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1 **LPS structure influences protein secretion in *Salmonella enterica***

2

3 Running title: *Salmonella* LPS mutants and protein secretion

4

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7

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20

21 & contributed equally to this work

22

23 **Abstract**

24 In this study we have compared protein secretion in the wild type of *S. Typhimurium* and the  
25 *rfaC* mutant. We found out that the *rfaC* mutant was defective in protein secretion. In  
26 addition, the *rfaC* mutant was defective in its invasion into an IPEC-J2 porcine epithelial cell  
27 line and also in motility in semisolid agar. Consistent with this, reduced flagella numbers  
28 were observed in the *rfaC* mutant. In the *rfaC* mutant, there were no defects in flagellin  
29 expression as detected by western blot and immune electron microscopy which demonstrated  
30 equal amounts of flagellin in the cytoplasm of both the *rfaC* mutant and the wild-type *S.*  
31 *Typhimurium*. However, in the wild-type strain only, the flagellin was assembled to spatially  
32 restricted areas on the inner side of cytoplasmic membrane. The oligosaccharide core of LPS  
33 is therefore required for the assembly of flagella and T3SS secretion machinery followed by  
34 protein secretion.

35

36 **Keywords**

37 *Salmonella*; LPS; *rfaC*; protein secretion; motility; SPI-1

38

## 39 Introduction

40 *Salmonella enterica* is a pathogen of many bird and mammalian species including humans. Its  
41 pathogenicity is dependent on type III secretion systems (T3SS) and nearly all the proteins  
42 found in nutrient rich media after *S. enterica* culture are those transported by T3SS  
43 (Komoriya et al., 1999). There are 3 different T3SS in *S. enterica* (Hautefort et al., 2008). The  
44 first one is used for the transport of flagellar proteins and the assembly of flagella on the  
45 surface of bacterial cells. The remaining two T3SS are tightly associated with *S. enterica*  
46 virulence. The T3SS encoded by *Salmonella* pathogenicity island 1 (SPI-1) is involved in *S.*  
47 *enterica* invasion into epithelial cells (Kaniga et al., 1995) and the last T3SS encoded by  
48 *Salmonella* pathogenicity island 2 (SPI-2) is involved in intracellular survival of *S. enterica*  
49 (Cirillo et al., 1998).

50  
51 To protect humans or farm animals against *Salmonella* infections, different live or inactivated  
52 vaccines have been tested. Besides the vaccination against *Salmonella* infections, *S. enterica*  
53 is also frequently used as a vector for the expression and delivery of heterologous antigens  
54 (Panthel et al., 2005). However, while the attenuation and immunogenicity have been tested  
55 experimentally many times, the pattern of protein secretion has not been addressed  
56 thoroughly. It is known that mutants defective in the synthesis of full-sized LPS show  
57 impaired motility (Parker et al., 1992; Karasova et al., 2009) which may indicate a defect in  
58 the secretion of flagellar proteins. In this study we were therefore interested in the secretion of  
59 flagellar and SPI-1-encoded T3SS proteins by *rfaC* mutant of *Salmonella enterica* serovar  
60 Typhimurium (*S. Typhimurium*). This mutation conveys defect in the synthesis of the  
61 oligosaccharide core of LPS and therefore produce an oligosaccharide core free LPS (Parker  
62 et al., 1992; Sirisena et al., 1992; Genevaux et al., 1999). We found out that secretion of  
63 flagellar and SPI-1-encoded proteins required the presence of an intact oligosaccharide core

64 of LPS and that this defect is caused most likely by the inability to set up the T3SS  
65 translocation machinery.

66

## 67 **Material and Methods**

### 68 *Bacterial strains and growth condition*

69 *S. Typhimurium* strain LT2, its *rfaC* mutant and the *rfaC* mutant complemented with a  
70 recombinant plasmid encoding *rfaC* has been used in this study. The deletion of *rfaC* in *S.*  
71 *Typhimurium* was constructed exactly as described previously for *S. Enteritidis* (Sebkova et  
72 al., 2008). For complementation, the *rfaC* gene was amplified by PCR using forward  
73 (CTCGGCAAAGACCATGAAG) and reverse (TCCACAATAGGTTTGGGATG) primers  
74 and the resulting PCR product was cloned into pCR2.1 using the TOPO cloning system  
75 according to the instructions of the manufacturer (pCR2.1 TOPO Cloning kit, Invitrogen).  
76 After selection by PCR using primers flanking the plasmid-insert junction, the pRfaC plasmid  
77 was electroporated into *S. Typhimurium rfaC* mutant. All the strains were stored in LB broth  
78 with 10% glycerol at -70 °C and when in use, the strains were grown in LB broth or LB agar  
79 at 37 °C supplemented with antibiotics if appropriate. Motility was determined by inoculation  
80 of 1 µl of 18-hour-old culture into the centre of Petri plate with 0.3% LB agar and by  
81 measuring the diameter of bacterial growth 8 hours later.

82

### 83 *SDS-PAGE and protein identification*

84 Protein secretion was determined by SDS-PAGE. All the strains and mutants were grown for  
85 18 hours in LB medium at 37 °C to reach OD<sub>600nm</sub> 0.8. To characterise total cellular protein,  
86 120 µl of bacterial culture was pelleted by centrifugation (7000g, 15 min, 4 °C), resuspended  
87 in SDS-PAGE loading buffer (20% SDS, 25% glycerol, 0.5% β-mercaptoethanol, 0.06 M  
88 Tris-HCl, pH 6.8, 0.15% bromphenol blue), heated at 95 °C for 10 minutes and loaded onto

89 12% polyacrylamide gel. Secreted proteins were precipitated from 20 ml filtered (Millex GP  
90 filter 0.22  $\mu\text{m}$ , Millipore) bacterial culture supernatant with trichloroacetic acid (13% final  
91 concentration). After 1 hour incubation on ice, the precipitated proteins were centrifuged  
92 (7000g, 10 min, 4 °C) and washed twice with ice-cold acetone. Finally the protein pellet was  
93 air dried and dissolved in 200  $\mu\text{l}$  of 10% SDS. Total protein was determined by BCA assay  
94 and protein samples were diluted to 100  $\mu\text{g}/\text{ml}$  concentration. In the case of proteins secreted  
95 by the *rfaC* mutant we were unable to get proteins at a concentration of 100  $\mu\text{g}/\text{ml}$  and all the  
96 proteins available were therefore loaded onto polyacrylamide gel. SDS-PAGE was performed  
97 using Mini Protean II system (Bio-Rad) and gels were stained with Coomassie brilliant blue.  
98 Individual protein bands were cut out from the polyacrylamide gel and identified by MALDI-  
99 TOF/TOF exactly as described previously (Straskova et al., 2009).

100

#### 101 *Invasion tissue culture cells*

102 The IPEC-J2 porcine epithelial cell line was grown on a 24-well microplate at 37 °C under  
103 5% CO<sub>2</sub> atmosphere. When the cultures reached semiconfluent growth, *S. Typhimurium* and  
104 the *rfaC* mutant from the 6 hour old LB culture were added at a multiplicity of infection equal  
105 to 5. The numbers of adhered and invaded *S. Typhimurium* cells were determined by a  
106 standard gentamicin protection assay – counts of total adhered and invaded bacteria were  
107 determined 2 hours post *Salmonella* inoculation after lysing the cells with Triton X-100 (1%  
108 v/v) and the counts were expressed as percentage of the adhered bacteria out of inoculum. To  
109 determine the counts of invaded bacteria, gentamicin (final concentration 100  $\mu\text{g}/\text{ml}$ ) was  
110 added to kill extracellular bacteria for 2 hours and after that, the tissue culture cells were lysed  
111 with 1% Triton X-100 to release intracellular bacteria. The released bacteria were serially  
112 diluted and plated on LB agar plates for overnight culture at 37 °C.

113

114 *Western blot analysis*

115 SDS-PAGE-separated proteins were electroblotted onto a Hybond<sup>TM</sup>-P PVDF membrane  
116 according to the instructions of the manufacturer (Amersham, GE Healthcare). After blocking  
117 with 0.1 % Tween and 0.5% casein hydrolysate in PBS, the membrane was incubated with  
118 primary monovalent H:i antibody (Bio-Rad) diluted 1:100 in PBS for 1 hour. After washing  
119 with 0.1 % Tween in PBS, the membrane was incubated for another 1 hour with a secondary  
120 anti-rabbit antibody conjugated with horse-radish peroxidase. 3,3'-Diamino-benzidin-  
121 tetrahydrochlorid (Serva) was finally used for the colorimetric signal development.

122

123 *Microarray analysis*

124 Microarray chips were prepared by spotting 5' amino linker modified 70mer oligonucleotides  
125 covering both the *S. Typhimurium* and *S. Typhi* genomes (The Salmonella Genus AROS  
126 V1.0, Operon, Germany). The oligonucleotides were resuspended in Micro Spotting Solution  
127 Plus buffer (Telechem International, Inc., Sunnyvale, USA) and spotted onto Nexterion Slide  
128 E Epoxysilane Coated Substrate microarray glasses (Nexterion, Jena, Germany). For RNA  
129 preparation, both strains were grown statically in 10 ml of LB broth for 16 hours at 37 °C,  
130 bacterial cultures were pelleted and total RNA was purified using RNeasy Mini kit (Qiagen).  
131 At least 5 µg of RNA was reverse transcribed with Cy3 or Cy5-labeled dCTP and hybridised  
132 to the microarray slides, which were subsequently washed and scanned exactly as described  
133 previously (Sebkova et al., 2008). Expression profiles were obtained from 3 independent  
134 experiments, each performed in dye-swap design. Data were normalised using statistical  
135 software R (Wit and McClure, 2004). Only the spots flagged as of good quality were included  
136 into the analysis and were further examined with three approaches: i) t-test with subsequent q-  
137 value package for p-value multiple adjustment (Dabney and Storey, 2002), ii) *limma* linear  
138 modeling package (Smyth, 2004) and iii) *smida* package of Significance Analysis of



139 Microarrays (Tusher et al., 2001). Full raw microarray data have been deposited in the NCBI  
140 GEO database under accession number GSE19878.

141

142 *Negative staining of bacterial cells from liquid culture*

143 Bacterial strains from 18-hour-old LB cultures grown at 37 °C were centrifuged at 2500g and  
144 washed with 0.1 M HEPES pH 7.4. A single drop of culture was placed on formvar-coated  
145 copper grids and the bacteria were allowed to attach for 5 min. In the next step, the bacteria  
146 were stained with 1% ammonium molybdate and observed with a Philips EM 208  
147 transmission electron microscope under an acceleration of 90 kV. At least 150 different cells  
148 were observed and the numbers of cells with zero, 1-2, and 3 or more flagella was  
149 determined.

150

151 *Detection of flagellin in ultrathin sections*

152 One ml of 18-hour-old culture was centrifuged at 2500g, washed with HEPES and the pellet  
153 was resuspended in 100 µl of 4% gelatine warmed to 45 °C. After gelatine solidification, each  
154 sample was cut into 1 to 3 mm<sup>3</sup> blocks which were fixed with a mixture of 3% glutaraldehyde  
155 and 2% paraformaldehyde (1:1), and prestained with 0.25% ruthenium red for one hour.  
156 Samples were then dehydrated with acetone and embedded in Lowicryl K4M (Sigma).  
157 Embedded samples were UV photopolymerized for 2 days at room temperature and 100 nm  
158 ultrathin sections were prepared using a LKB ultramicrotome. The ultrathin sections were  
159 transferred to formvar-coated copper grids and incubated for 10 min in blocking solution  
160 (0.5M NaCl containing 0.1% BSA, 0.05% Tween and 5% fetal bovine serum, pH 7.5) for 10  
161 min. The grids were first allowed to react with 10x diluted rabbit monovalent H:i antiserum  
162 (BioRad) for 20 min followed by rinsing 3 times with distilled water. In the next step, the  
163 grids were incubated for 20 min with a drop of anti-rabbit antibody conjugated with 10 nm

164 colloidal gold (Sigma) diluted 1:10 in blocking solution. Finally, the labeled ultrathin sections  
165 were stained with uranyl acetate and lead citrate and observed with a transmission electron  
166 microscope.

167

## 168 **Results**

### 169 *Protein secretion in the rfaC mutant of S. Typhimurium*

170 Major secreted proteins identified by MALDI-TOF/TOF in the wild type strain included  
171 SipA, FlgK, FliC, FliD, SipC, InvJ and FlgL, similar to Komoriya et al. (1999). On the other  
172 hand, none of the flagellar or SPI-1-encoded proteins were detected in culture supernatants of  
173 the *rfaC* mutant. Complementation with the pRfaC plasmid restored the ability of the mutant  
174 to secrete proteins, although the profile of the complemented mutant was slightly different  
175 from the secretion profile of the wild type *S. Typhimurium* (Fig. 1). Similar to observations of  
176 Sheng et al. (2008), complementation affected the size of secreted flagellin but we did not  
177 study this in more detail.

178

### 179 *Motility and flagella expression*

180 Since defects in secretion of flagellar proteins should correlate with defects in motility,  
181 motility in 0.3% agar was tested in next experiment. The *S. Typhimurium rfaC* mutant  
182 exhibited a defect in motility as the diameter of growth 8 hours post inoculation was around  
183 20 mm while the diameter of the growth halo of the wild type *S. Typhimurium* reached values  
184 around 65 mm (Fig. 2). Complementation with pRfaC plasmid only partially restored motility  
185 of the *rfaC* mutant in 0.3% agar (Fig. 2) which could be caused by the overexpression of the  
186 RfaC from the multicopy plasmid pCR2.1.

187

188 In the next step we therefore studied the presence of flagella on the surface of the *rfaC* mutant  
189 directly by electron microscopy. Majority of the wild type *S. Typhimurium* expressed flagella  
190 on the bacterial surface whilst only around 8 % cells of the *rfaC* mutant expressed flagella and  
191 the number of non-flagellated cells in the *rfaC* mutant reached 92 %. Complementation with  
192 pRfaC plasmid fully restored flagella expression on the surface of the cell (Fig. 3). In  
193 addition, a distinct electron dense capsule was observed in the *rfaC* mutant, consistent with  
194 previous reports on the overexpression of capsule in rough mutants (Parker et al., 1992).

195

#### 196 *Cell invasion*

197 Since defects in secretion of SPI-1-encoded proteins should correlate with defects in cell  
198 invasion, invasion of IPEC-J2 cell line was tested in the next experiment. The *rfaC* mutant  
199 tended to be more adherent to the IPEC-J2 cell line, consistent with our previous report  
200 (Matiasovic et al., 2011). The *rfaC* mutant of *S. Typhimurium* was also disabled in its  
201 invasion of the same cell line and this defect could be complemented with the pRfaC plasmid  
202 (Fig. 4).

203

#### 204 *Gene expression in S. Typhimurium rfaC mutant*

205 Next we were interested whether the decrease in protein secretion could be caused by a  
206 decrease in gene transcription since reports of altered transcription in rough mutants have  
207 been published (Bauer and Welch, 1997; Nagy et al., 2006). To test this, we compared gene  
208 expression in the wild type *S. Typhimurium* and the *rfaC* mutant by microarray analysis.  
209 Although fluorescent signals of 3963 spots (out of 6144 spots present on chips) were  
210 classified by the ScanArray Express software as of good quality, none of them was  
211 differentially expressed in the wild type *S. Typhimurium* and the *rfaC* mutant after their 18-  
212 hour growth in LB broth.

213

214 *Western blot analysis*

215 The absence of protein secretion with a pattern of maintained transcription suggested that the  
216 secreted proteins may accumulate in the bacterial cytoplasm. To address this, we separated  
217 secreted and whole cellular proteins of *S. Typhimurium* by SDS-PAGE, and performed  
218 western blot analysis using anti-flagella antibodies. Western blot confirmed that significantly  
219 more FliC protein was secreted by the wild type strain than by the *rfaC* mutant, as expected  
220 based on a visual inspection of Coomassie blue stained polyacrylamide gels. However, when  
221 the FliC protein was detected by western blot in total cellular protein, less FliC was observed  
222 in the wild type *S. Typhimurium* than in the *rfaC* mutant (Fig. 5).

223

224 *Immune electron microscopy of S. Typhimurium rfaC mutant*

225 Our results suggested that the defect in the *rfaC* mutant was at the level of protein  
226 translocation across the inner and outer membranes. To test this, we finally analysed the  
227 spatial distribution of flagellin inside *S. Typhimurium* cells using ultrathin sections and  
228 immune electron microscopy. Specific gold-labeled antibodies confirmed that both the wild  
229 type *S. Typhimurium* and the *rfaC* mutant expressed flagella antigens since these could be  
230 detected in both these strains. However, while the flagellin in the *rfaC* mutant was randomly  
231 distributed along the inner surface of the cytoplasmic membrane, in the wild type *S.*  
232 *Typhimurium* the flagellin was clustered to spatially discerned loci (Fig. 6). This indicated  
233 that incomplete LPS of the *rfaC* mutant did not allow the flagellar machinery to set up  
234 properly and transport flagellin out of the cell.

235

236 **Discussion**

237 In this study we have shown that mutation in *rfaC* gene cause defects in protein secretion,  
238 motility or cell invasion in *S. Typhimurium*. Similar to many previous reports (Parker et al.,  
239 1992; Genevaux et al., 1999; Nagy et al., 2006; Sheng et al., 2008), but different from  
240 Toguchi et al. (2000) and Inoue et al. (2007), the *rfaC* mutant was defective in motility. The  
241 different results in the two studies are likely to be associated with a different strain  
242 background and particular experimental procedures used. Mutant in *rfaC* produce an  
243 oligosaccharide core free LPS (Parker et al., 1992; Sirisena et al., 1992; Genevaux et al.,  
244 1999) suggesting that the oligosaccharide LPS core is important for the assembly of flagella  
245 and protein secretion including cell invasion. Using microarray analysis, we excluded the  
246 hypothesis of significantly altered RNA transcription in the *rfaC* mutant, although this was  
247 surprising considering the many defects associated with *rfaC* mutations (Nagy et al., 2006;  
248 Karasova et al., 2009) and the fact that Nagy et al. (2006) determined the expression of *rfaH*  
249 and *rfaG* mutants in *S. Typhimurium* by microarray analysis and observed a decrease of SPI-1  
250 and flagellar gene expression. We therefore checked the quality of spots containing the probes  
251 complementary to SPI-1 gene mRNA individually and even this additional verification step  
252 did not indicate any difference in the expression of these genes in the wild type *S.*  
253 *Typhimurium* and the *rfaC* mutant. We concluded that there is no or very small decrease in  
254 the expression of SPI-1 genes in the *rfaC* mutant, and that the differences in the results of  
255 microarray analysis can be explained by different culture conditions and time of growth prior  
256 to RNA purification when shaken cultures were used by Nagy et al. whereas we used a static  
257 overnight culture instead.

258

259 Using western blot analysis and immune electron microscopy we next excluded the  
260 hypothesis of significantly altered translation or protein stability in the *rfaC* mutant because  
261 western blot analysis clearly indicated that the amount of the FliC protein was either the same

262 or even slightly higher in the cytoplasm of the *rfaC* mutant than in the cytoplasm of the wild  
263 type *S. Typhimurium*. This was also confirmed by immune electron microscopy using  
264 ultrathin sections and we therefore excluded defects at the level of protein expression as a  
265 major reason for the aberrant behavior of the *rfaC* mutant in protein secretion. The only  
266 difference could be seen in the distribution of flagellin inside the cells of the wild type strain  
267 and the *rfaC* mutant. In the wild type *S. Typhimurium*, the flagellin was localised to several  
268 spatially restricted areas while no such clustering was evident in the *rfaC* mutant suggesting  
269 that the absence of flagellar protein secretion could be caused by the inability of flagellar  
270 transport apparatus to assemble correctly.

271  
272 The presence of FliC in the absence of protein secretion is quite puzzling since based on the  
273 current knowledge of hierarchal control of gene expression in flagella assembly, the FliC  
274 should not be expressed before the antisigma factor FlgM is secreted out of a cell through a  
275 polymerised flagellar hook (Karlinsky et al., 2000b). The size of the LPS molecule is around  
276 10 nm, of which 2.4 nm is the size of lipid A anchoring the LPS molecule into the outer  
277 membrane (Kastowsky et al., 1992). The flagellar hook extends approx. 50 nm from the  
278 bacterial surface (Moriya et al., 2006) and the SPI1 injectisome is of approx. the same size as  
279 the flagellar hook (Kubori et al., 2000). This means that if the injectisome or hook  
280 polymerisation was initiated, the process would continue above the LPS molecule and  
281 therefore independent of its structure. It is therefore likely that incomplete LPS did not allow  
282 initiation of injectisome or flagellar hook polymerisation immediately at the outer membrane  
283 of *S. Typhimurium* where it can be influenced by LPS structure. Furthermore, if the hook-  
284 basal body is not formed, FlgK protein cannot be secreted and may remain associated with the  
285 FlgN chaperone (Bennett et al., 2001). Interestingly, if the FlgN is associated with FlgK, it is  
286 not available for positive regulation of FlgM expression (Karlinsky et al., 2000a), which may

287 result in an expression of FliC. FliC can therefore be expressed and accumulate in the  
288 cytoplasm even in the tightly controlled flagella expression system, as has been documented  
289 for the double *flgG-L* and *flgN* mutant (Karlinsky et al., 2000a).

290

## 291 **Conclusion**

292 In this study we have found that mutant in *rfaC* gene exhibited defects in protein secretion via  
293 T3SS. The oligosaccharide core of LPS was required for the assembly of flagella and the SPI-  
294 1 encoded secretion apparatus.

295

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302

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384 **Figure legends**

385 Figure 1. Protein secretion in *S. Typhimurium* LT2, its *rfaC* mutant and the *rfaC* mutant  
386 complemented with pRfaC. Sizes of molecular weight marker (lane M) and proteins identified  
387 by MALDI-TOF/TOF are indicated.

388

389 Figure 2. Motility of *S. Typhimurium* LT2, its isogenic *rfaC* mutant and the mutant  
390 complemented with recombinant pRfaC plasmid in 0.3% LB agar. Since more strains were  
391 inoculated in a single plate for comparison, the assay in this figure was interrupted 3 hours  
392 post inoculation when the *rfaC* mutant still appeared as completely non-motile. However,  
393 after 8 hours of motility assay a small amount of growth similar to that described by  
394 Genevoux et al. (1999) was observed.

395

396 Figure 3. Presence of flagella on the surface of *S. Typhimurium*, *rfaC* mutant and the *rfaC*  
397 mutant complemented by recombinant pRfaC plasmid determined by electron microscopy  
398 (Panel A). Panel B, numbers of cells without any flagella (white columns), cells expressing 1  
399 or 2 flagella (grey columns), and cells expressing 3 or more flagella (black columns).

400

401 Figure 4. Adhesion and invasion of the *rfaC* mutant of *S. Typhimurium* into an IPEC-J2  
402 porcine epithelial cell line. Panel A, adhesion of *S. Typhimurium* *rfaC* mutant and the mutant  
403 complemented by the pRfaC plasmid. Panel B, invasion of *S. Typhimurium* *rfaC* mutant and  
404 the mutant complemented by the pRfaC plasmid. \* - significantly different by t-test ( $P < 0.05$ )  
405 from appropriate wild type strain control.

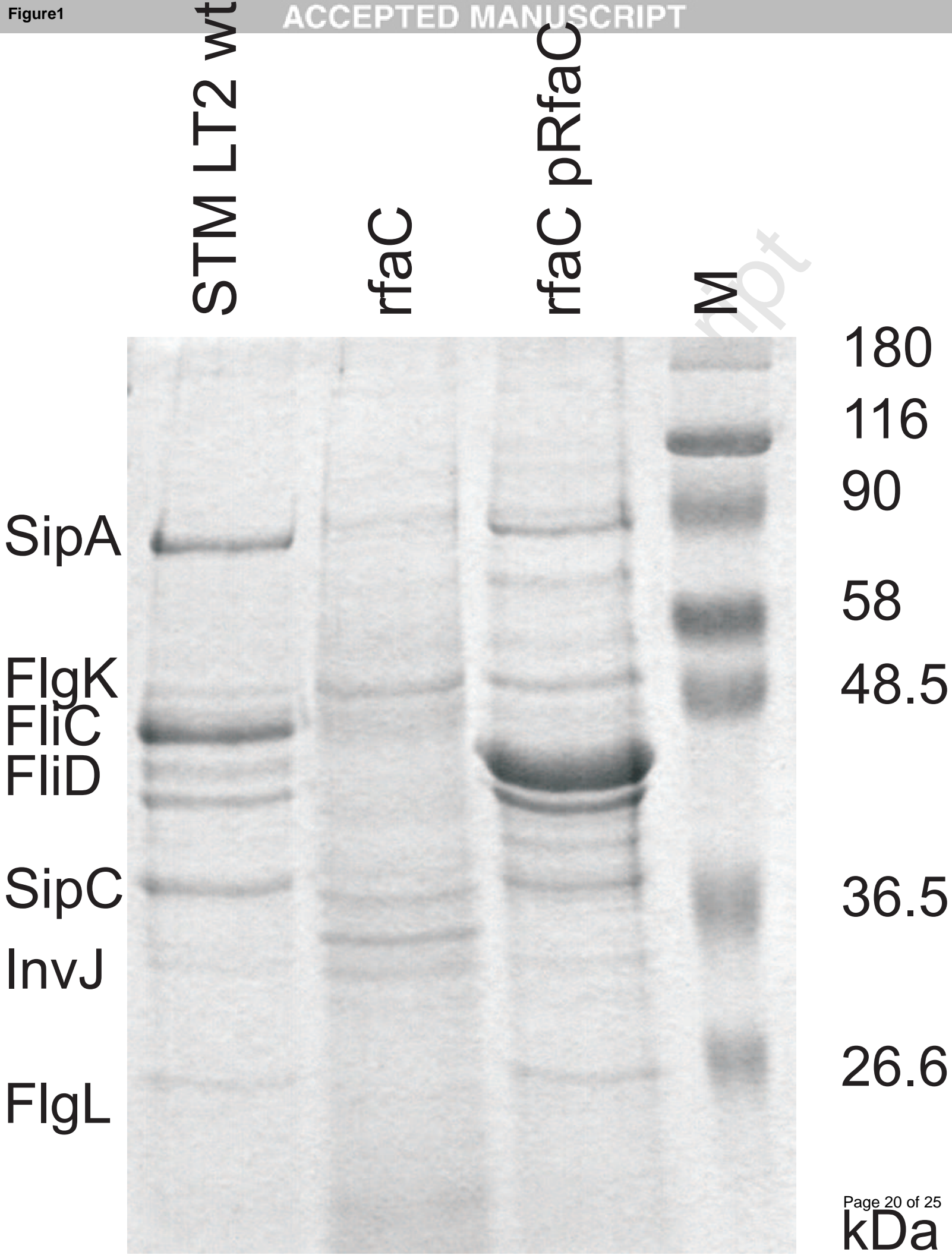
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408 Figure 5. SDS-PAGE (Panel A) and western blot analysis using anti flagellin H:i antibody  
409 (Panel B) of secreted and total cellular proteins in wild type *S. Typhimurium* (STM LT2 wt),  
410 *rfaC* mutant and *rfaC* mutant complemented with recombinant pRfaC plasmid. Lane M,  
411 molecular weight standard.

412

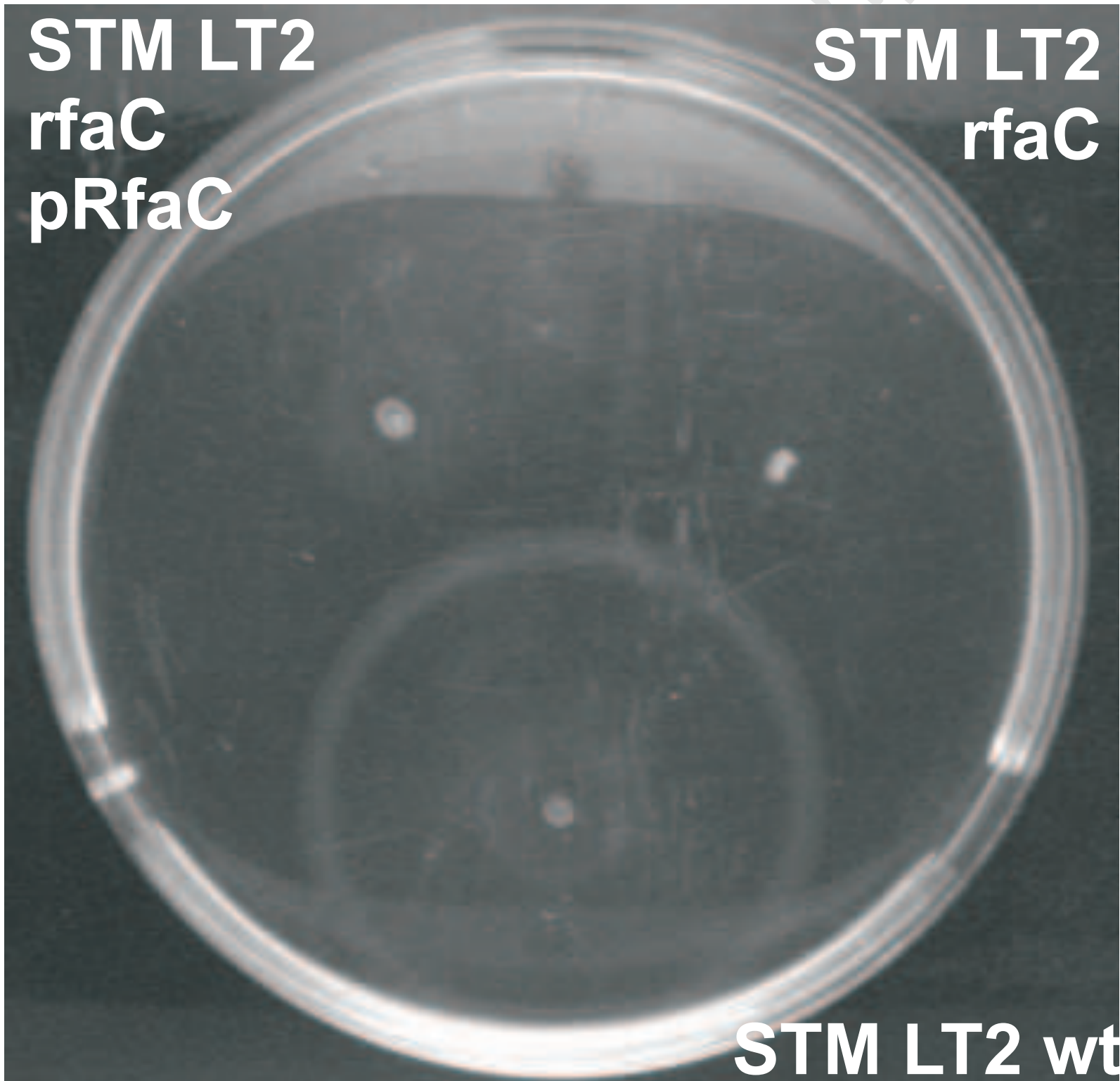
413 Figure 6. Immune electron microscopy in ultrathin section of the wild type *S. Typhimurium*  
414 (panel A) and its isogenic *rfaC* mutant (Panel B) using anti-flagella antibody. Unlike the *rfaC*  
415 mutant, in the wild type strain, spatially restricted clusters of flagellin indicated by arrows  
416 were observed.



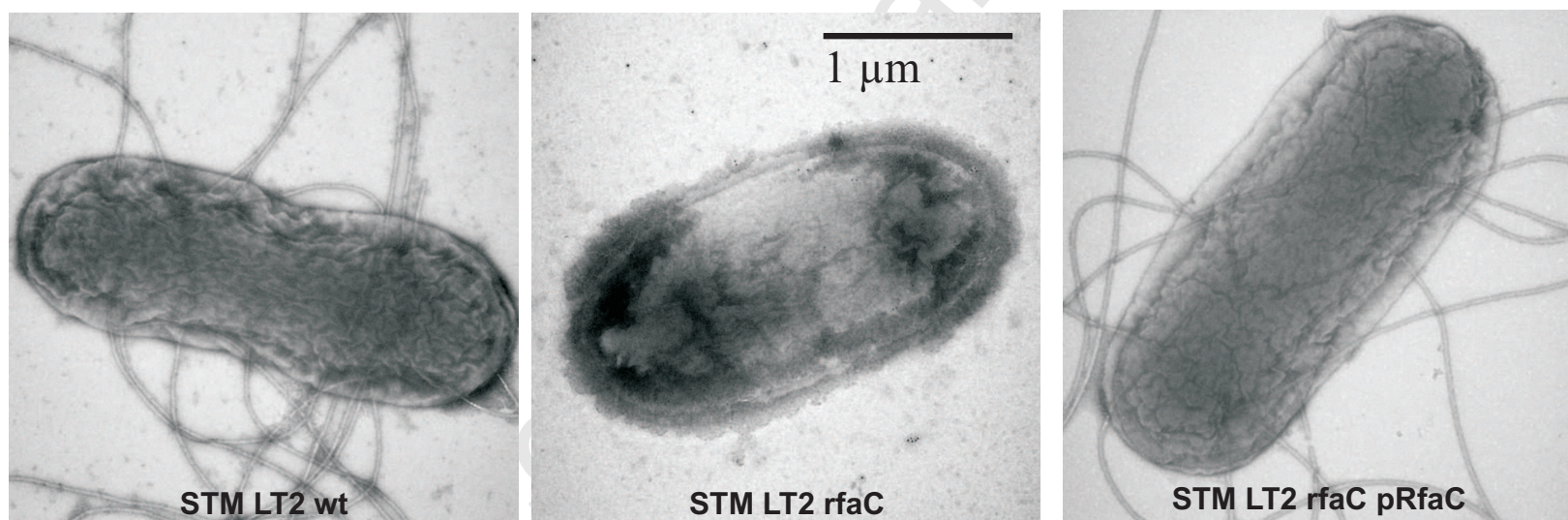
**STM LT2**  
**rfaC**  
**pRfaC**

**STM LT2**  
**rfaC**

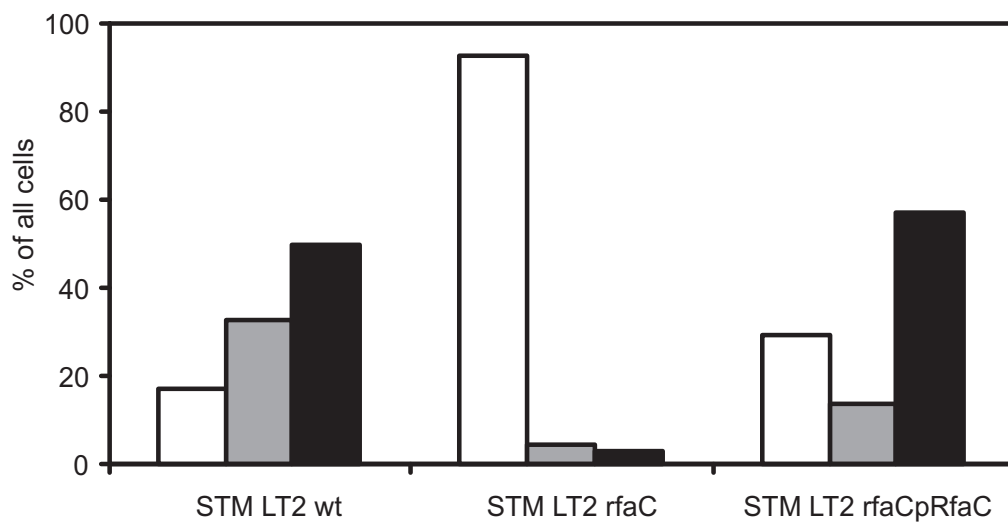
**STM LT2 wt**



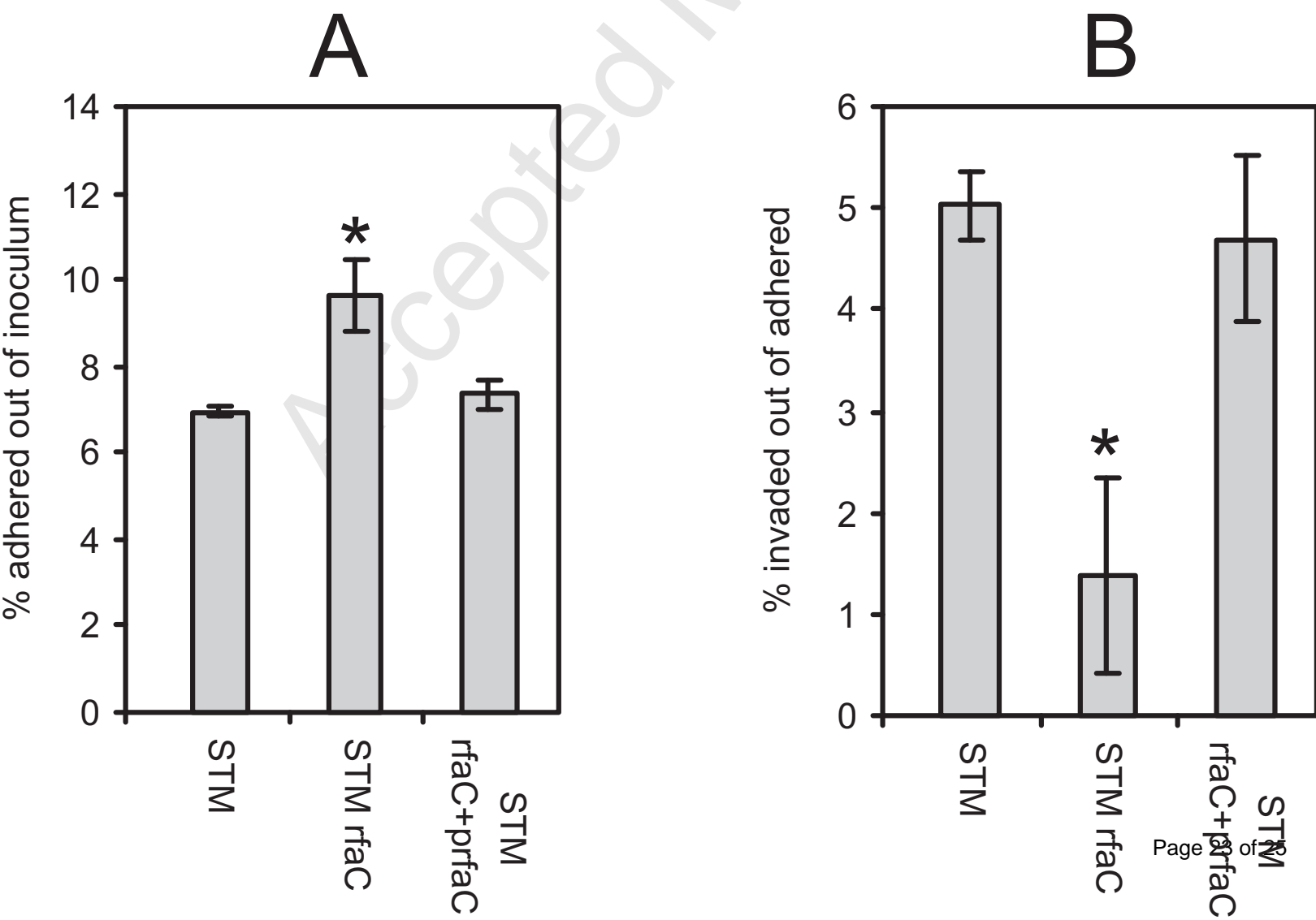
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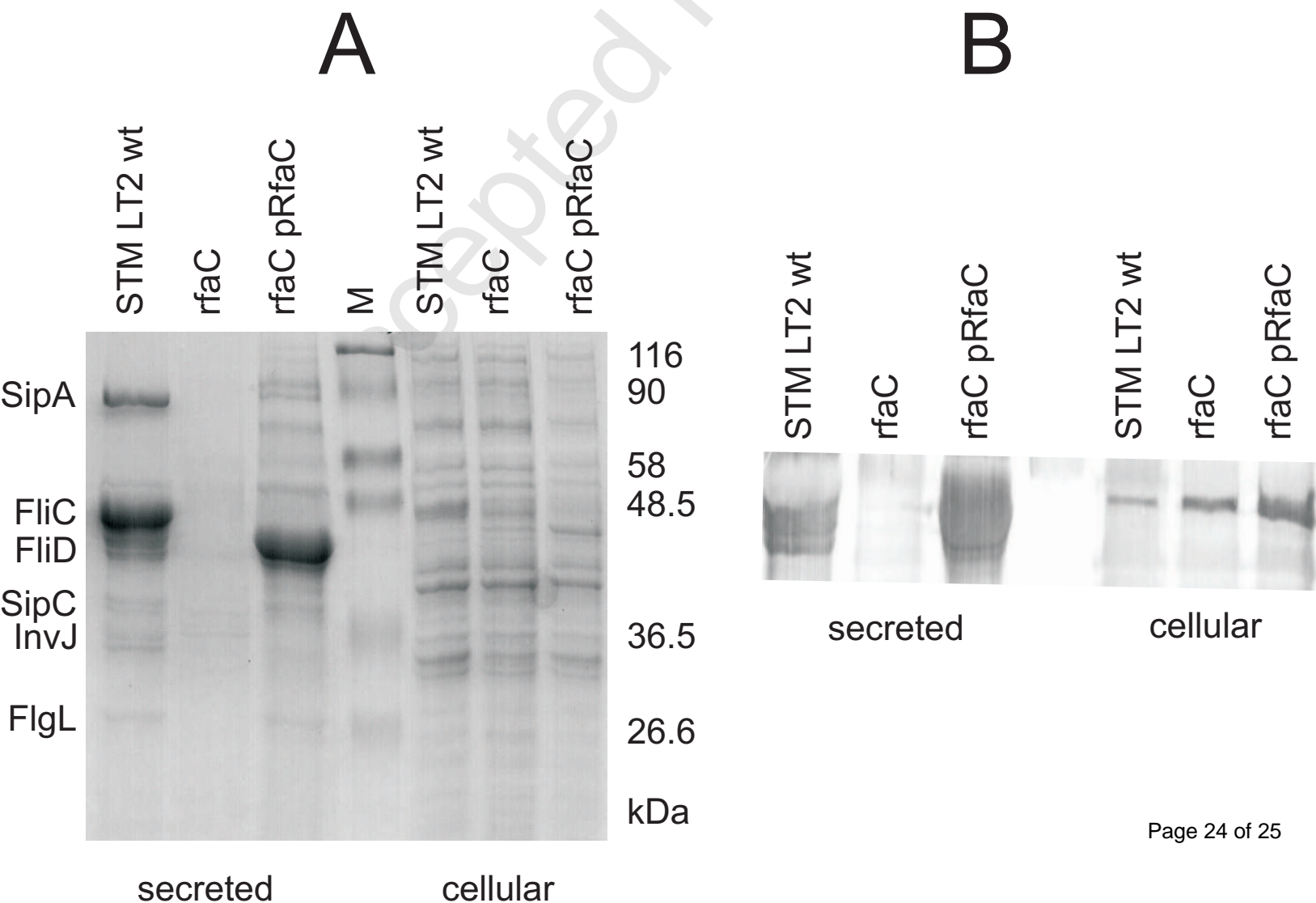


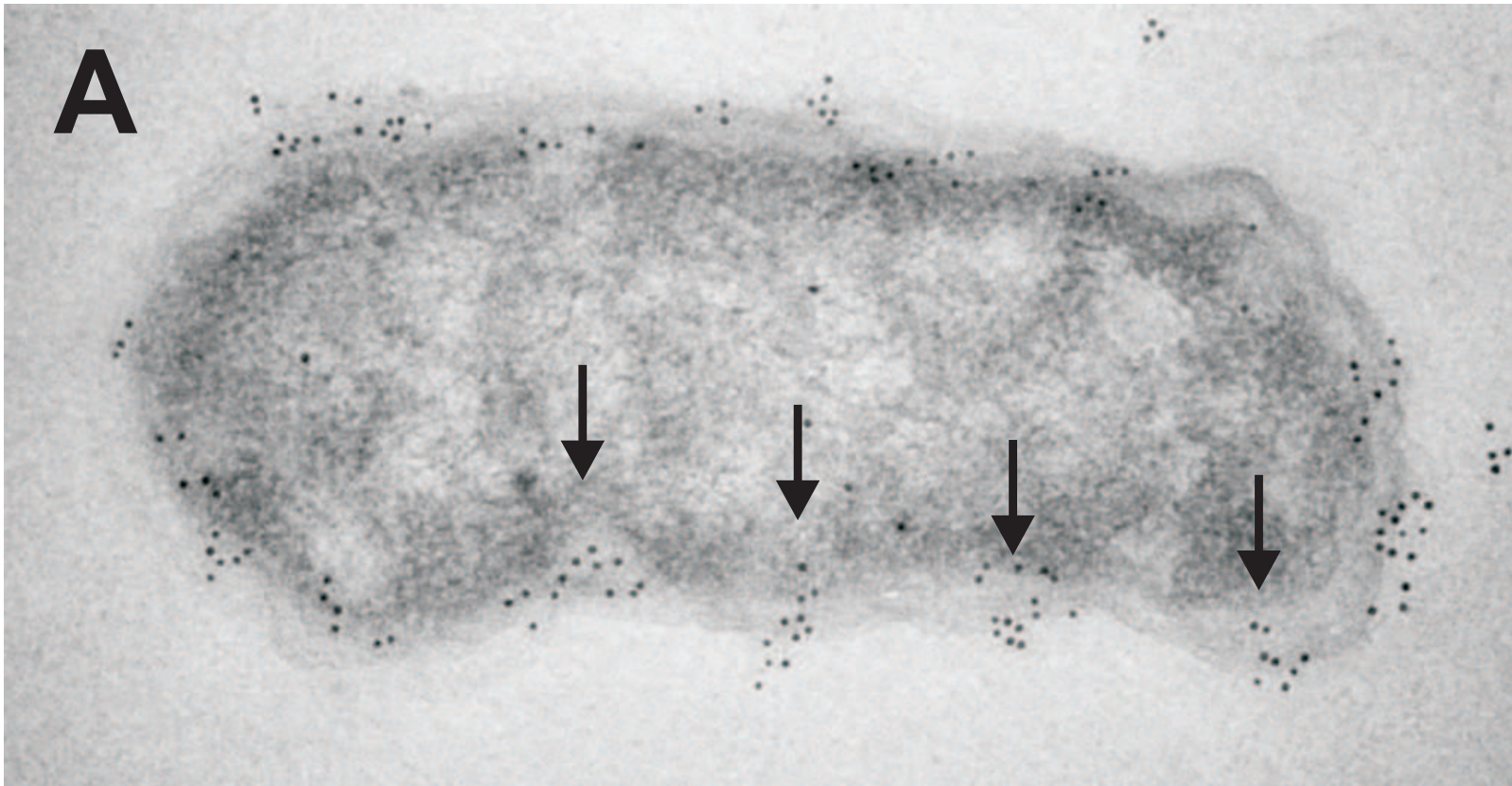
B











1  $\mu\text{m}$