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# 1 **Flagella methylation promotes bacterial adhesion and** 2 **host cell invasion**

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35 **Abstract:**

36 The flagellum is the motility device of many bacteria and the long external filament is  
37 made of several thousand copies of a single protein, flagellin. While posttranslational  
38 modifications of flagellin are common among bacterial pathogens, the role of lysine  
39 methylation remained unknown. Here, we show that both flagellins of *Salmonella*  
40 *enterica*, FliC and FliB, are methylated at surface-exposed lysine residues. A  
41 *Salmonella* mutant deficient in flagellin methylation was outcompeted for gut  
42 colonization in a gastroenteritis mouse model. In support, methylation of flagellin  
43 promoted invasion of epithelial cells *in vitro*. Lysine methylation increased the surface  
44 hydrophobicity of flagellin and enhanced flagella-dependent adhesion of *Salmonella* to  
45 phosphatidylcholine vesicles and epithelial cells. In summary, posttranslational flagellin  
46 methylation constitutes a novel mechanism how flagellated bacteria facilitate adhesion  
47 to hydrophobic host cell surfaces and thereby contributes to efficient gut colonization  
48 and successful infection of the host.

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55 **Keywords:** host-pathogen interaction, *Salmonella*, post-translational modification,  
56 protein methylation, flagella, flagellin, bacterial motility, invasion, hydrophobicity, lipids

## 57 **Introduction:**

58 The Gram-negative enteropathogen *Salmonella enterica* uses a variety of strategies to  
59 successfully enter and replicate within a host. In this respect, bacterial motility enables  
60 the directed movement of the bacteria towards nutrients or the target site of infection. A  
61 rotary nanomachine, the flagellum, mediates motility of many bacteria, including  
62 *Salmonella enterica*<sup>1</sup>. Flagella also play a central role in other infection processes,  
63 involving biofilm formation, immune system modulation and adhesion<sup>2-4</sup>.

64 The eukaryotic plasma membrane plays an important role in the interaction of  
65 flagellated bacteria with host cells during the early stages of infection<sup>5</sup>. The flagella of *S.*  
66 *enterica*, *Escherichia coli* and *Pseudomonas aeruginosa* can function as adhesion  
67 molecules<sup>6-8</sup> mediating the contact to various lipidic plasma membrane components,  
68 including cholesterol, phospholipids, sulfolipids and the gangliosides GM1 and aGM1<sup>9-</sup>  
69 <sup>12</sup>.

70 Structurally, the flagellum consists out of three main parts: the basal body embedded  
71 within the inner and outer membranes of the bacterium, a flexible linking structure - the  
72 hook, and the long, external filament, which functions as the propeller of the motility  
73 device<sup>13</sup>. The filament is formed by more than 20,000 subunits of a single protein,  
74 flagellin. Many *S. enterica* serovars express either of two distinct flagellins, FliC or FliB,  
75 in a process called flagellar phase variation<sup>14,15</sup>. FliC-expressing bacteria display a  
76 distinct motility behavior on host cell surfaces and a competitive advantage in  
77 colonization of the intestinal epithelia compared to FliB-expressing bacteria<sup>16</sup>. However,  
78 while the structure of FliC has been determined previously<sup>17</sup>, the structure of FliB  
79 remained unknown.

80 The many thousand surface-exposed flagellin molecules are a prime target of the host's  
81 immune system. Accordingly, many flagellated bacteria have evolved mechanisms to  
82 prevent flagellin recognition, *e.g.* by posttranslational modifications of flagellin. Flagellin  
83 glycosylation is relatively common among *Enterobacteriaceae*<sup>18</sup>, in *Campylobacter*,  
84 *Aeromonas* and *Pseudomonas* species<sup>19–21</sup> and plays a critical role in adhesion, biofilm  
85 formation or mimicry of host cell surface glycans<sup>22,23</sup>.

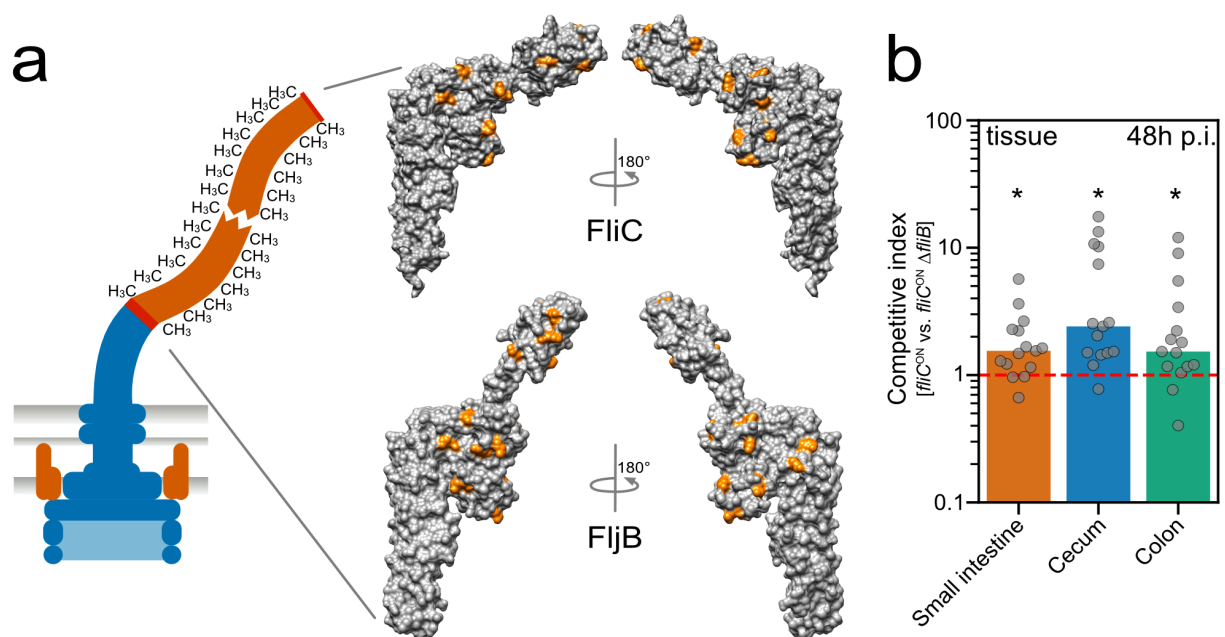
86 *S. enterica* does not posttranslationally glycosylate its flagellins. However,  $\epsilon$ -N-  
87 methylation at lysine residues of flagellin via the methylase FliB has been reported<sup>24–26</sup>.  
88 Although flagellin methylation was first reported in 1959<sup>25</sup>, the physiological role of the  
89 methylation remained elusive. Previous studies indicated that the absence of FliB had  
90 no significant effect on swimming and swarming motility suggesting that flagellin  
91 methylation might be required for virulence of *Salmonella*<sup>27,28</sup>.

92 In the present study, we analyzed the role of flagellin methylation for motility and  
93 virulence of *S. enterica in vivo* and *in vitro*. Our results demonstrate that *S. enterica*  
94 exploits methylated flagella to adhere to hydrophobic host cell surfaces. Thus, the  
95 posttranslational methylation of flagellin plays an important role for invasion of host  
96 cells, and accordingly, productive colonization of the host's epithelium.

## 97 Results

98 Previous studies suggested that the flagellins of *S. enterica* are posttranslationally  
99 methylated, however, the identity of the methylated lysine residues remained largely  
100 unknown<sup>25,26,29</sup>. We performed mass spectrometry analyses with high sequence  
101 coverage of both flagellins FliC and FljB isolated from *S. enterica* genetically locked in  
102 expression of FliC (*fliC<sup>ON</sup>*) or FljB (*fljB<sup>ON</sup>*), respectively, and isogenic mutants of the  
103 methylase FliB ( $\Delta$ *fliB*) (Supplementary Fig. S1). In order to map the identified  $\epsilon$ -N-  
104 methyl-lysine residues to the structure of both flagellins, we determined the crystal  
105 structure of FljB (Supplementary Fig. S2, Supplementary Text S1). The tertiary structure  
106 of FljB resembles, similar to FliC, a boomerang-shape with one arm formed by the D1  
107 domain and the other formed by D2 and D3<sup>17</sup>. However, in FljB compared with FliC the  
108 variable D3 domain is rotated about 90° around the axis defined by the D2-D3 arm,  
109 resulting in the widening angle of about 20° between the two boomerang's arms (Fig.  
110 1a, Supplementary Fig. S2). Interestingly, the methylated lysine residues are primarily  
111 located in the surface-exposed D2 and D3 domains of both flagellins (Fig. 1a,  
112 Supplementary Fig. S2d). In FliC, 16 out of 28 lysine residues and in FljB, 19 out of 30  
113 lysine residues were methylated. We note that the methylation of 15 lysines of FliC and  
114 18 lysines of FljB was dependent on the presence of FliB, while only one lysine in FljB  
115 and one in FliC was methylated in the absence of FliB ( $\Delta$ *fliB*). 10 of the identified lysines  
116 were methylated in both FliC and FljB flagellins, while 6 and 9 methylation sites were  
117 unique to FliC and FljB, respectively (Supplementary Fig. S2d). Interestingly, conserved

118 lysines were methylated in a FliB-dependent manner in both flagellins except for Lys<sup>396</sup>  
119 in FliB that is not modified in the corresponding Lys<sup>385</sup> in FliC.



120  
121 **Fig. 1: Surface-exposed methylation of flagellin contributes to efficient colonization of the murine**  
122 **intestine.** (a) Schematic of a methylated flagellar filament and surface representation of the structure of  
123 FliC (top) and FliB (bottom). FliB-dependent methylation sites are highlighted in orange. (b) Streptomycin  
124 pre-treated C57BL/6 mice were infected with 10<sup>7</sup> CFU of the FliC-expressing WT (*fliC<sup>CON</sup>*) and isogenic  
125  $\Delta$ *fliB* mutant, each harboring a different antibiotic resistant cassette. The organ burden (small intestine,  
126 colon and cecum lumen and tissue, respectively) was determined two days post-infection and used to  
127 calculate the competitive indices (CI). Each mouse is shown as an individual data point and significances  
128 were analyzed by the Wilcoxon Signed Rank test. The bar graph represents the median of the data and  
129 asterisks indicate a significantly different phenotype to the value 1 (\* =  $p < 0.05$ ).

130  
131 We next aligned the amino acid sequences of FliB and FliC up- and downstream of the  
132 identified  $\epsilon$ -N-methyl-lysine residues ( $\pm 6$  residues, Supplementary Fig. S3). Although  
133 no clear consensus sequences could be determined, we found prevalence of small (Ala,

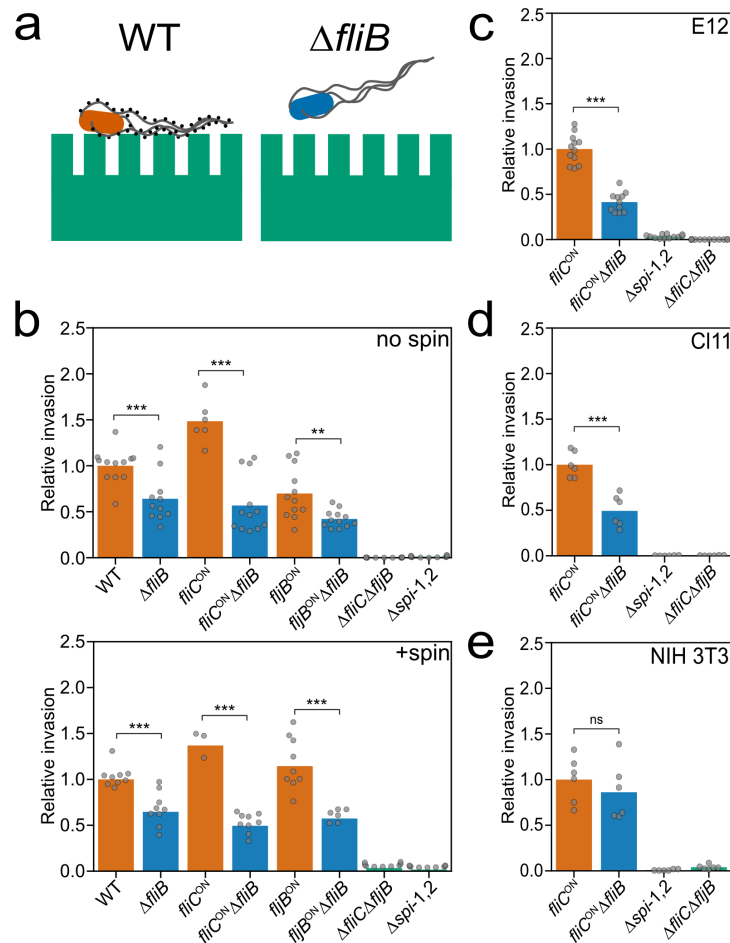
134 Gly, Thr, Val, Ser) and negatively charged (Asp) residues around the methylated  
135 lysines. Interestingly, a scan of the local sequences that surround methylated lysines  
136 using ScanProsite<sup>30</sup> matched the profile of the bacterial Ig-like domain 1 (Big-1) for 11  
137 and 10 FliB-dependent modifications in FliB and FliC, respectively, although with low  
138 confidence level (Supplementary Table S2). We note that the Big-1 domain is present in  
139 adhesion proteins of the intimin/invasin family, which are crucial in bacterial  
140 pathogenicity mediating host-cell invasion or adherence<sup>31–33</sup>.

141 Based on the weak homology of the methylation sites to the Big-1 domain and the  
142 absence of a motility phenotype in non-methylated flagellin mutants (Supplementary  
143 Fig. S4, Supplementary Text S2), we hypothesized that flagellin methylation might play  
144 a role in *Salmonella* virulence. We thus co-infected streptomycin-pre-treated mice<sup>34</sup> with  
145 the wildtype (WT) and an isogenic  $\Delta fliB$  mutant (Fig. 1b). Organ burden analysis two  
146 days post-infection revealed that the  $\Delta fliB$  strain was significantly outcompeted by the  
147 WT in the gastroenteritis mouse model, especially in the cecal tissue (Fig. 1b,  
148 competitive indices >1), suggesting that methylated flagella play an important role for  
149 efficient colonization of the intestinal epithelium.

150 We next tested if invasion of epithelial cells *in vitro* was also dependent on flagellin  
151 methylation (Fig. 2a). We first infected murine MODE-K epithelial cells with the WT and  
152 *S. enterica* strains deficient in the methylase FliB and determined the number of  
153 intracellular bacteria. Invasion was reduced about 50% for the  $\Delta fliB$  mutant strain  
154 independently of the flagellin type (Fig. 2b, top). We also observed a similar invasion  
155 defect for the  $\Delta fliB$  mutant when we forced contact of the bacteria with the epithelial



156 cells using centrifugation (Fig. 2b, bottom), suggesting that the invasion defect of the  
157  $\Delta fliB$  mutant did not depend on active bacterial motility.



158  
159 **Fig. 2: Flagella methylation facilitates eukaryotic cell invasion.** (a) Schematic illustration of productive  
160 adhesion and invasion of eukaryotic epithelial cells dependent on methylated flagella. (b) Invasion of  
161 MODE-K murine epithelial cells depends on methylated flagella. Reported are relative invasion rates of  
162 MODE-K epithelial cells for various flagellin methylation mutants without (top: no spin) or with forced  
163 contact of the bacteria by centrifugation (bottom: +spin). (c-e) Relative invasion rates of different  
164 eukaryotic host cell types. The human epithelial cell line E12, the murine epithelial cell line CI11, and the  
165 murine fibroblast cell line NIH 3T3 were infected with *Salmonella* flagella methylation mutants as  
166 described above. The bar graphs represent the mean of the reported relative invasion rate data

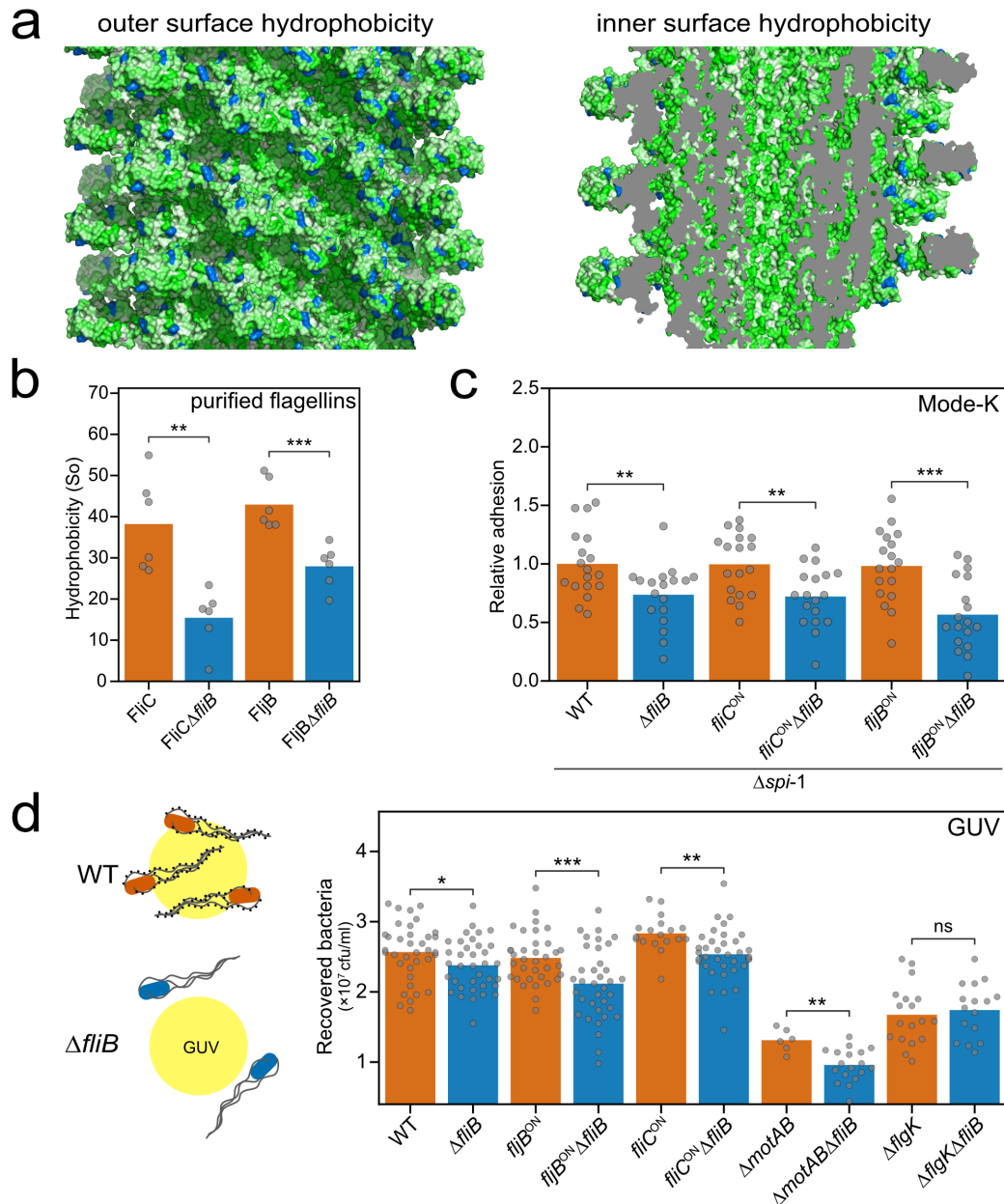
167 normalized to the inoculum. Replicates are shown as individual data points and statistical significances  
168 were determined by the Student's *t* test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; ns = not significant).

169  
170 We further analyzed if the methylation-dependent invasion phenotype was eukaryotic  
171 cell-line specific (Fig. 2c-e, Supplementary Fig. S5). The human epithelial cell line E12<sup>35</sup>  
172 and murine intestinal epithelial cell line CI11 mimic the native intestinal environment *in*  
173 *vitro*. Similar to the observed invasion rate of MODE-K cells, a  $\Delta fliB$  mutant strain  
174 displayed a two-fold decreased invasion rate of the human and murine epithelial cell  
175 lines compared with the WT. Similarly, in murine epithelial-like RenCa cells, the invasion  
176 rate of a  $\Delta fliB$  mutant was decreased. Invasion into the murine fibroblast cell lines NIH  
177 3T3 and CT26, however, was independent of flagellin methylation, suggesting that the  
178 observed invasion phenotype is cell type-specific for epithelial-like cells.

179 We next confirmed that the observed invasion phenotype was due to the lack of *fliB* by  
180 complementing expression of *fliB* from an inducible  $P_{tet}$  promoter at its native  
181 chromosomal locus. Addition of anhydrotetracycline (AnTc) induced *fliB* expression  
182 comparable to levels of the WT and restored invasion of MODE-K epithelial cells  
183 (Supplementary Fig. S6). We further tested if the observed invasion defect was  
184 dependent on the assembly of the methylated flagellar filament. A hook deletion mutant  
185 ( $\Delta flgE$ ) does not express flagellin, whereas a mutant of the hook-filament junction  
186 proteins ( $\Delta flgKL$ ) expresses and secretes flagellin, but does not assemble the flagellar  
187 filament. The methylase FliB is expressed in both  $\Delta flgE$  and  $\Delta flgKL$  mutant  
188 backgrounds<sup>27</sup>. We observed in neither the  $\Delta flgE$  nor the  $\Delta flgKL$  mutant a difference in  
189 MODE-K epithelial cell invasion in the presence or absence of FliB, suggesting that

190 methylated flagellin must assemble into a functional flagellar filament in order to  
191 facilitate epithelial cell invasion (Supplementary Fig. S7).

192 Our results presented above demonstrate that the presence of an assembled,  
193 methylated flagellar filament, but not the ability to move *per se*, contributes to the  
194 observed defect of *Salmonella* to invade epithelial cells. We thus hypothesized that  
195 adhesion to epithelial cells was facilitated through methylated flagella. In particular, we  
196 reasoned that the addition of hydrophobic methyl groups to surface-exposed lysine  
197 residues (Fig. 1) would affect the hydrophobicity of the flagellar filament. Consistently,  
198 the surface hydrophobicity  $S_o$  of purified FliC and FljB flagella was significantly reduced  
199 in the absence of lysine methylation (Fig. 3a+b, Supplementary Fig. S8). These results  
200 suggested that methylated flagella might promote adhesion to hydrophobic molecules  
201 present on the surface of epithelial cells. We therefore investigated adhesion of *S.*  
202 *enterica* to MODE-K epithelial cells. In order to dissect flagella methylation-dependent  
203 adhesion from methylation-dependent invasion of the epithelial cells, we employed  
204 *Salmonella* mutants deleted for the *Salmonella* pathogenicity island-1 (*spi-1*), which  
205 renders the bacteria unable to invade epithelial cells in an injectisome-dependent  
206 manner. We found that adhesion of  $\Delta spi-1$  *Salmonella* mutants to MODE-K epithelial  
207 cells was reduced up to 50% for strains deficient in flagellin methylation (Fig. 3c). This  
208 result suggested that methylated flagella promote adhesion to the hydrophobic surface  
209 of epithelial cells or surface-exposed proteinaceous receptors or glycostructures.



210

211 **Fig. 3: Flagella methylation mediates adhesion to hydrophobic surfaces.** (a) Methylation increases

212 hydrophobicity of the flagellar filament outer surface. Surface hydrophobicity distribution of the outer (left)

213 and the inner surface (right) of the FliC flagellar filament<sup>36</sup> according to the Eisenberg scale<sup>37</sup> (from green

214 to white indicates increasing hydrophobicity) with FliB-dependent methylation sites highlighted in blue. (b)

215 Measured surface hydrophobicity (So) of methylated and non-methylated ( $\Delta$ fliB) flagellins using PRODAN

216 on purified flagellar filaments. (c) Adhesion of *S. enterica* to MODE-K epithelial cells is reduced in the

217 absence of flagella methylation. Adhesion was monitored using *S. enterica* strains deleted for *spi-1* in

218 order to prevent invasion of the eukaryotic host cells. (d) Adhesion of *S. enterica* to giant unilamellar  
219 vesicles (GUV) consisting of phosphatidylcholine from egg chicken is dependent on the presence of  
220 methylated flagella. Left: schematic illustration of the adhesion of *Salmonella* to GUVs dependent on  
221 methylated flagella. Right: Quantified adhesion of *Salmonella* mutants to GUVs. The bar graphs represent  
222 the mean of the reported data. Replicates are shown as individual data points and statistical significances  
223 were determined by the Student's *t* test (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; ns = not significant).

224

225 However, we did not observe a significant flagella methylation-dependent effect on  
226 adhesion of *Salmonella* to various extracellular matrix proteins, nor to the  
227 oligosaccharide mannose, which has previously been shown to mediate adhesion of  
228 *Salmonella* and *E. coli* to eukaryotic cells using type I fimbriae<sup>38–41</sup> (Supplementary Fig.  
229 S9). We next tested the possibility that the increased hydrophobicity of methylated  
230 flagella might promote adhesion of the bacteria to the hydrophobic plasma membrane of  
231 epithelial cells (Fig. 3d). We therefore analyzed the binding of *Salmonella* to giant  
232 unilamellar vesicles (GUV) consisting of phosphatidylcholine, the most abundant  
233 phospholipid in animal tissues. Interestingly, we observed a reduction in bacterial  
234 adhesion to the GUV for *S. enterica* strains deficient in flagellin methylation, but not for  
235 the non-flagellated  $\Delta flgK$  mutants. In addition, non-motile, but flagellated bacteria  
236 ( $\Delta motAB$ ) were less adherent, which supports previous observations that actively  
237 rotating flagella are important for the initial interaction with surfaces before biofilm  
238 formation<sup>41</sup>.

## 239 **Discussion**

240 Flagella-dependent motility is crucial for *Salmonella* pathogenesis by enabling directed  
241 movement towards host epithelial cells. However, flagella not only play a role in  
242 bacterial motility, but also in colonization, adhesion, and biofilm formation<sup>41–44</sup>. In case  
243 of flagella-mediated adhesion to host cells, the primary interactions with the epithelial  
244 tissue occur *via* the external filament made of several thousand copies of flagellin and  
245 thus represents an excellent adhesion molecule.

246 Here, we describe a novel mechanism of flagella-dependent adhesion to surface-  
247 exposed hydrophobic molecules. This adhesion phenotype is dependent on methylation  
248 of surface-exposed lysine residues of flagellin by the methylase FliB. Flagellin  
249 methylation was first described in *Salmonella* in 1959<sup>24–26</sup>, however, the physiological  
250 relevance remained elusive. We demonstrate that FliB-mediated flagellin methylation is  
251 crucial for *Salmonella* pathogenesis in the mouse model and contributes significantly to  
252 adhesion and thus invasion of epithelial cells *in vitro*, but does neither affect swimming  
253 motility nor flagella assembly (Supplementary Text S3). Analysis of the surface  
254 hydrophobicity of purified flagella revealed that methylation of the filament subunits  
255 increases the hydrophobicity of the outer surface of the flagellar filament, while the  
256 lumen of the flagellar filament seems not to be affected (Fig. 3a+b, Supplementary Fig.  
257 S8). We note that the preferential methylation of surface-exposed lysine residues  
258 implicates a FliB methylation mechanism involving flagellin assemblies formed in the  
259 bacterial cytosol prior to secretion. Further, we found that a single flagellin molecule  
260 contains 16 or 19 surface-exposed methylation sites. Since a flagellar filament is made  
261 up of up to 20,000 flagellin copies, the methylation of flagellin subunits might

262 substantially increase the overall hydrophobicity of the flagellum. Consistently, we found  
263 that adhesion to the surface of epithelial host cells and phospholipid vesicles was  
264 dependent on the flagella methylation status. In support, flagella have been recently  
265 implicated to mediate adhesion to abiotic surfaces through hydrophobic interactions<sup>45,46</sup>.  
266 We thus speculate that bacteria use flagella to explore the host cell surface as  
267 suggested previously<sup>47</sup> and actively rotating flagella might be able to penetrate the lipid  
268 bilayer and interact with the fatty acids buried inside the plasma membrane. Increasing  
269 the surface hydrophobicity of the flagellar filament through methylation might improve  
270 those hydrophobic interactions for productive adhesion to eukaryotic host cells.  
271 Flagellin Methylation Islands (FMI) and thus modifications of flagellins by methylation  
272 are common in *Enterobacteriaceae*<sup>18</sup>. In addition to *Salmonella*, many bacterial species  
273 including *Yersinia*, *Enterobacter*, *Franconibacter*, and *Pantoea* contain chromosomal  
274 FMI loci, which encode orthologues of FliB. In summary, FliB-dependent methylation of  
275 flagella might represent a general mechanism facilitating adhesion to hydrophobic host  
276 cell surfaces in a broad range of bacterial species.

## 277 **Methods**

278

### 279 **Ethics statement**

280 All animal experiments were performed according to guidelines of the German Law for  
281 Animal Protection and with permission of the local ethics committee and the local  
282 authority LAVES (Niedersächsisches Landesamt für Verbraucherschutz und  
283 Lebensmittelsicherheit) under permission number 33.19-42502-04-13/1191.

284

### 285 **Strains, media and bacterial growth**

286 All bacterial strain used in this study are listed in Supplementary Table S3 and were  
287 derived from SL1344. Bacteria were grown in lysogeny broth (LB)<sup>48</sup> at 37 °C and growth  
288 was measured by optical density at 600 nm. For transductional crosses the generalised  
289 transducing phage P22 *HT105/1 int-201* was used<sup>49</sup>. Gene deletions or replacements  
290 were produced as previously described<sup>50</sup>. All bacterial strains are available upon  
291 request.

292

### 293 **Cloning and purification of FljB for structural analysis**

294 The truncated gene *fljB* encoding for the protein residues 55-462 was amplified from *S.*  
295 *Typhimurium* (SL1344) by standard PCR method and cloned into the expression vector  
296 pET28a(+) using the restriction sites NheI and XhoI to obtain N-terminal His-tagged  
297 protein. The mutation A190V was found by sequencing. Standard conditions were



298 applied to express His-tagged FliB<sub>55-462</sub> in BL21(DE3). The protein was purified from the  
299 soluble fraction using HisTrap HP and Superdex 75 columns (GE Healthcare) in 50 mM  
300 HEPES (pH 7.4), 150 mM NaCl.

301

## 302 **Crystallization and data collection**

303 FliB<sub>55-462</sub> was concentrated to 12-15 mg/mL and crystals were grown at 18 °C by  
304 hanging drop vapour diffusion against 0.1 M Tris (pH 8.5), 20% (w/v) PEG4000, 24%  
305 (v/v) isopropanol. Diffraction data were collected using crystals flash-frozen in  
306 crystallization buffer. Measurements were carried out at the beamLine BL14.1 at the  
307 Helmholtz-Zentrum Berlin synchrotron Bessy II, at the wavelength 0.918 Å and  
308 temperature 100 K, obtaining a data set at 2.00 Å resolution. Crystals belong to space  
309 group C2, with one FliB molecule in the asymmetric unit (solvent content 51.6%).  
310 Indexing, integration, merging and scaling were done using the program XDS<sup>51</sup>.

311

## 312 **Crystal structure determination**

313 The structure was phased by molecular replacement, using the structure of the F41  
314 fragment of FliC flagellin as search model (PDB ID 1IO1<sup>17</sup>). Cycles of manual building  
315 and refinement using Coot<sup>52</sup> and CNS version 1.3<sup>53</sup> led to the final structure, which  
316 includes residues 55-459 of FliB with the mutation A190V and the residue S54 present  
317 in the crystallised construct. 299 water molecules were also placed. Structural  
318 comparison between FliB and FliC has been done with the server PDBeFold v2.59<sup>54</sup>.

319 Molecular structure figures were generated using UCSF Chimera 1.13.1<sup>55</sup> and PyMol  
320 2.2.3 (Schroedinger, LLC (2018). Alignment Fig. S2d was generated with the server  
321 ESPript (<https://esript.ibcp.fr>)<sup>56</sup>.

322

## 323 **Mass spectrometry**

### 324 *Sample preparation*

325 FliC and FliB purified from the WT and a  $\Delta fliB$  mutant were separated using SDS-  
326 PAGE. The corresponding bands were cut from the gel and each was cut into 1x1 mm  
327 pieces. An in-gel digestion was performed. For destaining the gel pieces, a 50 mM  
328 ammonium bicarbonate (AmBiCa) in 50% acetonitrile (ACN) solution was added and  
329 incubated 30 min at room temperature to dehydrate the gel. After removal of the  
330 supernatant, 50 mM AmBiCa was added and incubated for 30 min at room temperature  
331 to rehydrate the pieces. This step was repeated two times. Disulfide bonds were  
332 reduced using 10 mM DTT in 50 mM AmBiCa for 30 min at 56 °C. After cooling to room  
333 temperature and removal of the supernatant, the reduced cysteines were alkylated  
334 using 55 mM iodoacetamide in 50 mM AmBiCa for 30 min at room temperature in the  
335 dark. After removal of the supernatant the gel pieces were dried *in vacuo*. Ice cold 50  
336 mM AmBiCa in 10% ACN containing 12.5 ng/ $\mu$ L trypsin was added and digested  
337 overnight. Peptides were extracted by transferring the supernatant to a fresh collection  
338 tube and adding 50 mM AmBiCa in 10% ACN to the gel pieces and transferring the  
339 second supernatant into the same collection tube. Peptides were dried *in vacuo* and  
340 stored at -20 °C. Before measuring the peptides were reconstituted in 10  $\mu$ L 0.1%

341 formic acid (FA) and 1  $\mu$ L was injected for measurement. All chemicals were purchased  
342 from Sigma-Aldrich. Trypsin was purchased from Promega.

343

#### 344 *Mass Spectrometry*

345 Peptides were measured on a tandem mass spectrometer (Fusion, Thermo Fisher  
346 Scientific) coupled to a nano UPLC system (Ultimate 3000 RSLCnano, Thermo Fisher  
347 Scientific) with a nano-spray source. Peptides were trapped on a C18 reversed-phase  
348 trap column (2 cm x 75  $\mu$ m ID; Acclaim PepMap trap column packed with 3  $\mu$ m beads,  
349 Thermo Fisher Scientific) and separated on a 25 cm C18 reversed-phase analytical  
350 column (25 cm x 75  $\mu$ m ID, Acclaim PepMap, 3  $\mu$ m beads, Thermo Fisher Scientific).  
351 The column temperature was kept constant at 45 °C. Peptides were separated using a  
352 2-step gradient starting with 3% buffer B (0.1% FA in ACN) and 97% buffer A (0.1% FA  
353 in H<sub>2</sub>O) with a steady increase to 28% buffer B over 20 min and a second increase to  
354 35% over 5 min with a subsequent ramping to 90% buffer B for 10 min followed by a 20  
355 min equilibration to 3% buffer B at a constant flow rate of 300 nL/min. Eluting peptides  
356 were injected directly into the mass spectrometer. Data were acquired in positive ion  
357 mode using data dependent acquisition (DDA) with a precursor ion scan resolution of  
358  $1.2 \times 10^5$  at 200  $m/z$  in a range of 300-1500  $m/z$  with an automatic gain control (AGC)  
359 target of  $2 \times 10^5$  and a maximum injection time of 50 ms. Peptides were selected for  
360 fragmentation using the “TopSpeed” method with a threshold of 5000 intensity and a  
361 dynamic exclusion time of 30 sec. Peptides were fragmented using higher-energy  
362 collision dissociation (HCD) in the C-Trap and fragment spectra were detected in the ion

363 trap. Fragment spectra were recorded using the “Rapid” setting with a maximum  
364 injection time of 35 ms and an AGC target of  $1 \times 10^4$  with the first mass set at 110 *m/z*.

365

### 366 *Data analysis*

367 Data were analyzed using the ProteomeDiscoverer 2.0 (Thermo Fisher Scientific)  
368 software. Spectra were identified using the Sequest HT search engine with precursor  
369 mass tolerance set to 10 ppm and the fragment mass tolerance set to 0.5 Da.  
370 Carbamidomethylation on cysteine was set as fixed modification and oxidation on  
371 methionine, acetylation on protein N-terminus as well as mono-, di- and tri-methylation  
372 on lysine were set as variable modifications. Trypsin was set as enzyme and 3 missed  
373 cleavages were allowed with a minimum peptide length of 6 amino acids. Spectra were  
374 searched against a *Salmonella* Typhimurium FASTA database obtained from UniProt in  
375 June 2016 containing 1821 entries and a contaminant database containing 298 entries.  
376 Sequence coverage maps were created using PatterLab for proteomics 4.0<sup>57</sup>.

377

### 378 **Protein secretion assay**

379 Protein secretion into the culture supernatant was analyzed as described before<sup>16</sup>.  
380 Samples were fractionated under denaturing conditions on SDS-gels (200 OD units)  
381 and immunoblotting was performed using primary  $\alpha$ -FliC/FliB and secondary  $\alpha$ -rabbit  
382 antibodies.

383

### 384 **Motility assay and immunostaining of flagella**

385 Swimming motility was analyzed in semi-solid agar plates as described before<sup>58</sup>. For  
386 immunostaining of flagella, logarithmically grown cells were fixed on a poly-L-lysine  
387 coated coverslip by 2% formaldehyde and 0.2% glutaraldehyde. Flagellin was stained  
388 using polyclonal  $\alpha$ -FliC (rabbit, 1:1000 in 2% BSA/PBS) and secondary  $\alpha$ -rabbit  
389 AlexaFluor488 (goat, 1:1000 in PBS). DNA was stained using DAPI (Sigma-Aldrich).  
390 Images were taken as described before<sup>28,59</sup>.

391

### 392 **Mouse infection studies**

393 Intragastrical infection of seven weeks old C57BL/6 mice (Janvier) was performed as  
394 described in<sup>16</sup>. Briefly, mice were infected with  $10^7$  CFU of two strains containing an  
395 antibiotic resistance cassette. Small intestine, cecum and colon were isolated 2 days  
396 post-infection and competitive indices (CI) were calculated.

397

### 398 **Invasion and adhesion assays**

399 The murine epithelial cell lines MODE-K<sup>60</sup> and CI11, the murine epithelial-like cell line  
400 Renca (CRL-2947), the human epithelial cell line HT29-MTX-E12 (E12)<sup>35</sup>, and the  
401 mouse fibroblast cell lines NIH-3T3 (CRL-1658) and CT26 (CRL-2638) were used for  
402 invasion assays. The immortalization and characterization of the muGob (CI11) cells will  
403 be described elsewhere (Truschel et al., in preparation). Briefly, murine intestinal  
404 organoids were plated and infected with different lentiviruses encoding the CI-SCREEN

405 gene library<sup>61</sup>. After transduction, the clonal cell line muGob (CI11) was established,  
406 which has integrated the following recombinant genes of the CI-SCREEN library: Id1,  
407 Id2, Id3, Myc, Fos, E7, Core, Rex (Zfp42). The muGob (CI11) cell line was cultivated on  
408 fibronectin/collagen-coated (InSCREENeX GmbH, Germany) well plates in a humidified  
409 atmosphere with 5% CO<sub>2</sub> at 37 °C in a defined muGob medium (InSCREENeX GmbH,  
410 Germany). 2.5x10<sup>5</sup> cells/mL were seeded in 24-well plates. *Salmonella* strains were  
411 added for infection at a MOI of 10 for 1 h. External bacteria were killed by addition of  
412 100 µg/mL gentamycin for 1 h and cells were lysed with 1% Triton X-100. Serial  
413 dilutions of the lysate were plated to calculate the CFU/mL. All values were normalised  
414 to the control strain. To test adhesion to MODE-K cells, cells were seeded and infected  
415 with strains lacking *spi-1* to prevent injectisome-dependent invasion. After infection, the  
416 MODE-K cells were washed extensively and lysed as described above.

417

#### 418 **RNA isolation and quantitative real-time PCR**

419 Strains were grown under agitating growth conditions in LB medium and total RNA  
420 isolation was performed using the RNeasy Mini kit (Qiagen). For removal of genomic  
421 DNA, RNA was treated with DNase using the TURBO DNA-free kit (Ambion). Reverse  
422 transcription and quantitative real-time PCRs (qRT-PCR) were performed using the  
423 SensiFast SYBR No-ROX One Step kit (Bioline) in a Rotor-Gene Q Lightcycler  
424 (Qiagen). Relative changes in mRNA levels were analyzed according to Pfaffl<sup>62</sup> and as  
425 described before<sup>28</sup>.

426

## 427 **ECM adhesion assays**

428 For ECM protein adhesion assays, a 96-well plate pre-coated with a variety of ECM  
429 proteins was used (EMD Millipore; Collagen I, II, IV, Fibronectin, Laminin, Tenascin,  
430 Vitronectin). Wells were rehydrated according to the user's manual and  $5 \times 10^7$  cells/mL  
431 were added. After incubation for 1 h at 37 °C, wells were washed extensively and 1%  
432 Triton X-100 was added. Colony forming units (CFU)/mL were calculated after plating of  
433 serial dilutions and normalised to the inoculum and BSA control.

434

## 435 **Mannose binding assay**

436 Binding to mannose was determined as described before<sup>63</sup> with minor modifications. A  
437 black 96-well plate was coated with BSA or mannose-BSA (20  $\mu$ g/mL in 50 mM  
438 bicarbonate buffer pH 9.5) for 2 h at 37 °C, followed by blocking with BSA (10 mg/mL)  
439 for 1 h at 37°C. Adjusted bacterial cultures (OD<sub>600</sub> 0.6) harboring the constitutive  
440 fluorescent plasmid pFU228-*P<sub>gapdh</sub>*-mCherry were added to the wells to facilitate  
441 binding. After 1 h incubation at 37°C, wells were washed with 1x PBS and fluorescence  
442 was measured with a Tecan plate reader (excitation 560 nm; emission 630 nm).  
443 Fluorescence relative to the binding to BSA was calculated from three technical  
444 replicates and the type-I fimbriae-inducible strain *P<sub>tet</sub>-fimA-F* served as positive control.

445

## 446 **Flagellar filament isolation**

447 Flagellar filaments were isolated similar as described<sup>64</sup>. Briefly, bacterial cultures were  
448 grown in LB media at 37 °C for 16 h in an orbital shaker incubator (Infors HT) at 80 rpm.

449 Cells were harvest by centrifugation at 2,000 x g for 20 min. Cell pellets were re-  
450 suspended in TBS buffer pH 7.4 at 4 °C. The flagella were sheared off with a magnetic  
451 stirrer at 500 rpm for 1 h, followed by centrifugation at 4,000 x g for 30 min.  
452 Supernatants were collected and ammonium sulfate was slowly added while stirring to  
453 achieve two-thirds saturation. After overnight incubation, the flagella were harvest by  
454 centrifugation at 15,000 x g for 20 min and pellet was re-suspended in TBS buffer at pH  
455 7.4. The quality of the purified flagella was checked by SDS-PAGE and transmission  
456 electron microscopy of negatively stained samples (microscope Talos L120C, Thermo  
457 Fisher Scientific).

458

#### 459 **Hydrophobicity determination**

460 Protein surface hydrophobicity was measured according to a modification of the method  
461 of Kato and Nakai<sup>65</sup> using PRODAN<sup>66</sup>. A stock solution of 1mM PRODAN (prepared in  
462 DMSO) was used, 8  $\mu$ L was added to successive samples containing 1 mL of diluted  
463 flagella in 20 mM HEPES (pH 7.4), 150 mM NaCl. After homogenization by pipetting,  
464 the samples were incubated 10 min in the dark and the relative fluorescence intensity  
465 was measured. All fluorescence measurements were made with a Cary Eclipse (Varian  
466 now Agilent) spectrofluorometer. Excitation and emission wavelengths were 365 nm  
467 and 465 nm, the slit widths were 5 and 5 nm. For standardization, BSA was used.  
468 Surface hydrophobicity ( $S_o$ ) values were determined using at least duplicate analyses.  
469 Five measures per sample repeated three times were performed and the mean was  
470 used.



## 471 **Bacterial adhesion to liposomes**

472 Giant unilamellar vesicles (GUV) were prepared according to the polyvinyl alcohol  
473 (PVA)-assisted swelling method<sup>67</sup>. Gold-coated glass slides were obtained by thermal  
474 evaporation under vacuum (Evaporator Edwards model Auto 306,  $0.01 \text{ nm}\cdot\text{s}^{-1}$ ,  $2\text{-}3 \times 10^{-6}$   
475 mbar). A gold layer of  $10 \pm 1 \text{ nm}$  was deposited on top of a chromium adhesion layer  
476 of  $1 \pm 0.5 \text{ nm}$ . Prior to GUV formation in HEPES buffered saline solution (HEPES 20  
477 mM pH 7.4, NaCl 150 mM), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-  
478 [PDP(polyethylene glycol)-2000] (DSPE-PEG-PDP) (Sigma-Aldrich) was mixed with L-  
479  $\alpha$ -phosphatidylcholine from egg chicken (Sigma-Aldrich) at a 3 % mass ratio that allows  
480 a direct covalent coupling of GUV onto gold surfaces. For the bacterial adhesion assay,  
481 a  $5 \mu\text{g}/\text{mL}$  GUV solution was deposited onto a gold-coated glass substrate and  
482 incubated one hour for immobilization. Then, the surface was gently rinsed with buffer to  
483 remove non-immobilized liposomes. Subsequently bacterial culture at  $10^8 \text{ CFU}/\text{mL}$   
484 resuspended in HEPES buffer was carefully deposit on the surface and incubated for  
485 one hour. Non-adherent bacteria were eliminated by buffer washes. Finally, the  
486 adherent bacteria were detached by pipetting several times directly onto the  
487 immobilized liposomes with PBS pH 7.4. The collected samples were serially diluted  
488 and plated on LB agar for viable bacterial counts. Averages and standard deviations  
489 were calculated from six independent experiments.

490

## 491 **Data availability**

492 The data that support the findings of this study are available from the corresponding  
493 authors upon request. The coordinates of the flagellin FliB have been deposited in the  
494 RCSB PDB under accession numbers 6RGV.

## 495 References

- 496
- 497
- 498 1. Adler, J. & Templeton, B. The effect of environmental conditions on the motility of  
499 *Escherichia coli*. *J Gen Microbiol* **46**, 175-184 (1967).
- 500 2. Duan, Q., Zhou, M., Zhu, L. & Zhu, G. Flagella and bacterial pathogenicity. *J Basic*  
501 *Microbiol* **53**, 1-8 (2013).
- 502 3. Chaban, B., Hughes, H. V. & Beeby, M. The flagellum in bacterial pathogens: For  
503 motility and a whole lot more. *Semin Cell Dev Biol* **46**, 91-103 (2015).
- 504 4. Rossez, Y., Wolfson, E. B., Holmes, A., Gally, D. L. & Holden, N. J. Bacterial  
505 flagella: twist and stick, or dodge across the kingdoms. *PLoS Pathog* **11**, e1004483  
506 (2015).
- 507 5. Tawk, C. et al. Stress-induced host membrane remodeling protects from infection  
508 by non-motile bacterial pathogens. *EMBO J* **37**, (2018).
- 509 6. Lillehoj, E. P., Kim, B. T. & Kim, K. C. Identification of *Pseudomonas aeruginosa*  
510 flagellin as an adhesin for Muc1 mucin. *Am J Physiol Lung Cell Mol Physiol* **282**,  
511 L751-6 (2002).
- 512 7. Girón, J. A., Torres, A. G., Freer, E. & Kaper, J. B. The flagella of enteropathogenic  
513 *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol* **44**, 361-379  
514 (2002).
- 515 8. Roy, K. et al. Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between  
516 flagella and host cells. *Nature* **457**, 594-598 (2009).
- 517 9. Crawford, R. W., Reeve, K. E. & Gunn, J. S. Flagellated but not hyperfimbriated  
518 *Salmonella enterica* serovar Typhimurium attaches to and forms biofilms on  
519 cholesterol-coated surfaces. *J Bacteriol* **192**, 2981-2990 (2010).
- 520 10. Rossez, Y. et al. Flagella interact with ionic plant lipids to mediate adherence of  
521 pathogenic *Escherichia coli* to fresh produce plants. *Environ Microbiol* **16**, 2181-  
522 2195 (2014).
- 523 11. Feldman, M. et al. Role of flagella in pathogenesis of *Pseudomonas aeruginosa*  
524 pulmonary infection. *Infect Immun* **66**, 43-51 (1998).
- 525 12. Adamo, R., Sokol, S., Soong, G., Gomez, M. I. & Prince, A. *Pseudomonas*  
526 *aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like  
527 receptor 2 as well as toll-like receptor 5. *Am J Respir Cell Mol Biol* **30**, 627-634  
528 (2004).
- 529 13. Berg, H. C. The rotary motor of bacterial flagella. *Annu Rev Biochem* **72**, 19-54  
530 (2003).
- 531 14. Aldridge, P. D. et al. Regulatory protein that inhibits both synthesis and use of the  
532 target protein controls flagellar phase variation in *Salmonella enterica*. *Proc Natl*  
533 *Acad Sci U S A* **103**, 11340-11345 (2006).
- 534 15. Lederberg, J. & lino, T. Phase variation in *Salmonella*. *Genetics* **41**, 743 (1956).
- 535 16. Horstmann, J. A. et al. Flagellin phase-dependent swimming on epithelial cell  
536 surfaces contributes to productive *Salmonella* gut colonisation. *Cell Microbiol* **19**,  
537 e12739 (2017).

- 538 17. Samatey, F. A. et al. Structure of the bacterial flagellar protofilament and  
539 implications for a switch for supercoiling. *Nature* **410**, 331-337 (2001).
- 540 18. De Maayer, P. & Cowan, D. A. Flashy flagella: flagellin modification is relatively  
541 common and highly versatile among the Enterobacteriaceae. *BMC Genomics* **17**,  
542 377 (2016).
- 543 19. Logan, S. M. Flagellar glycosylation - a new component of the motility repertoire.  
544 *Microbiology* **152**, 1249-1262 (2006).
- 545 20. Miller, W. L. et al. Flagellin glycosylation in *Pseudomonas aeruginosa* PAK  
546 requires the O-antigen biosynthesis enzyme WbpO. *J Biol Chem* **283**, 3507-3518  
547 (2008).
- 548 21. Szymanski, C. M., Logan, S. M., Linton, D. & Wren, B. W. *Campylobacter*--a tale of  
549 two protein glycosylation systems. *Trends Microbiol* **11**, 233-238 (2003).
- 550 22. Power, P. M. & Jennings, M. P. The genetics of glycosylation in Gram-negative  
551 bacteria. *FEMS Microbiol Lett* **218**, 211-222 (2003).
- 552 23. Merino, S. & Tomás, J. M. Gram-negative flagella glycosylation. *Int J Mol Sci* **15**,  
553 2840-2857 (2014).
- 554 24. Stocker, B. A. D., McDonough, M. W. & Ambler, R. P. A Gene Determining  
555 Presence or Absence of  $\epsilon$ -N-Methyl-Lysine in *Salmonella* Flagellar Protein. *Nature*  
556 **189**, 556-558 (1961).
- 557 25. Ambler, R. P. & Rees, M. W. Epsilon-N-Methyl-lysine in bacterial flagellar protein.  
558 *Nature* **184**, 56-57 (1959).
- 559 26. Tronick, S. R. & Martinez, R. J. Methylation of the flagellin of *Salmonella*  
560 *typhimurium*. *J Bacteriol* **105**, 211-219 (1971).
- 561 27. Frye, J. et al. Identification of new flagellar genes of *Salmonella enterica* serovar  
562 Typhimurium. *J Bacteriol* **188**, 2233-2243 (2006).
- 563 28. Deditius, J. A. et al. Characterization of Novel Factors Involved in Swimming and  
564 Swarming Motility in *Salmonella enterica* Serovar Typhimurium. *PLoS One* **10**,  
565 e0135351 (2015).
- 566 29. Kanto, S., Okino, H., Aizawa, S. & Yamaguchi, S. Amino acids responsible for  
567 flagellar shape are distributed in terminal regions of flagellin. *J Mol Biol* **219**, 471-  
568 480 (1991).
- 569 30. de Castro, E. et al. ScanProsite: detection of PROSITE signature matches and  
570 ProRule-associated functional and structural residues in proteins. *Nucleic Acids*  
571 *Res* **34**, W362-5 (2006).
- 572 31. Kelly, G. et al. Structure of the cell-adhesion fragment of intimin from  
573 enteropathogenic *Escherichia coli*. *Nat Struct Biol* **6**, 313-318 (1999).
- 574 32. Hamburger, Z. A., Brown, M. S., Isberg, R. R. & Bjorkman, P. J. Crystal structure  
575 of invasins: a bacterial integrin-binding protein. *Science* **286**, 291-295 (1999).
- 576 33. Luo, Y. et al. Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor  
577 complex. *Nature* **405**, 1073-1077 (2000).
- 578 34. Barthel, M. et al. Pretreatment of mice with streptomycin provides a *Salmonella*  
579 *enterica* serovar Typhimurium colitis model that allows analysis of both pathogen  
580 and host. *Infect Immun* **71**, 2839-2858 (2003).

- 581 35. Navabi, N., McGuckin, M. A. & Lindén, S. K. Gastrointestinal cell lines form  
582 polarized epithelia with an adherent mucus layer when cultured in semi-wet  
583 interfaces with mechanical stimulation. *PLoS One* **8**, e68761 (2013).
- 584 36. Maki-Yonekura, S., Yonekura, K. & Namba, K. Conformational change of flagellin  
585 for polymorphic supercoiling of the flagellar filament. *Nat Struct Mol Biol* **17**, 417-  
586 422 (2010).
- 587 37. Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R. Analysis of membrane and  
588 surface protein sequences with the hydrophobic moment plot. *J Mol Biol* **179**, 125-  
589 142 (1984).
- 590 38. Hanson, M. S. & Brinton, C. C. Identification and characterization of *E. coli* type-1  
591 pilus tip adhesion protein. *Nature* **332**, 265-268 (1988).
- 592 39. Ofek, I., Mirelman, D. & Sharon, N. Adherence of *Escherichia coli* to human  
593 mucosal cells mediated by mannose receptors. *Nature* **265**, 623-625 (1977).
- 594 40. Duguid, J. P. & Campbell, I. Antigens of the type-1 fimbriae of *salmonellae* and  
595 other enterobacteria. *J Med Microbiol* **2**, 535-553 (1969).
- 596 41. Pratt, L. A. & Kolter, R. Genetic analysis of *Escherichia coli* biofilm formation: roles  
597 of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* **30**, 285-293 (1998).
- 598 42. Josenhans, C. & Suerbaum, S. The role of motility as a virulence factor in bacteria.  
599 *Int J Med Microbiol* **291**, 605-614 (2002).
- 600 43. Danese, P. N., Pratt, L. A., Dove, S. L. & Kolter, R. The outer membrane protein,  
601 antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Mol*  
602 *Microbiol* **37**, 424-432 (2000).
- 603 44. Danese, P. N., Pratt, L. A. & Kolter, R. Exopolysaccharide production is required  
604 for development of *Escherichia coli* K-12 biofilm architecture. *J Bacteriol* **182**,  
605 3593-3596 (2000).
- 606 45. Friedlander, R. S., Vogel, N. & Aizenberg, J. Role of Flagella in Adhesion of  
607 *Escherichia coli* to Abiotic Surfaces. *Langmuir* **31**, 6137-6144 (2015).
- 608 46. Bruzaud, J. et al. Flagella but not type IV pili are involved in the initial adhesion of  
609 *Pseudomonas aeruginosa* PAO1 to hydrophobic or superhydrophobic surfaces.  
610 *Colloids Surf B Biointerfaces* **131**, 59-66 (2015).
- 611 47. Friedlander, R. S. et al. Bacterial flagella explore microscale hummocks and  
612 hollows to increase adhesion. *Proc Natl Acad Sci U S A* **110**, 5624-5629 (2013).
- 613 48. Bertani, G. Lysogeny at mid-twentieth century: P1, P2, and other experimental  
614 systems. *J Bacteriol* **186**, 595-600 (2004).
- 615 49. Sanderson, K. E. & Roth, J. R. Linkage map of *Salmonella typhimurium*, edition  
616 VII. *Microbiol Rev* **52**, 485-532 (1988).
- 617 50. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in  
618 *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640-  
619 6645 (2000).
- 620 51. Kabsch, W. XDS. *Acta Crystallogr D Biol Crystallogr* **66**, 125-132 (2010).
- 621 52. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta*  
622 *Crystallogr D Biol Crystallogr* **60**, 2126-2132 (2004).

- 623 53. Brünger, A. T. et al. Crystallography & NMR system: A new software suite for  
624 macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* **54**,  
625 905-921 (1998).
- 626 54. Krissinel, E. & Henrick, K. Secondary-structure matching (SSM), a new tool for fast  
627 protein structure alignment in three dimensions. *Acta Crystallogr D Biol Crystallogr*  
628 **60**, 2256-2268 (2004).
- 629 55. Pettersen, E. F. et al. UCSF Chimera--a visualization system for exploratory  
630 research and analysis. *J Comput Chem* **25**, 1605-1612 (2004).
- 631 56. Robert, X. & Gouet, P. Deciphering key features in protein structures with the new  
632 ENDscript server. *Nucleic Acids Res* **42**, W320-4 (2014).
- 633 57. Carvalho, P. C. et al. Integrated analysis of shotgun proteomic data with  
634 PatternLab for proteomics 4.0. *Nat Protoc* **11**, 102-117 (2016).
- 635 58. Wozniak, C. E., Lee, C. & Hughes, K. T. T-POP array identifies EcnR and Pefl-  
636 SrgD as novel regulators of flagellar gene expression. *J Bacteriol* **191**, 1498-1508  
637 (2009).
- 638 59. Erhardt, M. Fluorescent Microscopy Techniques to Study Hook Length Control and  
639 Flagella Formation. *Methods Mol Biol* **1593**, 37-46 (2017).
- 640 60. Vidal, K., Grosjean, I., evillard, J. P., Gespach, C. & Kaiserlian, D. Immortalization  
641 of mouse intestinal epithelial cells by the SV40-large T gene. Phenotypic and  
642 immune characterization of the MODE-K cell line. *J Immunol Methods* **166**, 63-73  
643 (1993).
- 644 61. Kuehn, A. et al. Human alveolar epithelial cells expressing tight junctions to model  
645 the air-blood barrier. *ALTEX* **33**, 251-260 (2016).
- 646 62. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-  
647 PCR. *Nucleic Acids Res* **29**, e45 (2001).
- 648 63. Hansmeier, N. et al. Functional expression of the entire adhesiome of *Salmonella*  
649 *enterica* serotype Typhimurium. *Sci Rep* **7**, 10326 (2017).
- 650 64. Ibrahim, G. F., Fleet, G. H., Lyons, M. J. & Walker, R. A. Method for the isolation of  
651 highly purified *Salmonella* flagellins. *J Clin Microbiol* **22**, 1040-1044 (1985).
- 652 65. Kato, A. & Nakai, S. Hydrophobicity determined by a fluorescence probe method  
653 and its correlation with surface properties of proteins. *Biochim Biophys Acta* **624**,  
654 13-20 (1980).
- 655 66. Alizadeh-Pasdar, N. & Li-Chan, E. C. Comparison of protein surface  
656 hydrophobicity measured at various pH values using three different fluorescent  
657 probes. *J Agric Food Chem* **48**, 328-334 (2000).
- 658 67. Weinberger, A. et al. Gel-assisted formation of giant unilamellar vesicles. *Biophys*  
659 *J* **105**, 154-164 (2013).

660  
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671

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694

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701

## 702 **Competing interests**

703 The authors declare no competing interests.

704



705 **Figure legends**

706

707 **Fig. 1: Surface-exposed methylation of flagellin contributes to efficient**

708 **colonization of the murine intestine.** (a) Schematic of a methylated flagellar filament

709 and surface representation of the structure of FliC (top) and FljB (bottom). FliB-

710 dependent methylation sites are highlighted in orange. (b) Streptomycin pre-treated

711 C57BL/6 mice were infected with  $10^7$  CFU of the FliC-expressing WT (*fliC<sup>ON</sup>*) and

712 isogenic  $\Delta$ *fliB* mutant, each harboring a different antibiotic resistant cassette. The organ

713 burden (small intestine, colon and cecum lumen and tissue, respectively) was

714 determined two days post-infection and used to calculate the competitive indices (CI).

715 Each mouse is shown as an individual data point and significances were analyzed by

716 the Wilcoxon Signed Rank test. The bar graph represents the median of the data and

717 asterisks indicate a significantly different phenotype to the value 1 (\* =  $p < 0.05$ ).

718

719 **Fig. 2: Flagella methylation facilitates eukaryotic cell invasion.** (a) Schematic

720 illustration of productive adhesion and invasion of eukaryotic epithelial cells dependent

721 on methylated flagella. (b) Invasion of MODE-K murine epithelial cells depends on

722 methylated flagella. Reported are relative invasion rates of MODE-K epithelial cells for

723 various flagellin methylation mutants without (top: no spin) or with forced contact of the

724 bacteria by centrifugation (bottom: +spin). (c-e) Relative invasion rates of different

725 eukaryotic host cell types. The human epithelial cell line E12, the murine epithelial cell

726 line CI11, and the murine fibroblast cell line NIH 3T3 were infected with *Salmonella*

727 flagella methylation mutants as described above. The bar graphs represent the mean of

728 the reported relative invasion rate data normalized to the inoculum. Replicates are

729 shown as individual data points and statistical significances were determined by the  
730 Student's *t* test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; ns = not significant).

731  
732 **Fig. 3: Flagella methylation mediates adhesion to hydrophobic surfaces.** (a)  
733 Methylation increases hydrophobicity of the flagellar filament outer surface. Surface  
734 hydrophobicity distribution of the outer (left) and the inner surface (right) of the FliC  
735 flagellar filament<sup>36</sup> according to the Eisenberg scale<sup>37</sup> (from green to white indicates  
736 increasing hydrophobicity) with FliB-dependent methylation sites highlighted in blue. (b)  
737 Measured surface hydrophobicity ( $S_o$ ) of methylated and non-methylated ( $\Delta fliB$ )  
738 flagellins using PRODAN on purified flagellar filaments. (c) Adhesion of *S. enterica* to  
739 MODE-K epithelial cells is reduced in the absence of flagella methylation. Adhesion was  
740 monitored using *S. enterica* strains deleted for *spi-1* in order to prevent invasion of the  
741 eukaryotic host cells. (d) Adhesion of *S. enterica* to giant unilamellar vesicles (GUV)  
742 consisting of phosphatidylcholine from egg chicken is dependent on the presence of  
743 methylated flagella. Left: schematic illustration of the adhesion of *Salmonella* to GUVs  
744 dependent on methylated flagella. Right: Quantified adhesion of *Salmonella* mutants to  
745 GUVs. The bar graphs represent the mean of the reported data. Replicates are shown  
746 as individual data points and statistical significances were determined by the Student's *t*  
747 test (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ; ns = not significant).

748