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

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1 **A novel flagellar sheath protein, FcpA, determines filament coiling, translational motility**

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## Summary

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

## Introduction

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

Spirochete PF are similar in structure and function to flagella of externally flagellated bacteria, as each consists of a basal body complex or motor, a flexible hook and a flagellar filament (Charon & Goldstein, 2002, Limberger, 2004). Whereas PF from non-*Leptospira* 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  leptospires 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.

## Results



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

  *L. interrogans* serovar Copenhageni strain Fiocruz LV2756 was isolated from a Brazilian

patient with pulmonary hemorrhage syndrome due to leptospirosis (Gouveia *et al.*, 2008).  Two

colony morphologies, large and small, were observed after plating the isolate onto solid EMJH

media. The phenotype was confirmed by measuring the ability of the cells to grow in motility

plate assays (Fig. 1A), confirming the previous observation.  Sub-culturing of larger colonies

yielded leptospires, which had phenotypes similar to wild-type (WT) *L. interrogans* with respect

to the characteristic hook-end cell morphology (Figs. 1B and 1C) and translational motility

(Video S1).  In contrast, sub-cultures of small colonies yielded leptospires that lacked the

terminal hook-ends (Figs. 1B and 1C), and did not produce translational motility (Video S2).

Leptospires from small colonies retained the characteristic corkscrew cell body morphology,

were able to gyrate their ends, forming spiral waveforms at their terminal ends.  We also

observed that a proportion of these leptospires grew as long chains with incomplete division

planes (Video S2).  Sub-cultures of large and small colonies were named motile and motility-

deficient strains, respectively.

  Velocity measurements of both strains showed a statistically significant decrease of the

mean velocity for the motility-deficient strain ($2.77 \pm 1.7 \mu m/s$ ) when compared with the motile

strain cells ($11.4 \pm 4.47 \mu m/s$, $p<0.0001$) (Fig. S2A). The path of individuals cells shows that the

Fiocruz LV2756 motility-deficient strain lacks translational motility, indicating that the residual

velocity measured most-likely derives from gyration-related movement combined with Brownian

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 $16.3 \pm 2.9$ nm) were significantly smaller than those from motile

120    strain (mean $21.5 \pm 2.2$ nm, p <0.0001). However, there was no difference in the length of the

121    flagella between motile strain ($2.14 \pm 0.57 \mu$m) and motility-deficient strain ($2.17 \pm 0.52 \mu$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 $LD_{50}$ (Table S1). Complementation restored the virulence phenotype (Table 1)

211    and $LD_{50}$ 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 ± 2.3nm; L1-130 WT, mean 22.8 ± 3.8nm; 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 ± 2.1nm), 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.

## Discussion

Our study provides strong evidence that the leptospiral PF structure determines the cell shape, specifically the hooked end morphology, and in turn, the spirochete's ability to generate translational motility and ultimately virulence. Furthermore, we found that a novel protein, FcpA, is a key component of the PF sheath, and that FcpA is the molecule mediating these phenotypes. In the 1960s, studies of motility-deficient leptospires described the loss of the hooked-end cell morphology and its correlation with small colony phenotype (Simpson & White, 1964) and virulence attenuation (Faine & Vanderhoeden, 1964). However no association between cell morphology and virulence was reported at that time. Bromley et al. characterized a motility-deficient mutant obtained by chemical mutagenesis, which had straight cell ends and yielded uncoiled PF after purification. The authors proposed that the PF contributed to the hooked end cell morphology (Bromley & Charon, 1979), but could not rule out the possibility of secondary mutations. More recently, studies showed that loss of *fliY* or *flaA2* in *L. interrogans* was associated with attenuated motility and virulence yet, complementation of the gene and rescue of these phenotypes were not performed (Lambert *et al.*, 2012, Liao *et al.*, 2009) . In this study, the construction of *fcpA* knockout and complemented mutants provided the opportunity to apply Koch's molecular postulates, thus demonstrating that a novel flagellar structural protein, FcpA, is essential for PF structure, cell morphology and translational motility. Inactivation of *fcpA* resulted in a more than seven-fold increase ($\leq 10$ to $\geq 10_8$) in the $LD_{50}$ of *L. interrogans* in the hamster model of leptospirosis, while complementation of *fcpA* restored the $LD_{50}$ to that observed for the WT strain. Further indicating that that motility is a key virulence determinant 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 leptospires, 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 leptospires 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.

## Experimental Procedures

**Bacterial strains and whole-genome sequencing**

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

**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* β2163 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 μg/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 *Himar1* 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 $5 \times 10_8$ 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-x 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 80μL

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 $MgSO_4$ 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 $H_2O$, 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 $H_2O$ 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-

443      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 (100μL) was diluted into 900μL of 1% methyl cellulose (MP

479    Biomedicals) in 0.1μm-filtered ultrapure water (Sigma) and mixed by inverting gently. 10μL 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 leptospires 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 leptospires were excluded from tracking, as were leptospires 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 leptospires 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 μm defocus with a cumulative dose of $\sim 100$ e-/Å$_2$ distributed

512    over 87 images, covering an angular range from -64° to +64°, 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 × 192 × 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 $2 \times 10_5$ cells in 500 µl

528    of DMEM were seeded onto 12-mm-diameter Transwell filter units with 3- µm 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 300Ω/cm2, as

531    measured with an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, Fla.).

532    The TER for polycarbonate filters without cells was approximately 100Ω/cm2. The upper

533    chamber of the transwell apparatus was inoculated with a multiplicity of infection (MOI) of 100

534    leptospires by adding 500µL 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 (100µl) at 2, 4, 6 and 24 hours and the

537    number of leptospires were counted in triplicate by using the Petroff-Hausser counting chamber

538    (Fisher Scientific). The ability of leptospires to translocate MDCK polarized monolayers was

539   determined by calculating the proportion of leptospires in the lower chamber in comparison to

540   the initial inoculums 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 ($10^8$ leptospires) in 1ml of EMJH medium. For

549   the $LD_{50}$ experiments (Reed & Muench, 1938) , two groups of 4 animals were inoculated IP with

550   doses of $10^3$, $10^2$ and 10 leptospires, 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 $10^7$ leptospires. 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 $CO_2$.

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   leptospires 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 leptospires for 10 minutes at 1000rcf and using an inoculum of

561   $10^8$ leptospires in 10µl 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 $CO_2$ 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 -80ºC 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-phophate 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 ($10_0$–$10_7$

596 leptospires) 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 leptospires 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.

## Acknowledgments

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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 *fcpA-/+* § | 100❖ | 6,6,8,8,9,9,10,10❖ |
| Fiocruz L1-130 *WT* | 100 | 6,6,6,8,8,8,8,8 |
| Fiocruz L1-130 *fcpA-* | 0¶⌘ | -¶ |
| Fiocruz L1-130 *fcpA-/+* § | 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.

**FIGURE LEGENDS**

776 **Figure 1. Phenotypes of *WT*, *fcpA-* mutant and complemented *L. interrogans* strains.** (A)

777 Motility assay for which $10_5$ 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 $10_8$ 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.