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

Amélie Dewitte, Elisabeth Werkmeister, François Pierre, Florent Sebbane,  
Sébastien Bontemps-Gallo

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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

4 Amélie Dewitte<sup>a</sup>, Elisabeth Werkmeister<sup>a,b</sup>, François Pierre<sup>a</sup>, Florent Sebbane<sup>a#</sup>, Sébastien  
5 Bontemps-Gallo<sup>a#</sup>

6

7 <sup>a</sup> Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019-UMR9017-  
8 CIIL-Centre d'Infection et d'Immunité de Lille, Lille, France

9 <sup>b</sup> Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, US41-UMS2014 -  
10 PLBS, Lille, France

11

12

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

14

15 #Address correspondence to Sébastien Bontemps-Gallo, sebastien.bontemps-  
16 gallo@cns.fr and Florent Sebbane, florent.sebbane@inserm.fr.

17 F.S. and S.B-G are co-senior authors.

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  $\beta$ 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  $1.17 \times 10^7$  arbitrary units (AU) ( $1.04 \times 10^7$  AU –  $1.31 \times 10^7$  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  $1.32 \times 10^7$  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 ( $5.62 \times 10^7$  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 $\Delta hmsHFERS$ strain of *Y. pestis***

228 Although the *hmsHFERS* operon is essential for flea blockage (9), the literature data  
229 indicate that a  $\Delta hmsHFERS$  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*<sup>+</sup> strain of *Y. pestis* is firmly anchored to the proventriculus, thanks  
246 to the synthesis of a  $\beta$ -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  $5 \times 10^8$  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 dH<sub>2</sub>O  
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.  $\lambda_{exc} = 488$   
392 nm,  $\Delta\lambda_{em} = 9$  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  $1.32 \times 10^7$  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

482