log
210 increase in the LD₅₀ (Table S1). Complementation restored the virulence phenotype (Table 1)
211 and LD₅₀ to values observed for WT strains (Table S1), showing that motility of leptospires is an
212 essential determinant for virulence and that FcpA plays a key role in this process. Furthermore,
213 complementation of the *fcpA* gene restored the ability to translocate across cell monolayers (Fig.
214 S1). Knockout and complementation studies thus demonstrate that motility is essential for
215 pathogen penetration and entry into the host, and the leptospires' ability to traverse tissue
216 barriers.

217

218 **FcpA is essential for the formation of the *Leptospira* flagellar sheath**

219 Diameters of PF from strains with mutations in *fcpA* were significantly smaller than WT
220 strains (L1-130 *fcpA*-, mean 17.6 ± 2.3 nm; L1-130 WT, mean 22.8 ± 3.8 nm; $p < 0.0001$).

221 Reintroducing the wild-type *fcpA* restored diameters of PF in complemented strains (LV2756
222 *fcpA*-/+, mean 22.1 ± 1.7 nm; Fiocruz L1-130 *fcpA*-/+, mean 22.1 ± 2.1 nm), similar to that of the
223 motile strains, demonstrating that expression of FcpA protein is required to generate PF with
224 appropriate thickness, in addition to maintaining its coiled structure.

225 Cryo-electron tomography of intact leptospires confirmed that *in situ* diameters of PF for
226 WT, *fcpA*- mutant and complemented strains were similar to those obtained for purified PF
227 preparations. PF of *fcpA*- (Fig. 5E) were uniformly thinner than PF from WT and complemented
228 strains (Figs. 5D and 5F). *In situ* three-dimensional reconstructions of intact organisms were
229 generated for *fcpA*- mutant (Video S7) and complemented strains (Video S8) confirming this

230 finding (Fig. 5). There were no differences found in the *fcpA*- mutant when compared to the WT
231 or complemented strains with respect to cell morphology or the helical pitch of the flagella along
232 the cell axis.

233 Immuno-electron microscopy demonstrated that anti-FcpA antibodies specifically labeled
234 the surface of PF from WT strains and did not bind to PF from *fcpA*- strains (Fig. 6).
235 Furthermore, labeling was evenly distributed along the length of wild-type PF. Although
236 antibodies to FlaB1 strongly bound to the respective moiety in immunoblotting assays (Fig. 3B),
237 this antibody did not label PF from WT and *fcpA*- strains, confirming that FlaB1 is not expressed
238 on the PF surface. These findings, together with the reduced PF diameter observed in *fcpA*
239 mutants, indicate that FcpA protein is a major component of the leptospiral flagellar sheath.

240 **Discussion**

241 Our study provides strong evidence that the leptospiral PF structure determines the cell
242 shape, specifically the hooked end morphology, and in turn, the spirochete's ability to generate
243 translational motility and ultimately virulence. Furthermore, we found that a novel protein,
244 FcpA, is a key component of the PF sheath, and that FcpA is the molecule mediating these
245 phenotypes. In the 1960s, studies of motility-deficient leptospire described the loss of the
246 hooked-end cell morphology and its correlation with small colony phenotype (Simpson & White,
247 1964) and virulence attenuation (Faine & Vanderhoeden, 1964). However no association
248 between cell morphology and virulence was reported at that time. Bromley et al. characterized a
249 motility-deficient mutant obtained by chemical mutagenesis, which had straight cell ends and
250 yielded uncoiled PF after purification. The authors proposed that the PF contributed to the
251 hooked end cell morphology (Bromley & Charon, 1979), but could not rule out the possibility of
252 secondary mutations. More recently, studies showed that loss of *fliY* or *flaA2* in *L. interrogans*
253 was associated with attenuated motility and virulence yet, complementation of the gene and
254 rescue of these phenotypes were not performed (Lambert *et al.*, 2012, Liao *et al.*, 2009) . In this
255 study, the construction of *fcpA* knockout and complemented mutants provided the opportunity to
256 apply Koch's molecular postulates, thus demonstrating that a novel flagellar structural protein,
257 FcpA, is essential for PF structure, cell morphology and translational motility. Inactivation of
258 *fcpA* resulted in a more than seven-fold increase (≤ 10 to $\geq 10^8$) in the LD₅₀ of *L. interrogans* in
259 the hamster model of leptospirosis, while complementation of *fcpA* restored the LD₅₀ to that
260 observed for the WT strain. Further indicating that that motility is a key virulence determinant
261 among spirochetal pathogens.

262 We also found that *fcpA*- mutant strains were unable to induce infection, as ascertained by
263 PCR detection, when applied to mucous membranes of the conjunctiva, which mimics a natural
264 mode of transmission (Bolin & Alt, 2001, Evangelista & Coburn, 2010). *fcpA*- mutants were
265 unable to translocate *in vitro* across polarized mammalian cell monolayers, in contrast to WT and
266 complemented strains (Fig. S1). These finding support the assertion that motility is required for
267 the key initial infection event of host penetration.

268 FcpA is essential for the formation of the hook-shaped ends of leptospire, but more
269 importantly it appears to interfere in the generation of the spiral waveform during translational
270 motility. Prior studies showed that counterclockwise rotation of PF at the terminal end, as
271 viewed from the center of the cell, creates a backward motion of the spiral wave, which in turn,
272 causes the cylinder to roll clockwise across the body axis (Goldstein & Charon, 1988, Kan &
273 Wolgemuth, 2007, Goldstein & Charon, 1990, Berg *et al.*, 1978). Therefore, in a low viscosity
274 medium leptospire achieve translational motility through counter-clockwise rotation of the PF,
275 gyration of the leading, spiral-shaped end, and generation of a left-handed waveform that travels
276 opposite to the swimming direction. In viscous gel-like media, such as connective tissue, the
277 clockwise roll of the cell cylinder allows the organisms to swim with no slippage (Li *et al.*,
278 2000b, Berg *et al.*, 1978, Charon *et al.*, 1991, Kan & Wolgemuth, 2007, Goldstein & Charon,
279 1990). A recent study concluded that the change in the hook-shaped end rotation rate occurs in
280 response to the change in the spiral-shaped end rotation rate, indicating that the hook-shaped end
281 of the cells does not contribute to the translation motion of the cell (Nakamura *et al.*, 2014).
282 Therefore, the phenotype observed in our mutants reflects a disturbance on the gyration of the
283 spiral-shaped end of the cell with concomitant inhibition of rolling of the cell cylinder.

284 Genetic manipulation of flagellar genes of the spirochete *B. hyodysenteriae* demonstrated
285 that stiffer PF deform the cell cylinder and that larger deformations produce more thrust (Li *et*
286 *al.*, 2008). Although *fcpA*- mutants were able to generate spiral-shaped ends (Videos S2 and S5),
287 gyration of the spiral-shaped ends is either too slow or not large enough to yield sufficient thrust
288 for the bacteria to translate, similar to what is proposed to *T. phagedenis* in low-viscosity media
289 (Charon *et al.*, 1991). Thus, the perturbed interactions between the PF and cell cylinder due to
290 loss of FcpA, result in qualitatively different effects depending on the direction of rotation.
291 Although speculative, rotation-dependent conformational changes in the PF, due to interactions
292 influenced by FcpA protein within the flagellar assembly, may explain the differences in the
293 spiral and hook-end morphologies observed during translational motility in *Leptospira*.

294 We found that FcpA, which was previously found as an abundant protein (Malmstrom *et*
295 *al.*, 2009), is a major component of the *Leptospira* flagellar sheath and may play a key role in the
296 interaction between PF and cell cylinder. The loss of FcpA generated a flagellar structure that
297 lost its super-coiled form when purified. We demonstrated that PF from *fcpA*- mutants were
298 significantly thinner than those from the WT and complemented strains (Fig. 5). The
299 observation of a smaller PF diameter was consistent with a peripheral location of FcpA, which
300 we confirmed in immuno-EM analyses to be surface exposed (Fig. 6). *B. hyodysenteriae* with a
301 mutation in the *flaA* gene showed similar results, with the mutant having significant thinner
302 flagella (19.6nm) when compared to the WT strain (25nm) (Li *et al.*, 2000a). Furthermore, the
303 measured diameter for the WT strain (22.8nm) was consistent with previous results for
304 *Leptospira* spp. (18-25nm) (Nauman *et al.*, 1969). Taking together these results corroborate
305 previous observations of thicker PF in spirochetes compared to *E. coli* and *Salmonella* spp.
306 (20nm) (Macnab, 1996). Finally, FcpA appears to play a similar role for PF structure, cell

307 morphology and motility across *Leptospira* spp. since the same phenotypes were observed in a
308 *fcpA*- mutant generated in the saprophyte *L. biflexa* (data not shown).

309 The function of flagellar sheath likely appears to be highly heterogeneous in spirochetes
310 given the variation in sheath composition. *B. hyodysenteriae* FlaA influences the shape, stiffness
311 and helicity of PF (Li *et al.*, 2000a, Li *et al.*, 2008). In contrast, preliminary evidence indicates
312 that *B. burgdorferi* FlaA is located on the surface of the PF proximal to the basal body (Ge *et al.*,
313 1998, Sal *et al.*, 2008). A recent study found that the two FlaA proteins are not involved in the
314 formation of the flagellar sheath in *Leptospira* (Lambert *et al.*, 2012). Similarly, our immuno-
315 EM studies did not detect expression of FlaB1 protein on the surface of PF purified from WT or
316 *fcpA*- mutant strains (Fig. 6). Mutants deficient in FlaA proteins produced PF containing the
317 same pattern of expression of FcpA (data not shown), but lacked hook-shaped ends and
318 translational motility and yielded straight PF when purified (Lambert *et al.*, 2012). Our results
319 showed that the lack of FcpA expression influence the expression of FlaA proteins, thus
320 indicating that FlaA proteins and FcpA contribute to the coiled characteristics of the PF.
321 Exposure of proteins, other than the flagellin ortholog FlaB, on the surface of PF filaments may
322 serve as the structural basis for a sheath, which would adopt the topology of a continuous
323 envelope covering a FlaB core, constituted by four different FlaB isoforms (Malmstrom *et al.*,
324 2009). This model could be thought of as concentric layers, each one composed homogenously
325 of a distinct set of protein species. The findings we are now reporting suggest that the formation
326 of coiled flagella and hook-shaped ends, and their involvement in translational motility, require a
327 complex set of proteins and interactions. Nevertheless, as in other spirochetes (Li *et al.*, 2008),
328 the distribution of these proteins within the core and sheath of *Leptospira* PF has not been

329 delineated. Further studies are required to fully elucidate the 3D architecture of the PF filaments
330 from *Leptospira*, unraveling the exact composition, stoichiometry and network of interactions.

331 Rotation of the PF leads to changes in the cell shape caused by resistive forces between
332 PF and cell body, which in turn drive the movement of spirochetes (Yang *et al.*, 2011). For that
333 reason, perturbation in the flagellar structure itself and/or the interaction of the flagella with the
334 cell body will generate impaired motility. Our findings generate the hypothesis that partial or
335 total loss of the flagellar sheath lead to a reduced tensile strength of PF and hence inability of
336 *fcpA*- mutants to generate sufficient thrust. However, mathematical modeling of *B. burgdorferi*
337 motility proposed the existence of a fluid layer separating the PF and peptidoglycan layer (PG)
338 and that thrust occurs as a result of the resistance created by fluid drag, rather than friction (Yang
339 *et al.*, 2011). Considering that the flagella sheath constitutes the expected interface by which the
340 PF interacts with PG, it is conceivable, as a second mechanistic hypothesis, that the loss of the
341 sheath, whether partial or total, may lead to an impaired adherence of those two structures, which
342 in turn prevents them from engaging properly and thus compromising cell end gyration. It is
343 unclear if the phenotype observed in our mutants is due to either one or both of these posited
344 mechanisms.

345 Given their unique morphology and structure, spirochetal motility is unusual and by far
346 one of the most complex motility systems among bacteria. In this study, we identified a novel
347 *Leptospira* protein that is an abundant component of the flagellar sheath and is required for
348 maintenance of the PF structure, as well as its function in determining cell morphology and
349 translational motility. The lack of such an important structural protein clearly affects proper
350 flagellar assembly, and impacts also on the expression of other protein species that constitute
351 such a complex supramolecular assembly. The work presented here is just a starting point, as we

352 are currently pursuing continued efforts to better understand the exact composition and
353 interactions within the flagellar assembly of *Leptospira*. These data, while completing the
354 puzzle, shall deliver invaluable information about the precise way by which the flagellar
355 structure influences the spirochete's biology. In turn a full structural description may yield new
356 paradigms of flagella-associated motility systems in bacteria.

357 **Experimental Procedures**

358

359 **Bacterial strains and whole-genome sequencing**

360 The original clinical isolate and wild-type, knockout and complemented strains were
361 grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Johnson & Harris,
362 1967) at 29°C, and observed under dark-field microscopy. Strains were plated onto 1% agar
363 supplemented with EMJH medium and incubated at 29°C for a period of 4 weeks to obtain
364 colonies. *E. coli* strains were grown in Luria-Bertani (LB) medium. When necessary,
365 spectinomycin and/or kanamycin were added to culture media at a concentration of 50 µg/mL.
366 For all virulence studies, the correct number of *Leptospira* was determined by counting the cells
367 in triplicate using the Petroff-Hausser counting chamber (Fisher Scientific). We performed dark-
368 field microscopy of leptospiral strains with a Zeiss AxioImager.M2 microscope outfitted with an
369 AxioCam MRm camera and analyzed images using the AxioVision 4.8.2 software (Carl Zeiss
370 Microscopy LLC). Genomic DNA was extracted from a pellet of 5mL cultures using the
371 Maxwell®16 (Promega Corporation, Madison, WI). Solexa sequencing was performed to obtain
372 the genome sequence for motile and non-motile strains. SNPs and indels between genomes were
373 identified using SAMtools (<http://samtools.sourceforge.net/>) and CLC (<http://www.clcbio.com/>),
374 respectively, after assembly of reads using Stampy software (Lunter & Goodson, 2011). Genome
375 analysis was performed on the 569 genomes of the genus *Leptospira* which have been sequenced
376 to date (<http://www.ncbi.nlm.nih.gov/assembly/?term=leptospira>).

377

378 **Construction of mutant and complemented strains**

379 We obtained knockout *fcpA*- mutants and complemented strains by allelic exchange
380 (Croda *et al.*, 2008) and Himar1 transposon mutagenesis (Murray *et al.*, 2009), respectively,
381 according to approaches previously described. We used conjugation to transfect plasmid
382 constructs into leptospiral strains (Picardeau, 2008) and selected transformant colonies after
383 plating strains on 1% agar plates of EMJH containing the appropriate antibiotic selection agent.
384 For allelic exchange of the *fcpA* gene, upstream and downstream regions of the gene were
385 amplified from the genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130
386 using primers FcpA_FlkAF and FcpA_FlkAR for the upstream region and FcpA_FlkBF and
387 FcpA_FlkBR for the downstream region. The PCR products of upstream and downstream region
388 were digested with BamHI and XbaI, and HindIII and SpeI, respectively. The Spectinomycin
389 resistance (SpcR) cassette was amplified using primers Spc_Xba5 and Spc_Hind3, and the PCR
390 product was digested with XbaI and HindIII. The three digested PCR products were transformed
391 into the non-replicative plasmid pSW29T (Picardeau, 2008), previously digested with BamHI
392 and SpeI. The final plasmid, containing the flanking regions of the *fcpA* gene and SpcR cassette
393 insertion, was transfected into the donor strain *E. coli* β 2163 cells, and introduced into the
394 Fiocruz L1-130 strain by conjugation, as previously described (Picardeau, 2008). After 4 to 6
395 weeks of plate incubation at 30°C, spectinomycin-resistant transformants were inoculated into
396 liquid EMJH supplemented with 50 μ g/mL of spectinomycin, and examined for allelic exchange
397 in the target gene by PCR, using primers FcpA_AscF and FcpA_AscR, and by Western blotting.
398 For complementation, the *fcpA* gene with its native promoter region (a 400bp-region upstream
399 the start codon as identified by the Softberry software;
400 <http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>), was
401 amplified with primers FcpA_AscF and FcpA_AscR from *L. interrogans* strain Fiocruz L1-130.

402 The amplified product, after digestion with *AscI*, was cloned into the suicide pSW29T_TKS2
403 plasmid (Picardeau, 2008), which carried a kanamycin-resistant *HimarI* transposon. Random
404 insertion mutagenesis by conjugation was carried out in *L. interrogans* strain Fiocruz L1-130
405 *fcpA*- and strain LV 2756 motility deficient, as previously described (Murray *et al.*, 2009). For
406 further characterization of the transposon insertion sites in transformants, semi-random PCR was
407 performed in a set of kanamycin-resistant clones obtained in Fiocruz LV2556 and L1-130 *fcpA*-
408 as previously described (Murray *et al.*, 2009). For complementation, we selected clones that had
409 the transposon inserted in non-coding regions for further analysis.

410 In Fiocruz LV2556 *fcpA*-/+, the transposon was inserted between genes LIC12898 and LIC12899,
411 which encode for a hypothetical and a cytoplasmic membrane protein, respectively. In Fiocruz
412 L1-130 *fcpA*-/+, the transposon was inserted between genes LIC11818 and LIC11819, both
413 encoding for hypothetical proteins.

414

415 **Flagella purification and protein analysis**

416 PF were purified using a protocol modified from that described by Trueba *et al.* (Trueba *et*
417 *al.*, 1992) and subsequently analysed by SDS-PAGE, MS and electron microscopy. Briefly,
418 300mL of a broth culture of late-logarithmic-phase cells (approximately 5×10^8 cells/mL) were
419 harvested and centrifuged at $8,000 \times g$ for 20 min at 4°C. The cell pellet was re-suspended and
420 washed in 28mL of PBS. The cell pellet was then re-suspended in 30mL of sucrose solution (0.5
421 M sucrose, 0.15 M Tris, pH 8.0), and centrifuged at $8,000 \times g$ for 15 minutes. Pellet was re-
422 suspended in 8mL of sucrose solution, and stirred on ice for 10 minutes. To remove the
423 spirochete outer membrane sheath, 0.8 mL of a 10% Triton X-100 solution (1% final
424 concentration) was added, the mixture was stirred for 30 minutes at room temperature, and 80 μ L

425 solution of Lysozyme (10mg/mL) was added slowly and stirred on ice for 5 minutes. Before a 2h
426 stirring at room temperature, 0.8mL of EDTA solution (20mM, pH 8.0) was added slowly.
427 Afterwards, 160µL of MgSO₄ solution (0.1M), and 160µL of EDTA solution (0.1M, pH 8.0)
428 were added, both with intervals of 5 minutes with stirring at room temperature. The suspension
429 was centrifuged at 17,000 x g for 15 minutes, and the supernatant fluid was mixed well with
430 2mL of PEG 8000 solution (20%) in 1M NaCl), and kept on ice for 30 minutes. After
431 centrifugation at 27,000 x g for 30 minutes, the pellet was re-suspended in 3mL of H₂O, and a
432 new centrifugation was performed, at 80,000 x g for 45 minutes. The final pellet, consisting of
433 purified PF, was suspended in 1mL of H₂O and stored at 4C. SDS/PAGE and Western blotting of
434 leptospiral cell lysates and purified PF were carried out as previously described (Croda *et al.*,
435 2008, Lourdault *et al.*, 2011). Western blot analyses were performed with polyclonal antibodies
436 prepared against recombinant proteins of leptospiral flagellar components. Quantitative analysis
437 of protein expression was done using Image Lab™ Software (Bio-Rad). Mass spectrometry
438 analysis (MS + MS/MS) of the whole cell lysates and PF preparations of the *L. interrogans* strain
439 LV 2756 motile and strain LV 2756 motility-deficient were carried out by analyzing fragments
440 of the polyacrylamide gel stained with coomassie blue, according to protocols of the Proteomics
441 Platform of the Institute Pasteur, Paris, France
442 ([http://www.pasteur.fr/ip/easysite/pasteur/fr/recherche/plates-formes-](http://www.pasteur.fr/ip/easysite/pasteur/fr/recherche/plates-formes-technologiques/proteopole/modules/pf3-proteomique)
443 [technologiques/proteopole/modules/pf3-proteomique](http://www.pasteur.fr/ip/easysite/pasteur/fr/recherche/plates-formes-technologiques/proteopole/modules/pf3-proteomique)). Two independent experiments for each
444 sample and strain were performed.

445

446 **Transmission electron microscopy**

447 Late log-phase cultures (5mL) were centrifuged at 3.000 rpm for 15 min at 4°C. The
448 supernatant was removed and 5 mL of fixative containing 2% glutaraldehyde and sodium
449 cacodylate buffer (pH 7.4) 0,1M was added to the pelleted cells. The cells were fixed for 1h at
450 4°C and then placed on coverslips treated with poly-L-lysine. After this step, the cells were post-
451 fixed with 1% osmium tetroxide and treated with a graded series of ethanol solutions. The
452 samples were subjected to critical point drying and sputter coating with gold and then examined
453 using a JEOL 6390LV scanning electron microscopy (SEM).

454 Purified PF (10µL) were allowed to adsorb for 60s onto a copper grid coated with
455 Formvar 400 mesh. The grid was washed three times with 0.1M Sodium Cacodylate and then
456 negatively stained with 2% (w/v) phosphotungstic acid (PTA) pH 7.2. Grids were observed using
457 a JEOL JEM1230 transmission electron microscope (TEM) operating at 80keV. For the diameter
458 and length measurement of the flagella, twenty random pictures were taken from each group on
459 the same magnification (200.000 x), using Gatan camera and software DigitalMicrograph® for
460 acquisition. For PF thickness, four different measurements were taken from each strain, using the
461 ImageJm1.45s software. For PF length, measurement was taken from 20 different flagella of
462 each strain using Illustrator CS 5.5 (Adobe). Mean values and standard deviations of
463 measurements were used for comparison between groups.

464

465 **Immuno-electron microscopy**

466 Purified PF (15µL) were allowed to adsorb for 10min in glow-discharged copper grids
467 coated with Formvar 300 mesh. Immediately the grids were blocked for 2min in 0.1% BSA, and
468 incubated for 20min with 1:10 dilution (0.1% BSA) of primary antibody. Polyclonal antibodies
469 anti-FlaA1, FlaA2, anti-FlaB1, and FcpA were used as primary antibodies. Grids were washed 3

470 times with ultrapure water, and blocked again in 0.1% BSA for 2min. Secondary antibody 5nm
471 gold-conjugated Protein A (PAG) was used in a dilution of 1:50 (0.1% BSA), incubated for
472 20min. Grids were washed three times with ultrapure water and then negatively stained with 2%
473 PTA pH 7. Grids were observed using a Philips TECNAI 12 BioTwin II operating at 80keV.
474 Images were acquired on Soft Imaging System Morada camera using iTEM image acquisition
475 software.

476

477 **Dark-field video microscopy**

478 Log phase cultures (100 μ L) was diluted into 900 μ L of 1% methyl cellulose (MP
479 Biomedicals) in 0.1 μ m-filtered ultrapure water (Sigma) and mixed by inverting gently. 10 μ L of
480 the 1:10 dilution was transferred to a glass microscope slide (Thermo Scientific), an 18x18mm
481 glass coverslip (Carl Zeiss) was applied, and the edges of the coverslip were sealed with clear
482 nail polish (LA Colors) to prevent drying. The slides were immediately viewed in an Axio
483 Imager.M2 motorized dark-field microscope (Carl Zeiss). Videos for qualitative analysis were
484 recorded at 100X (EC Plan-Neofluar 100x/1.30 Oil) under oil immersion (Zeiss Immersol 518F)
485 on an AxioImagerM3 camera (Carl Zeiss) and analyzed using AxioVision 4.8.2 software (Carl
486 Zeiss).

487

488 **Video tracking analysis**

489 Digital high-speed videos for tracking were recorded at 200ms intervals for up to 10
490 seconds (50 frames at 5 fps; digital gain = 1; sensitivity = 100%; image orientation = flipped
491 vertically) and all videos recorded were analyzed using the AxioVision Tracking Module (Carl
492 Zeiss). Inclusion criteria for tracking consisted of all leptospire whose search area was entirely

493 within the field of view at the start frame and in the plane of focus at the start frame. Aggregates
494 or chains of two or more leptospire were excluded from tracking, as were leptospire whose
495 tracks could not be followed by the computer algorithm. The instantaneous velocity of each
496 tracked leptospire was recorded by the tracking software at each frame by comparison to the
497 preceding frame, and the mean velocity for each leptospire was calculated by averaging the
498 instantaneous velocities of that particular leptospire and reported by the software as a mean
499 velocity for each of the individual leptospire tracked in a given video.

500

501 **Cryo-electron tomography and 3D reconstruction**

502 Viable bacterial cultures were centrifuged to increase the concentration to $\sim 2 \times 10^9$
503 cells/ml. Five-microliter samples were deposited onto freshly glow-discharged holey carbon
504 grids for 1 min. The grids were blotted with filter paper and rapidly frozen in liquid ethane using
505 a gravity-driven plunger apparatus as previously described (Raddi *et al.*, 2012). The resulting
506 frozen-hydrated specimens were imaged at -170°C using a Polara G2 electron microscope (FEI
507 Company, Hillsboro, OR) equipped with a field emission gun and a 4K x 4K charge-coupled-
508 device (CCD) (16-megapixel) camera (TVIPS; GMBH, Germany). The microscope was operated
509 at 300 kV with a magnification of x 31,000, resulting in an effective pixel size of 5.6 Å after 2 x
510 2 binning. Using the FEI “batch tomography” program, low-dose single-axis tilt series were
511 collected from each bacterium at $-6 \mu\text{m}$ defocus with a cumulative dose of $\sim 100 \text{ e}/\text{Å}^2$ distributed
512 over 87 images, covering an angular range from -64° to $+64^\circ$, with an angular increment of 1.5° .
513 Tilted images were aligned and then reconstructed using IMOD software package (Kremer *et al.*,
514 1996). In total, 10, 15 and 17 reconstructions were generated from WT, fcpA mutant and
515 complemented strains, respectively.

516 A total of 1392 segments ($192 \times 192 \times 96$ voxels) of flagellar filaments were manually
517 identified and extracted from 42 reconstructions. The initial orientation was determined using
518 two adjacent points along the filament. Further rotational alignment is performed to maximize
519 the cross-correlation coefficient. Averaging is carried out with a merging procedure in reciprocal
520 space (Raddi *et al.*, 2012). Tomographic reconstructions were visualized using IMOD (Kremer *et*
521 *al.*, 1996). Reconstruction of cells were segmented using 3D modeling software Amira (Visage
522 Imaging). 3D segmentations of the cytoplasmic, outer membranes and flagellar filaments were
523 manually constructed.

524

525 ***In vitro* translocation assays with polarized MDCK cell monolayers**

526 We performed a translocation assay according to a protocol modified from that described
527 by Figueira *et al.* (Figueira *et al.*, 2011). MDCK cells at a concentration of 2×10^5 cells in 500 μ l
528 of DMEM were seeded onto 12-mm-diameter Transwell filter units with 3- μ m pores
529 (COSTAR). Monolayers were incubated at 37°C in 5% CO₂ for 3 to 4 days with daily changes
530 in media until the transepithelial resistance (TER) reached a range of 200 and 300 Ω /cm², as
531 measured with an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, Fla.).
532 The TER for polycarbonate filters without cells was approximately 100 Ω /cm². The upper
533 chamber of the transwell apparatus was inoculated with a multiplicity of infection (MOI) of 100
534 leptospire by adding 500 μ L of bacteria, which were resuspended in 1:2 v/v ratio of DMEM and
535 EMJH media. Duplicate transwell chamber assays were performed for each leptospiral strain
536 tested. Aliquots were removed from lower chamber (100 μ l) at 2, 4, 6 and 24 hours and the
537 number of leptospire were counted in triplicate by using the Petroff-Hausser counting chamber
538 (Fisher Scientific). The ability of leptospire to translocate MDCK polarized monolayers was

539 determined by calculating the proportion of leptospire in the lower chamber in comparison to
540 the initial inoculum for duplicate assays at each time point.

541

542 **Virulence studies.**

543 Animal experiments were conducted according to National Institutes of Health guidelines
544 for housing and care of laboratory animals and protocols, which were approved by the Yale
545 University Institutional Animal Care and Use Committee (Protocol # 2014-11424). All the
546 experiments were performed using 3-6 week-old Golden Syrian male hamsters. For the
547 experiments of virulence, one group of 8-10 animals for each of the six strains was inoculated
548 intraperitoneally (IP) with a high-dose inoculum (10^8 leptospire) in 1ml of EMJH medium. For
549 the LD₅₀ experiments (Reed & Muench, 1938), two groups of 4 animals were inoculated IP with
550 doses of 10^3 , 10^2 and 10^1 leptospire, for motile LV2756, LV2756 *fcpA*^{-/+}, Fiocruz L1-130 WT
551 and Fiocruz L1-130 *fcpA*^{-/+}. For motility-deficient LV2756 and Fiocruz L1-130 *fcpA*⁻ strains,
552 animals were infected with doses of 10^8 and 10^7 leptospire. In all experiments, animals were
553 monitored twice daily for clinical signs of leptospirosis and death, up to 21 days post-infection.
554 Moribund animals presenting difficulties to move, breath or signs of bleeding or seizure were
555 immediately sacrificed by inhalation of CO₂.

556 In experiments evaluating leptospiral dissemination, one group of six animals for strains
557 LV2756 motile and LV2756 motility deficient was inoculated intraperitoneally with 10^8
558 leptospire in 1ml of EMJH medium. After 1 hour and 4 days post-infection, sub-groups of two
559 animals were euthanized. With the same strains, a conjunctival infection was performed by
560 centrifugation of 30ml culture of leptospire for 10 minutes at 1000rcf and using an inoculum of
561 10^8 leptospire in 10 μ l of EMJH medium instilled in the left eye conjunctiva using a

562 micropipette. Groups of four animals were infected and two were euthanized after 7 days of
563 infection for each strain tested. In those experiments, a group of two animals were left as positive
564 controls.

565 The necropsy for the dissemination study was performed as follows. Animals were
566 sacrificed by inhalation of CO₂ and placed on their backs slightly inclined in the dissecting tray.
567 After sterilization of the abdomen with alcohol 70% and using sets of sterile instruments, the
568 internal organs were exposed, including the heart and lungs. All blood was collected directly
569 from the heart in a Vacutainer® K2 EDTA Tubes (BD Diagnostics) and Glass Serum Tubes,
570 using a 5ml syringe with a 21G needle. A 21G butterfly needle affixed to a 60ml syringe
571 containing sterile saline 0.85% was then inserted into the left ventricle. The right atrium was
572 snipped to allow the residual blood and normal saline to leave the body during the perfusion.
573 Each hamster was perfused with 100ml of saline solution. After perfusion, right pulmonary lobe,
574 right dorsocaudal hepatic lobe, spleen, right kidney and right eye were carefully removed. All the
575 tissues were collected into cryotubes and immediately placed into liquid nitrogen before being
576 stored at -80°C until extraction. Blood, kidney, liver, lung, spleen and eye were analyzed. Using
577 scissors and scalpels, 25mg of lung, liver, kidney cortex, and eye, 10mg of the spleen, and 200µl
578 of blood were aseptically collected. DNA was extracted using the Maxwell®16 Tissue DNA
579 purification Kit (Promega Corporation, Madison, WI), after homogenization with Bullet Blender
580 (Next Advance, Averill Park, NY).

581

582 **Quantitative real-time PCR evaluation of bacterial load**

583 Quantitative Real-time PCR assays were performed using an ABI 7500 (Applied
584 Biosystems, Foster City, CA) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen

585 Corporation, Carlsbad, CA). The *lipL32* gene was amplified using the set of primers and probe
586 (Table S1), according to protocol previously described (Stoddard *et al.*, 2009). We performed
587 amplifications of hamster housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)
588 as a control to monitor nucleic acid extraction efficiency and to evaluate for potential inhibition
589 of the amplification reaction. GAPDH_F and GAPDH_R primers were designed to amplify a
590 fragment that was detected by the probe, GAPDH_P. A sample with a threshold cycle (Ct) value
591 between 16 and 21 was considered as positive and further analyzed by real-time PCR targeting
592 *lipL32*. In case of sample for which the *gapdh* gene sequence did not amplify, a new DNA
593 extraction of the sample was performed and analyzed by PCR. For each organ, the DNA was
594 extracted from one sample and the Real Time PCR was performed in duplicate, including a
595 standard curve of genomic DNA from *L. interrogans* serovar Fiocruz L1-130 (10⁰–10⁷
596 leptospire) and 12 negative control wells (water) per plate. Considering the amount of tissue
597 that was used for DNA extraction, an equation was applied to express the results as the number
598 of leptospire per gram of tissue or per mL of blood/water.

599

600 **Statistical analysis**

601 Fisher's exact test and analysis of variance (ANOVA) were performed to assess statistical
602 significance of differences between pairs of groups and multiple groups, respectively. A p value
603 of <0.05 was considered to be statistically significant.

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761

762 **TABLE**

763

Table 1. Virulence of wild-type, *fcpA*- mutant and complemented strains of *Leptospira interrogans* in the hamster model of leptospirosis*

| Strains | Mortality (%) | Time to death (days) |
|---|---------------|----------------------|
| Motile Fiocruz LV2756 | 100 | 6,6,6,6,6,6,6,8 |
| Motility-deficient Fiocruz LV2756 | 0¶ | -¶ |
| Fiocruz LV2756 <i>fcpA</i> ^{-/+} § | 100❖ | 6,6,8,8,9,9,10,10❖ |
| Fiocruz L1-130 <i>WT</i> | 100 | 6,6,6,8,8,8,8,8 |
| Fiocruz L1-130 <i>fcpA</i> ⁻ | 0¶⌘ | -¶ |
| Fiocruz L1-130 <i>fcpA</i> ^{-/+} § | 100❖ | 6,6,6,8,8,8,8,8❖ |

764 * Results are shown for one representative experiment among a total of three, which were
765 performed. Groups of 8 animals were inoculated intraperitoneally with 10⁸ bacteria for each
766 strain and then followed for 21 days.

767 ¶ Mortality and survival was significantly ($p < 0.0001$) decreased and increased, respectively,
768 compared with motile Fiocruz LV2756 and Fiocruz L1-130 strains.

769 § Strains were genetically complemented with *fcpA* gene.

770 ❖ Mortality and survival was significantly ($p < 0.0001$) increased and decreased, respectively,
771 compared with motility-deficient Fiocruz LV2756 and Fiocruz L1-130 *fcpA*⁻ strains.

772 ⌘ Mortality was 37.5% in one of the three experiments but was significantly lower ($p = 0.026$)
773 than the mortality (100%) for hamsters infected with the Fiocruz L1-130 strain.

774 **FIGURE LEGENDS**

775

776 **Figure 1. Phenotypes of WT, *fcpA*- mutant and complemented *L. interrogans* strains.** (A)
777 Motility assay for which 10⁵ bacteria were inoculated on 0.5% agarose plates [each square, 1
778 cm²] and incubated for 10 days at 29°C; (B) Dark-field microscopy [bar=10µm]; (C) Scanning
779 electron microscopy [bar=2µm]; and (D) Transmission electron microscopy of negatively
780 stained, purified periplasmic flagella [bar=100nm]. Motility-deficient Fiocruz LV2756 strain
781 was isolated from a clinical isolate. Fiocruz L1-130 *fcpA*- strain was generated by allelic
782 exchange and complemented strains Fiocruz LV2756 *fcpA*^{-/+} and Fiocruz L1-130 *fcpA*^{-/+} were
783 obtained by reintroducing the *fcpA* gene. See also Videos S1-S6.

784

785 **Figure 2. Dissemination of motile and motility-deficient *Leptospira interrogans* strains**
786 **during hamster infection.** Hamsters were inoculated with 10⁸ bacteria of the motile (white
787 columns) and motility-deficient (gray columns) strains by intraperitoneal (A and B) or
788 conjunctival (C) routes. Quantitative PCR analysis was performed on blood and tissues
789 harvested one (A) and four (B) days after intraperitoneal inoculation and 7 days (C) after
790 conjunctival inoculation. Geometric mean values and standard deviations are shown for genome
791 equivalents of leptospiral DNA per ml of blood and gram of tissue, which were obtained in two
792 independent experiments. Leptospiral DNA load for the motile strain was significantly
793 (p<0.0001) higher than the motility-deficient strain for all tissues and time points. ND, not
794 detected. See also Fig. S1.

795

796 **Figure 3. Expression of FcpA protein in purified periplasmic flagella of WT, *fcpA*- mutant**
797 **and complemented *L. interrogans* strains.** (A) Coomassie-stained SDS-PAGE of purified

798 flagella from motile Fiocruz LV2756 [lane 1], motility-deficient Fiocruz LV2756 [lane 2],
799 Fiocruz LV2756 *fcpA*^{-/+} [lane 3], *WT* Fiocruz L1-130 [lane 4], Fiocruz L1-130 *fcpA*⁻ [lane 5], and
800 Fiocruz L1-130 *fcpA*^{-/+} [lane 6] strains. In Fig. 3A, arrows indicate the position of FcpA and
801 FlaB1 proteins, which were identified by mass spectroscopy. (B) Immunoblotting analysis of
802 purified flagella incubated with a mixture of polyclonal antibodies against FcpA and control
803 antibodies against flagella-associated proteins, FlaA1 and FlaA2. Arrows indicate the positions
804 of these three proteins in Fig. 3B.

805

806 **Figure 4. Inactivation of the *fcpA* gene in *Leptospira interrogans*.** The figure is a schematic
807 representation of the *fcpA* loci in the *WT* Fiocruz L1-130 strain and Fiocruz L1-130 *fcpA*⁻ strain.
808 The expanded circle shows the site of the frameshift mutation, which occurred in the *fcpA* gene
809 of the motility-deficient Fiocruz LV2756 strain.

810

811 **Figure 5. Cell morphology and structural characterization of periplasmic flagella (PF) *in***
812 ***situ* for wt, *fcpA*⁻ mutant and complemented *Leptospira interrogans* strains.** Cryo-electron
813 tomography was performed for Fiocruz L1-130 *WT* (A), Fiocruz L1-130 *fcpA*⁻ (B) and Fiocruz
814 L1-130 *fcpA*^{-/+} (C) strains. Panels D, E and F show one slice of a tomographic reconstruction for
815 the regions (boxes in panels A, B and C) of *WT* Fiocruz L1-130, Fiocruz L1-130 *fcpA*⁻ and
816 Fiocruz L1-130 *fcpA*^{-/+} strains, respectively. Arrows indicate the location of PF. Inserts in panel
817 D, E and F depict averaged maps of PF segments for each of the strains. The diameter of the
818 flagellar filament in Fiocruz L1-130 *fcpA*⁻ mutant was 15.7 nm, whereas the diameter of
819 filaments in the *WT* and complemented strains was 20.5 nm. Surface renderings of the
820 corresponding 3-D reconstructions of Fiocruz L1-130 *fcpA*⁻ (G) and Fiocruz L1-130 *fcpA*^{-/+} (H)

821 strains are shown, with prominent structural features including the outer membrane (OM),
822 cytoplasmic membrane (IM) and flagellar filament. See also Videos S7 and S8.

823

824 **Figure 6. Immuno-electron microscopy of periplasmic flagella (PF) from wt and *fcpA*-**

825 **mutant of *Leptospira interrogans* strains.** PF from wt Fiocruz L1-130 [bar=200nm] and

826 Fiocruz L1-130 *fcpA*- [bar=500nm] strains were purified and labeled with antibodies against

827 FcpA (α -FcpA) and FlaB1 (α -FlaB1). Anti-rabbit IgG anti-sera conjugated with 5nm gold

828 nanoparticles were used to detect bound antibodies. PF were visualized using 2% PTA negative

829 staining.