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A Widefield Light Microscopy-Based Approach Provides Further Insights into the Colonization of the Flea Proventriculus by *Yersinia pestis*

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1 A widefield light microscopy-based approach provides further insights into the
2 colonization of the flea proventriculus by *Yersinia pestis*.

3

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13 Running Head: Quantification of PV colonization by *Y. pestis*

14

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18

19 **ABSTRACT**

20 *Yersinia pestis* (the agent of flea-borne plague) must obstruct the flea's proventriculus to
21 maintain transmission to a mammalian host. To this end, *Y. pestis* must consolidate a
22 mass that entrapped *Y. pestis* within the proventriculus very early after its ingestion. We
23 developed a semi-automated fluorescent image analysis method and used it to monitor
24 and compare colonization of the flea proventriculus by a fully competent flea-blocking *Y.*
25 *pestis* strain, a partially competent strain, and a noncompetent strain. Our data suggested
26 that flea blockage results primarily from the replication of *Y. pestis* trapped in the anterior
27 half of the proventriculus. However, consolidation of the bacteria-entrapping mass and
28 colonization of the entire proventricular lumen increased the likelihood of flea blockage.
29 The data also showed that consolidation of the bacterial mass is not a prerequisite for
30 colonization of the proventriculus but allowed *Y. pestis* to maintain itself in a large flea
31 population for an extended period of time. Taken as the whole, the data suggest that a
32 strategy targeting bacterial mass consolidation could significantly reduce the likelihood
33 of *Y. pestis* being transmitted by fleas (due to gut blockage), but also the possibility of
34 using fleas as a long-term reservoir.

35 **IMPORTANCE**

36 *Yersinia pestis* (the causative agent of plague) is one of the deadliest bacterial pathogens.
37 It circulates primarily amongst rodent populations and their fleas. Better knowledge of
38 the mechanisms leading to the flea-borne transmission of *Y. pestis* is likely to generate
39 strategies for controlling or even eradicating this bacillus. It is known that *Y. pestis*
40 obstructs the flea's foregut so that the insect starves, frantically bites its mammalian host,
41 and regurgitates *Y. pestis* at the bite site. Here, we developed a semi-automated
42 fluorescent image analysis method and used it to document and compare foregut
43 colonization and disease progression in fleas infected with a fully competent flea-
44 blocking *Y. pestis* strain, a partially competent strain, and a noncompetent strain. Overall,
45 our data provided new insights into *Y. pestis*' obstruction of the proventriculus for
46 transmission but also the ecology of plague.

47

48 INTRODUCTION

49 Plague is caused by the bacillus *Yersinia pestis*. This disease remains active in several
50 countries worldwide, including Madagascar and the USA (1). In 1898, Simond
51 discovered that plague is transmitted by fleas – a mechanism confirmed by Gauthier and
52 Raybaud in 1903 (2). Eleven years later, Bacot and Martin developed the first model of
53 the transmission of *Y. pestis* by this hematophagous insect (3). Since then, several
54 researchers have deepened and refined our knowledge of the pathophysiological
55 mechanisms leading to flea-borne transmission of *Y. pestis* (4-10).

56 In the current model, the flea bites a septicemic mammalian host and ingests blood
57 infected by *Y. pestis*. During the blood meal, the infected blood is pulled through the
58 esophagus and the proventriculus (a valve that prevents blood regurgitation but that opens
59 periodically to allow blood ingestion) and then arrives in the midgut (8). Within a few
60 hours of the blood meal, the infection causes the production a soft bactericidal matrix that
61 traps *Y. pestis* and entirely fills the lumen of the proventriculus (4). Due to its softness,
62 this “proventricular cast” can be totally or partially dislodged by the following blood
63 meal; a new *Y. pestis*-containing proventricular cast then forms and fills the lumen of the
64 proventriculus. The new cast can again be partially dislodged and replaced by subsequent
65 feeding (4). During these "cast destruction-reconstruction" cycles, *Y. pestis* replicates in
66 the cast and produces a polymer of β 1-6-N-acetyl-glucosamine that progressively
67 consolidates the initially soft mass (3, 4, 9, 11). Eventually, the mass becomes so hard
68 that it blocks the proventriculus and prevents the flea from ingesting blood. The so-called
69 “blocked” flea then regurgitates some drawn blood at the biting site and continues to
70 attempt to feed until it starves to death. However, it is during one of the many

71 unsuccessful feeding attempts that the blocked flea will regurgitate *Y. pestis*-containing
72 blood into the host's dermis and thus inoculates the host with the plague bacillus (12-14).
73 In 1996, Hinnebusch et al. used fluorescent *Y. pestis* to demonstrate that the hemin
74 storage (*hmsHFRS*) gene locus is essential for blockage of the flea's proventriculus (9). It
75 is therefore possible to use microscopy to monitor the progression of foregut colonization
76 by fluorescent *Y. pestis*. Indeed, we have previously acquired fluorescence microscopy
77 images of the proventriculus from infected fleas and have classify them according to the
78 approximate number of bacteria present (*i.e.* none, very few, few, or many) (4). Next, we
79 developed an algorithm that analyzed the fluorescence microscopy images and measured
80 the surface area occupied by fluorescent bacteria within each individual proventriculus
81 (15, 16). Although this computerized approach is more accurate than the visual method, it
82 still provides only a rough estimation of the state of proventriculus colonization; only the
83 area occupied by the bacteria is taken into account, and the technique does not consider
84 the fluorescence signal's intensity in the various part of the proventriculus. We have
85 therefore developed a semi-automatic method that estimates (i) the percentage of the
86 proventriculus infected, (ii) the percentage of the proventriculus surface occupied by
87 bacteria, and (iii) the change over time in bacterial density (*i.e.* growth or not) during the
88 proventriculus infection. We used the new method to gain insights in the mechanisms
89 responsible for the colonization and blockage of the flea proventriculus by *Y. pestis*.

90

91 **RESULTS AND DISCUSSION**

92 **Choice of the filter set used to acquire fluorescence microscopy images of the**
93 **proventriculus.**

94 The proventriculus is covered with inward-pointing spines that auto-fluoresce at all
95 wavelengths of the visible spectrum (Fig. 1A). Despite the interference from auto-
96 fluorescence, it is possible to discern *Y. pestis* expressing the green fluorescent protein
97 (GFP) from *Aequorea coerulea* (excitation max =475nm, emission max= 505 nm)
98 located in the proventriculus (Fig. 1B). We used a raster graphics editor to make the
99 bacteria appear in blue while conserving the green color of the proventricular spines (4,
100 15). To avoid this image processing step (which is a source of bias), it is possible to use a
101 long-pass B2-A cube filter (Nikon) to expand the absorption window for fluorophores
102 that are excited at 470/40 nm (Fig. 1B) (17). This filter transmitted all wavelengths above
103 500 nm (*i.e.*, green, yellow, and red light), whereas the bandpass GFP cube filter
104 transmitted only wavelengths between 500 and 560 nm (*i.e.*, green). By combining this
105 technical change with a dichromic mirror of 505 nm for B2-A (instead of 500 nm for
106 GFP), almost all of the autofluorescence in the proventriculus appears in orange and the
107 GFP bacteria still fluoresce in green. We therefore selected the B2-A filter and GFP-*Y.*
108 *pestis* for our quantification of the surface area occupied by the bacteria in the
109 proventriculus and as the relative bacterial load.

110

111 **Semi-automated quantitative analysis of the proventriculus infected by *Y. pestis*.**

112 After infection, *Y. pestis* remains confined to the midgut and/or the proventriculus (4, 8).
113 Therefore, bacteria located in the midgut must be excluded from the acquired image
114 before the signal from proventriculus can be processed. In other words, it is necessary to
115 manually select the proventriculus, which we delineated as the zone between the lower

116 spines (a marker of the proventriculus/midgut interface) and the top of the spines or the
117 edge of a bacterial mass overflowing into the esophagus (if present) (Fig. 2).

118 To determine the surface area occupied by the bacteria (*i.e.* the area in green), a color
119 threshold is applied in order to remove the orange color (corresponding to the spines of
120 the proventriculus) (Fig. 2). To this end, we defined a hue range (47 to 255, on our
121 microscope and with our settings) and applied it throughout the automatic analysis. Next,
122 a binary white mask (representing the presence of bacteria in green) was generated.
123 Lastly, the surface area of the white mask was calculated as a proportion of the total
124 surface area of the proventriculus. This process was also used to estimate the number of
125 proventriculi infected with bacteria.

126 We also sought to estimate the bacterial load within the lumen of the proventriculus. To
127 this end, we selected the elements within the chosen hue range, split the three RGB
128 channels of the image, and analyzed the fluorescence intensity on a 32-bit, floating-point
129 grayscale mask (Fig. 2). Lastly, the mean fluorescence intensity (relative to the surface
130 area occupied by the bacteria) was calculated.

131 The method described above is time-consuming if performed manually: 4 hours are
132 needed to analyze 20 proventriculi. We therefore generated an ImageJ macro
133 (supplemental material) that performed the analysis automatically. ImageJ is a free and
134 public program developed by the National Institutes of Health and can be run on Mac,
135 Windows and Linux. This automation reduced the time required by a factor of 16 (*i.e.*, 15
136 min, rather than 4 hours). After launching ImageJ (Fig. 2), the user must click on
137 "Plugins" menu, then select "Run". The software asks to select the macro file then to
138 select the folder in which the images of the proventriculus to be analyzed are stored.

139 Next, the user will be requested to manually select the area of interest (*i.e.*, the area
140 occupied by the spines of the proventriculus, as described above). Once the region of
141 interest has been selected, the percentage of the surface area occupied by the bacteria in
142 the proventriculus and the mean fluorescence intensity were determined automatically.
143 Lastly, all the processed images and measured values are saved in a folder called “mask”.

144

145 **Colonization by a strain fully competent for flea blockage.**

146 In a proof-of-principle experiment, we studied the course of proventriculus colonization
147 in fleas fed on blood contaminated with a *Y. pestis* strain known to be fully competent for
148 flea blockage. However, in order to define the limits of detection (*i.e.*, the noise), we first
149 analyzed 20 proventriculi from uninfected fleas. The median (range) values of the noise
150 for “colonized surface area” and “fluorescence intensity” were 0.3% (0.014% – 0.807%)
151 and 1.17×10^7 arbitrary units (AU) (1.04×10^7 AU – 1.31×10^7 AU), respectively (Figs.
152 3C and D). We then arbitrarily set the thresholds (above which we consider a signal to be
153 positive) to 1% for “colonized surface area” and 1.32×10^7 AU for “fluorescence
154 intensity”.

155 Next, we applied our method to images of proventriculi acquired 1, 2, 6, 13 and 27 days
156 (D) after infection (Fig. 3). A surface area analysis showed that all fleas were colonized
157 on D1, D2 and D6 but that one was no longer colonized on D13 and D27 (Fig. 3B). The
158 data revealed that approximately half of the surface area of the proventriculus was
159 colonized by bacteria within two days of the infection (median: 51% on D1, and 65% on
160 D2) (Fig. 3A and 3C). At the end of the first week of infection and on D13 post-infection,
161 ~90% of the surface area of the proventriculus had been colonized by *Y. pestis*. After 27

162 days of infection, the colonized surface area had fallen slightly but was still high in
163 absolute terms (median: ~70%). As expected, the pattern of surface area occupation by *Y.*
164 *pestis* followed that of the fluorescence intensity (Figs. 3C and 3D). The median
165 fluorescence intensity rose from D1 to a maximum value on D6 (5.62×10^7 AU), which
166 persisted until at least D13 and then started to decrease slightly. However, the
167 fluorescence intensity was still high on D27 post-infection. This decrease in fluorescence
168 intensity was not related to a loss of the GFP-expressing plasmid. Indeed, the plasmid
169 pAcGFP1 is highly stable for at least 4 weeks in the absence of any antibiotic selection
170 pressure (B.J. Hinnebusch, personal communication).

171 The results of the semi-automatic analysis were strongly correlation with those obtained
172 by eye ($R^2=0.9882$), except for D1 post-infection (Table 1). In fact, the fluorescence
173 intensity strongly underestimated the number of infected proventriculi, which was not the
174 case when considering the surface area. Therefore, we could only use surface area values
175 to measure the number of infected proventriculi throughout the experiment. However, it
176 should be borne in mind that the surface area analysis might have slightly overestimated
177 the number of infected proventriculi (16 out of 16), relative to a by-eye analysis (14 out
178 of 16). This overestimation might conceivably have been due to background noise from
179 the proventriculus spines. However, the automatic analysis of 20 additional uninfected
180 proventriculi showed that none was colonized. Hence, the computer-based image analysis
181 might have detected a few bacteria that could not be detected by eye, due to their
182 particular orientation. Taken as a whole, these results indicate that (i) our semi-automatic
183 method provides reliable data throughout the infection, and (ii) a quick comparison of the

184 data obtained automatically and those validated by eye is nevertheless advisable on D1
185 post-infection.

186 We found that the bacterial load in the proventriculus peaked at D6 post-infection (Fig.
187 3). Interestingly, we had reported previously that the bacterial load in the flea as a whole
188 (*i.e.*, in the proventriculus and the midgut) peaked on D1 post-infection (4, 5) –
189 suggesting that *Y. pestis* colonizes the proventriculus more slowly than the midgut. In
190 turn, slower colonization might indicate that the proventriculus is a more hostile
191 environment than the midgut. Alternatively, the slower colonization might be due to a
192 “flushing” effect: during the first week of infection, the bacteria located in the
193 proventriculus would be more easily flushed out by the act of feeding than those located
194 in the midgut; feeding occurred once (on D2 post-infection) in both experiments. This
195 “flushing” hypothesis is consistent with the fact that (i) the *Y. pestis*-containing
196 proventricular cast anchored to the proventriculus is partially or totally flushed out by the
197 act of feeding, and (ii) all or most of the cast released during the act of feeding
198 accumulates in the midgut because it too large to be excreted in the feces (4).

199 Although the proventriculus appears to be colonized more slowly than the midgut during
200 the first week of infection, this is not the case for the first two days of infection (*i.e.*
201 between D1 of infection and the first uninfected meal on D2). Indeed, bacterial loads in
202 the whole gut and in the proventriculus were respectively lower and greater just before
203 the meal on D2 than immediately after infection (16) (Fig. 3D). This difference may be
204 due to the digestion process, which involves the release of enzymes, nutrient-sequestering
205 molecules and bactericidal molecules into the midgut (8, 15, 16, 18). In other words,
206 digestion might generate a bactericidal environment in the midgut. Furthermore, only *Y.*

207 *pestis* present in the midgut will be excreted in the feces. This is possible because (i) *Y.*
208 *pestis* does not bind to cells in the midgut, and (ii) although fleas are able to defecate for
209 two days post-infection, the *Y. pestis* entrapped in the cast remained firmly attached
210 unless blood is drawn.

211 Further analysis of images of infected proventriculi collected at the onset of infection (on
212 D1) revealed that the few bacteria present are consistently located in the anterior half
213 region of the proventriculus and not in the posterior half (Fig. 3A). Once bacteria are
214 present in both the anterior and posterior halves of the proventriculus (from D2 onwards),
215 the former region always contains a much larger number of bacteria (Fig. 3A). The
216 apparent anterior-to-posterior progression of bacterial colonization of the proventriculus,
217 the rising bacterial load in the proventriculus, and the decreasing bacterial load in the
218 midgut suggest that the proventriculus is colonized by bacteria trapped in this organ soon
219 after infection rather than those returning from the midgut. This hypothesis is consistent
220 with a previous model in which the anterior half of the anterior region of the
221 proventriculus is the initial site of infection; this leads to recurrent colonization and,
222 ultimately, blockage of the proventriculus (4). However, we cannot yet fully rule out a
223 midgut origin for the bacteria present in the anterior half of the proventriculus at the start
224 of the infection and there intend to carry out further (non-trivial) experiments addressing
225 this question.

226

227 **Colonization by the $\Delta hmsHFRS$ strain of *Y. pestis***

228 Although the *hmsHFRS* operon is essential for flea blockage (9), the literature data
229 indicate that a $\Delta hmsHFRS$ mutant can persist in the proventriculus for at least 13 days

230 post-infection (4). We therefore used our method to study the progression of the infection
231 and the status of proventriculi infected with a $\Delta hmsHFERS$ strain (relative to the wild-type
232 strain), in order to confirm the literature data, extend the observation beyond D13 post-
233 infection and thus better characterize the *hmsHFERS* operon's role in the colonization of
234 the proventriculus.

235 We found that the $\Delta hmsHFERS$ mutant colonizes and persists in the proventriculus up to
236 27 days post-infection (Fig. 3). However, the median colonized surface area of the
237 proventriculus was about half that seen in the wild-type strain (~45%) on D6 post-
238 infection and remained so until D27 post-infection (Fig. 3C). The median fluorescence
239 signal intensity remained very low throughout the experiment (Fig. 3D). Furthermore,
240 21% and 55% of the proventriculi collected respectively on D2 and D27 post-infection
241 apparently contained no bacteria (Figs. 3A and B), suggesting that the mutant is slowly
242 flushed out of the proventriculus. Taken as a whole, our results show that the *hmsHFERS*
243 operon is not essential for infecting the proventriculus but enables the maintenance of *Y.*
244 *pestis* in the proventriculus for a long period in a large population of insects. This is
245 because the *hmsHFERS*⁺ strain of *Y. pestis* is firmly anchored to the proventriculus, thanks
246 to the synthesis of a β -N-acetyl-glucosamine polymer; the latter consolidates the mass
247 and traps the bacteria in the proventriculus (4, 9). Therefore, the *hmsHFERS* operon's role
248 is to increase the incidence of both proventriculus infection and flea blockage. In this
249 context, one can hypothesize that *hmsHFERS* enables *Y. pestis* to use the flea as a long-
250 term reservoir for transmission.

251 Interestingly, several proventriculi were colonized by the *hmsHFERS* mutant to much the
252 same extent (in terms of the percentage surface area and fluorescence intensity) as by the

253 wild-type strain on D6, D13 and D27 post-infection (Fig. 3). This observation raises the
254 question of whether the mutant could block a very low proportion and/or be transmitted
255 more than a month after infection. To answer this question, one would need to study a
256 very large population and measure (i) the blockage rate, (ii) the colonization of the
257 proventriculus (using the fluorescence method), (iii) the bacterial load in the
258 proventriculus before and after feeding, and (iv) the regurgitation of bacteria. However,
259 this type of study is far from trivial and requires the sacrifice of fleas; this might bias the
260 results by the removal of nonblocked insects that might have become blocked later in the
261 experiment.

262

263 **Colonization by the $\Delta ompR-envZ$ strain of *Y. pestis***

264 After applying our method to a mutant unable to block fleas, we studied a strain that has a
265 partial impact on the flea blockage rate. We focused on the OmpR-EnvZ phosphorylated
266 regulatory system because it activates the transcription of genes required for flea
267 blockage - presumably after having sensed nutrient depletion (15). Therefore, we
268 analyzed the proventriculi of fleas infected with the $\Delta ompR-envZ$ and the wild-type
269 strains in the same infection experiment (Fig. 3).

270 The results showed that the median surface area of the proventriculus colonized by the
271 $\Delta ompR-envZ$ mutant was 20% on D1 post-infection and peaked at 40% on D13 post-
272 infection (Fig. 3C). Furthermore, the maximum surface area occupied by the mutant in
273 some proventriculi was consistently lower than that seen for the wild-type strain (~70%
274 vs ~95%, respectively) at the time points when several fleas are diagnosed as blocked
275 (i.e., D6, D13 and D27 post-infection). Consistently, the median fluorescence intensity

276 was very low at all post-infection timepoints (Fig. 3D). It is noteworthy that the
277 fluorescence intensity values in two proventriculi collected on D6, D13 and D27 post-
278 infection were similar to those measured with the wild-type strain, although the surface
279 area of the proventriculus occupied by the mutant strain did not reach that observed with
280 the wild-type strain (Fig. 3C and D). Taken as a whole, our present data and our previous
281 findings (showing that the proportion of fleas blocked by the $\Delta ompR-envZ$ mutant is half
282 that of the wild-type strain) suggest that colonization of the entire surface area of the
283 proventriculus and a high bacterial load in the proventriculus are not prerequisites for flea
284 gut blockage. Therefore, the consolidation of the mass that entraps *Y. pestis* soon after
285 infection in the proventriculus might be more important for flea blockage (and therefore
286 the transmission of *Y. pestis*) than having a high bacterial load in the entire
287 proventriculus. Furthermore, the anterior part of the proventriculus (i.e. close to the
288 esophagus) is certainly the most important site for consolidation and blockage, since the
289 $\Delta ompR-envZ$ mutant is mostly located there (Fig. 3A). Lastly, given that the wild-type
290 strain blocks more fleas, colonizes a greater surface area of the proventriculus and results
291 in a high bacterial load in the proventriculus (relative to the $\Delta ompR-envZ$ mutant), one
292 can hypothesize that full colonization of the proventriculus maximize *Y. pestis*' chances
293 of blocking the flea.

294

295 **CONCLUSION**

296 We developed a user-friendly, semi-automated method for studying the infection of the
297 flea proventriculus by *Y. pestis*. The method is based on the analysis of fluorescent
298 images but does not require any empirical analyses and/or manipulation of the images. It

299 quantifies the number of proventriculi colonized by *Y. pestis*, the colonized surface area
300 in each individual proventriculus, and the estimated bacterial growth. Previous studies
301 showed that images of proventriculus of *Ctenocephalides felis*, *Oropsylla montana*,
302 *Oropsylla hirsuta* infected with *Y. pestis* GFP strain and using a B2-A filter display the
303 same color as with *X. cheopis* (17, 22, 23). In other words, our method will be useful to
304 compare colonization by *Y. pestis* of different flea species. We also expect this tool to
305 greatly facilitate the characterization of the roles of genes required for flea blockage and
306 to better bridge *in vitro* experiments with *in vivo* flea infection experiments.

307 The implementation of our method with *X. cheopis* fed on mouse blood contaminated
308 with the wild-type strain, a $\Delta hmsHFRS$ strain or a $\Delta ompR-envZ$ strain provided further
309 insights into how *Y. pestis* produces a transmissible infection in fleas. In particular, our
310 results suggest that the infectious process leading to flea blockage results primarily from
311 *Y. pestis* entrapment and replication in a cast in the anterior part of the proventriculus, in
312 conjunction with consolidation of this mass early after infection. The flea blockage rate
313 appears to be increased by extension of the colonization from the anterior part of the
314 proventriculus to the posterior part, with the lumen being completely filled by the
315 consolidated mass.

316 Lastly, our data indicate that *Y. pestis*' ability to consolidate the mass in the
317 proventriculus and thus block fleas is not a prerequisite for colonization of this organ.
318 However, this consolidation allows *Y. pestis* to maintain itself in a larger population of
319 fleas for a longer period of time. In other words, the consolidation of the proventricular
320 mass by *Y. pestis* not only increases the likelihood of bacterial transmission via gut
321 blockage but also constitutes a long-term reservoir for the bacteria.

322

323

324 **MATERIALS AND METHODS**

325 **Ethics statement**

326 All experiments complied with the national and institutional regulations and ethical
327 guidelines, and all procedures were approved by the regional animal care and use
328 committee.

329

330 **Strains**

331 The bacterial strains used in this study were the wild-type Kim 6+ strain (19), the
332 $\Delta ompR-envZ::dfrB_{\#2}$ mutant (15), and the $\Delta hmsHFRS::aphA3'$ mutant (4) harboring the
333 pAcGFP1 plasmid (Takara Bio Compagny, Mountain View, CA). The pAcGFP1 plasmid
334 is stable in *Y. pestis* regardless of the genetic background according the following
335 protocol: populations were propagated by 1% serial transfer every 24h for a total of 3
336 transfers. After dilution and plating onto LB Agar, 60 single colonies per populations
337 were patched on LB supplemented with and without carbenicillin. All the colonies grew
338 on both media. Bacteria were cultured without shaking in brain heart infusion broth
339 (211059, BD Difco, Franklin Lakes, NJ) supplemented with carbenicillin (100 ug/mL, to
340 ensure the retention of the pAcGFP1 plasmid).

341

342 **Flea rearing and infection**

343 Oriental rat fleas (*Xenopsylla cheopis*) were allowed to feed on heparinized blood from
344 OF1 female mice (Charles River Laboratories, Saint Germain Nuelles, France) containing

345 5×10^8 bacteria/mL (grown overnight first at 28°C and then at 37°C in brain heart infusion
346 broth), using an artificial feeding apparatus (13, 20). One hour later, cohorts of fleas were
347 collected and disposed on a chill table under a dissecting microscope. Only those that had
348 taken a blood meal (*i.e.* confirmed by the presence of fresh, red blood in the flea's
349 foregut) were selected for use in the study. Fleas were maintained at 21°C and 75%
350 relative humidity and were fed on D2, D6, D9, D13, D16, D20 and D23 post-infection.

351

352 **Imaging of GFP-tagged bacteria during proventriculus colonization**

353 The guts from 16-20 randomly selected fleas were immediately mounted in dH₂O
354 between a glass slide and a glass coverslip (thickness: 1.5 mm) after dissection and were
355 then observed under an Eclipse CiS microscope through a 20x objective (Plan fluor 20x,
356 NA 0.5). The GFP (Ex. 470/40, Em. 535/50), and B2-A (Ex. 470/40, Em. 500)
357 fluorescent filter cubes (Nikon, Melville, NY) were used. Bright-field and fluorescence
358 images of the gut were acquired with a Sight DS-F1c camera (Nikon). Fluorescence
359 images were analyzed using ImageJ software (21).

360

361 **Spectra**

362 The spectrum was scanned with a laser scanning confocal microscope (Zeiss
363 AxioobserverZ1, LSM880) with a 40x objective (EC Plan Neofluar 40x, NA 1.3, oil
364 immersion) and the Quasar Detector. In lambda mode, we acquired 22 successive
365 intensity images within a spectral bandwidth of 9 nm each, ranging from 504 nm to 690
366 nm. Excitation at 488 nm was provided by an argon ion laser.

367

368 **Statistical analysis**

369 One-way analyses of variance with Tukey's post-test for multiple comparisons were
370 performed with Prism 9 software (GraphPad Software, La Jolla, CA).

371

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384

385 A.D., F.S. and S.B.-G. conceived the experiments. A.D., S.B.-G. performed the
386 experiments. A.D., S.B.-G., F.S. analyzed and interpreted the data. E.W. developed the
387 ImageJ macro. F.P. reared the mice. S.B.-G. and F.S. wrote the manuscript, with input
388 from A.D. and E.W.

389

390 **LEGENDS**

391 **Fig. 1.** (A) A fluorescence emission spectrum of an uninfected proventriculus. $\lambda_{\text{exc}} = 488$
392 nm, $\Delta\lambda_{\text{em}} = 9$ nm (B) Uninfected and *Y. pestis*-infected proventriculi, observed by
393 confocal light microscopy and a GFP filter or a B2-A filter. Objective: 20X.

394

395 **Fig. 2.** A sketch of the procedure for measuring the surface area of the proventriculus
396 colonized by bacteria and for estimating the bacterial load from the fluorescence intensity
397 (using ImageJ software). Created with BioRender.com

398

399 **Fig. 3.** Colonization of the *X. cheopis* flea proventriculus by the wild-type *Y. pestis* strain,
400 the $\Delta hmsHFRS$ strain, and the $\Delta ompR-envZ$ strain. (A) Fluorescence images of the
401 proventriculus (in orange) infected with the different strains (in green) acquired at 1, 2, 6,
402 13, and 27 days post-infection (B2-A filter, objective 20X). (B) The percentage of
403 proventriculi colonized (in green) or not (in orange) by *Y. pestis*. (C) Changes over time
404 in the surface area colonization (%) of the proventriculus and (D) the mean fluorescence
405 intensity (arbitrary units AU) in the proventriculus of fleas that had fed on blood infected
406 with the wild-type strain (in pink), the $\Delta hmsHFRS$ strain (in blue) or the $\Delta ompR-envZ$
407 strain (in green). The data were obtained from the images shown in (A); each dot
408 corresponds to an individual flea (n=16-20). The data obtained from uninfected
409 proventriculi are also shown (black circles). The dotted line represents the threshold
410 value above which the fluorescence value (AU) indicates the presence of bacteria. The
411 thresholds were set to 1% for the surface area and 1.32×10^7 AU for the fluorescence
412 intensity. Open circles (regardless of the color) represent values below the threshold. The
413 black line represents the median of all the values above the threshold.

414

415 **SUPPLEMENTAL MATERIAL**

416 SUPPLEMENTAL FILE 1, the macro

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- 479
480

481 **Table 1.** The number of fleas colonized, according to each of the three approaches.

Day post-infection	Fleas studied (n)	Number of fleas colonized, according to:		
		By eye	Surface area occupied	Fluorescence intensity
1	16	14	16	10
2	20	20	20	19
6	20	20	20	20
13	20	19	19	19
27	20	19	19	18

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