

1 **Title**

2 A conserved protein, BcmA, mediates motility, biofilm formation, and host colonisation in  
3 Adherent Invasive *Escherichia coli*

4 **Short Title**

5 BcmA is a novel modulator of motility in AIEC

6 **Authors**

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## 24 **Abstract**

25 Adherent Invasive *Escherichia coli* (AIEC) is a non-diarrhoeagenic intestinal *E. coli* pathotype  
26 associated with Crohn's Disease. AIEC pathogenesis is characterised by biofilm formation,  
27 adhesion to and invasion of intestinal epithelial cells, and intracellular replication within  
28 epithelial cells and macrophages. Here, we identify and characterise a protein in the  
29 prototypical AIEC strain LF82 which is required for efficient biofilm formation and dispersal –  
30 LF82\_p314. LF82  $\Delta$ LF82\_314 have defective swimming and swarming motility, indicating  
31 LF82\_p314 is important for flagellar-mediated motility, and thus surface colonisation and  
32 biofilm dispersal. Flagellar morphology and chemotaxis in liquid appear unaffected by  
33 deletion of LF82\_314, suggesting LF82\_p314 does not elicit an effect on flagella biogenesis or  
34 environmental sensing. Flagellar motility has been implicated in AIEC virulence, therefore we  
35 assessed the role of LF82\_p314 in host colonisation using a *Caenorhabditis elegans* model.  
36 We found that LF82  $\Delta$ LF82\_314 have an impaired ability to colonise the *C. elegans* compared  
37 to wild-type LF82. Phylogenetic analysis showed that LF82\_314 is conserved in several major  
38 enterobacterial pathogens, and suggests the gene may have been acquired horizontally in  
39 several genera. Our data suggests LF82\_p314 may be a novel component in the flagellar  
40 motility pathway and is a novel determinant of AIEC colonisation. Our findings have potential  
41 implications not only for the pathogenesis of Crohn's Disease, but also for the course of  
42 infection in several major bacterial pathogens. We propose a new designation for LF82\_314,  
43 *biofilm coupled to motility A*, or *bcmA*.

## 44 **Author summary**

45 Adherent Invasive *Escherichia coli* (AIEC) are a group of bacteria implicated in the  
46 pathogenesis of Crohn's Disease, a chronic inflammatory bowel disease with no cure. Critical  
47 to the process of many bacterial infections is the ability of bacteria to swim towards and  
48 colonise the host surface using specialised, propeller-like appendages called flagella. In this  
49 paper, we describe a novel protein – LF82\_p314 (BcmA) – which is required for efficient  
50 flagella-mediated motility and surface colonisation in AIEC. Using a nematode worm  
51 (*Caenorhabditis elegans*) infection model, we show that LF82\_p314 enables effective  
52 colonisation of the *C. elegans* gut, suggesting a role for the protein during human infection.

53 These findings indicate BcmA is significant for initial colonisation of the human gut by AIEC,  
54 and therefore the onset of Crohn's Disease.

## 55 Introduction

56 Crohn's Disease (CD) is a chronic and relapsing inflammatory bowel disease presenting with  
57 frequent bloody diarrhoea, bowel obstruction, abdominal pain, and extraintestinal  
58 manifestations affecting the eyes, skin, joints, and liver (reviewed in [1–3]). CD is a complex  
59 syndrome which is understood as an unchecked and inappropriate inflammatory response to  
60 intestinal bacteria, potentiated by carriage of one or several of over 180 predisposing  
61 immune-related alleles [4–13] and their interaction with environmental risk factors such as  
62 smoking [14–16]; consumption of a “western” high-fat, low-fibre diet [17–20]; colonisation  
63 by a low-complexity, pro-inflammatory microbiome [21–23]; and carriage of CD-associated  
64 pathobionts *Mycobacterium avium* subsp. *paratuberculosis* [24,25] and Adherent Invasive  
65 *Escherichia coli* (AIEC) [26–34]. An increasing body of evidence suggests AIEC can act as a key  
66 aetiological component of CD. AIEC strains are found present in the ileal mucosa of up to  
67 51.9% CD patients compared to 16.7% healthy controls [26,30,33], and have been shown to  
68 induce inflammation and colitis in mice carrying CD-associated *TLR5* deletions [35–37]; mice  
69 fed CD-associated “western” diets [19]; and mice with infection-associated intestinal  
70 inflammation and microbiome perturbations [37–39]. Indeed, the recent demonstration that  
71 AIEC alone can perturb simple microbiomes and instigate inflammation in a *TLR5*<sup>-/-</sup> mouse  
72 model [37] raises the possibility that – given a set of predisposing factors – AIEC infection may  
73 serve as a first step towards triggering the CD inflammatory cascade.

74 AIEC pathogenesis is classically characterised by adherence to, invasion of, and  
75 replication within intestinal epithelial cells (IECs) and macrophages [40–43]. Despite its  
76 significance in CD aetiology, however, the molecular pathogenesis of AIEC infection is  
77 comparatively poorly understood. AIEC are thought to use flagella to swim through the mucus  
78 layer in the gut [44–46], and secrete the mucolytic Vat-AIEC protease [47] to gain access to  
79 the intestinal epithelial surface. AIEC bind the epithelial surface via long polar fimbriae [48]  
80 and interactions between type 1 pili and CEACAM6 [40,49–51], a host adhesin over-expressed  
81 by CD patient intestinal epithelial cells. Epithelium-associated AIEC may be transcytosed by  
82 microfold cells into Peyer's Patches to be phagocytosed by macrophages, may actively invade

83 IECs, or may alternatively form biofilms on the luminal surface of the gut. Invasion is mediated  
84 by microtubule polymerisation and actin recruitment [41], and is thought in part to be  
85 facilitated *via* uncharacterised effector delivery in outer-membrane vesicles (OMVs) [52,53],  
86 and by a putative oxidoreductase, *ibeA* [54]. However, inhibited OMV release and *ibeA*  
87 deletion do not fully abrogate invasion, suggesting other, unknown factors may be involved.  
88 The mechanisms of intracellular replication remain to be elucidated, with only one protein –  
89 the oxidoreductase *dsbA* – known to be required [55]. In addition to canonical adhesion and  
90 invasion traits explored in the first descriptions of AIEC, biofilm formation [56–58], motility  
91 [44–46,59], and the ability to utilise short-chain fatty acids (SCFA's) as carbon sources [59–61]  
92 are becoming understood as determinants of AIEC pathogenesis. The discovery of elevated  
93 mucosa-associated biofilms in CD patients [29] suggests biofilms may be of specific  
94 importance in AIEC pathogenesis, warranting further investigation.

95 We previously conducted a high-throughput heterologous expression screen to  
96 identify putative effectors in the prototypical AIEC strain, LF82 [62]. AIEC-specific putative  
97 virulence genes were selected by comparison of the LF82 genome to several pathogenic and  
98 commensal *E. coli* reference genomes, and expressed in a HeLa cell line as GFP fusions.  
99 Automated microscopy and image analysis of putative effector-GFP fusions expressing HeLa  
100 cells allowed identification of protein subcellular localisations and co-localisations. From this  
101 screen, we identified a conserved hypothetical protein of unknown function – *LF82\_314* –  
102 which self-assembles into large filaments (Fig. S1). *LF82\_314* is widely conserved, and  
103 bioinformatic analysis (Table S1) suggested the gene is co-inherited with components of the  
104 General Secretion Pathway (GSP). The GSP is required for secretion of extracellular proteins,  
105 including pili (reviewed in [63]). We therefore hypothesised that *LF82\_314* may encode either  
106 a novel, self-assembling pilin, or an amyloid-like biofilm matrix component. Using established  
107 biofilm and motility assays, and a *Caenorhabditis elegans* infection model, we established that  
108 LF82\_p314 is required for efficient biofilm formation, motility, and host colonisation in LF82.  
109 Furthermore, bioinformatic analysis reveals *LF82\_314* is conserved in a range of  
110 enterobacterial genomes, many of which are human pathogens. Because of the roles  
111 *LF82\_314* plays in infection, and the potential significance of this novel virulence factor in  
112 diverse enterobacterial pathogens, we propose a new designation for *LF82\_314*, *biofilm*  
113 *coupled to motility A*, or *bcmA*.

## 114 Results

### 115 LF82\_p314 promotes biofilm formation

116 To identify a role for LF82\_p314 in biofilm formation, we created a clean, markerless deletion  
117 in *LF82\_314*, LF82  $\Delta$ *LF82\_314*. Using a microtitre plate-based crystal violet assay, we  
118 established that LF82  $\Delta$ *LF82\_314* has a marked biofilm formation defect with incomplete  
119 dispersal upon biofilm maturation, when compared to wild-type LF82 (Fig. 1A). Episomal  
120 expression of LF82\_p314 from p*LF82\_314* complemented *LF82\_314* deletion. Microscopic  
121 analysis of LF82 biofilms formed on glass cover slips revealed LF82  $\Delta$ *LF82\_314* form patchier,  
122 less complete biofilms than wild-type LF82, a defect which can be also complemented by  
123 LF82\_p314 expression (Fig. 1B). We theorised that the biofilm formation defect may be due  
124 to defective initial surface attachment, intercellular adhesion, or altered extracellular matrix  
125 architecture. If intercellular adhesion or extracellular matrix formation is altered by *LF82\_314*  
126 deletion, LF82  $\Delta$ *LF82\_314* biofilm formation may be complemented in *trans* by co-culture  
127 with wild-type LF82. We therefore conducted a *trans*-complementation assay, in which the  
128 biofilm formation of wild-type LF82 and LF82  $\Delta$ *LF82\_314* mixed in a 1:1 ratio was assessed.  
129 We found that the LF82:LF82  $\Delta$ *LF82\_314* mix formed biofilms of intermediate mass when  
130 compared to LF82 and LF82  $\Delta$ *LF82\_314* biofilms (Fig 1C). To characterise the architecture of  
131 these mixed biofilms, LF82 and LF82  $\Delta$ *LF82\_314* strains expressing sGFP2 and mScarlet-I,  
132 respectively, were generated for fluorescence microscopy of biofilms (Fig. 1D). LF82-sGFP2  
133 and LF82  $\Delta$ *LF82\_314*-mScarlet-I biofilms appear similar in extant and structure to those  
134 generated by non-fluorescent, parental strains. When mixed in a 1:1 ratio, LF82-sGFP2 and  
135 LF82  $\Delta$ *LF82\_314*-mScarlet-I form biofilms composed of distinct, strain-exclusive islands,  
136 suggesting initial attachment and biofilm growth of LF82 and LF82  $\Delta$ *LF82\_314* are  
137 independent of one another. Taken together with Fig. 1C, this demonstrates that the biofilm  
138 formation defect observed in LF82  $\Delta$ *LF82\_314* cannot be complemented in *trans*, suggesting  
139 that LF82\_p314 is unlikely to have a direct role in intercellular adhesion or biofilm matrix  
140 architecture.

141 To define whether LF82\_p314 is likely to function as a pilin, adhesin required for initial  
142 attachment, or extracellular matrix component, we characterised the cellular localisation of  
143 LF82\_p314 in biofilms and planktonic cells. Fluorescence microscopy of biofilms formed by

144 LF82 expressing an LF82\_p314-mEmerald fusion protein showed LF82\_p314 localises as cell-  
145 associated filaments – as in HeLa cells (Fig. 1E) – which align with the long axis of the  
146 bacterium. To assess the subcellular localisation of the LF82\_p314 filaments, we stained live,  
147 planktonic LF82 p*LF82\_314-mEmerald* with an amine-reactive succinimidyl ester dye  
148 conjugate (CF™ 633, Sigma Aldrich) to define the outer membrane, fixed the dyed cells, and  
149 them imaged by fluorescence microscopy. Pearson correlation analysis of pixel intensities was  
150 performed using CellProfiler and demonstrated a very weak correlation between CF633 and  
151 LF82\_p314-mEmerald fluorescence (Pearson correlation coefficient, mean  $r = 0.135 \pm 0.032$   
152 (95% CI)). Furthermore, cross-sectional analysis of fluorescence intensity (Fig. 1F) shows two  
153 peaks of CF633 intensity, representing the cell membranes, and one peak of LF82\_p314-  
154 mEmerald intensity between these peaks, suggesting that LF82\_p314 filaments localise  
155 intracellularly. We also note that in cells imaged 1 hour post induction, LF82\_p314 filaments  
156 are shorter than in biofilms imaged at 16 h, and localise near the cell pole, suggesting  
157 interactions with intracellular, pole-localised proteins. LF82\_p314 is therefore unlikely to be  
158 a pilin or extracellular matrix component, and the biofilm defect observed in LF82  $\Delta$ *LF82\_314*  
159 is not due to aberrant pilin-mediated attachment or extracellular matrix architecture.

#### 160 **LF82\_p314 modulates flagella-mediated motility *via* an uncharacterised mechanism**

161 In the absence of evidence for an adhesin or extracellular matrix function for LF82\_p314, we  
162 reasoned that a motility defect might confer a surface colonisation defect, manifesting in an  
163 apparent biofilm formation defect, as has been shown elsewhere [64]. Accordingly, we used  
164 established soft agar motility assay methods to analyse the swimming and swarming  
165 behaviour of LF82, LF82  $\Delta$ *LF82\_314*, and LF82  $\Delta$ *LF82\_314* p*LF82\_314*. We found that LF82  
166  $\Delta$ *LF82\_314* has notable swimming (Fig. 2A) and swarming (Fig. 2B) defects (One-way ANOVA  
167 with multiple comparisons to wild-type LF82; swim, LF82 vs LF28  $\Delta$ *LF82\_314*,  $p = 0.0004$ ;  
168 swarm, LF82 vs LF28  $\Delta$ *LF82\_314*,  $p = 0.0001$ ) when compared to wild-type LF82 at 10 and 24h  
169 post-inoculation, respectively. No significant difference was observed between LF82 and the  
170 *LF82\_314*-expressing strain, LF82  $\Delta$ *LF82\_314* + p*LF82\_314*, demonstrating these defects are  
171 fully complemented by *LF82\_314* expression. We noted that at 24h post-inoculation, both  
172 LF82 and LF82  $\Delta$ *LF82\_314* on swimming plates had reached the edge of the plate; however,  
173 these plates lack the characteristic chemotactic rings observed on wild-type LF82 and LF82  
174  $\Delta$ *LF82\_314* + p*LF82\_314* plates (Fig. 2A), and also often showed swarming behaviour in the

175 centre. We therefore theorised that the motility and/or chemotaxis systems may be defective  
176 in LF82  $\Delta LF82_{314}$ , leading to a slower rate of swimming, and/or an inappropriate response  
177 to wetness conditions. To test the chemotactic response of LF82  $\Delta LF82_{314}$ , we conducted a  
178 simple capillary-based chemotaxis assay using media with or without glucose as a  
179 chemoattractant (Fig. 2C). We found both LF82 and LF82  $\Delta LF82_{314}$  are more enriched in  
180 capillaries containing glucose than without, and no statistically significant difference was  
181 observed, suggesting chemotaxis is intact in LF82  $\Delta LF82_{314}$ . We also assessed whether the  
182 number per cell or morphology of flagella was affected by deletion of  $LF82_{314}$ , using Kodaka  
183 staining [65] and transmission electron microscopy (TEM). Kodaka staining (Fig. 2D) confirmed  
184 the presence of flagella on both wild-type LF82 and LF82  $\Delta LF82_{314}$ . Negative-stain TEM  
185 demonstrated no gross morphological differences in flagella between wild-type LF82 and  
186 LF82  $\Delta LF82_{314}$  flagella (Fig. 2E). Flagella counts from 30 TEM micrographs (Fig. 2F) revealed  
187 no difference between the numbers of flagella per flagellated cell. These data demonstrate  
188  $LF82_{314}$  is required for efficient flagella-mediated motility; however, gross behavioural and  
189 morphological traits such as in-liquid chemotaxis and flagella biosynthesis are intact in LF82  
190  $\Delta LF82_{314}$ , suggesting LF82\_p314 elicits its effect via a more subtle, uncharacterised  
191 mechanism.

## 192 **LF82\_p314 is required for optimal *C. elegans* gut colonisation**

193 Non-motile AIEC have significantly reduced virulence in *in vivo* models [45,46,66], and host-  
194 adapted AIEC are hyper-motile [59], suggesting flagella motility is critical in AIEC virulence.  
195 We therefore assayed the *in vivo* virulence of LF82 and LF82  $\Delta LF82_{314}$  using an established  
196 *C. elegans* survival assay [66]. The *C. elegans* food source strain *E. coli* OP50 was used as a  
197 negative control. We found LF82 was capable of “slow killing” *C. elegans*, and that deletion of  
198  $LF82_{314}$  does not improve or abrogate the survival of infected *C. elegans* (Fig. 3A),  
199 suggesting  $LF82_{314}$  does not directly contribute to *C. elegans* killing by AIEC in this model.  
200 We noted, however, that worms fed wild-type LF82 consistently begin to die 1-2 days before  
201 those fed LF82  $\Delta LF82_{314}$ , and reasoned that this may be due to less efficient colonisation of  
202 the *C. elegans* gut by the less motile LF82  $\Delta LF82_{314}$ , prolonging the time required to fully  
203 establish infection. We therefore chose to assess the number of bacteria stably colonising the  
204 *C. elegans* gut at daily intervals. In worms fed on lawns containing exclusively LF82 or LF82  
205  $\Delta LF82_{314}$ , we found no statistically significant deviation between wild-type and mutant CFU



206 recovered per worm gut (Fig. 3B), although mean LF82 CFU per worm gut was higher at 3 and  
207 4 days post-infection (d.p.i.).

208 Reasoning that an assay in which *C. elegans* are continuously fed up to  $10^{11}$  CFU/mL  
209 of one bacterial strain may not represent a realistic infection scenario, and that this could  
210 mask a colonisation defect, we conducted competition assays to detect whether *LF82\_314*  
211 deletion impacts fitness against wild-type LF82. LF82 and LF82  $\Delta LF82_314$  carrying the  
212 Kanamycin-resistant pBAD18 (LF82-Kan<sup>R</sup> and LF82  $\Delta LF82_314$ -Kan<sup>R</sup>) or Chloramphenicol-  
213 resistant pBAD33 (LF82-Cm<sup>R</sup> and LF82  $\Delta LF82_314$ -Cm<sup>R</sup>) were used to allow differential  
214 selection of CFU recovered from *C. elegans*. Worms were fed on 1:1 mixes of LF82-Kan<sup>R</sup>:LF82  
215  $\Delta LF82_314$ -Cm<sup>R</sup> or LF82-Cm<sup>R</sup>:LF82  $\Delta LF82_314$ -Kan<sup>R</sup> as above. The Competitive Indices (CI) for  
216 LF82  $\Delta LF82_314$  in this assay (Fig. 3C) are significantly below a “no-disadvantage” CI ratio of  
217 1 throughout infection (one-tailed Wilcoxon match-pairs signed rank test, 1 d.p.i.,  $p = 0.0195$ ;  
218 day 3 d.p.i.,  $p = 0.0004$ ; day 6 d.p.i.,  $p = 0.0011$ ), showing LF82  $\Delta LF82_314$  has a gut  
219 colonisation disadvantage to wild-type LF82. To visualise the infection process, LF82-sGFP2  
220 and LF82  $\Delta LF82_314$ -mScarlet-I were used to infect worms either alone, or mixed in a 1:1  
221 ratio as above. Fluorescence microscopy of infected worms (Fig. 3D) shows that at 1 d.p.i.,  
222 both LF82-sGFP2 and LF82  $\Delta LF82_314$ -mScarlet-I colonise the worm mouth. Fluorescence in  
223 worms fed a LF82-sGFP2:LF82  $\Delta LF82_314$ -mScarlet-I mix was below background levels. At 3  
224 d.p.i. and 6 d.p.i, LF82-sGFP2 and LF82  $\Delta LF82_314$ -mScarlet-I successfully colonise the head  
225 and gut of worm in both mono- and co-feeding conditions, with penetration into the  
226 pseudocoelom at 6 d.p.i.; however, in concordance with Fig. 3C, LF82-sGFP2 appears to  
227 outcompete LF82  $\Delta LF82_314$ -mScarlet-I when co-fed to *C. elegans*. Taken together, our data  
228 suggests that although deletion of *LF82\_314* does not attenuate “slow killing” of *C. elegans* in  
229 mono-feeding conditions, *LF82\_314* is required for efficient colonisation of the *C. elegans* gut.

### 230 ***LF82\_314* is a widely-conserved, and may be horizontally transmissible**

231 *LF82\_314* is encoded by 468 DNA bases, annotated as encoding the 155 residue protein  
232 LF82\_p314 [67]. All results returned by blastp and JACKHMMER searches designate *LF82\_314*  
233 as a “hypothetical protein,” “conserved hypothetical protein,” “MULTISPECIES: hypothetical  
234 protein,” “uncharacterised protein,” or “conserved uncharacterised protein”. *LF82\_314* is  
235 located proximal to a tRNA site (*asnV*) in a region of the LF82 genome (Fig. 4A) which encodes  
236 predicted transposases (*LF82\_309* and *yhhI*), integrases (*LF82\_309* and *LF82\_311*), a toxin-



237 antitoxin addiction module (*LF82\_312* and *LF82\_313*), a transcription factor (*LF82\_774*), an  
238 endonuclease (*LF82\_317*), and a helicase (*LF82\_318*). The putative components of this  
239 genome neighbourhood and its proximity to a common transposable element insertion site  
240 (tRNA) led us to theorise that *LF82\_314* may be encoded on an active or former mobile  
241 genetic element (MGE). MGEs are significant sources of horizontally acquired virulence  
242 factors, notable examples of which include the Shiga toxin – which is transmissible among *E.*  
243 *coli* strains by the *stx* bacteriophage, generating highly virulent Shiga Toxin-producing *E. coli*  
244 (STEC; reviewed in [68]) – and the *Salmonella* Typhi pathogenicity island, SPI-7 – a mosaic of  
245 conjugative elements and temperate bacteriophage insertions which encodes genes for Vi  
246 capsule synthesis and the Type III Secretion System effector, *sopE* [69,70].

247 We therefore sought to assess the distribution of *LF82\_314* homologues in related  
248 phyla. We harvested the top 100 DNA sequences of *LF82\_314* homologues returned by blastn  
249 discontinuous megablast (Table S3), and curated this list to remove strains for which 16S rRNA  
250 sequences were not readily available. This produced a list of 77 *LF82\_314* homologues  
251 encoded in 68 enterobacterial genomes. Many of the strains returned by our search strategy  
252 are human pathogens (see Fig 4C). Of note are several *E. coli* strains which belong to an  
253 emergent clonal, pandemic urinary tract infection (UTI) -associated Extraintestinal Pathogenic  
254 *E. coli* (ExPEC) clade, ST131 [71]. Interestingly, blast search strategies excluding the order  
255 enterobacteriales did not return any significant results, suggesting *LF82\_314* homologues are  
256 restricted to this order. We obtained 16S rRNA sequences from the 68 selected strains from  
257 SILVA, and used these to build a Maximum Likelihood phylogenetic tree. Comparison of this  
258 16S rRNA tree (Fig. S2) with a Maximum Likelihood tree created from *LF82\_314* homologues  
259 showed marked differences (Figure 4C). For example, in the *LF82\_314* homologue tree,  
260 *Salmonella* sp. have a fragmented phylogeny rather than clustering as a distinct phylogenetic  
261 group as in the 16S rRNA tree. Similarly, *Shigella boydii* strains, and AIEC LF82 and NRG 857c,  
262 cluster together away from the *E. coli* ST131 clade in the *LF82\_314* tree, however in the 16S  
263 rRNA tree one *E. coli* group is formed. These data suggest that *LF82\_314* may have been  
264 introduced into these strains horizontally, raising the possibility that in some conditions,  
265 *LF82\_314* may become a transmissible virulence factor.

## 266 Discussion

267 Flagella-mediated motility is a critical virulence factor in a wide variety of gram-negative  
268 bacterial pathogens. In AIEC LF82 and the closely related strain NRG 857c, flagella motility has  
269 been shown to be required for host colonisation, cell invasion, and persistence in *in vivo*  
270 models [45,46,59,66]. Flagellar biosynthesis in AIEC is regulated in a canonical fashion by the  
271 master flagellar regulators, *flhCD* and *fliA* [44,72]. The *E. coli* quorum sensing system, QseBC,  
272 is involved in regulation of *flhCD* function [73], and a novel transcription factor, NrdR, has  
273 been implicated in flagellar biosynthesis and regulation of chemotaxis gene expression [45].  
274 The AIEC LF82 genome encodes a full complement of chemotaxis genes [67], and typical  
275 chemotactic responses have been observed in LF82 in the literature and this study, suggesting  
276 environmental sensing and motile responses are conserved.

277 We describe in this study a conserved hypothetical gene – *LF82\_314* – which has novel  
278 functions in biofilm formation and host colonisation, which are mediated by a role in flagellar  
279 motility. Although we initially theorised LF82\_p314 may function as a self-assembling pilin or  
280 biofilm extracellular matrix protein, we have found that LF82\_p314 localises within bacterial  
281 cells, and is therefore likely a cytoplasmic or periplasmic protein. We have demonstrated that  
282 *LF82\_314* is required for efficient swimming and swarming in soft agar motility assays; we  
283 note, however, in a soft agar swimming assay, that both LF82 and LF82  $\Delta$ *LF82\_314* reach the  
284 edge of the plate at 24h post-inoculation. When considered with the defect observed at 8h  
285 post-inoculation, this observation suggests that LF82  $\Delta$ *LF82\_314* swims and swarms more  
286 slowly than wild-type LF82, a phenotype which may be mediated by defective chemotaxis or  
287 flagella. LF82  $\Delta$ *LF82\_314* swim plates lack chemotactic rings, suggesting aberrant chemotaxis  
288 may be responsible for the observed defect. However, the in-liquid chemotactic response to  
289 glucose, and flagella biosynthesis, are indistinguishable from wild-type LF82 in LF82  
290  $\Delta$ *LF82\_314*, suggesting neither gross morphological differences nor defective chemotactic  
291 signalling can account for the observed motility defect. LF82\_p314 must therefore elicit a  
292 more subtle effect which nevertheless manifests as a notable motility defect in soft agar.  
293 Currently no model of LF82\_p314 function exists. We hypothesise that LF82\_p314 may be  
294 involved in modulating bacterial velocity in high-viscosity environments, or may be involved  
295 in surface sensing and transitioning in-liquid motility to surface-associated motility and  
296 adhesion. Further study towards a molecular understanding of LF82\_p314's function –

297 including mapping LF82\_p314 protein interactions, and studying the effects of LF82\_p314 on  
298 the AIEC transcriptome – are ongoing in our laboratory.

299 We demonstrate that *LF82\_314* is required for efficient biofilm formation in LF82.  
300 Biofilms play a role in Crohn’s Disease pathology [29], and infection-associated biofilms are  
301 often sources of persistence, antibiotic resistance, and tolerance [74,75]. Antibiotic therapy  
302 is routinely used as an intervention in CD, and is known to temporarily ameliorate symptoms  
303 in the majority of patients [76]. However, relapse during treatment is common and reportedly  
304 universal [77] when treatment is halted, suggesting inflammation in relapsing CD may be due  
305 to outgrowth of antibiotic resistant or surviving, tolerant bacteria, such as those in mucosa-  
306 associated AIEC biofilms. LF82\_p314 may therefore be of some interest as a potential anti-  
307 virulence target which might potentiate more successful antibiotic treatment in CD, by  
308 breaking down or inhibiting formation of drug-tolerant AIEC biofilms.

309 Of particular significance, our work demonstrates *LF82\_314* is required for effective  
310 colonisation of the gut in a *C. elegans* infection model. We did not observe decreased  
311 virulence or colonisation by LF82  $\Delta$ *LF82\_314* when worms were fed on one strain exclusively,  
312 but were able to detect a clear defect when LF82  $\Delta$ *LF82\_314* was in competition with wild-  
313 type LF82. However, this does not imply that *LF82\_314* has only a marginal effect on LF82 gut  
314 colonisation. In the assay we have adapted from [66], worms are constantly fed on plates  
315 prepared with bacterial concentrations between  $10^{10}$  and  $10^{11}$  CFU per ml of culture. In such  
316 mono-feeding experiments, it is likely that bacteria at this density saturate the worm gut,  
317 bringing equal numbers of wild-type and mutant bacterial cells in contact with the gut surface,  
318 thus masking colonisation defects. Indeed, the colonisation defect observed in the  
319 competition assay, which still saturates the gut with bacteria, suggests that in more  
320 biologically relevant scenarios – such as a substantially reduced total infectious dose of LF82  
321 competing against an established microbiome – LF82  $\Delta$ *LF82\_314* may have a marked  
322 colonisation defect. Further study to establish the role of *LF82\_314* in AIEC colonisation in  
323 complex polymicrobial contexts is required to test this hypothesis; however, our data  
324 provides strong evidence to suggest that LF82\_p314-mediated motility plays an important  
325 role in host colonisation.

326 Finally, we report that *LF82\_314* is widely distributed throughout the  
327 Enterobacteriaceae, including several significant human pathogens, and that the gene

328 appears to be laterally inherited. Our analysis was limited to the top 100 blastn results;  
329 however, the wide distribution of closely related *LF82\_314* homologues presented in our  
330 analysis suggests that this novel virulence factor is likely to be present in an even greater  
331 range of enterobacterial pathogens. Although our analysis does not show that the putative  
332 *LF82\_314* mobile genetic element can be mobilised in the strains we have analysed, it is  
333 conceivable that this element may be transmissible from a strain not included in our analysis.  
334 This is of particular interest in the context of some of the strains we analysed, such as those  
335 belonging to the *E. coli* ST131 clade. ST131 is a clade of ExPEC associated with antibiotic-  
336 resistant recurrent UTIs, which was first identified in 2008 [71]. Among the pathogenic  
337 characteristics of ST131 are increased biofilm formation and adhesion to epithelial cells, both  
338 processes which require flagella motility, and which may be potentiated by *LF82\_p314*. It is  
339 thought that many UTIs are seeded from a gut reservoir and colonisation of both epithelial  
340 surfaces occurs via similar mechanisms [78]. An ST131 *LF82\_314* homologue may play a role  
341 both in establishment of a gut niche, as well as subsequent infection of the urinary epithelium.  
342 The presence of *LF82\_314* in the genomes of numerous strains representing an emergent  
343 pathogen raises the possibility that acquisition of *LF82\_314* may have been an important step  
344 in becoming such a successful pathogen.

345 Further work is required to understand the molecular function of *LF82\_p314*; to assess  
346 its significance in higher-complexity infection systems; and to characterise fully its  
347 distribution, and whether this novel virulence factor is transmissible. What is clear is  
348 *LF82\_314* is a novel player in flagellar-mediated motility with significance for host colonisation  
349 and biofilm formation, and is conserved in a range of important human pathogens. We  
350 therefore suggest a new designation for *LF82\_314* and its homologues – *bcmA* (biofilm  
351 coupled to motility A) – to facilitate future work without diverse nomenclature confusing the  
352 literature.

## 353 **Materials and Methods**

### 354 **Strains and media**

355 *E. coli* LF82, XL-1, and OP50, were grown in Lysogeny Broth (LB) or on LB agar with  
356 supplements, antibiotics, and agitation as appropriate, and incubated at 37°C unless  
357 otherwise stated. *E. coli* S17-1 carrying pMRE-Tn7-XXX plasmids were maintained at 25°C. C.

358 *C. elegans* SS104 [*glp-4(bn2)I.*] obtained from the *Caenorhabditis* Genetics Centre were cultured  
 359 as in [79]. Strains used in this study are listed in Table 1.

360 **Table 1**

Strain	Genotype / Description	Source / Reference
<i>Caenorhabditis elegans</i> SS104	[ <i>glp-4(bn2)I.</i> ]	Caenorhabditis Genetics Centre
<i>E. coli</i> LF82	Prototypical AIEC type strain. Genome sequenced.	Arlette Darfeuille-Michaud, Université Clermont Auvergne
<i>E. coli</i> LF82 $\Delta$ LF82_314	LF82 with 428 bases of the LF82_314 CDS deleted	This study
<i>E. coli</i> LF82 $\Delta$ LF82_314 pLF82_314	<i>E. coli</i> LF82 $\Delta$ LF82_314 with an LF82_314-3xFLAG construct on pBAD18	This study
<i>E. coli</i> LF82-sGFP2	LF82 with chromosomally inserted sGFP2	This study
<i>E. coli</i> LF82 $\Delta$ LF82_p314-mScarlet-I	LF82 $\Delta$ LF82_314 with chromosomally inserted mScarlet-I	This study
<i>E. coli</i> LF82-Kan <sup>R</sup>	LF82 carrying pBAD18	This study
<i>E. coli</i> LF82-Cm <sup>R</sup>	LF82 carrying pBAD33	This study
<i>E. coli</i> LF82 $\Delta$ LF82_314-Kan <sup>R</sup>	LF82 $\Delta$ LF82_314 carrying pBAD18	This study
<i>E. coli</i> LF82 $\Delta$ LF82_314-Cm <sup>R</sup>	LF82 $\Delta$ LF82_314 carrying pBAD33	This study
<i>E. coli</i> OP50	Uracil auxotrophic <i>C. elegans</i> food source. Genome sequenced. Tet <sup>R</sup>	Steve Atkinson, University of Nottingham
<i>E. coli</i> S17-1 $\lambda$ pir	Strain for conjugation of pMre-Tn7-XXX plasmids into LF82 strains	AddGene, (81)
<i>E. coli</i> XL-1 blue	Common cloning strain	

361 Strains used in this study. Cm<sup>R</sup> = Chloramphenicol resistant; Kan<sup>R</sup> = Kanamycin resistant; Tet<sup>R</sup>  
 362 = Tetracycline resistant.

363 **Genetic manipulation**

364 Genes of interest were amplified by PCR amplification using Phusion-HF DNA Polymerase  
 365 (NEB). Sequences were inserted into pBAD18 or pBAD33 by restriction digest using EcoRI,  
 366 KpnI, and XbaI restriction enzymes (NEB), and ligation using T4 DNA ligase (NEB). LF82

367  $\Delta LF82\_314$  was generated from LF82 wild-type using the CRISPR-Cas9-based no-SCAR  
368 strategy [80]. Deletion was confirmed by Sanger sequencing, and strains were fully validated  
369 by whole genome Illumina sequencing (MicrobesNG, Birmingham, UK). LF82-sGFP2 and LF82  
370  $\Delta LF82\_314$ -mScarlet-I were constructed using pMRE-Tn7-132 and pMRE-Tn7-135  
371 respectively, as in [81]. See Table 2 for a list of plasmids used in this study, and Table 3 for a  
372 list of primers.

373 **Table 2**

Plasmid	Description
pBAD18	Expression plasmid; Kan <sup>R</sup>
pBAD33	Expression plasmid; Cm <sup>R</sup>
pBAD - <i>LF82_314</i> -mEmerald	Expression plasmid for LF82_p314-mEmerald fusion protein; Cm <sup>R</sup>
pCas9-cr4	Cas9- expressing plasmid for no-SCAR deletion strategy; Tet <sup>R</sup>
pCMV-3xFLAG- <i>LF82_314</i>	Plasmid for expression of FLAG-tagged LF82_p314 in mammalian cells; Amp <sup>R</sup>
pCMV-mEmerald-4GS	Plasmid for expression of mEmerald-tagged proteins in mammalian cells; Amp <sup>R</sup>
pCMV-mEmerald- <i>LF82_314</i>	Plasmid for expression of mEmerald-tagged LF82_p314 in mammalian cells; Amp <sup>R</sup>
pKD-sgRNA-p314	$\lambda$ -Red recombinase- and <i>LF82_314</i> -targeting sgRNA-expressing plasmid for no-SCAR deletion strategy; Spc <sup>R</sup>
p <i>LF82_314</i>	Complementation plasmid for LF82 $\Delta LF82\_314$ encoding an LF82_p314-3xFLAG protein on pBAD18; Kan <sup>R</sup>
pMRE-Tn7-132	Conjugative suicide plasmid for transposon cloning of sGFP2 construct into chromosomal sites; Amp <sup>R</sup> , Cm <sup>R</sup>
pMRE-Tn7-135	Conjugative suicide plasmid for transposon cloning of mScarlet-I construct into chromosomal sites; Amp <sup>R</sup> , Cm <sup>R</sup>

374 Plasmids used in this study. Amp<sup>R</sup> = Ampicillin resistant; Cm<sup>R</sup> = Chloramphenicol resistant;  
375 Kan<sup>R</sup> = Kanamycin resistant; Spc<sup>R</sup> = Spectinomycin resistant; Tet<sup>R</sup> = Tetracycline resistant.

376

377 **Table 3**

Primer	Sequence	Description
3xFLAG F	GAggtaccGACTACAAGGACGACGATG	Amplifying 3xFLAG tag for insertion into pBAD plasmids
3xFLAG R	GActtagactaGCCCCCTCCACCAATTCG	
LF82_314 F	GCgaatccAGGAGGAtaaataATGGCTACGATCCCCAC	Amplifying <i>LF82_314</i> from LF82 genome for insertion into pBAD plasmids
LF82_314 R	CCggtaccTGCTTTGGCCTCCACACC	
mEmerald F	TAggtaccATGGTGAGCAAGGGCGAG	Amplifying mEmerald for insertion into pBAD plasmids
mEmerald R	GCtctagaCTACTTGTACAGCTCGTCCATG	
p314_del F	GAGGAACATCAGCGATAGC	Confirming <i>LF82_314</i> deletion
p314_del R	GCGCGCTTAGCTACACC	
pTET_com	CCAATTGTCCATATTGCATCA	Amplifying pKD-sgRNA-p314 in two parts
pTET_p314	GTCTGACTCTGCTGCAACCCGTGCTCAGTATCTCTATCACTGA	
sgRNA_com	TTTATAACCTCCTTAGAGCTCGA	Amplifying pKD-sgRNA-p314 in two parts
sgRNA p314	GGGTTGCAGCAGAGTCAGACGTTTTAGAGCTAGAAATAGCA AG	

378 Primers used in this study. Upper-case letters denote complimentary sequences; lower-case  
379 bases represent restriction sites

### 380 **Light microscopy**

381 Light microscopy was conducted using an Olympus BX51 microscope at appropriate  
382 magnifications, using  $\mu$ Manager software [82].

### 383 **Electron microscopy**

384 EM images were captured using a Tecnai T12 BioTwin Transmission Electron Microscope at  
385 an accelerating voltage of 100 kV. Images were captured using a Megaview III Soft Imaging  
386 System (SIS) camera.

### 387 **Biofilm assays**

388 Crystal Violet (CV) biofilm assays were adapted from [83]. Overnight bacterial cultures were  
389 diluted 1:100 in LB, and 100  $\mu$ l diluted culture was inoculated into each well of a 96-well  
390 microtitre plate before static incubation at 37°C. For *trans*-complementation assays, diluted



391 cultures were mixed in specified ratios before inoculation. At appropriate intervals,  
392 planktonic bacteria were removed from the plate, and biofilms were washed three times with  
393 phosphate buffered saline (PBS). Washed biofilms were stained with 0.1% CV dissolved in  
394 water. CV was removed, and stained biofilms were washed four times with PBS, before the  
395 plates were dried in a laminar flow cabinet. Dry stain was solubilised in 30% glacial acetic acid  
396 and moved to a clean 96-well plate. The OD<sub>550</sub> of solubilised CV was read using an automated  
397 plate reader. Each experiment contained 3-4 technical replicates of 4 biological replicates.

### 398 **Biofilm microscopy**

399 Biofilms were grown for microscopy on acid-washed coverslips. Coverslips were placed in 12-  
400 well plates, which were then inoculated with 500 µl bacterial culture diluted as above.  
401 Inoculated plates were inclined at a 45° angle to ensure the air-liquid interface bisected the  
402 coverslip, and were incubated at 37°C for 16 h. At 16 h, culture media was aspirated, and  
403 biofilms were fixed in 4% formaldehyde in PBS for 1 h. Fixed biofilms were washed three times  
404 with PBS, and coverslips were mounted on slides in a 90% glycerol mounting medium with  
405 0.1% DABCO (Sigma), before imaging at 40x and 100x magnification.

### 406 **Motility assays**

407 Motility was assessed using established soft agar protocols. 5µl of saturated overnight culture  
408 was inoculated into the centre of soft LB agar plates, solidified with either 0.15% (swimming)  
409 or 0.25% (swarming) agar (Sigma) supplemented with 0.4% glucose. Plates were incubated at  
410 37°C. At appropriate intervals, the maximum diameter of the resulting bacterial cloud or  
411 swarm was determined, and plates were imaged using a handheld camera.

### 412 **Chemotaxis assays**

413 A chemotaxis assay was modified from [84]. 75mm Haemocrit capillary tubes (Hawksley &  
414 Sons Ltd, catalogue no. 01604-00) were sealed at one end in a Bunsen flame, before being  
415 passed quickly through the flame several times to heat the glass. Heated capillaries were  
416 immediately placed open-end down into LB with or without 0.4% (w/v) glucose, and left to  
417 draw in media for 15 minutes. Overnight cultures were diluted 1:100 in fresh LB, and  
418 inoculated into the wells of a 96-well plate. Media-loaded capillaries were placed into  
419 inoculated wells, and the plate was incubated in a laminar flow cabinet at room temperature

420 for 1 h. To recover bacteria, the outside of capillaries were washed with water, and the sealed  
421 ends were broken over tubes containing fresh LB to catch escaping culture. Remaining culture  
422 was removed by pipetting. Recovered bacteria were then plated at appropriate dilutions for  
423 colony forming unit (CFU) enumeration.

#### 424 **Flagella staining**

425 To prepare bacteria for light microscopy, overnight cultures were diluted 1:33 in fresh LB and  
426 incubated at 37°C with agitation for 3 hours, before being spread onto glass slides and stained  
427 as in [65]. Stained bacteria were then mounted in immersion oil under a cover slip, sealed  
428 with nail varnish, and imaged at 100x magnification. TEM samples were prepared in a protocol  
429 modified from [85]. Bacterial cultures were prepared as above, before being absorbed onto  
430 carbon-coated copper grids (EM Resolutions) for 10 minutes. Excess fluid was blotted away,  
431 and bacteria were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 minutes.  
432 Fixed samples were washed three times with 0.1 M sodium cacodylate buffer for 10 seconds.  
433 Samples were then stained using 2% phosphotungstic acid before imaging at 6000x  
434 magnification as above.

#### 435 **Surface staining**

436 To stain the outer surface of LF82, we employed an amine-reactive dye conjugation, modified  
437 from [86]. An overnight culture of LF82 p*LF82\_314-mEmerald* was sub-cultured as for flagella  
438 staining, and LF82\_p314 expression was induced with 0.1% arabinose. At appropriate  
439 intervals, 1 ml culture was spun at 1200 x *g* for 10 minutes in a 15ml round-bottom tube,  
440 washed twice in 1 ml PBS, and resuspended in 100 µl PBS using minimal agitation. Cells were  
441 stained at 37°C using 300 µg/ml CF-633 succinimidyl ester (Sigma) for 30 minutes with  
442 agitation at 100 rpm, then washed in PBS. Finally, bacteria were fixed in 4% formaldehyde in  
443 PBS for 20 minutes, washed, and mounted on 2% agarose pads for microscopy. Images were  
444 captured using a 100x lens.

#### 445 ***C. elegans* kill assays**

446 A *C. elegans* infection model was modified from [66]. To preclude data being confounded by  
447 progeny, *C. elegans* SS104 cultures were synchronised as in [79], and maintained at 25°C to  
448 ensure development of sterile adult worms. To prepare kill plates, 100 µl 10x concentrated

449 overnight cultures was spread onto NGM agar plates with appropriate antibiotics, and  
450 incubated at 37°C overnight. 30 synchronised L4 or young adult worms were transferred to  
451 the prepared kill plates, and incubated at 25°C for up to 14 days. Plates were scored every 24  
452 hours for death, and dead worms were removed. To prevent contamination, worms were  
453 transferred to freshly prepared kill plates every 4 days.

#### 454 ***C. elegans* colonisation assays**

455 *C. elegans* colonisation assays were modified from [66]. Colonisation plates were prepared as  
456 above, and 50 synchronised L4 or young adult worms were transferred to each plate, before  
457 being incubated at 25°C. To assay stable gut colonisation, 10 worms were transferred to  
458 freshly prepared OP50 lawns daily, and incubated at 25°C for 1 h to “wash” transient bacteria  
459 from the gut and worm exterior. Worms were then picked and suspended in 1ml M9 buffer,  
460 before being washed three times by pelleting at 1200 x *g* for 1 minute before removal of 750  
461 µL buffer, which was replaced with fresh M9 buffer. To determine external bacterial numbers  
462 following washing, a sample from the final wash was plated at appropriate dilutions on  
463 selective agar. To release gut contents, the worms were homogenised by vortexing with  
464 approximately 400 mg sterile 1mm diameter glass beads (BioSpec Products Inc., catalogue  
465 no. 11079110) in 1% Triton-X (Sigma) in M9 buffer for 2 minutes, before plating the  
466 homogenate at appropriate dilutions on selective agar. CFU per worm gut was defined as:

$$467 \quad CFU \text{ per worm gut} = \text{Homogenate CFU per worm} - \text{External CFU per worm} \quad (2)$$

468 Colonisation experiments were conducted at least twice, with three separate  
469 biological replicates per experiment.

#### 470 ***C. elegans* competition assays**

471 Plates for competition assays were prepared as above, using 1:1 ratios of LF82-Kan<sup>R</sup>:LF82  
472  $\Delta$ LF82\_314-Cm<sup>R</sup> or LF82-Cm<sup>R</sup>:LF82  $\Delta$ LF82\_314-Kan<sup>R</sup>. Plates with an input ratio substantially  
473 different from 1 were discarded. CFU per worm gut was assessed as above, and a competitive  
474 index (CI) was calculated. CI was defined as:

$$475 \quad CI = \frac{(CFU \text{ per worm gut})_{LF82 \Delta LF82_314}}{(CFU \text{ per worm gut})_{LF82}} \quad (2)$$

476 Competition assays were conducted three times, with three separate biological  
477 replicates of each mix per experiment.

478 To image competition assays, infected worms were “washed” on OP50 lawns as  
479 above, and immobilised in a 0.1% NaN<sub>3</sub> solution on a 2% agarose 0.05% NaN<sub>3</sub> pad on a glass  
480 slide, which was sealed under a cover slip. Slides were imaged at 10x magnification.

#### 481 **Data analysis**

482 Statistical analyses were conducted in GraphPad Prism 7. Error bars in graphs represent  
483 standard deviation, unless stated otherwise. Light microscopy images were analysed and  
484 processed using FIJI. Fluorescence co-localisation data analysis and Pearson correlation  
485 analysis was conducted in CellProfiler. Raw *r* values were converted to *z*' values by Fisher's Z-  
486 Transformation and used to calculate mean correlations and 95% CIs, before transformation  
487 back to Pearson's *r* values for interpretation. Cross-sectional intensity measurements were  
488 taken using ImageJ.

#### 489 **Bioinformatics**

490 Protein and nucleotide sequences were retrieved from NCBI [87]. 16S rRNA sequences were  
491 harvested from SILVA [88]. Protein homology searches were conducted using BLASTp and  
492 JACKHMMER [89,90]. Nucleotide sequences for phylogenetic analysis were retrieved by  
493 discontinuous megablast [91]. Multiple sequence alignments were generated using MAFFT  
494 [92–94], before submission to PhyML [95] for automated tree generation. Trees were  
495 visualised in PRESTO (Phylogenetic tRE viSualisaTiOn, available at [http://www.atgc-](http://www.atgc-montpellier.fr/presto/)  
496 [montpellier.fr/presto/](http://www.atgc-montpellier.fr/presto/)).

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816 **Fig 1. *LF82\_314* is required for optimal biofilm formation**

817 (A) *LF82 ΔLF82\_314* do not form biofilms as strongly as wild-type *LF82*, and *LF82 ΔLF82\_314*  
818 biofilms do not appear to mature and disperse as readily as wild-type biofilms. Wild-type  
819 biofilm formation and dispersal behaviour is restored by episomal expression of *LF82\_314*.  
820 Coloured asterisks represent significant difference between mutant (magenta) and  
821 complemented (black) groups, and *LF82* (two-way ANOVA with multiple comparisons to *LF82*;  
822 see Table S2 for significance levels). (B) Microscopic analysis of biofilms at 16 h shows *LF82*  
823 *ΔLF82\_314* form patchy, less dense biofilms than wild-type *LF82*, and that this phenotype can  
824 be complemented by *LF82\_314* expression. (C) To assess whether *LF82\_p314* functions in  
825 *trans*, we assessed the biofilm formation of wild-type *LF82*, *LF82 ΔLF82\_314*, and a 1:1 mix of  
826 *LF82:LF82 ΔLF82\_314*. An *LF82 ΔLF82\_314* biofilm defect was observed as in (A); however, a  
827 1:1 mix of *LF82:LF82 ΔLF82\_314* displayed an intermediate phenotype. Coloured asterisks  
828 represent significant difference between mutant (magenta) and mixed (black) cultures, and  
829 *LF82* (two-way ANOVA with multiple comparisons to *LF82*; see Table S1 for significance  
830 levels). (D) *LF82-sGFP2* (green) and *LF82 ΔLF82\_314\_mScarlet-I* (magenta) form biofilms  
831 comparable to non-fluorescent *LF82* and *LF82 ΔLF82\_314*. A 1:1 co-culture of *LF82-sGFP2*  
832 and *LF82 ΔLF82\_314\_mScarlet-I* show that mixed biofilm are composed of strain-exclusive  
833 islands. (E) Biofilms in which *LF82* express an *LF82\_p314-mEmerald* fusion protein (green)  
834 shows *LF82\_p314* forms cell-associated filaments, as observed in HeLa cells [62]. (F)  
835 Fluorescence intensity cross-section analysis of *LF82* expressing *LF82\_314-mEmerald* with  
836 CF633 succinimidyl ester-stained outer membrane proteins shows the extracellular stain and  
837 mEmerald fluorescence intensity peaks do not overlap, demonstrating an intracellular  
838 localisation for *LF82\_314* filaments. White lines represent the 30 pixels of the cross-section.  
839 Cyan = CF633; green = *LF82\_p314-mEmerald*.

840 **Fig. 2. *LF82\_314* promotes flagella-mediated motility via an uncharacterised mechanism.**

841 *LF82 ΔLF82\_314* has notable defects in (A) swimming and (B) swarming motility (One-way  
842 ANOVA, \*\*\* =  $p \leq 0.001$ ), which are complemented by *LF82\_314* expression. Each dot  
843 represents one technical replicate, or plate; separate colours represent biological replicates.  
844 Swim plates were measured at 10 h post-inoculation, and swarm plates at 24h. Motility plates  
845 imaged at 24 h post-inoculation show *LF82 ΔLF82\_314* have atypical swimming motility  
846 lacking chemotactic rings observed on wild-type plates, and the swarming defect. (C) A

847 capillary-based chemotaxis assay demonstrated increased recovery of both LF82 and LF82  
848  $\Delta LF82\_314$  CFU from media supplemented with glucose compared to LB alone, with no  
849 significant difference between strains in CFU recovered in either condition. Each dot  
850 represents one biological replicate. Bright-field microscopy of Kodaka stained (D) and  
851 negative stain TEM (E) of LF82 and LF82  $\Delta LF82\_314$  demonstrates no differences in flagella  
852 morphology, and flagella counts from 30 TEM micrographs (F) show no difference in flagella  
853 numbers per flagellated cell, suggesting flagella biosynthesis is intact in both strains. TEM  
854 scale bar represents 5  $\mu\text{m}$ .

855 **Fig. 3. *LF82\_314* promotes gut colonisation in *Caenorhabditis elegans***

856 (A) Survival of *C. elegans* SS104 is significantly decreased when cultivated on LF82 or LF82  
857  $\Delta LF82\_314$ , compared to OP50, however no significant difference was noted between  
858 survival on LF82 or LF82  $\Delta LF82\_314$ . (B) No significant difference in stable colonisation of the  
859 *C. elegans* gut by LF82 or LF82  $\Delta LF82\_314$  was found when worm were fed on each strain  
860 exclusively. (C) A competition assay demonstrated LF82  $\Delta LF82\_314$  has a competitive  
861 colonisation disadvantage compared to LF82 throughout the course of infection (one-tailed  
862 Wilcoxon match-pairs signed rank test, \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$ . CI's were  
863 compared to an ideal "no-disadvantage" CI = 1). CI ratios below 1 represent a competitive  
864 disadvantage. Data was pooled from 3 independent experiments. Error bars show 95%  
865 confidence intervals. (D) Fluorescence microscopy of *C. elegans* fed LF82-sGFP2 and LF82  
866  $\Delta LF82\_314$ -mScarlet-I alone demonstrates both LF82 and LF82  $\Delta LF82\_314$  are capable of  
867 establishing gut and pseudocoelomic infections; however, when fed to worms in a 1:1 ratio,  
868 LF82-sGFP2 appears to outcompete LF82  $\Delta LF82\_314$ -mScarlet-I, mirroring Fig. 3C. These data  
869 suggest that *LF82\_314* is required for efficient host colonisation. Scale bars represent 50  $\mu\text{m}$ .

870 **Fig. 4. *LF82\_314* is conserved among enterobacterial pathogens**

871 *LF82\_314* is encoded in a region of the LF82 genome (A) containing several ORFs with  
872 predicted transposase and integrase functions, and a toxin-antitoxin addiction module (B),  
873 suggesting the region may represent an MGE. (C) A ML tree of 77 *LF82\_314* homologues from  
874 68 strains reveals that *LF82\_314* is conserved in a wide variety of pathogenic enterobacteria,  
875 and that *LF82\_314* homologue-derived phylogenies do not recapitulate expected  
876 phylogenetic relationships (see Fig. S2). Of note is the large clade *E. coli* strains, which

877 represents members of the clonal, UTI-associated ExPEC, ST131. Red = human pathogen; gold  
878 = human and animal or zoonotic pathogen; green = plant pathogen; brown = commensal; blue  
879 = environmental; black = insufficient data. Scale bar represents number of substitutions per  
880 site.

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901 **Supporting information**

902 **S1 Fig. *LF82\_314* self-assembles into large filaments**

903 When expressed heterologously in HeLa cells, *LF82\_314* forms large filaments. Filament formation is  
904 independent of the tag used for visualisation, suggesting this phenotype is not a tag-dependent  
905 artefact. See S3 File Supplementary methods for protocol.

906 **S2 Fig. *LF82\_314* homologue distribution does not mirror true phylogenies**

907 The distribution of *LF82\_314* homologues represented in the *LF82\_314* ML phylogenetic tree (A)  
908 differs significantly from the 16S rRNA phylogenies (B). For example, in (A), *E. coli* LF82 and NRG 857c  
909 cluster with *Shigella boydii* Sb227 and ATCC 9210 in a separate clade to other *E. coli* isolates; however,  
910 in (B), LF82, NRG 857c, Sb227, and ATCC 9210 cluster as expected with *E. coli* in a distinct clade.  
911 Similarly, (A) suggests a loose relationship exists between *Salmonella* strains, whereas in (B), a distinct  
912 phylogenetic group is generated. These data suggest *LF82\_314* may be horizontally inherited. Strains  
913 are coloured by genus. Scale bars = substitutions per site.

914 **S1 File *LF82\_314* is co-inherited with the general secretion pathway**

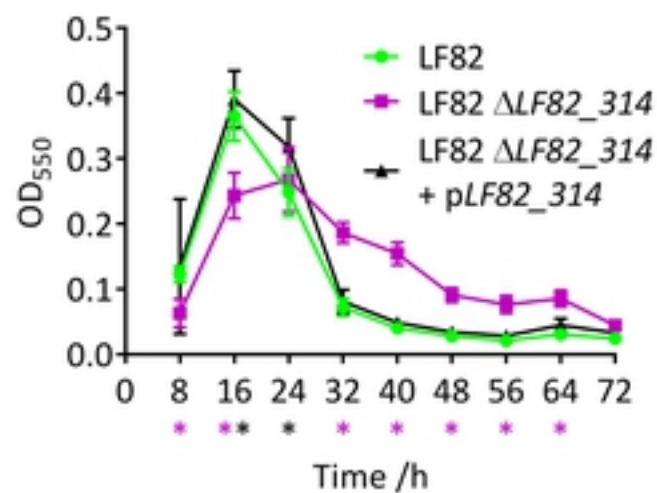
915 **S2 File *LF82\_314* homologues from blastn discontinuous megablast**

916 **S3 File Supplementary methods**

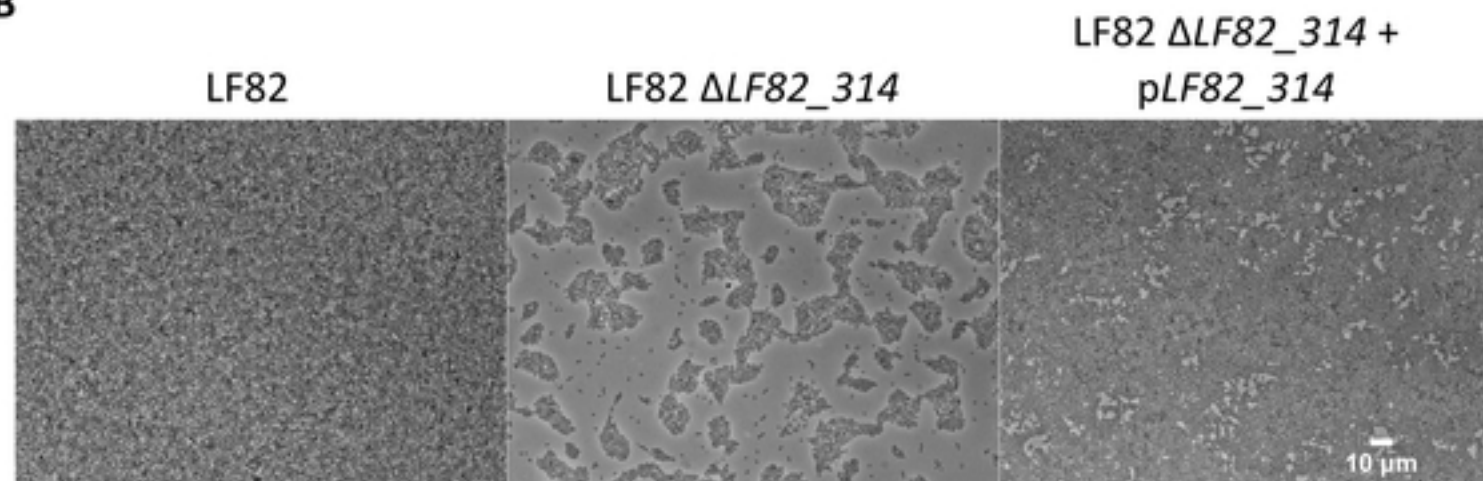
917 **S1 Table p-values for Fig. 1**

Fig. 1

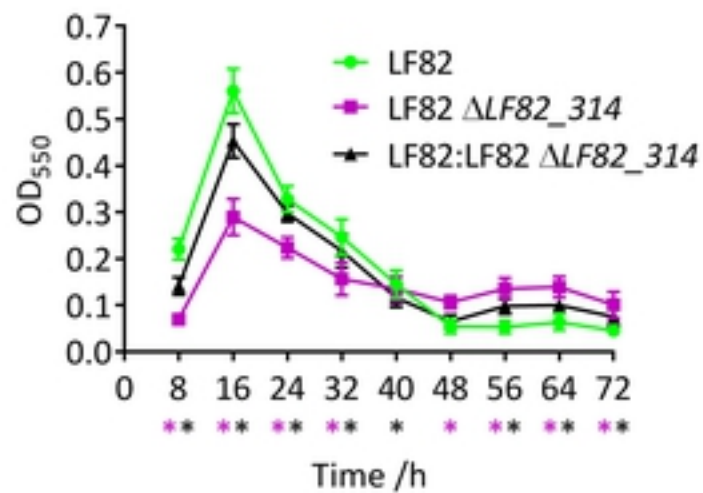
A



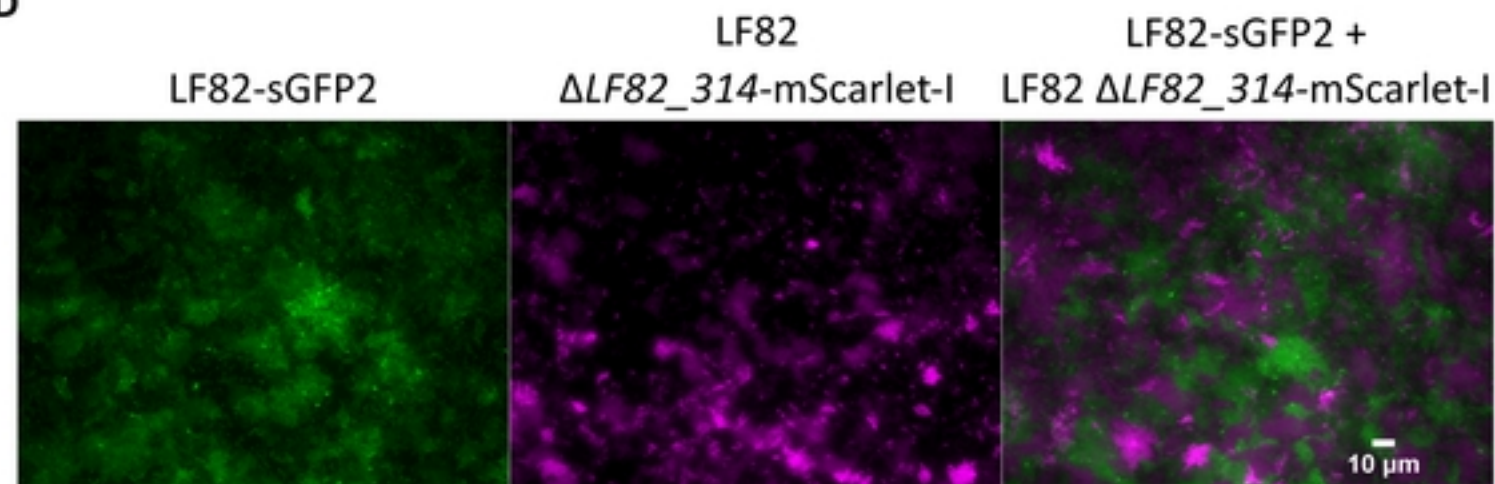
B



C

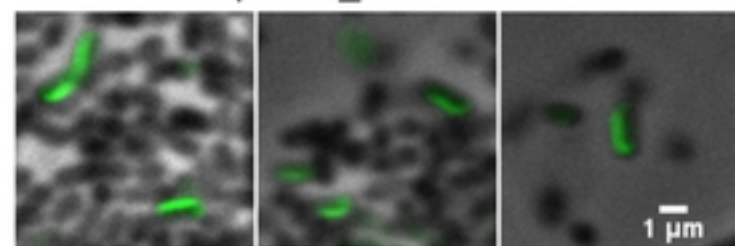


D



E

LF82 pLF82<sub>314</sub>-mEmerald



F

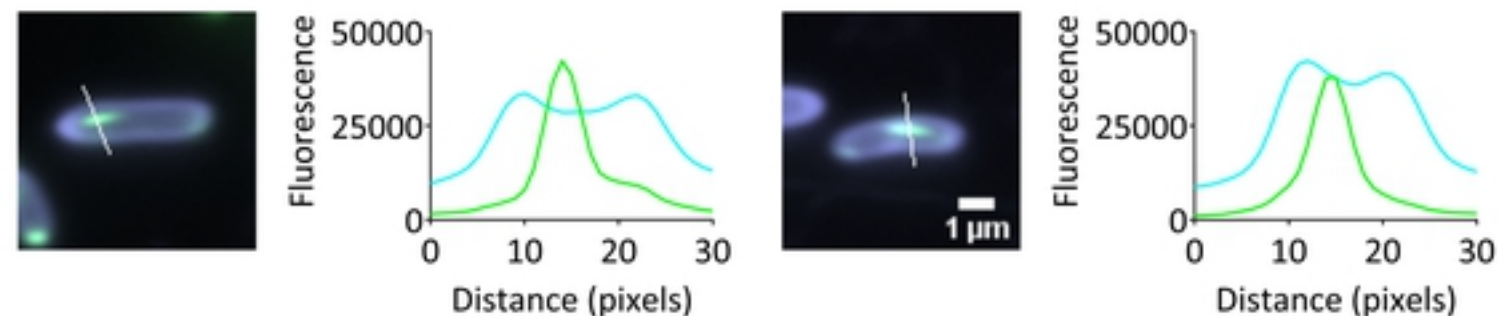


Figure 1



Fig. 2

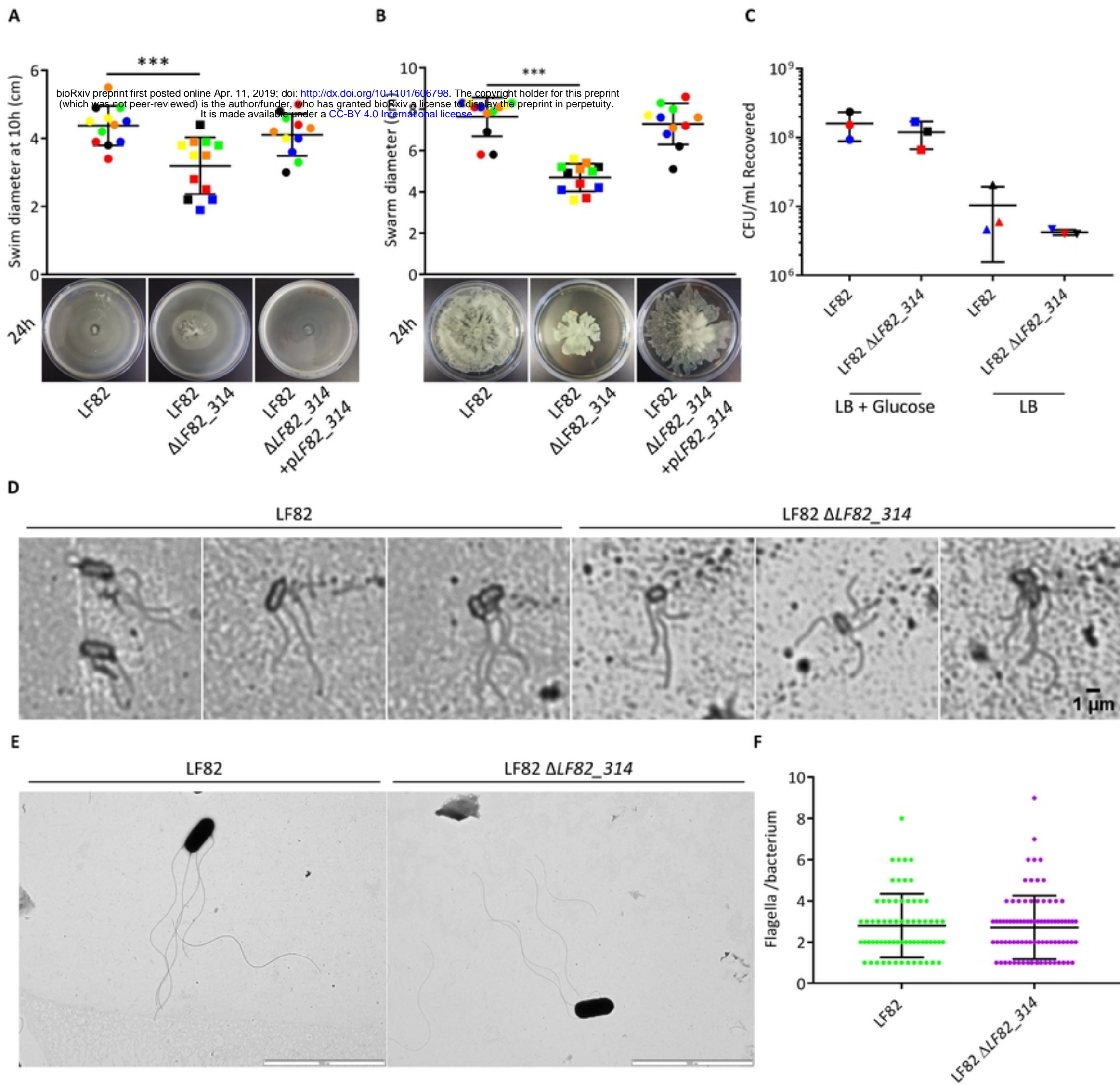


Figure 2



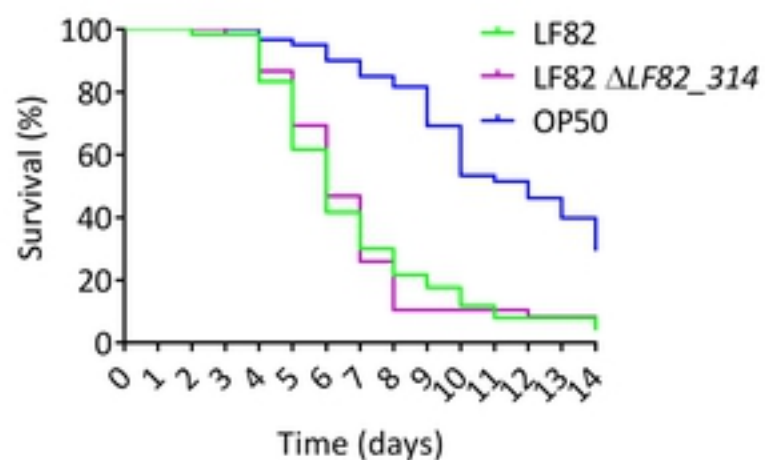
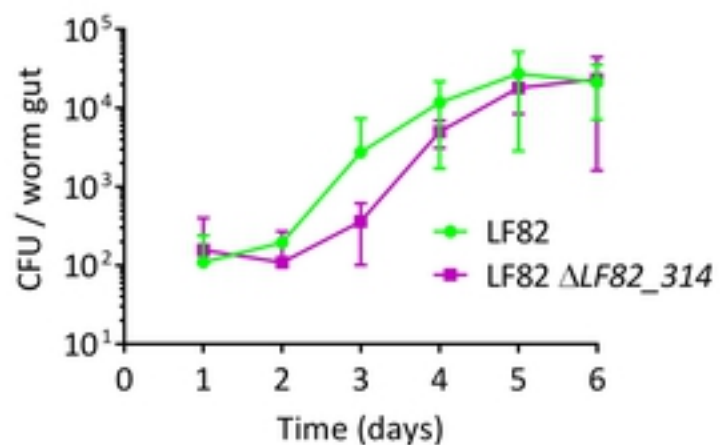
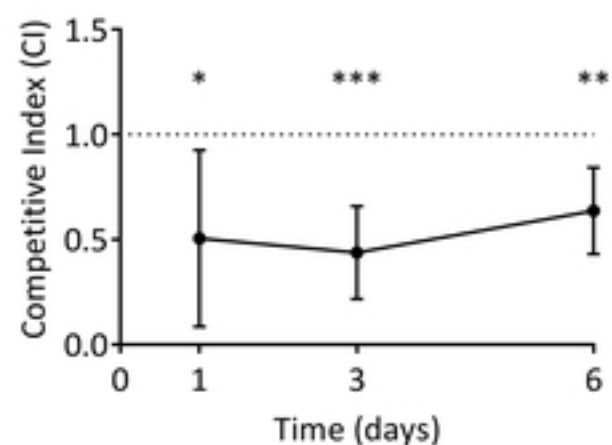
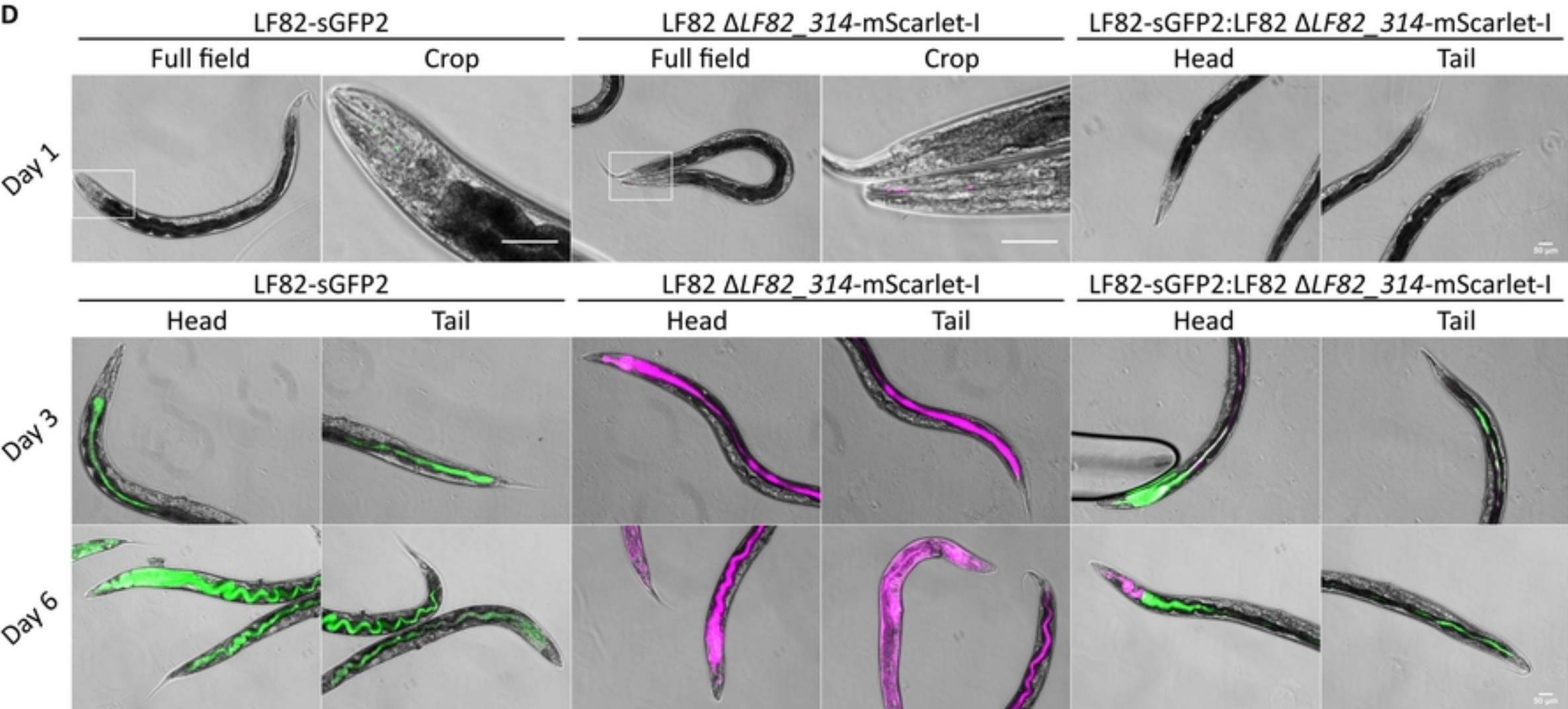
**Fig. 3****A****B****C****D****Figure 3**

Fig. 4

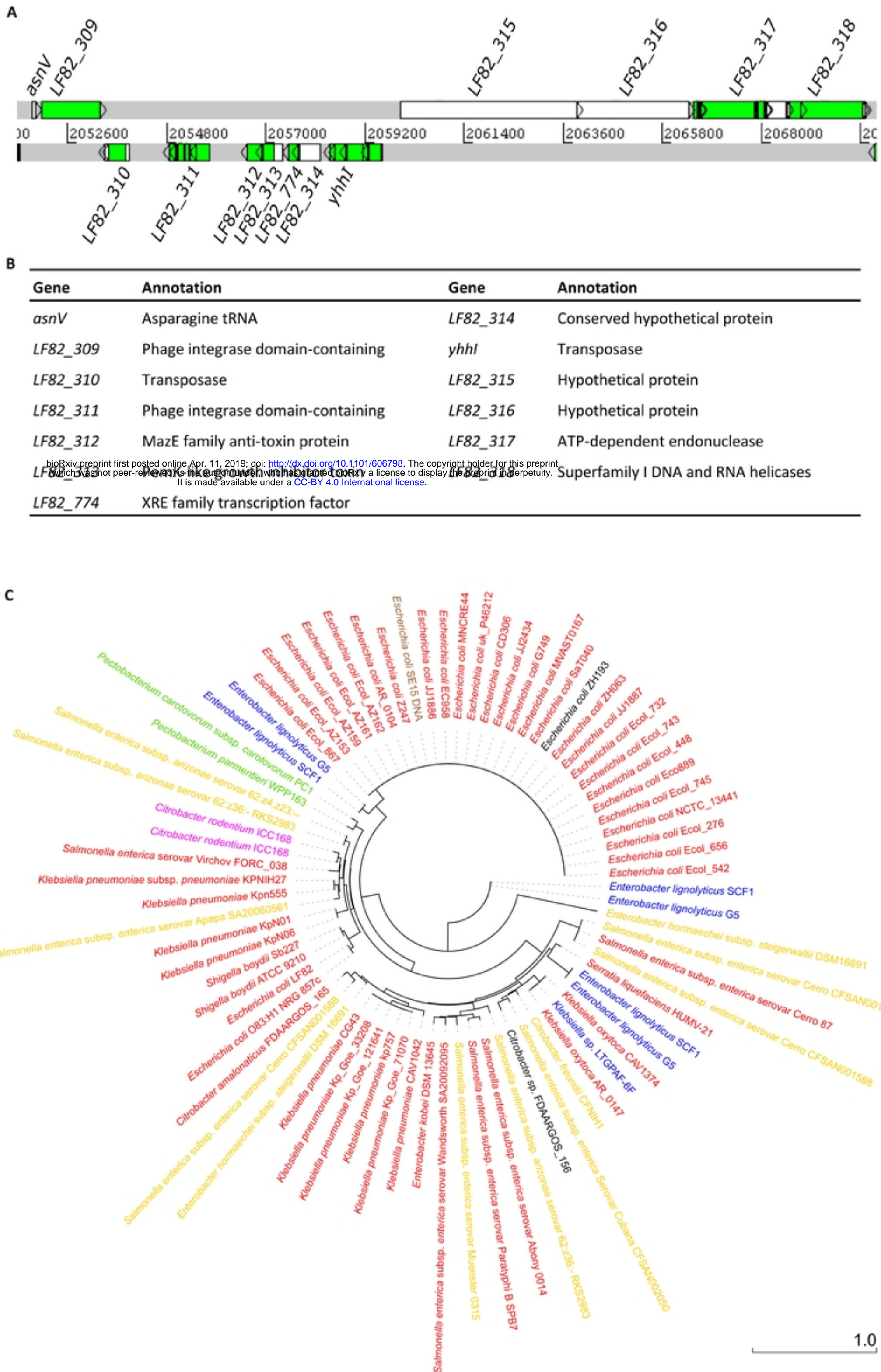


Figure 4

1.0