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1 **Variable membrane protein A of flavescence dorée phytoplasma binds the midgut**
2 **perimicrovillar membrane of *Euscelidius variegatus* and promotes adhesion to its**
3 **epithelial cells**

4

5 Running title: Phytoplasma adhesion to insect vector cells

6

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17

18 **Abstract**

19 Phytoplasmas are uncultivated plant pathogens and cell wall-less bacteria and are transmitted
20 from plant to plant by hemipteran insects. Phytoplasmas' circulative propagative cycle in
21 insects requires the crossing of the midgut and salivary glands, and primary adhesion to cells
22 is an initial step towards the invasion process. The flavescence dorée phytoplasma possesses a
23 set of variable membrane proteins (VmPs) exposed to its surface, and this pathogen is
24 suspected to interact with insect cells. The results showed that VmpA is expressed by the
25 flavescence dorée phytoplasma present in the midgut and salivary glands. Phytoplasmas
26 cannot be cultivated at present, and no mutant can be produced to investigate the putative role
27 of VmPs in the adhesion of phytoplasma to insect cells. To overcome this difficulty, we
28 engineered the *Spiroplasma citri* mutant G/6, which lacks the adhesins ScARPs, for VmpA
29 expression and used VmpA-coated fluorescent beads to determine if VmpA acts as an adhesin
30 in *ex vivo* adhesion assays and *in vivo* ingestion assays. VmpA specifically interacted with
31 *Euscelidius variegatus* insect cells in culture and promoted the retention of VmpA-coated
32 beads to the midgut of *E. variegatus*. In this latest case, VmpA-coated fluorescent beads were
33 localized and embedded in the perimicrovillar membrane of the insect midgut. Thus, VmpA
34 functions as an adhesin that could be essential in the colonization of the insect by the FD
35 phytoplasmas.

36 **Importance**

37 Phytoplasmas infect a wide variety of plants, ranging from wild plants to cultivated species,
38 and are transmitted by different leafhoppers, planthoppers and psyllids. The specificity of the
39 phytoplasma-insect vector interaction has a major impact on the phytoplasma plant host
40 range. As entry into insect cells is an obligate process for phytoplasma transmission, the
41 bacterial adhesion to insect cells is a key step. Thus, studying surface-exposed proteins of
42 phytoplasma will help to identify the adhesins implicated in the specific recognition of insect

43 vectors. In this study, it is shown that the membrane protein VmpA of the flavescente dorée
44 phytoplasma acts as an adhesin that is able to interact with cells of *Euscelidius variegatus*, the
45 experimental vector of the FD phytoplasma.

46

47 INTRODUCTION

48

49 Phytoplasmas are bacteria responsible for diverse epidemic diseases in various
50 cultivated and ornamental plants (1, 2). Characterized by the absence of a cell wall and no
51 peculiar morphology, they belong to the class *Mollicutes* in the order *Acholeplasmatales*,
52 “*Candidatus* genus Phytoplasma”. These bacteria are exclusively located in the sieve
53 elements of plant hosts and are propagated by numerous insect vectors from the Order
54 Hemiptera (3). In hosts, plants and insects, phytoplasmas are found intracellularly. In insects,
55 these bacteria colonize different organs, such as the intestinal tract, muscles and salivary
56 glands (4). Phytoplasma members of the 16SrV-C and V-D taxonomic subgroups cause a
57 severe epidemic disease of grapevine called flavescente dorée (FD) and have therefore been
58 classified as quarantine pests. These phytoplasmas are propagated within and from vineyard
59 to vineyard by the Deltocephalinae leafhopper *Scaphoideus titanus* Ball (5), which was
60 introduced in France well before 1950 (6). These grapevine-specialized insects, from the first
61 nymphal to imago stages (7), acquire phytoplasmas while feeding on infected grapevines and
62 subsequently become infectious after a latency period. The use of insecticide treatments
63 against the vector is one of three main ways to control flavescente dorée, with the other two
64 being planting phytoplasma-free material for planting and removing infected grapes.
65 However, chemical treatments cause unwanted economic, social and environmental impacts
66 and must be reduced. To strengthen such an improvement in FD management, a better

67 understanding of the mechanisms leading to phytoplasma transmission, especially the
68 acquisition phase, is necessary.

69 In the insect vector, the cycle is persistent and multiplicative (8). This property implies
70 the crossing of the two barriers represented by the intestine epithelium and the salivary gland
71 cells but also the multiplication of bacteria into insects. Phytoplasmas have the capacity to
72 multiply into a wide variety of cellular types, such as the intestine, particularly the muscle
73 layer of the midgut, and salivary glands (9, 10), but FD phytoplasmas have not been detected
74 in the sexual organs (11). Passing through intestinal and salivary gland cells is clearly
75 mediated by endocytosis; next, there is movement into the cytoplasm and then exocytosis, as
76 is the case for *Spiroplasma citri*, another plant pathogen of the class *Mollicute* transmitted by
77 leafhoppers (12, 13). These steps imply the direct interactions between phytoplasma and
78 eukaryotic cell proteins to promote endocytosis by cells that are not specialized in
79 phagocytosis. Lacking specialized organelles for mobility or kinetic cytoskeleton,
80 phytoplasmas have to move from apical to basal membranes and leave the host cell by
81 exocytosis after multiplication. All of these steps must be achieved without altering tissue
82 integrity to avoid toxicity to their vector. Several genomes of “*Ca. Phytoplasma*” species are
83 available from which proteins that are predicted to be secreted or surface-exposed are
84 tentatively selected for functional studies. However, notably few protein functional studies
85 have been described in relation with insect transmission. Among the three types of
86 immunodominant membrane proteins (IDPs) that are the major membrane proteins of
87 phytoplasmas (14), the Amp of “*Ca. P. asteris*” was observed to interact with the three main
88 proteins of the microfilament complex, *i.e.*, the actin and myosin light and heavy chains of the
89 intestinal smooth muscle, as well as with the ATP synthase of leafhopper vector (15, 16).
90 Although Amp is essential for transmission by insect vectors (17), Amp interacts with insect
91 proteins after phytoplasmas have become intracellular and could be implicated in the

92 movement of phytoplasmas across these cells. However, phytoplasma adhesins, which are
93 necessary for the promotion of the adhesion of phytoplasma to cells in the digestive tract and
94 salivary glands, have not been identified to date. Several immunogenic membrane proteins
95 that are present at the surface of the phytoplasmas, such as the variable membrane protein
96 Vmp1 of “*Ca. P. solani*”, are targets of strong selective pressures (18). This finding suggests
97 the proteins’ implication in interactions with host molecules. VmpA, similar to Vmp1, is a
98 variable membrane protein predicted to be destined to the FD phytoplasma (FD-P) surface by
99 the Sec-dependent pathway to be finally anchored to the membrane by a C-term
100 transmembrane segment (19). Several other genes found in the genome of the FD-P encode
101 variable membrane proteins, including VmpB, which share the same structure (20). The
102 structure of FD-P VmpA also contains a hydrophilic central that possesses 3 complete repeats
103 of 78 amino acids exposed to the phytoplasma surface. This finding is consistent with the
104 possible role of VmpA in the FD-P adhesion to insect cells, as repeated domains are
105 commonly found in bacterial proteins involved in cell recognition (21). Such adhesins have
106 been characterized in *S. citri* (22–24), and in *Mycoplasma agalactiae* (25, 26). Thus, the role
107 of VmpA in the adhesion of the phytoplasma to insect cells was examined.

108 The *in vitro* culture of leafhopper vector cells provides an experimental tool to study
109 the phytoplasma-insect interaction at the cellular level. For example, it had been demonstrated
110 that the ability of *S. citri* to invade insect cells *ex vivo* is correlated to its ability to be
111 transmitted by the leafhopper vector *Circulifer haematoceps* (27). Additionally, a useful
112 experimental cycle was done to transmit FD-P to the broad bean *Vicia faba* using the
113 leafhopper *Euscelidius variegatus* (28), which similar to *S. titanus*, belongs to the
114 Deltocephalinae subfamily. This prompted us to use cultured cells of *E. variegatus* to explore
115 the implication of the strain FD92 (FD92-P) VmpA in the adhesion process of FD-P to insect
116 cells. In this study, antibodies were used to ascertain the VmpA expression by FD92-P in the

117 insect *E. variegatus*, and we measured the adhesion to *E. variegatus* cells of recombinant
118 spiroplasmas expressing VmpA and fluorescent latex beads coated with His₆-tagged VmpA.
119 The interaction of VmpA-His₆-coated beads with the apical surface of midgut epithelial cells
120 was assessed in *in vivo* ingestion assays.

121

122 **RESULTS**

123

124 **1- VmpA protein is expressed by FD92 phytoplasmas in insects**

125 To assess VmpA expression by FD92-P in the intestinal tract and the salivary glands,
126 indirect immunofluorescence labeling and confocal observations were used. VmpA proteins
127 were visualized in the phytoplasmas in midguts 2 weeks after feeding acquisition with
128 infected broad beans, and they were still detected five weeks after infection (Fig 1). Bacteria
129 were located in intestine cells (arrows), and a number were observed considerably closer to
130 actin filaments of the muscle fibers covering the basal lamina (arrowheads). VmpA was also
131 detected in the salivary glands of some insects, already two weeks after feeding acquisition
132 (Fig 2) and in the majority of insects after a longer latency period of 4 and 5 weeks (Fig 2).
133 No labeling was observed in the midgut and salivary glands of healthy insects. The detection
134 of VmpA by immuno-labeling showed that the FD92-P infecting *E. variegatus* produced
135 VmpA both in the midgut and salivary glands.

136

137 **2- Euva-1 cell line**

138 A cell line from *E. variegatus* was established to study the cellular and molecular
139 interactions between phytoplasma proteins and insect cells. Ten months of the continuous
140 culturing of cells isolated from embryos of *E. variegatus* resulted in the Euva-1 cell line. Cell
141 morphology was examined by light microscopy using methylene blue staining and the

142 fluorescent labeling of actin filaments and nuclei. Based on cellular morphology and
143 colorations, the Euva-1 cell line possessed three main cellular types. The first cells were the
144 largest ones and had only their nucleus stained with methylene blue (Fig. 3, asterisk). The
145 second cell type had its nucleus and cytosol colored (Fig. 3, arrow), while the third was the
146 smallest in size and was darker colored by methylene blue (Fig. 3, arrowhead). Actin
147 coloration showed that the type 2 and 3 cells had clear attachment fibers and filopodia,
148 enabling them to adhere to the flask (Fig. 3B). The two first cell types resembled epithelial
149 cells, whereas the nature of the third was unknown. The interaction experiments were
150 performed with cells cultivated between passages 15 and 21.

151 The sequencing of the mitochondrial marker cytochrome oxidase subunit I (COI) was
152 used to confirm the leafhopper origin of the Euva-1 cell line. Sequences from the
153 mitochondrial marker amplified from Euva-1 cell DNA and from the *E. variegatus* insect
154 DNA were found to be identical (data not shown). A BLAST search in the GenBank database
155 revealed that this sequence shared 80% nucleotide identity with the COI gene of the
156 Cicadellinae *Acrogonia virescens* , which may be its closest relative.

157

158 **3- VmpA-coated beads adhere to *E. variegatus* leafhopper cells in culture**

159 As a phytoplasma mutant cannot be engineered at present, we used recombinant
160 VmpA proteins to test the interaction between VmpA and the insect cells *ex vivo*. For that
161 purpose, we covalently linked VmpA-His₆ recombinant protein and GFP, which served as
162 negative control, to NH₂-beads instead of COOH-beads to better mimic the surface exposition
163 of the VmpA N-terminal part. In these adherence assays, fluorescent beads were incubated for
164 1 h with Euva-1 cells and counted by epifluorescence observation (Fig 4A). The adhesion of
165 beads to Euva-1 cells significantly augmented with increasing concentrations of VmpA, and

166 the median number of adherent beads was 3-fold higher when beads were linked with 9 nmol
167 of VmpA than the control beads coated with GFP only (Fig 4B).

168 To evaluate the specificity of VmpA adhesion to insect cells, we used competitive and
169 inhibition adhesion assays. The adhesion of the fluorescent VmpA-His₆-coated beads was
170 strongly decreased in the presence of anti-His₆-VmpA antibodies (PAb) in a dose-dependent
171 manner (Fig. 4C), although a small but significant increase of bead adhesion was measured
172 when few antibodies were used (1/1000 dilution). When anti-spiraline PABs were used as a
173 negative baseline control, as expected, the adhesion rate was not significantly changed. No
174 visible aggregation of the VmpA-His₆-coated beads was observed in the presence of anti-
175 His₆-VmpA PABs (data not shown). The results of competitive adhesion assays show that the
176 presence of an increasing quantity of His₆-VmpA overlaying the leafhopper cells decreased
177 the adhesion of VmpA-His₆-coated beads in a concentration-dependent manner (Fig. 4D). In
178 parallel experiments, we used the other predicted surface-exposed protein VmpB, which is
179 also expressed in the insect *E. variegatus* (Fig. S1). No reduction was observed when the cells
180 were pre-incubated with the His₆-VmpB recombinant protein. Taken together, these results
181 strongly suggest that VmpA was able to specifically interact *in vitro* with the cells of the FD-
182 P experimental insect vector *E. variegatus*, as an adhesin would do.

183

184 **4- VmpA allows the adhesion of recombinant *S. citri* to leafhopper cells in culture**

185 To complete functional studies on phytoplasma proteins, spiroplasmas that express
186 VmpA at their surface were engineered to measure the impact of VmpA on bacterial adhesion
187 to insect cells (19). We first verified that the recombinant *S. citri* G/6 strain still produced the
188 protein VmpA after several passages before the adhesion assay. A comparison of VmpA
189 expression in recombinant *S. citri* was conducted in the presence and absence of antibiotic
190 selection pressures to promote the stability of pSTVA1. Two clones of *S. citri* G/6 carrying

191 pSTVA1 (clones 5 and 6) were plated on SP4 agar, and different sub-clones were cultivated
192 for 5 passages. The pSTVA1 plasmid was easily detected in the presence of tetracycline,
193 while in the absence of the antibiotic, it was visualized in the sub-clone 6g only, but the
194 restriction map was incorrect, suggesting a deletion (Figure 5A). Thus, PCR amplifications
195 and sequencing were performed to verify the presence of a correct *vmpA* sequence. In the case
196 of sub-clone 6g, a deletion of approximately 700 bp was observed, corresponding to the size
197 of the repeat domains that contain the VmpA protein, and confirmed the plasmid profile after
198 *HindIII* restriction. In the case of the sub-clones 5e and 5h, amplicons were observed after
199 electrophoresis, which suggests that pSTVA1 plasmids were present in these two sub-clones.
200 No deletion or mutation in the *vmpA* gene was observed in clones propagated in the presence
201 of the antibiotic and in the sub-clones 5e and 5h propagated without the antibiotic. When the
202 expression of VmpA was monitored by Western blot (Figure 5A), differences in VmpA
203 expression were observed between spiroplasmas cultivated in the presence or absence of
204 tetracycline. When the antibiotic was omitted, the production of VmpA was abolished. In
205 contrast, the culture of the sub-clones in the presence of tetracycline allowed for the strong
206 detection of VmpA. Thus, second culture passage of recombinant *S. citri* was performed only
207 in the presence of tetracycline for adhesion assays, and the production of VmpA was verified
208 by colony blot.

209 Adhesion assays were performed by comparing the *S. citri* G/6 strain carrying the
210 plasmid pSTP2 (vector without *vmpA*) to G/6 carrying pSTVA1. The colony blots showed
211 that 50 to 100% of the spiroplasmal colonies were expressing VmpA, depending on the
212 replicates, and as revealed by immunoblot. A significant increase of adhesion was observed
213 when VmpA was expressed by the recombinant spiroplasmas (Fig 5B). These results
214 reinforce the previous results to show that VmpA acted as an adhesin binding to Euva-1 cells.

215

216 **5- Interaction of VmpA with the midgut**

217 To assess the role of VmpA in the adhesion of phytoplasmas to intestinal epithelial
218 cells, we used fluorescent beads to localize and count the VmpA-His₆-coated beads in the
219 midguts of *E. variegatus* in *in vivo* experiments. After ingestion by insects, the fluorescent
220 latex beads were only observed in the midgut and occasionally in the filter chamber but were
221 never detected in the Malpighian tubules (Fig. 6A). A high variability of the number of
222 retained beads could be observed between insects (Fig. 6B), but it was reproducible among 3
223 independent experiments. Regardless of the amount of VmpA-His₆ bound to the
224 fluorospheres, the number of beads per surface unit was observed to be higher in the anterior
225 midgut compared to the middle midgut. Furthermore, the higher the quantity of VmpA-His₆
226 coupled to the beads, the greater amount of beads was attached to the midgut surface. When
227 VmpA was in excess compared to BSA, the beads attached to the anterior midgut were too
228 numerous to be accurately quantified. For this reason, counting was performed only at the
229 middle midgut level. As shown in figure 6B, VmpA-His₆-coated fluorescent beads were more
230 greatly retained in midguts than BSA-coated beads did at one, two and four days after feeding
231 acquisition. Seven days post-ingestion, the number of VmpA-His₆-coated beads fell and
232 showed values similar to those of BSA-coated beads four days after ingestion. These results
233 suggest that VmpA, unlike BSA, is more strongly retained in the luminal surface of midgut
234 cells.

235

236 **6- VmpA-His₆-coated beads are localized and embedded in the perimicrovillar**

237 **membrane of midguts**

238 To more precisely localize the VmpA-His₆-coated beads in the midgut at the cellular
239 level, we used transmission electron microscopy (TEM). Midguts dissected from leafhoppers
240 that had ingested VmpA-His₆-coated beads in HEPES-sucrose for two days and then having

241 fed healthy broad bean for one day were compared to leafhoppers that were only fed healthy
242 broad bean (Fig. 7). Bacteria-like particles were visualized in the lumen of the midgut and in
243 the anterior and middle midgut of insects, regardless of whether the leafhoppers were fed
244 (Fig. 7, arrowheads). The particles were often associated with a structure that resembles the
245 perimicrovillar membrane in the anterior and medium parts of midgut. In the midgut of
246 insects that had ingested beads coated with VmpA-His₆, the beads were clearly visible in the
247 lumen (asterisks in Fig. 7B and 7C). The beads were found alone or in groups, embedded in
248 the perimicrovillar membrane, and certain beads were clearly in contact with the microvilli of
249 epithelial cells (Fig. 7C, arrow). In the anterior part of the midgut, the VmpA-His₆-coated
250 beads were present in a larger quantity than in the medium midgut, as previously observed by
251 fluorescence microscopy. Beads were observed in the same gut lumen section where bacteria-
252 like particles were also visualized. No beads were seen inside cells, regardless of where the
253 observation was conducted. Other small dense unidentified particles could also be seen within
254 epithelial cells. As a control, microscopy observations did not show differences between
255 leafhoppers having been fed HEPES-sucrose or healthy broad bean (data not shown). Taken
256 together, TEM observations suggest an affinity of VmpA for the perimicrovillar membrane
257 that covers the apical surface of epithelial cells.

258

259 **DISCUSSION**

260

261 Because phytoplasmas have not been cultivated *in vitro* to date, no defective mutants
262 are available to study candidate genes putatively implicated in phytoplasma adhesion to insect
263 cells. Fortunately, *Spiroplasma citri*, another plant pathogen also transmitted by Cicadellidae
264 insects, is amenable to genetic manipulation. The *S. citri* GII3 mutant G/6, devoid of the
265 adhesins ScARPs (24, 29), was transformed to express the VmpA protein of the FD-P strain

266 FD92 at the spiroplasmal surface (19). We used the mutant *S. citri* G/6, as it is deficient for
267 the adhesion to insect cells (24), to express VmpA with the aim of increasing the spiroplasmal
268 adhesion to the *E. variegatus* cells. To screen the adhesion-like properties of phytoplasma
269 surface proteins, the recipient cell line Euva-1 was established from the experimental vector
270 *E. variegatus* of the FD92 phytoplasmas. The percentages of Euva-1 cells with adherent
271 spiroplasmas G/6 measured in this study were low and similar to those observed when this
272 defective strain was incubated with Ciha-1 cells, a cell line derived from one of *S. citri* natural
273 vectors, *Circulifer haematoceps* (24). The expression of VmpA at the surface of this G/6
274 mutant resulted in recombinant spiroplasmas to significantly increase their adhesion to Euva-1
275 cells. These results are reinforced by experiments that show an enhanced adhesion of VmpA-
276 coated fluorescent beads to the same cells. Taken together, these data demonstrate that VmpA
277 acts as an adhesin, regardless of the support used. Thus, *S. citri* was confirmed to be a good
278 model to functionally characterize adhesins or other surface proteins of phytoplasmas. By
279 extension, it should constitute a reliable experimental platform for the simultaneous
280 expression of sets of phytoplasma proteins acting in concert in the adhesion and entry
281 phenomenon. However, the results also indicated that an antibiotic pressure was necessary to
282 maintain the VmpA expression encoded by the pSTVA1 plasmid. The pSTVA1 plasmid is a
283 derivative of the plasmid pSci21NT, a modified-pSci natural plasmid of *S. citri* GII3 (19), and
284 it was stably expressed in the non-transmissible *S. citri* strain 44 (30). The plasmids pScis are
285 present in 10 to 14 copies per cell (31), which might also be the case for pSci derivatives. The
286 instability of pSTVA1 could therefore result from an increase in the metabolic energy
287 necessary for plasmid maintenance and function or from the general deleterious effect on the
288 bacterial growth rate (32, 33). There are many reports that show that overexpressed
289 heterologous membrane proteins can affect the bacterial growth rate by imposing a metabolic
290 burden, an overload of the membrane biogenesis machinery, a membrane stress or local

291 membrane disruptions in bacteria (33–35). Thus, one possible explanation for the lack of
292 VmpA detection in the absence of tetracycline is that expression of this protein decreases *S.*
293 *citri* fitness and produces counter selection, leading to the loss of VmpA expression. Such
294 instability of viral-derived vector has previously been observed for *S. citri* viral vectors
295 engineered to express a fragment of a mycoplasma adhesin (35).

296 As shown in the current paper, phytoplasmas expressing VmpA were observed in the
297 intestinal cells of *E. variegatus*, a necessary condition to envisage its role in the adhesion to
298 intestine cells. In addition, the ingested VmpA-His₆-coated beads were more greatly retained
299 in the midgut than those predominantly coated with BSA, especially when the beads were
300 coated with a higher amount of VmpA. This validates the hypothesis that VmpA could play a
301 major role in midgut colonization. Ingested coated beads have been observed to be embedded
302 in a matrix associated with the apical surface of microvilli, occasionally with bacteria in the
303 anterior midgut. One of these bacteria might be the congenitally-transmitted enterobacterium
304 previously observed by Cheung and Purcell (36, 37). The bead location was similar to that of
305 maize bushy stunt phytoplasma cells in the midgut lumen of *Dalbulus maidis* (37). Packed
306 maize bushy stunt phytoplasmas were observed by the authors in the lumen near the
307 microvilli and appeared to be surrounded by a slightly electron-dense structure resembling the
308 structure in which the VmpA-coated beads were detected. The precise composition of the *E.*
309 *variegatus* matrix observed in the lumen of *E. variegatus* since 1993 has not been deciphered
310 to date (36). In our observations, the size of this structure is similar to that observed by these
311 authors and is approximately 2-6 μm wide. This matrix had been called glycocalyx by
312 Cheung and Purcell, but according to recent studies on hemipteran insects, we prefer calling it
313 perimicrovillar membrane (39). Unlike lepidopterans and coleopterans, euhemipterans lack a
314 peritrophic membrane (PM). Their perimicrovillar membrane (PMM) is partly composed of
315 glycoproteins (40). In euhemipterans, this PMM seems to act as a protective barrier against

316 invasive microbes and could have diverse functions in the digestion and absorption of
317 nutrients. Microorganisms blocked by the PMM and that use adhesins to stick to the PMM
318 and escape the feeding bowl flow must cross through to reach the apical surface of gut
319 epithelial cells to finally undergo midgut colonization. One example is *Trypanosoma cruzi*,
320 which is attached to the PMM of the Chagas disease vector bug *Rhonius prolixus*. This
321 attachment is mediated via lectin-like proteins of *T. cruzi* to glycoproteins of the midgut
322 PMM (42). Similar to the surface lectin spiralin of *S. citri* (43, 44), VmpA enables *S. citri* and
323 fluorescent beads to adhere to insect cells in culture and to the PMM, which is rich in
324 glycoconjugates. VmpA has also been detected on phytoplasmas attached to the salivary
325 glands, the surface of which is glycosylated like the different lobes of *Circulifer haematoceps*
326 salivary glands (41). Because of these analogies between the two models, a lectin activity for
327 VmpA could therefore be hypothesized and should be further investigated. In the case of
328 another pathosystem, the TnGV granulosis virus encodes the metalloprotease enhancin that
329 alters the structural integrity and porosity of the lepidopteran PM and results in an increased
330 movement of the virus (43). Regarding the structural and functional domains found in VmpA,
331 a PepSY motif that is implicated in regulation of peptidase activity (44) was found upon *in*
332 *silico* analysis. In this regard, VmpA could promote the local degradation of the PMM protein
333 component, allowing phytoplasmas to reach the apical membrane of the midgut epithelium.
334 Nevertheless, these two VmpA activities remained to be investigated.

335 During their cycle within their vectors, phytoplasmas have to invade diverse types of
336 cells or different vectoring insects. These successive steps must involve different phytoplasma
337 membrane-associated proteins, as this has been deciphered in the *Spiroplasma* models (22, 24,
338 41, 42, 45). In the case of FD92-P, VmpB, which shows a similar structure, is a potential
339 candidate to have a similar function. Competition adhesion assays showed that VmpB does
340 not inhibit the adhesion of VmpA to insect cells, suggesting that if VmpB interacts with insect

341 proteins, it is probably not targeting the same receptor(s). The recent deciphering of the
342 FD92-P (20) chromosome will help to compile the list of the potential actors in phytoplasma-
343 insect vector interactions. The use of the Euva-1 cells and recombinant spiroplasmas should
344 be of great help in addressing this challenge.

345

346 **MATERIALS AND METHODS**

347

348 **Insects, bacterial strains and culture conditions**

349 Phytoplasma-free *Euscelidius variegatus* leafhoppers were reared in cages on broad
350 bean (*Vicia faba* var. aquadulce) and oat (*Avena sativa*) at 25°C. The phytoplasma strain
351 FD92 (FD92-P) was originally transmitted to broad bean (*Vicia faba* var. aquadulce) by
352 infected *Scaphoideus titanus* sampled on FD-diseased vineyards in southwest France (46, 47)
353 and was continuously maintained in broad bean by *Euscelidius variegatus* transmissions as
354 described by Caudwell and colleagues (48).

355 The *Spiroplasma citri* strain GII3 was originally isolated from its leafhopper vector
356 *Circulifer haematoceps* captured in Morocco (49). The low-passage, wild-type strain GII3
357 contains seven plasmids, pSciA and pSci1 to pSci6 (31). The *S. citri* GII3 mutant G/6 was
358 engineered through plasmid incompatibility curing and only contains the pSciA and pSci6
359 plasmids; therefore, it lacks ScARP genes (29). Spiroplasmas that expressed the FD92-P
360 VmpA at their cell surface were obtained by electro-transformation of the *S. citri* GII3 mutant
361 G/6 with 1-5 µg of purified plasmid pSTVA1, as described by Renaudin and colleagues in
362 (19). In summary, this plasmid carries the signal peptide depleted-*vmpA* coding sequence
363 fused to the signal peptide sequence of the adhesin ScARP3d and is under the control of the *S.*
364 *citri tuf* gene promoter and ribosome binding site (RBS). Spiroplasmas were cultivated at
365 32°C in SP4 medium from which the fresh yeast extract was omitted (50), and the medium

366 was supplemented with 5-10 $\mu\text{g}/\text{mL}$ tetracycline when necessary. Colonies were further
367 propagated in broth medium SP4 containing 5-10 $\mu\text{g}/\text{mL}$ tetracycline during 3 passages and
368 submitted to a dot blot immunoassay and Western blotting (see below) to reveal the
369 production of VmpA.

370

371 **Establishment of the Euva-1 cell line derived from the leafhopper *E. variegatus***

372 The cell line Euva-1 of the leafhopper *E. variegatus* was established according to a
373 previously described protocol (27). Primary cell cultures, originally established from eggs
374 with red eyespots, were maintained in monolayer culture at 25°C in culture medium made of
375 400 mL Schneider's *Drosophila* medium, 50 mL Grace's insect cell culture medium
376 (Invitrogen), 50 mL heat-inactivated fetal bovine serum (Eurobio), 3 mL G-5 supplement
377 (Invitrogen) supplemented with 1.25 $\mu\text{g}\cdot\text{mL}^{-1}$ fungizone (Invitrogen) and 50 $\mu\text{g}\cdot\text{mL}^{-1}$
378 penicillin/streptomycin (Invitrogen). After the cell line was established, leafhopper cells were
379 passed every 10 days with a 2/3 dilution with an additional change of the medium during the
380 week.

381

382 **DNA isolation, restriction and PCR**

383 To confirm the origin of the cells, the genomic DNA was extracted from 20 mL
384 culture of Euva-1 cells with the Wizard Genomic DNA purification kit (Promega). A
385 fragment of approximately 800 bp of the cytochrome oxidase subunits I and II (COI)
386 mitochondrial gene was amplified using the primers C1-J-2195 (5'-
387 TTGATTTTTTGGTCATCCAGAAGT-3') and TL2-N-3014 (5'-
388 TCCAATGCACTAATCTGCCATATTA-3') (51). PCR amplifications were performed
389 according to Bertin *et al.* (52) with 1 μL of DNA template in a final volume of 25 μL . The
390 sequencing of the PCR products from both the 5' and 3' end of purified PCR products was

391 performed by Beckman Coulters Genomics (Takeley, United Kingdom). The sequences were
392 deposited in ENA (accession numbers LT960658 and LT960628).

393 Plasmid DNA was purified from 10 mL cultures of *Spiroplasma citri* with the Wizard
394 SV Minipreps DNA Purification Kit (Promega). The plasmids were digested with *HindIII*,
395 and the digested products were analyzed on 0.8% agarose gels. The amplification of the *vmpA*
396 fragment was performed using the primers pSciF11 (5'-GTTATTGTGTGGGTCAGATG-3')
397 and VmpARev (5'-CCCTAGCTAACTGAATTCATGGATC-3'). The PCR conditions were
398 35 cycles of 30 s at 92°C, 30 s at 52°C, and 45 s at 68°C with the Taq polymerase (Biolab).

399

400 **Expression, purification of recombinant protein Vmps and production of antibodies**

401 The primers Cl-VmpA-F1 (5'-
402 ACAAACATATGAAAGCTATTACAGATTTGAGTGG -3'), Cl-VmpA-R1 (5'-
403 TCATTCTCGAGTTAACTTTTTTCTTAACAGTAAAC-3'), Cl-VmpB-92F (5'-
404 ATCAACATATGGCAGATAAAGAAAAACCATTATC-3') and Cl-VmpB-92R (5'-
405 TAATTCTCGAGTTAGATTCTGTAACGGTTTCG-3') were designed for the cloning of a
406 part of the *vmpA* (accession number LN680870) and *vmpB* genes (accession number
407 PRJEB22700), respectively, without the trans-membrane regions, as detailed in figure S2A
408 and S2B. The DNA fragments, 930 bp-long for VmpA (amino acids 38 to 347) and 756 bp-
409 long for VmpB (aa 34 to 285), were PCR amplified with the Phusion High-Fidelity DNA
410 polymerase (Finnzyme) from the total DNA of *V. faba* infected by FD92-P. For the
411 expression of the histidine-tagged proteins in *Escherichia coli*, the amplicons were cloned into
412 the pET28 expression system (Novagen, Madison, WI). *E. coli* BL21 Star (DE3) cells
413 (Invitrogen) were then transformed with pet28-His₆-VmpA, pet-VmpA-His₆ or with pet28-
414 His₆-VmpB, according to the manufacturer's protocol. Expression was induced with 1 mM
415 IPTG. The tagged proteins were purified as described previously (24) on HIS-Select Nickel

416 affinity gel-packed columns (Sigma). For VmpA, the nickel column was conditioned with 0.05
417 M sodium phosphate buffer at pH 7.4 with 0.2% Triton X-100 and for VmpB with 0.05 M
418 sodium phosphate buffer at pH 7.4 with 0.025 M imidazole and 0.2% Triton X-100.
419 Imidazole elution concentrations were respectively 0.25 M for His₆-VmpA and 0.5 M for His-
420 VmpB. The purification of each protein was monitored by sodium dodecyl sulfate-
421 polyacrylamide gel electrophoresis (SDS PAGE), and Western blotting was applied with anti-
422 FD monoclonal antibodies provided by the Sediag Company for His₆-VmpA/VmpA-His₆ and
423 with the anti-Histidine antibodies (Sigma) for His₆-VmpB. Rabbit polyclonal antibodies
424 (PABs) raised against the His₆-tagged recombinant VmpA (His₆-VmpA) (19) and the His₆-
425 tagged recombinant VmpB (His₆-VmpB) were produced by Covalab (Villeurbanne, France).

426

427 **Western immunoblotting and dot blot immunoassay**

428 Immunoblotting analysis of spiroplasmal proteins has been previously described (53).
429 Briefly, spiroplasmas were pelleted from 20 mL cultures by centrifugation at 25,000 g for 20
430 min and washed twice in HEPES-sucrose (HS) buffer (8 mM HEPES [pH 7.4] and 280 mM
431 sucrose). Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad,
432 Hercules, CA, USA). Protein preparations were mixed with one volume of 2× Laemmli
433 solubilization buffer and solubilized by heating at 80°C for 20 min. The preparation was
434 stored at -20°C until use or directly separated by 10% SDS-PAGE, and then, the proteins
435 were electro-transferred to a nitrocellulose membrane. For dot blotting, spiroplasmas from 2
436 mL culture were spotted onto nitrocellulose membrane after two washes in HEPES-sucrose
437 (HS) buffer. The presence of VmpA was revealed using anti-His₆-VmpA PABs (1:5000
438 dilution), goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and NBT-BCIP
439 (Sigmafast™, Sigma-Aldrich, St Louis, MO, USA) as the substrate.

440

441 **Coating of fluorescent beads**

442 The yellow-green fluorescent and amine-modified beads (4×10^9 beads at 1 μm)
443 (Invitrogen) were covalently coated with 10 nmol of a mix of recombinant VmpA-His₆ and
444 GFP or BSA, according to the supplier's instructions. The relative quantity of VmpA-His₆,
445 GFP and BSA varied according the experiments and are indicated under the graphs. The
446 coating of the beads was verified by immunofluorescence. VmpA-His₆-coated beads were
447 incubated with anti-VmpA PAbs diluted 1:500 in PBS-BSA solution (PBS containing 1%
448 BSA) for 30 min; after 3 washes with PBS, the beads were incubated for 30 min with Alexa
449 633-conjugated goat anti-rabbit antibodies (Invitrogen) diluted at 1:200. The beads were
450 included in the anti-fading ProLong Gold Reagent (Invitrogen), mounted with cover slips and
451 imaged using a TCS SP2 upright Leica confocal laser scanning microscope (CLSM), with a
452 63x oil immersion objective lens with a pixel size of 70 nm. Fluorochromes were detected
453 sequentially frame by frame. The coating of fluorescent beads was also verified for VmpA
454 and BSA by measuring the remaining uncoated proteins using the Bradford procedure.

455

456 **Coloration and microscopy of Euva-1 cells, salivary glands and midguts of *Euscelidius***
457 ***variegatus***

458 Euva-1 cells were grown on coverslips in 24-well plates for one day and then washed
459 in PBS. The cells were fixed with 4% paraformaldehyde and incubated with methylene blue
460 (0.1%) for one minute after 3 washes in water or with Alexa 568-Phalloidin (Thermo Fisher
461 Scientific) and DAPI (SIGMA). Samples were mounted with ProLong Gold antifade reagent
462 (Thermo Fisher Scientific) and imaged using a Nikon Eclipse E800 microscope with 40x and
463 20x objective lenses.

464 To infect *E. variegatus* with FD-P, 5th instar nymphs were fed phytoplasma-infected
465 broad bean for one week, corresponding to the acquisition period. The insects were

466 subsequently caged on healthy broad bean for different latency periods in a greenhouse. The
467 intestine and salivary glands were dissected from 10 insects. The organs were fixed with
468 paraformaldehyde 4%, washed in PBS, incubated with a 1:3000 dilution of anti-VmpA rabbit
469 serum (PABs) in PBS-BSA, washed and then incubated with Alexa 488-conjugated goat anti-
470 rabbit IgG (Thermo Fisher Scientific) at a 1:200 dilution. F-actin and nuclei were stained
471 using Alexa 568-Phalloidin (Thermo Fisher Scientific) and DAPI (SIGMA), respectively.
472 Immunofluorescent samples were finally mounted with ProLong Gold antifade reagent
473 (Thermo Fisher Scientific) and imaged using a TCS SP2 upright Leica confocal laser
474 scanning microscope (CLSM) with X40 water immersion and X20 objective lens.

475 For transmission electron microscopy (TEM), the insects were fed in microtubes as
476 described above with caps filled with HEPES-sucrose with or without VmpA-His₆-coated
477 beads (6.6 nmol of recombinant VmpA-His₆ with 3.3 nmol of BSA) for two days and caged
478 on healthy plants for one day. The dissected midguts were fixed in glutaraldehyde, post-fixed
479 in osmium tetroxide, and dehydrated in ethanol, and inclusion was performed in Epon resin as
480 described previously (24, 27). Micrographs were taken at 120 kV on an FEI Tecnai G2 Spirit
481 equipped with an Eagle 4K digital camera (FEI France, Lyon).

482

483 **Euva-1 adhesion assays**

484 Adhesion assays of yellow-green fluorescent and amine-modified beads were
485 performed as previously described (41). Briefly, Euva-1 cells cultivated on coverslips in 24-
486 well plates were incubated with 2×10^6 coated latex beads in Schneider's Drosophila medium
487 for 1 h at 25°C. After three washes, the cells were fixed with 4% paraformaldehyde, and the
488 cell nuclei were stained with $1 \mu\text{gml}^{-1}$ DAPI for 5 min. The samples were mounted in the
489 anti-fading ProLong Gold Reagent (Thermo Fisher Scientific), and immunofluorescent
490 samples were analyzed with a fluorescence microscope (Nikon Eclipse E800) at 40×

491 magnification. Each experiment was repeated three times independently in triplicate. For each
492 experiment, 20 to 25 fields with approximately 30 cells per field were observed randomly.
493 Counting of beads per cell was performed with the free software package ImageJ
494 (<http://imagej.nih.gov/ij/>). For the relative number of adherent beads per cells, the average of
495 the bead number in the control condition (0 in figures 4 B and C) corresponded to a value of
496 1. The relative values of bead numbers obtained in the different conditions were then
497 readjusted according to this endogenous standard. For the antibody inhibition assay, VmpA-
498 His₆-coated beads were pre-incubated in the presence of various concentrations of anti-His₆-
499 VmpA PABs (1/10 to 1/1 000) or anti-spiralin (54) for 1 h at room temperature. For the
500 competitive assay, Euva-1 cells were pre-incubated for 1 h at 25°C in the presence of various
501 quantities of His₆-VmpA (0.2 to 0.8 nmol) or His₆-VmpB (0.8 nmol). After one wash in PBS,
502 the fluorescent VmpA-His₆-coated beads were added to the Euva-1 cells as above. Each
503 experiment was repeated three times independently in triplicate.

504 The binding of spiroplasmas that express VmpA to Euva-1 cells were determined
505 essentially as described previously in (27). In brief, approximately 10⁵ Euva-1 cells per well
506 were infected with *S. citri* at a multiplicity of infection between 20 and 50 and incubated at
507 30°C for 3 h. The insect cells were trypsinized with TrypLE (Thermo Fisher Scientific) for 5
508 min. Serial dilutions were plated onto SP4 containing 1% noble agar for colony forming units
509 (cfu) counting. To calculate the relative percentage of adherent spiroplasmas, the value 100%
510 corresponded to the average of the adherent *S. citri* G/6/pSTP2 (control condition). Each
511 experiment was performed in four distinct wells and was repeated three times.

512

513 **Ingestion assays**

514 HEPES-sucrose solution (500 µL) containing 10⁵ coated beads was introduced in the
515 cap of 1.5 mL microtubes and closed with parafilm. Three young adults of *E. variegatus* were

516 introduced by tube containing a narrow band of Whatman paper to allow the insects to move
517 up to the cap, and a piece of gauze placed just below the cap to permit the insect to hold on to
518 it and eat. After 2 days at room temperature, to ensure insect adaptation to the artificial
519 feeding system, the 25 to 30 surviving insects (approximately 80%) were transferred into a
520 cage containing 2 broad beans. One, four and seven days later, 20 insects were dissected.
521 Other experiments with insects left two days on broad beans were independently conducted
522 beside the first set of experiments but were shown on the same graph in figure 6. The midguts
523 were fixed for 18 h with 4% paraformaldehyde in PBS containing 0.1% Triton X-100. Alexa
524 568-phalloidin (Invitrogen) was used to stain the actin filaments (diluted 1:200 in PBS-BSA
525 for 1 h) and DAPI (SIGMA) to stain nuclei (for 5 min in water). The organs were mounted in
526 anti-fading ProLong Gold Reagent (Invitrogen), and immunofluorescent samples were
527 imaged using the fluorescence microscope Nikon Eclipse E800. For each experiment,
528 approximately 15 midguts were observed per condition, and the experimentation was repeated
529 three times. Counting of beads per midgut and the determination of the area of midguts were
530 performed with the free software package ImageJ (<http://imagej.nih.gov/ij/>).

531

532 **Statistical analyses**

533 The similarities of deviations between independent experiments were checked with the
534 F-test first. Then, for the purposes of statistical evaluation, Student's t-test was used for
535 comparing two samples, and Student's z-test was used for comparing four samples. The
536 results of the statistical analyses using tests were considered to be significant if their
537 corresponding P values were less than 0.05 (*) and 0.001 (**).

538

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547

548 **References**

- 549 1. Lee I-M, Davis RE, Gundersen-Rindal DE. 2000. Phytoplasma: Phytopathogenic
550 Mollicutes. *Annu Rev Microbiol* 54:221–255.
- 551 2. Sugio A, MacLean AM, Kingdom HN, Grieve VM, Manimekalai R, Hogenhout SA.
552 2011. Diverse Targets of Phytoplasma Effectors: From Plant Development to Defense
553 Against Insects. *Annu Rev Phytopathol* 49:175–195.
- 554 3. Weintraub PG, Beanland L. 2006. Insect Vectors of Phytoplasmas. *Annu Rev Entomol*
555 51:91–111.
- 556 4. Hogenhout SA, Oshima K, Ammar E-D, Kakizawa S, Kingdom HN, Namba S. 2008.
557 Phytoplasmas: bacteria that manipulate plants and insects. *Mol Plant Pathol* 9:403–423.
- 558 5. Schvester D, Carle P, Moutous G. 1961. Sur la transmission de la flavescence dorée des
559 vignes par une cicadelle. *Comptes Rendus Académie Sci* 18:1021–1024.
- 560 6. EFSA Panel on Plant Health (PLH), Jeger M, Bragard C, Caffier D, Candresse T,
561 Chatzivassiliou E, Dehnen-Schmutz K, Gilioli G, Jaques Miret JA, MacLeod A, Navajas

- 562 Navarro M, Niere B, Parnell S, Potting R, Rafoss T, Rossi V, Urek G, Van Bruggen A,
563 Van Der Werf W, West J, Winter S, Bosco D, Foissac X, Strauss G, Hollo G, Mosbach-
564 Schulz O, Grégoire J-C. 2016. Risk to plant health of Flavescence dorée for the EU
565 territory. EFSA J 14:n/a-n/a.
- 566 7. Boudon-Padieu E, Larrue J, Caudwell A. 1989. ELISA and dot-blot detection of
567 flavescence dorée-MLO in individual leafhopper vectors during latency and inoculative
568 state. Curr Microbiol 19:357–364.
- 569 8. Maillet PL, Gouranton J. 1971. Etude du cycle biologique du mycoplasme de la
570 phyllodie du trèfle dans l’insecte vecteur, *Euscelis lineolatus* Brulle (Homoptera,
571 Jassidae). J Microsc.
- 572 9. Gouranton J, Maillet P. 1973. High-Resolution Autoradiography of Mycoplasma-like
573 Organisms Multiplying. J Invertebr Pathol 21:158–163.
- 574 10. Lefol C, Lherminier J, Boudonpadieu E, Larrue J, Louis C, Caudwell A. 1994.
575 Propagation of Flavescence Doree Mlo (mycoplasma-Like Organism) in the Leafhopper
576 Vector *Euscelidius-Variegatus* Kbm. J Invertebr Pathol 63:285–293.
- 577 11. Bressan A, Girolami V, Boudon-Padieu E. 2005. Reduced fitness of the leafhopper
578 vector *Scaphoideus titanus* exposed to Flavescence doree phytoplasma. Entomol Exp
579 Appl 115:283–290.
- 580 12. Fletcher J, Wayadande A, Melcher U, Ye F. 1998. The phytopathogenic mollicute-insect
581 vector interface: A closer look RID E-7160-2010. Phytopathology 88:1351–1358.
- 582 13. Liu H-Y. 1983. The Relationship of *Spiroplasma citri* and *Circulifer tenellus*.
583 Phytopathology 73:585.

- 584 14. Kakizawa S, Oshima K, Nishigawa H, Jung H-Y, Wei W, Suzuki S, Tanaka M, Miyata
585 S, Ugaki M, Namba S. 2004. Secretion of immunodominant membrane protein from
586 onion yellows phytoplasma through the Sec protein-translocation system in *Escherichia*
587 *coli*. *Microbiol Read Engl* 150:135–142.
- 588 15. Suzuki S, Oshima K, Kakizawa S, Arashida R, Jung H-Y, Yamaji Y, Nishigawa H,
589 Ugaki M, Namba S. 2006. Interaction between the membrane protein of a pathogen and
590 insect microfilament complex determines insect-vector specificity. *Proc Natl Acad Sci U*
591 *S A* 103:4252–4257.
- 592 16. Galetto L, Bosco D, Balestrini R, Genre A, Fletcher J, Marzachì C. 2011. The major
593 antigenic membrane protein of “*Candidatus Phytoplasma asteris*” selectively interacts
594 with ATP synthase and actin of leafhopper vectors. *PloS One* 6:e22571.
- 595 17. Rashidi M, Galetto L, Bosco D, Bulgarelli A, Vallino M, Veratti F, Marzachì C. 2015.
596 Role of the major antigenic membrane protein in phytoplasma transmission by two
597 insect vector species. *BMC Microbiol* 15:193.
- 598 18. Cimerman A, Pacifico D, Salar P, Marzachì C, Foissac X. 2009. Striking Diversity of
599 *vmp1*, a Variable Gene Encoding a Putative Membrane Protein of the Stolbur
600 Phytoplasma. *Appl Environ Microbiol* 75:2951–2957.
- 601 19. Renaudin J, Béven L, Batailler B, Duret S, Desqué D, Arricau-Bouvery N, Malembic-
602 Maher S, Foissac X. 2015. Heterologous expression and processing of the flavescence
603 dorée phytoplasma variable membrane protein *VmpA* in *Spiroplasma citri*. *BMC*
604 *Microbiol* 15:82.

- 605 20. Carle P, Malembic-Maher S, Arricau-Bouvery N, Desqué D, Eveillard S, Carrere S,
606 Foissac X. 2011. Flavescence doree' phytoplasma genome: a metabolism oriented
607 towards glycolysis and protein degradation. *Bull Insectology* 64:S13–S14.
- 608 21. Dramsi S, Dehoux P, Cossart P. 1993. Common features of Gram-positive bacterial
609 proteins involved in cell recognition. *Mol Microbiol* 9:1119–1122.
- 610 22. Yu J, Wayadande AC, Fletcher J. 2000. Spiroplasma citri Surface Protein P89
611 Implicated in Adhesion to Cells of the Vector *Circulifer tenellus*. *Phytopathology*
612 90:716–722.
- 613 23. Berg M, Melcher U, Fletcher J. 2001. Characterization of Spiroplasma citri adhesion
614 related protein SARP1, which contains a domain of a novel family designated sarpin.
615 *Gene* 275:57–64.
- 616 24. Béven L, Duret S, Batailler B, Dubrana M-P, Saillard C, Renaudin J, Arricau-Bouvery
617 N. 2012. The repetitive domain of ScARP3d triggers entry of Spiroplasma citri into
618 cultured cells of the vector *Circulifer haematoceps*. *PLoS ONE* 7:e48606.
- 619 25. Glew MD, Papazisi L, Poumarat F, Bergonier D, Rosengarten R, Citti C. 2000.
620 Characterization of a multigene family undergoing high-frequency DNA rearrangements
621 and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. *Infect*
622 *Immun* 68:4539–4548.
- 623 26. Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM. 2002.
624 Characterization of P40, a Cytadhesin of *Mycoplasma agalactiae*. *Infect Immun*
625 70:5612–5621.

- 626 27. Duret S, Batailler B, Danet J-L, Béven L, Renaudin J, Arricau-Bouvery N. 2010.
627 Infection of the *Circulifer haematoceps* cell line Ciha-1 by *Spiroplasma citri*: the non-
628 insect-transmissible strain 44 is impaired in invasion. *Microbiol Read Engl* 156:1097–
629 1107.
- 630 28. Caudwell A, Larrue J. 1977. La production de cicadelles saines et infectieuses pour les
631 épreuves d'infectivité chez les jaunisses à mollicutes des végétaux. L'élevage de
632 *Euscelidius variegatus* KBM et la ponte sur mousse de polyurethane. *Ann Zool Ecol*
633 *Anim* 9:443–456.
- 634 29. Breton M, Duret S, Danet J-L, Dubrana M-P, Renaudin J. 2010. Sequences essential for
635 transmission of *Spiroplasma citri* by its leafhopper vector, *Circulifer haematoceps*,
636 revealed by plasmid curing and replacement based on incompatibility. *Appl Environ*
637 *Microbiol* 76:3198–3205.
- 638 30. Breton M, Duret S, Arricau-Bouvery N, Béven L, Renaudin J. 2008. Characterizing the
639 replication and stability regions of *Spiroplasma citri* plasmids identifies a novel
640 replication protein and expands the genetic toolbox for plant-pathogenic spiroplasmas.
641 *Microbiol Read Engl* 154:3232–3244.
- 642 31. Saillard C, Carle P, Duret-Nurbel S, Henri R, Killiny N, Carrère S, Gouzy J, Bové J-M,
643 Renaudin J, Foissac X. 2008. The abundant extrachromosomal DNA content of the
644 *Spiroplasma citri* GII3-3X genome. *BMC Genomics* 9:195.
- 645 32. Aiba S, Tsunekawa H, Imanaka T. 1982. New approach to tryptophan production by
646 *Escherichia coli*: genetic manipulation of composite plasmids *in vitro*. *Appl Environ*
647 *Microbiol* 43:289–297.

- 648 33. Rai M, Padh H. 2001. Expression systems for production of heterologous proteins. *Curr*
649 *Sci* 80:1121–1128.
- 650 34. Steen A, Wiederhold E, Gandhi T, Breitling R, Slotboom DJ. 2011. Physiological
651 adaptation of the bacterium *Lactococcus lactis* in response to the production of human
652 CFTR. *Mol Cell Proteomics MCP* 10:M000052MCP200.
- 653 35. Hattab G, Suisse AYT, Ilioaia O, Casiraghi M, Dezi M, Warnet XL, Warschawski DE,
654 Moncoq K, Zoonens M, Miroux B. 2014. Membrane Protein Production in *Escherichia*
655 *coli*: Overview and Protocols, p. 87–106. *In* *Membrane Proteins Production for*
656 *Structural Analysis*. Springer, New York, NY.
- 657 36. Cheung WWK, Purcell AH. 1993. Ultrastructure of the digestive system of the Le
658 fopper *Euscelidius variegatus* Kirshbaum (Homoptera : Cicadellidae), with and without
659 congenital bacterial infections. *Int J Insect Morphol Embryol* 22:49–61.
- 660 37. García González J, Ossamu Tanaka FA, Spotti Lopes JR. 2015. First Findings in the
661 Route of the Maize Bushy Stunt Phytoplasma Within Its Vector *Dalbulus maidis*
662 (Hemiptera: Cicadellidae). *J Econ Entomol*.
- 663 38. Silva CP, Silva JR, Vasconcelos FF, Petretski MDA, Damatta RA, Ribeiro AF, Terra
664 WR. 2004. Occurrence of midgut perimicrovillar membranes in paraneopteran insect
665 orders with comments on their function and evolutionary significance. *Arthropod Struct*
666 *Dev* 33:139–148.
- 667 39. Gutiérrez-Cabrera AE, Córdoba-Aguilar A, Zenteno E, Lowenberger C, Espinoza B.
668 2016. Origin, evolution and function of the hemipteran perimicrovillar membrane with
669 emphasis on Reduviidae that transmit Chagas disease. *Bull Entomol Res* 106:279–291.

- 670 40. Alves CR, Albuquerque-Cunha JM, Mello CB, Garcia ES, Nogueira NF, Bourguignon
671 SC, de Souza W, Azambuja P, Gonzalez MS. 2007. Trypanosoma cruzi: Attachment to
672 perimicrovillar membrane glycoproteins of Rhodnius prolixus. Exp Parasitol 116:44–52.
- 673 41. Duret S, Batailler B, Dubrana M-P, Saillard C, Renaudin J, Béven L, Arricau-Bouvery
674 N. 2014. Invasion of insect cells by Spiroplasma citri involves spiralin relocalization and
675 lectin/glycoconjugate-type interactions. Cell Microbiol 16:1119–1132.
- 676 42. Killiny N, Castroviejo M, Saillard C. 2005. Spiroplasma citri Spiralin Acts In Vitro as a
677 Lectin Binding to Glycoproteins from Its Insect Vector Circulifer haematoceps.
678 Phytopathology 95:541–548.
- 679 43. Peng J, Zhong J, R. Granados R. 1999. A baculovirus enhancin alters the permeability of
680 a mucosal midgut peritrophic matrix from lepidopteran larvae. J Insect Physiol 45:159–
681 166.
- 682 44. Yeats C, Rawlings ND, Bateman A. 2004. The PepSY domain: a regulator of peptidase
683 activity in the microbial environment? Trends Biochem Sci 29:169–172.
- 684 45. Killiny N, Batailler B, Foissac X, Saillard C. 2006. Identification of a Spiroplasma citri
685 hydrophilic protein associated with insect transmissibility. Microbiol Read Engl
686 152:1221–1230.
- 687 46. Caudwell A, Kuszala C, Bachelier JC, Larrue J. 1970. Transmission de la Flavescence
688 dorée de la vigne aux plantes herbacées par l’allongement du temps d’utilisation de la
689 cicadelle Scaphoideus littoralis Ball et l’étude de sa survie sur un grand nombre
690 d’espèces végétales. Ann Phytopathol 1572(Hors série):181–9.

- 691 47. Angelini E, Clair D, Borgo M, Bertaccini A, Boudon-Padieu E. 2001. Flavescence doree
692 in France and Italy - Occurrence of closely related phytoplasma isolates and their near
693 relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. *VITIS -*
694 *J Grapevine Res* 40:79–86.
- 695 48. Caudwell A, Kuszla C, Larrue J, Bachelier JC. 1972. Transmission de la flavescence
696 doree de la feve a la feve par des cicadelles des genres *Euscelis* et *Euscedlidius*:
697 Intervention possible de ces insectes dans l'epidemiologie du bois noir en Bourgogne.
698 *Ann Phytopathol.*
- 699 49. Vignault JC, Bové JM, Saillard C, Vogel R, Faro A, Venegas L, Stemmer W, Aoki S,
700 McCoy R, Al-Beldawi A, Larue M, Tuzcu O, Ozsan M, Nhami A, Abassi M, Bonfils J,
701 Moutous G, Fos A, Poutiers F, Viennot-Bourgin G. 1980. Mise en culture de
702 spiroplasmes à partir de matériel végétal et d'insectes provenant de pays circum
703 méditerranéens et du Proche Orient. *C R Acad Sci Ser III* 290:775–780.
- 704 50. Tully JG, Whitcomb RF, Clark HF, Williamson DL. 1977. Pathogenic mycoplasmas:
705 cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* 195:892–894.
- 706 51. Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, Weighting,
707 and Phylogenetic Utility of Mitochondrial Gene-Sequences and a Compilation of
708 Conserved Polymerase Chain-Reaction Primers. *Ann Entomol Soc Am* 87:651–701.
- 709 52. Bertin S, Picciau L, Ács Z, Alma A, Bosco D. 2010. Molecular identification of the
710 *Hyalesthes* species (Hemiptera: Cixiidae) occurring in vineyard agroecosystems. *Ann*
711 *Appl Biol* 157:435–445.
- 712 53. Duret S, Berho N, Danet J-L, Garnier M, Renaudin J. 2003. Spiralin is not essential for
713 helicity, motility, or pathogenicity but is required for efficient transmission of

714 Spiroplasma citri by its leafhopper vector Circulifer haematoceps. Appl Environ
715 Microbiol 69:6225–6234.

716 54. Wróblewski H, Johansson KE, Hjérten S. 1977. Purification and characterization of
717 spiralin, the main protein of the Spiroplasma citri membrane. Biochim Biophys Acta
718 465:275–289.

719

720 **FIGURE LEGEND**

721 **Figure 1. Confocal micrographs of *Euscelidius variegatus*-infected leafhoppers' midgut**
722 **immunolabeled with anti-VmpA PAbs.** The midguts of leafhoppers that were fed healthy
723 broad bean or FD-P infected broad bean were observed with fluorescent and differential
724 interference contrast (DIC) microscopy. Actin was labeled with Alexa 568-phalloidin (red),
725 nuclei was labeled with DAPI (blue), and FD-P was labeled with anti-His₆-VmpA PAbs and
726 secondary Alexa 488-antibodies (green). Arrows indicate internal phytoplasmas, and
727 arrowheads show the phytoplasmas that were located close to actin filaments.

728

729 **Figure 2. Confocal micrographs of *E. variegatus*-infected leafhoppers' salivary glands**
730 **immunolabeled with anti-VmpA PAbs.** The salivary gland cells of leafhopper fed healthy
731 broad bean or FD-P infected broad bean were observed with fluorescent and differential
732 interference contrast (DIC) microscopy. Actin was labeled with Alexa 568-phalloidin (red),
733 nuclei were stained with DAPI (blue), and FD-P was labeled with anti-His₆-VmpA PAbs and
734 secondary Alexa 488-antibodies (green).

735

736 **Figure 3. Observation of Euva-1 cells cultured from *E. variegatus* embryos by phase**
737 **contrast (A) and epifluorescence (B) microscopy.** (A) Low magnification (x20, a) and high
738 magnification (x40, b) of the Euva-1 monolayer colored with methylene blue. Cells that have

739 their nucleus colored by methylene blue are indicated by red asterisks. Cells with both the
740 nucleus and cytosol colored by methylene blue are indicated by arrows for the larger ones or
741 arrowheads for the smaller ones. (B) Cellular actin was labeled with Alexa 568-phalloidin
742 (green), and nuclei were stained with DAPI (blue). (A) and (B) same magnification; scale bar,
743 50 μm (a) and 20 μm (b).

744

745 **Figure 4. Adhesion of VmpA-His₆-coated fluorescent beads to Euva-1 cells.** (A)
746 Observation of fluorescent VmpA-coated beads adherent to Euva-1 cells. (a) Fluorescent
747 VmpA-coated beads (green), (b) nuclei were stained with DAPI (light blue), (c) overlay, and
748 (d) same view by differential interference contrast microscopy. Scale bar 20 μm . (B).
749 Fluorescent beads were coated with different amounts of VmpA-His₆ and GFP before coming
750 in contact with insect cells in culture. ** and * indicates significant differences compared to
751 beads coated with 0 nmol of VmpA-His₆ and 10 nmol of GFP (Student's test, P<0.01 and
752 P<0.05, respectively). (C) The fluorescent beads coated with VmpA-His₆ were pre-incubated
753 with rabbit serum anti-His₆-VmpA (gray bars) or anti-spiralin (control, white bars) at the
754 indicated dilutions. * indicates a significant difference compared to non-treated beads (0)
755 (Student's test, P<0.05). (D) Euva-1 cells were pre-incubated with the recombinant protein
756 His₆-VmpA (gray bar) or His₆-VmpB (white bar) at the indicated concentrations. *,
757 significantly different from Euva-1 pre-incubated with medium alone (0) (Student's test,
758 P<0.05).

759

760 **Figure 5. Expression of VmpA by *Spiroplasma citri* and the adhesion of VmpA-**
761 **expressing *S. citri* to Euva-1 cells.** (A) Plasmid extractions and restriction with *Hind*III
762 enzyme (plasmid profile), PCR amplification of the *vmpA* gene and Western immunoblotting
763 of proteins from *S. citri* transformants. Sub-clones of the two clones 5 and 6 of VmpA

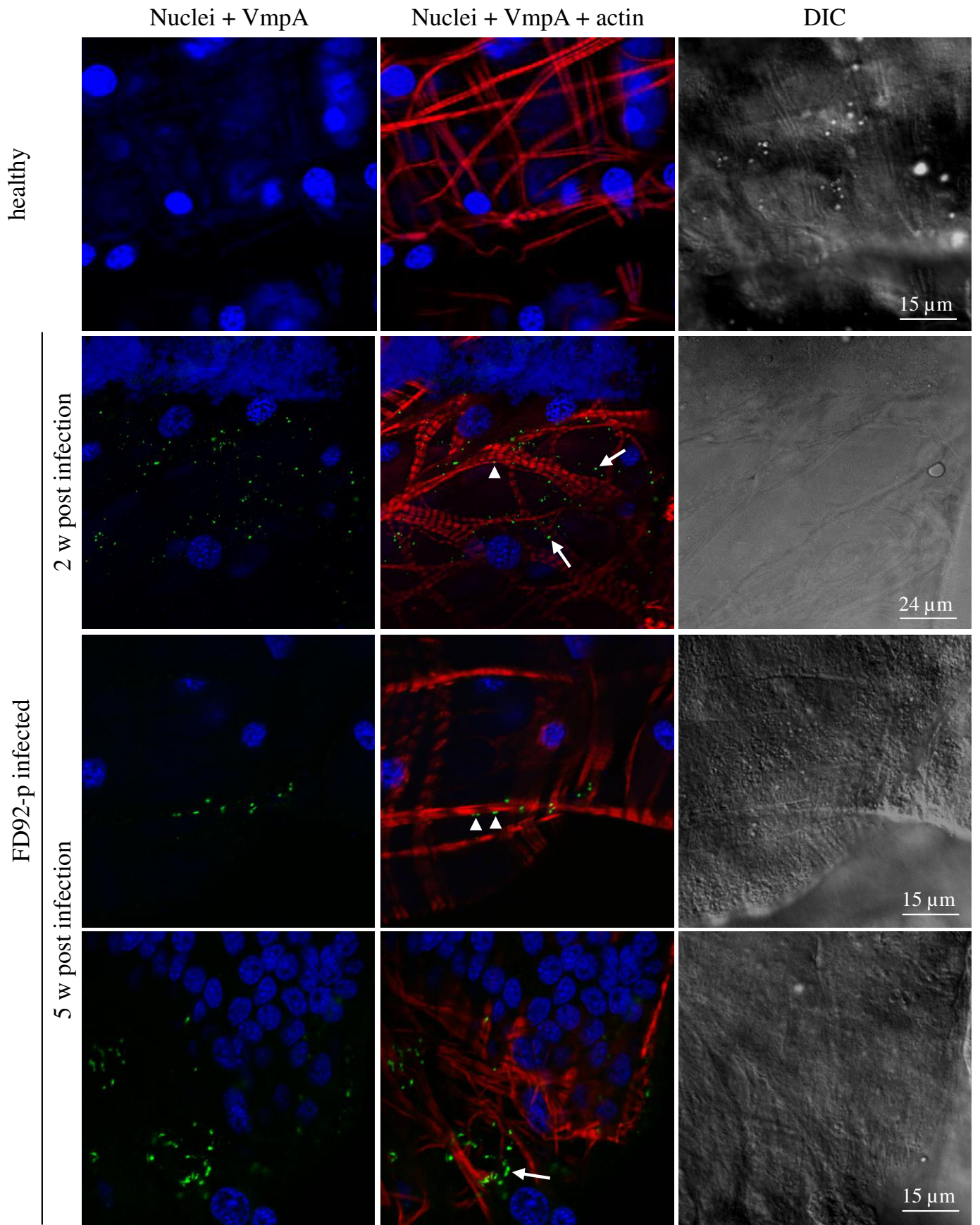
764 expressing *S. citri* were cultivated without tetracycline (-) or in the presence of tetracycline
765 (+) for 5 passages prior to extractions. The blot was probed with a 1:5000 dilution of anti-
766 His₆-VmpA rabbit serum. Lane 1 kb +: 1 kb Plus DNA ladder; lane +: plasmid pSTVA1; lane
767 MW: molecular weight in kDa; lane rVmpA: recombinant protein VmpA lacking the C-
768 terminal transmembrane segment. (B) The Adhesion of *S. citri* transformants to Euva-1 cells.
769 The 100% relative corresponds to the condition of cell adhesion with *S. citri* G/6 strain
770 carrying the plasmid pSTP2. **, significantly different from Euva-1 infected with *S. citri*
771 G/6/pSTP2 devoid of VmpA (Student's test, P<0.001).

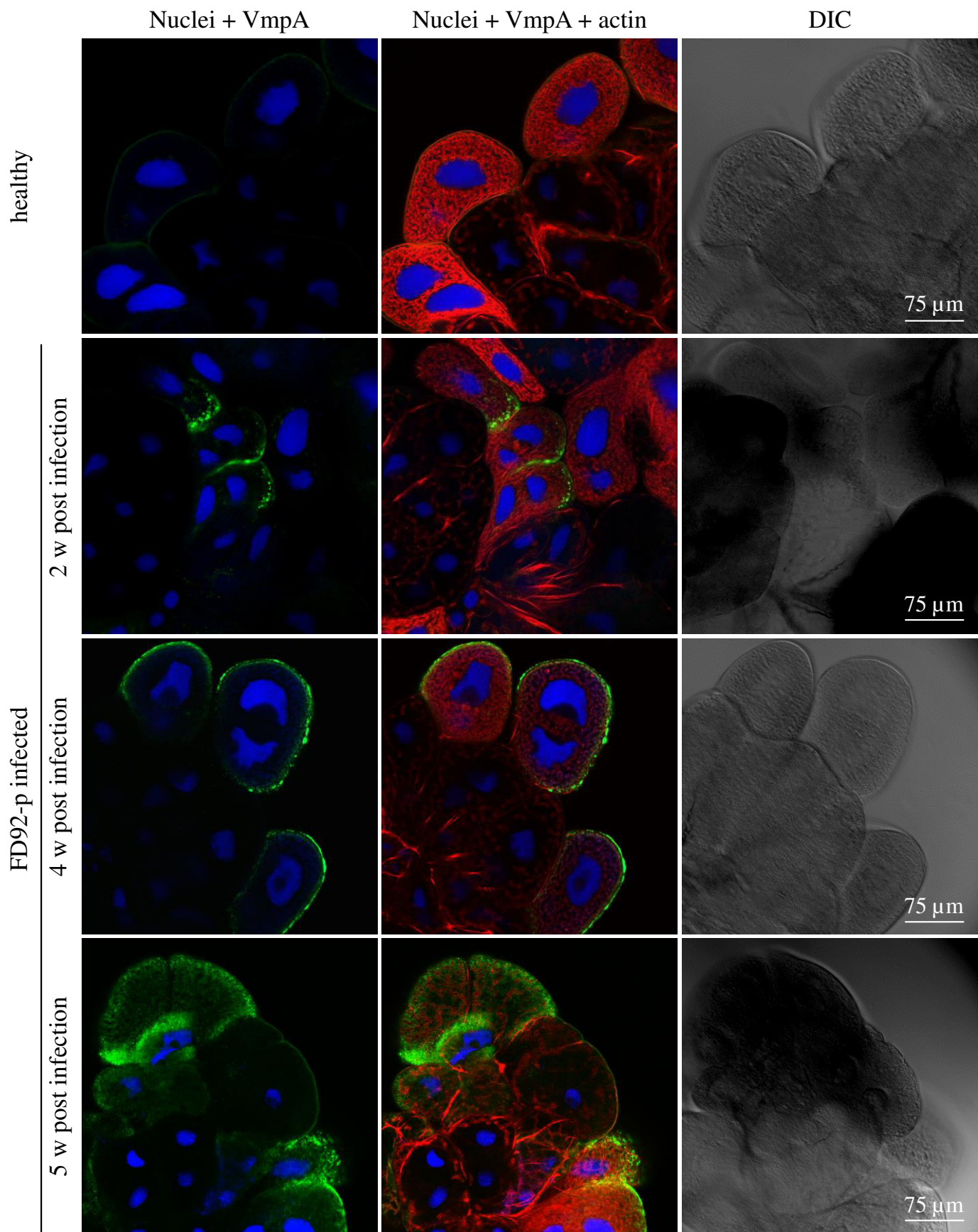
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773 **Figure 6. Number of VmpA-His₆-coated latex beads in *E. variegatus* midguts after**
774 **ingestion and fluorescent observations.** (A) The presence of fluorescent beads coated with
775 VmpA-His₆ in *E. variegatus* midgut after the ingestion of beads for two days. Low
776 magnification (x4) by fluorescence microscopy (a) showing the actin fluorescence (red, Alexa
777 568-phalloidin) of intestine cells and fluorescent beads (green) and by phase-contrast
778 microscopy overlaid with fluorescent image of beads (b). amg, anterior midgut; mmg, middle
779 midgut; pmg, posterior midgut; fc, filter chamber; mt, Malpighian tubules. (c, d and e) Higher
780 magnification showing VmpA-His₆-coated latex beads (green) (c), actin (red) and nuclei
781 (blue, DAPI) (d), and the overlay of green VmpA-His₆-coated latex beads and intestine tube
782 viewed by phase-contrast microscopy (e). (B) The number of VmpA-His₆-coated beads in the
783 *E. variegatus* middle midgut. After feeding HEPES-sucrose with BSA-coated beads (white
784 circles) or with VmpA-His₆-coated beads (black and gray circles), *E. variegatus* insects were
785 maintained one, two, four or seven days on broad bean before their intestines were dissected.
786 These assays were performed in three independent experiments, and 36 to 70 insects per
787 group were examined. Different lowercase letters above sets indicate statistically significant
788 differences calculated separately for each time on broad bean (Student's test, P<0.05).

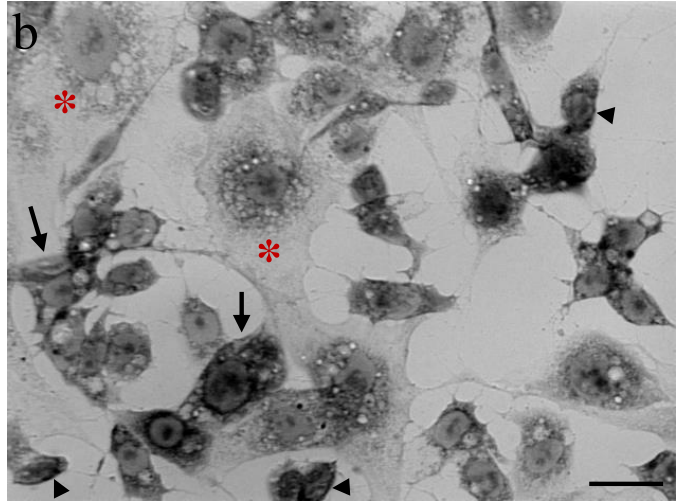
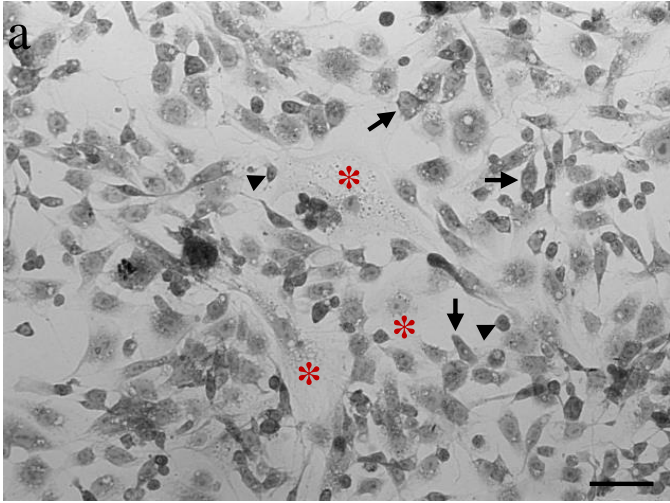
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790 **Figure 7. Transmission electron microscopy images of the dissected midgut of *E.***
791 ***variegatus* that have ingested VmpA-H₆-coated fluorescent beads.** (A) The anterior (a) and
792 middle (b and c) midgut of one healthy insect. (c) shows the high magnification of the boxed
793 part of (b). (B and C) A section of the anterior (B) and middle (C) midgut of an insect that
794 was fed VmpA-His6-coated beads in HEPES-sucrose for two days. (B b) and (B c) show
795 different magnifications of the boxed part of (B a). (C b) and (C c) represent higher
796 magnifications of the boxed parts of (C a). Beads are shown with asterisks and bacteria with
797 arrowheads. L, lumen; mv, microvillosity; pmm, perimicrovillar membrane.

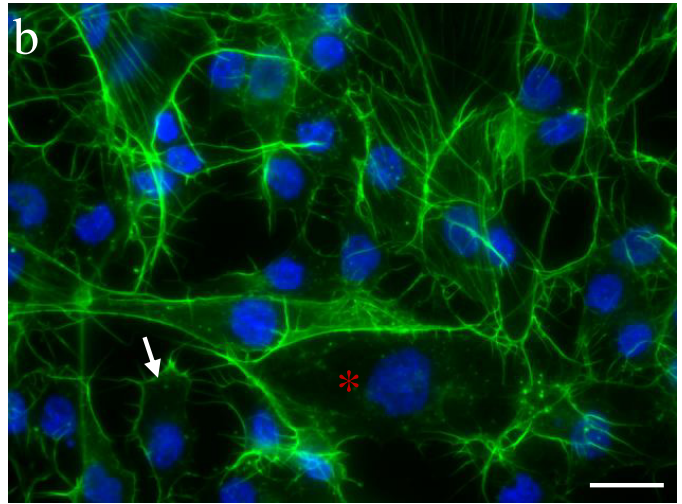
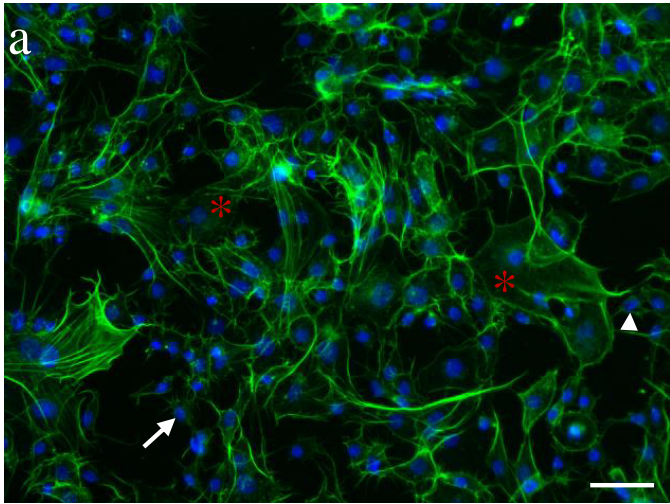


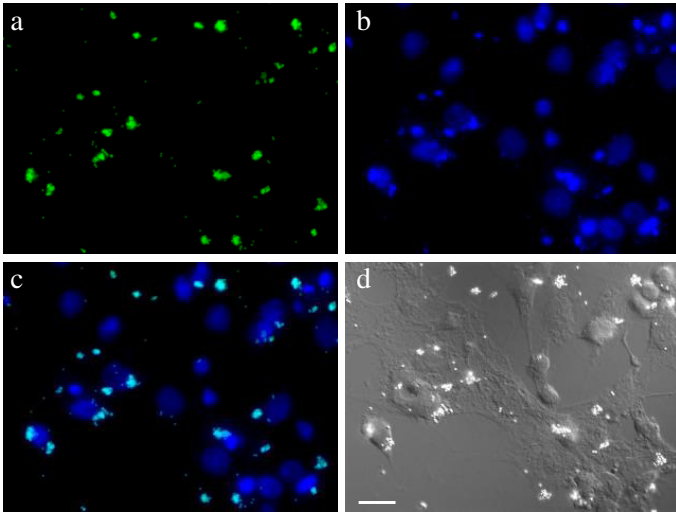
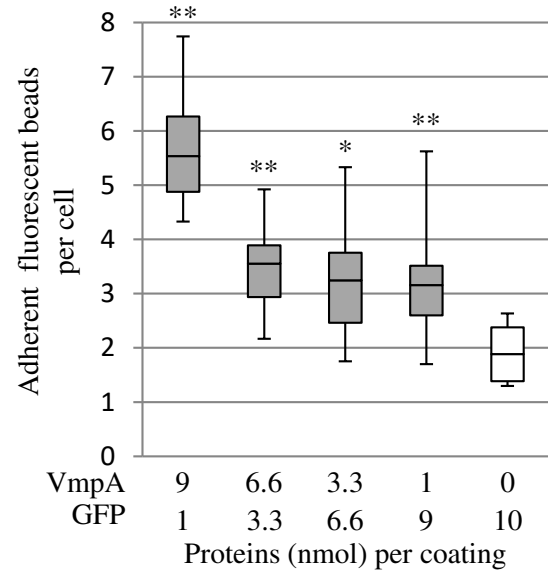
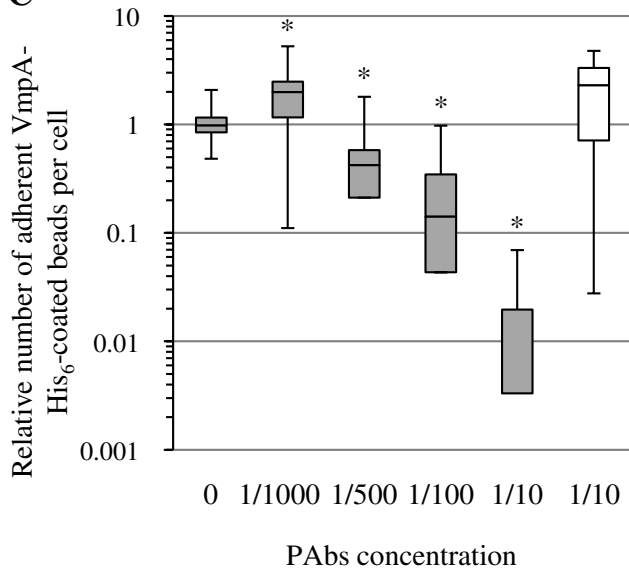
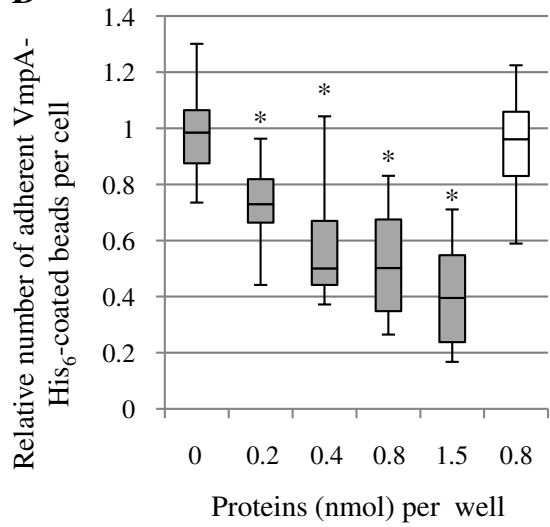


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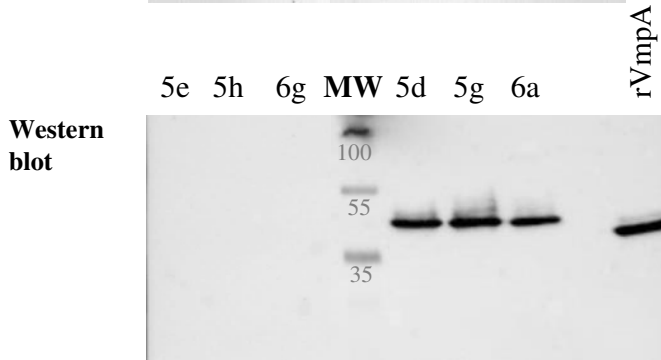
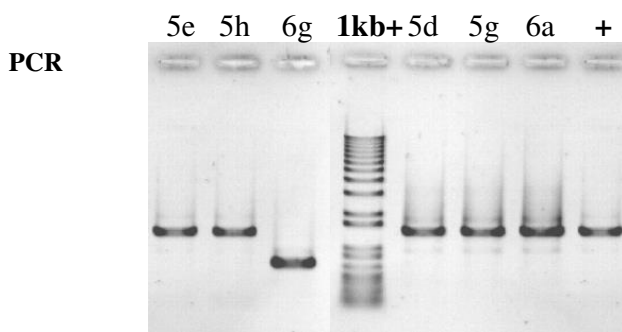
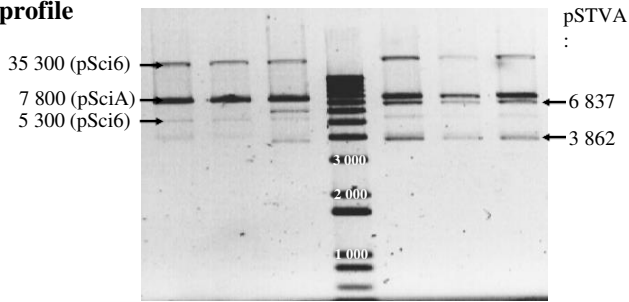
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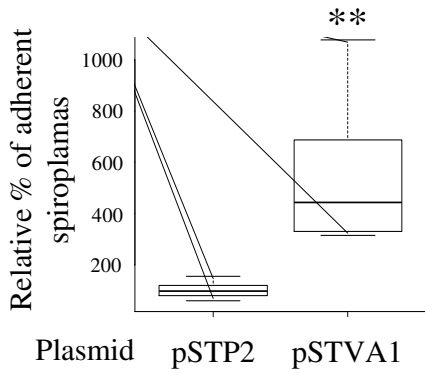
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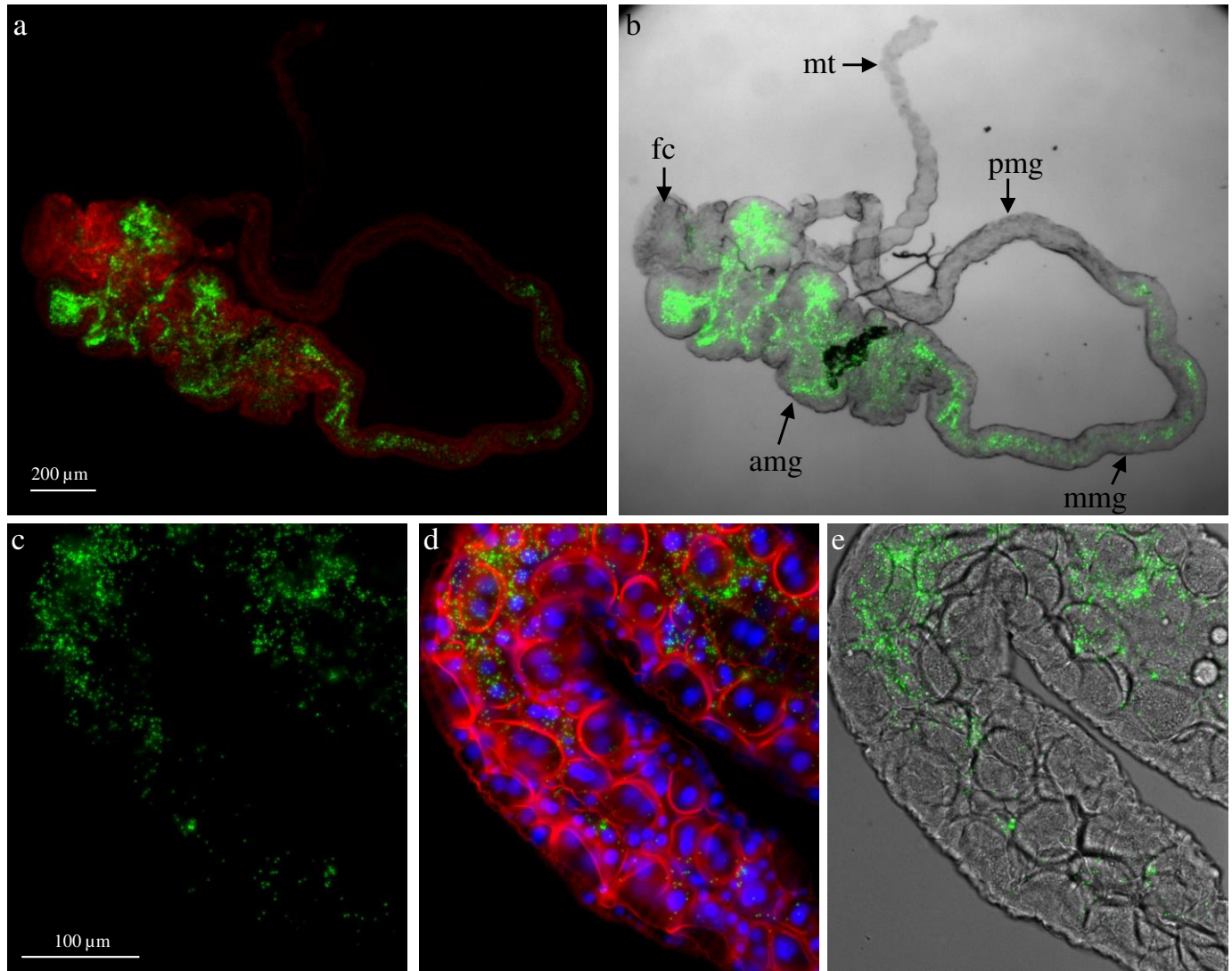
Antibiotic - - - + + +
 Sub-clone 5e 5h 6g 1kb+ 5d 5g 6a
Plasmid profile



B



A



B

