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Asaia symbionts interfere with infection by Flavescence dorée phytoplasma in leafhoppers

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1 **_Asaia_ symbionts interfere with infection by Flavescence dorée phytoplasma in leafhoppers**

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15

16 ## Abstract

17 The transmission of microbial pathogens by insect vectors can be affected by the insect's microbial
18 symbionts, which may compete in colonizing organs, express antagonistic factors or activate host
19 immune response. Acetic acid bacteria of the genus *Asaia* are symbionts of the leafhopper
20 *Scaphoideus titanus*, which transmits Flavescence dorée phytoplasma. These bacteria could be used
21 as control agents against the disease. Here, we experimentally investigated the interaction between
22 different strains of *Asaia* and phytoplasma transmission in the laboratory by using the model
23 leafhopper *Euscelidius variegatus* and the plant host *Vicia faba*. We found that uncultivable and low
24 concentrations of *Asaia* phylotypes were associated with *E. variegatus*. When we supplied different
25 *Asaia* strains isolated from other insects and exhibiting different phenotypes to *E. variegatus* orally,
26 the bacteria stably colonized the leafhopper, reached relatively higher densities and could then be
27 isolated from the host. We conducted transmission trials of Flavescence dorée phytoplasma with
28 individuals colonized with three exogenous *Asaia* strains. When the phytoplasma became established
29 in the bodies of *E. variegatus*, leafhoppers were able to transmit it to broad beans, with transmission
30 rates ranging from 33 to 76% in different experiments. However, leafhoppers that were colonized by
31 one of *Asaia* strains producing an air-liquid interface biofilm exhibited significantly reduced
32 phytoplasma acquisition, with infection rates at 5 to 28%, whereas they were 25 to 77% in control
33 insects. Although the mechanisms regulating this interference remain to be elucidated, our results
34 provide evidence of the potential use of *Asaia* as a biocontrol agent.

35

36 ## Key words

37 Flavescence dorée, Acetic acid bacteria, vector competence, symbiotic control

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Key messages

- The reduction of the vector competence of insects to impede the spread of vectored plant pathogens is a promising control strategy. The potential of microbial symbionts in insects to reduce such competence has been poorly explored in phytoplasma vectors, however.
- A strain in the genus *Asaia,* which is a symbiont of leafhopper vectors of phytoplasmas, was found to reduce infection by Flavescence dorée phytoplasma in the insect model *Euscelidius variegatus* under laboratory conditions.
- This work provides evidence of the potential for *Asaia* to be used as a biocontrol agent capable of reducing phytoplasma infection in leafhopper vectors.

Author contributions

EG, EC, AA, DD conceived and designed research. EG, EC, MM made experiments. AA, DD, MM provided reagents and analytical tools. EG analyzed data. EG, EC wrote manuscript. All authors read and approved.

57 # Introduction

58 Many insects carry and transmit microbial pathogens to their animal and plant hosts. The transmission
59 of such pathogens is usually carried out during the feeding process, often requiring incubation for
60 multiplication in the salivary glands. Stable colonization of the vector by the pathogen is necessary
61 for transmission and is successfully completed in only a subset of individuals in an insect population
62 (Weiss and Aksoy 2011; Galetto et al. 2011; Krugner et al. 2012; Ammar et al. 2016). Imperfect
63 vector competence is regulated by the ingested load of the pathogen, the insect-pathogen genotype
64 interaction, and many environmental factors (Tabachnick 2015). Other factors that limit pathogen
65 spread include the presence of organ-specific barriers in the host, insufficient host survival rates, or
66 the immune response of the insects (Alto et al. 2005; Galetto et al. 2009; Westbrook et al. 2010; Weiss
67 and Aksoy 2011, Mandrioli et al. 2015; Shapiro et al. 2016). Key to the transmission process are the
68 digestive tract and the salivary glands of the insect. In the midgut, interactions with the peritrophic
69 matrix and gut epithelia have been found to be crucial for microbial invasion of the insect's hemocoel
70 (Killiny et al. 2005; Lo et al. 2013; Rashidi et al. 2015; Buchon et al. 2016; Malta et al. 2016).
71 Insects are inhabited by a multitude of microbial symbionts capable of establishing variable beneficial
72 (mutualist) and neutral (commensal) interactions with their hosts. The microbial community hosted
73 by insects may influence their competence in transmitting pathogens as vectors (Weiss and Aksoy
74 2011; Kramer and Ciota 2015; Trivedi et al. 2016) through competitive exclusion, anti-pathogen
75 activity or immunomodulation. For example, both negative and positive correlations between various
76 symbiotic bacteria and the plant pathogen '*Candidatus* Liberibacter asiaticus' have been documented
77 in *Diaphorina citri* Kuwayama (Fagen et al. 2012); infection with Palm Creek virus has been shown
78 to control the presence of the West Nile virus in *Culex annulirostri*s Skuse (Hall-Mendelin et al.
79 2016). The manipulation of the microbial communities associated with pathogen vectors has been
80 proposed to improve the insect's performance related to interesting phenotypes (Mueller and Sachs
81 2015), possibly driving the evolution of the vector in the direction of reduced vector competence,

82 similarly to techniques designed for pathogen control based on genetic shifting (Powell and
83 Tabachnick 2014).
84 Phytoplasmas are pathogenic bacteria in plants belonging to the class Mollicutes. They are
85 transmitted from plant to plant by insect vectors, namely leafhoppers, planthoppers, and psyllids
86 (Maejima et al. 2014). More than 1,000 plant diseases are activated by phytoplasmas, causing losses
87 among economically important species worldwide (Maejima et al. 2014; Perilla-Henao et al. 2016).
88 One of the most bothersome phytoplasma-related diseases in Europe is Flavescence dorée (FD),
89 which affects grapevines. The symptoms associated with this disease can be severe and even lead to
90 the death of infected plants, causing extensive economic loss (Chuche and Thiéry, 2014). Based on
91 the analysis of 16SrRNA gene, the FD agents are included in two subgroups of 16SrV phytoplasma
92 group, namely subgroups –C and –D, both widespread in the mainly affected countries (Davis and
93 Delly, 2001; Martini et al., 2002). In nature, the vector of FD phytoplasmas (FDp) is the leafhopper
94 *Scaphoideus titanus* Ball. Because rearing the monovoltine *S. titanus* and maintaining FDp in
95 grapevines are both challenging under laboratory conditions, the cicadellid *Euscelidius variegatus*
96 Kirschbaum is commonly used as an experimental vector in the broad bean *Vicia faba* L. in the
97 laboratory (Bressan et al. 2005).
98 Even though the microbial communities of many phytoplasma vectors have been previously
99 described (Marzorati et al. 2006; Gonella et al. 2011; Wangkeeree et al. 2011, 2012; Iasur-Kruh et al.
100 2013; Ishii et al. 2013; Powell et al. 2015), little information on the influence of the symbiotic
101 microbiome on competence of the leafhopper vector of phytoplasmas is currently available (Trivedi
102 et al. 2016) despite the relevance to worldwide agriculture of these pathogens. Interactions between
103 symbiotic bacteria and phytoplasma inside the insects have been proposed (Ishii et al. 2013) but not
104 yet demonstrated. In *S. titanus*, symbiotic Acetic Acid Bacteria (AAB) of the genus *Asaia* have been
105 identified and proposed as potential symbiotic control candidates (Crotti et al. 2009). Indeed, this
106 symbiotic bacterium was reported to be dominant in the leafhopper and to be vertically and
107 horizontally transmitted; moreover *Asaia* is easy to cultivate and transform and it efficiently colonizes

108 various phylogenetically distant hosts (Crotti et al. 2009). *Asaia* strains with different phenotypic
109 traits have been isolated from plant tissues and have been frequently found in association with
110 different insect orders, including Hymenoptera, Diptera and Hemiptera (Crotti et al. 2010). Some of
111 these strains have been shown to inhibit the diffusion of other organisms, including vector-borne
112 pathogenic agents (Hughes et al. 2014; Sant'Anna et al. 2014). However, the potential of insect-
113 associated *Asaia* to inhibit FD transmission in insect vectors has never been tested.
114 Here, we evaluated the potential of different *Asaia* strains to interfere with the vector competence of
115 leafhoppers for FDp in the laboratory. For this purpose, we used the experimental vector *E. variegatus*
116 and the broad bean *Vicia faba* L. (Salar et al. 2013).

117

118 Materials and methods

119 Insect and plant material and bacterial strains

120 *E. variegatus* individuals used in this work were from a laboratory line reared on oat (*Avena sativa*
121 L.) in growth chambers at 25°C with a photoperiod of 16:8 (L:D) at the DISAFA laboratories. Broad
122 beans (*Vicia faba* L.) infected by FDp were obtained by exposing seedlings to *E. variegatus*
123 specimens infected by FDp (strain FD-C). Healthy broad bean and oat seedlings were grown in screen
124 houses that were separated from the insect rearing space and other phytoplasma sources.
125 *Asaia* colonization experiments in *E. variegatus* were performed using spontaneous rifampicin-
126 resistant mutants of *Asaia* SF2.1 (Favia et al., 2007), *Asaia* AE6.5 (Crotti et al., 2009) and *Asaia*
127 SF15.14. The latter was isolated from a female *Anopheles stephensi* Liston mosquito derived from a
128 colony reared in the insectary at the University of Camerino since 1988. Briefly, following a surface
129 sterilization procedure consisting of three washings with 0.9% NaCl, mosquitoes were homogenated
130 in 200 ml 0.9% NaCl. Twenty microlitres of the homogenate were inoculated into an enrichment
131 medium at pH 3.5, followed by plating on a $CaCO_3$-rich agarized medium, as reported by Favia et al.
132 (2007). The 16S rRNA gene of the SF15.14 strain was amplified using the 27F and 1495R primers

133 (Lane 1991), as described by Mapelli et al. (2013). The nearly full-length sequence was deposited in
134 the European Nucleotide Archive's database under accession number LT838398. Spontaneous
135 rifampicin-resistant mutants of *Asaia* were obtained by exposing well-grown cultures of *Asaia* strains
136 (SF2.1, AE6.5 and SF15.14) to 100 μg/ml rifampicin overnight. This process generated the SF2.1
137 Rif^R, AE6.5 Rif^R and SF15.14 Rif^R strains, respectively.
138 To evaluate the capability of the bacterial strains to produce an air-liquid interface (ALI) biofilm,
139 static cultures of the three *Asaia* strains were grown in tubes with 5 ml of modified potato dextrose
140 broth (PDB), containing 20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, 8 g/L potato infusion,
141 with pH 6. After overnight growth, 100 μl of bacterial cultures (diluted to OD 1) were inoculated into
142 the tubes and incubated at 30°C for 10-12 days.
143
144 **Assessment of *Asaia* infection in *E. variegatus***
145 To verify whether our population of *E. variegatus* was naturally infected by *Asaia*, 30 adults were
146 collected (15 males and 15 females) and submitted to the following molecular diagnostic analysis.
147 After DNA extraction (according to Gonella et al. 2012), *Asaia*-specific PCR was performed with
148 the Asafor/Asarev primer pair (Crotti et al. 2009). The sequence of the 16S rRNA of the *Asaia* strain
149 from all positive *E. variegatus* was extended with two additional specific PCRs to amplify the
150 flanking regions at the 5′ and 3′ ends of the fragment for *Asaia*-specific amplification. We used the
151 *Asaia*-specific primer Asafor in combination with the universal reverse primer 1495R and Asarev
152 with the universal forward primer 27F as described previously (Mapelli et al. 2013). The amplicons
153 were sequenced in both directions. The final sequence, obtained by assembling the two 16S rRNA
154 gene contigs, was used to analyse the phylogenetic position of the isolate. A phylogenetic tree was
155 constructed based on the maximum likelihood method with the software MEGA 7. Additionally, the
156 near full-length sequence of *Asaia* 16S rRNA gene from one specimen was deposited in the European
157 Nucleotide Archive's database under accession number LT838399.

158 Ten adult leafhoppers were used in attempts to isolate the *Asaia* bacteria. Insect surface sterilization
159 was performed by rinsing the insects with 1% sodium hypochlorite for one minute, followed by
160 washing with 0.9% NaCl for five minutes and a final rinse in 0.9% NaCl. Four single or two pooled
161 (3 insects in each pool) individuals were homogenized in 200 µl of 0.9% NaCl. One-hundred µl of
162 the homogenates and their serial dilutions were plated on mannitol agar (MAN) plates (Vacchini et
163 al. 2017), combined with 100 µg/ml cycloheximide and incubated at 30°C. Furthermore, 20 µl of the
164 homogenates were inoculated in TA1, YE and ABEM liquid media (Vacchini et al. 2017; Favia et
165 al., 2007), combined with 100 µg/ml cycloheximide and incubated at 30°C under aerobic conditions
166 with shaking, until turbidity in the medium was observed. Serial dilutions were then plated on MA
167 (1% D-glucose, 1% glycerol, 1% bactopeptone, 0.5% yeast extract, 0.7% $CaCO_3$, 1% ethanol, 1.5%
168 agar, pH 6.8) or ABEM (2% D-glucose, 0.5% ethanol, 0.8% yeast extract, 0.7% $CaCO_3$, 1.2% agar,
169 pH 7) agar plates as described by Vacchini et al. (2017) and Favia et al. (2007). Based on morphology,
170 several colonies were purified on agarized media, and pure strains were stored in 15% glycerol at -
171 80°C. Dereplication of the isolates was performed by comparing the isolates' Intergenic Transcribed
172 Spacer (ITS)-PCR profiles (Vacchini et al. 2017). From bacterial representatives for each ITS profile
173 group, a partial fragment of 16S rRNA gene was amplified (Vacchini et al., 2017); amplicons were
174 sequenced at Eurofin Genomics (Vimodrome, Milano). The partial 16S rRNA gene sequences from
175 representatives of each ITS group were deposited in the European Nucleotide Archive's database
176 under the accession numbers LT934417-LT934420 under the study ID PRJEB22871
177 (http://www.ebi.ac.uk/ena/data/view/LT934417-LT934420).
178
179 ***Asaia* colonization of *E. variegatus***
180 To evaluate the capability of *Asaia* isolates to colonize *E. variegatus* stably, we provided the isolates
181 to third instar nymphs following procedures described by Crotti et al. (2009). Briefly, cells were
182 grown at 30°C under the selection of rifampicin, harvested by centrifugation (10 min, 3000 *g*), washed
183 three times with 0.9% NaCl and adjusted to 10^8 cells/ml in 5% (w/v) sucrose solution in Tris-EDTA

184 at pH 8. Cell suspensions were provided to the nymphs and they were allowed to feed for 48 hours.
185 This artificial diet was provided to a total of 810 nymphs: 270 of them were fed with the SF2.1 Rif^R
186 strain, another 270 with the SF15.14 Rif^R strain, and the remaining 270 with the AE6.5 Rif^R strain.
187 An additional 270 specimens were not fed in the presence of any *Asaia* strain and were used as the
188 control. The total number of nymphs corresponding to each treatment was then divided into three
189 groups of 90. The first 90 insects in each group were immediately fed an equivalent, cell-free sugar
190 diet for 24 more hours to avoid possible contamination by transient bacterial cells. The remaining 180
191 nymphs were maintained in plastic cages (24 × 16 × 12 cm) covering FD-infected broad beans for
192 one week. Subsequently, 90 specimens were immediately collected, and the others were reared for a
193 latency access period (LAP) of 21 days on healthy oat plants.
194 At the end of every experimental step, 60 individuals were taken and preserved at -20°C for *Asaia*-
195 specific qPCR (Favia et al. 2007), whereas 30 nymphs were used for reisolation trials. In the
196 reisolation experiments, we took advantage of the rifampicin resistance trait to selectively isolate only
197 the strains that we previously provided to the insect and avoiding possible contamination with native
198 culturable bacterial symbionts. Insects were surface sterilized as described above and homogenized
199 in 900 µl of saline; these suspensions were then plated on MA medium (Vacchini et al. 2017)
200 combined with 100 µg/ml rifampicin. One-hunderd µl of the last saline washing solution used for
201 each treatment was plated in triplicate on a separate plate as a control. Plates were incubated at 30°C
202 for two days; total DNA was extracted from colonies by sodium dodecyl sulfate-proteinase K-
203 cethyltrimethyl ammonium bromide treatment (Sambrook et al., 1989) and stored at –20°C.
204 Intergenic transcribed spacer (ITS)-PCR fingerprinting was performed as described above. ITS-PCR
205 amplification patterns of all the isolates were visually compared to those produced by the pure strains
206 of *Asaia*.
207
208 **FDp transmission trials**

209 FDp transmission trials were set up under laboratory conditions to compare FDp acquisition and
210 transmission efficiencies of *E. variegatus* colonized by different *Asaia* strains (Table 1, Fig. S1). In
211 Experiment 1, the minimum transmission efficiency of *E. variegatus* individuals exposed to the three
212 tested *Asaia* strains was determined. Three groups of 70 third instar nymphs were fed with *Asaia*
213 strains SF2.1 Rif^R, SF15.14 Rif^R, or AE6.5 Rif^R, respectively, as described above; 70 specimens never
214 fed in the presence of any *Asaia* strain were used as a control. Subsequently, insects were reared on
215 FDp-infected broad beans for an acquisition access period (AAP) of one week and then moved to
216 healthy oat seedlings for a LAP of 21 days. At the end of this period, leafhoppers were collected and
217 individually reared on sterile sugar diets for 48 hours. Finally, 60 insects and their diets were collected
218 and preserved at -20°C for molecular analyses. The remaining 10 specimens in each group were
219 subjected to dissection of their midguts and preserved for Fluorescence *In Situ* Hybridization (FISH)
220 analysis (see the FISH section below for a detailed method description).
221 Experiments 2-4 were carried out to specifically compare the FDp transmission efficiency by
222 leafhoppers exposed or not exposed (control) to *Asaia* SF15.14 Rif^R. Groups of 60 individuals were
223 used in each treatment. In Experiment 2, the efficiencies in transmitting FDp to a plant were compared
224 using healthy broad beans as infection recipients; in Experiments 3 and 4, transmission efficiencies
225 when the leafhoppers were subjected to constant pathogen pressure were evaluated. In these three
226 experiments, the steps involving *Asaia* acquisition (not perfomed on individuals in control groups)
227 and FDp acquisition were the same as in Experiment 1. In Experiment 2, LAP was carried out under
228 the same conditions as those of Experiment 1; conversely, Experiments 3 and 4 were conducted with
229 a latency on FDp-infected broad beans. For the final phytoplasma inoculation, insects were singularly
230 maintained on sterile sugar diets for 48 hours (Experiment 3) or on healthy broad bean seedlings for
231 one week (Experiments 2 and 4), before being collected and preserved at -20°C for subsequent
232 analyses. The sugar diets were collected along with the insects for molecular analysis. Broad bean
233 leaf samples were collected after being grown in an insect-proof chamber (25°C, RH 70%) for three
234 weeks after the beginning of phytoplasma inoculation.

235

236 **DNA extraction and PCR-based analyses**

237 At the end of Experiments 1-4, total DNA was extracted from *E. variegatus* individuals and the
238 respective sugar diets or broad bean leaves. DNA extraction from the insects and artificial diets was
239 performed as described by Gonella et al. (2012). Plant DNA was extracted from leaf portions
240 previously ground with liquid nitrogen in a sterile mortar according to the DNeasy Plant Mini Kit
241 protocol (Qiagen, Italy) instructions.

242 Quantitative real-time PCR was performed to measure the presence and concentration of *Asaia* cells
243 in colonized E. *variegatus*, insects, artificial diet and plants at the end of the transmission trials; the
244 latter were tested for FDp as well. All qPCR reactions were performed on a Chromo4 real-time
245 instrument (Bio-Rad, Milan, Italy) using the SsoFast^{TM} EvaGreen$^{®}$ Supermix (Bio-Rad). In insects
246 used in the bacterial colonization studies, specific reactions targeting *Asaia* 16S rRNA gene were
247 carried out with the Asafor/Asarev primers according to Favia et al. (2007). On the samples used in
248 the FDp transmission trials, 16SrV group phytoplasma-specific reactions were performed with the
249 fAY/rEY primer pair (Marcone et al. 1996; Marzachì et al. 2001), under conditions described
250 elsewhere (Galetto et al. 2005). The average FDp Genome Units (GU) per sample were considered
251 to be the 16S rRNA gene copy numbers divided by two, because this gene was estimated to be in two
252 copies in the FDp's genome (Schneider and Seemüller 1994). *Asaia* GUs per sample were considered
253 as 16S rRNA gene copies divided by four, assuming that four rRNA gene copies per cell were present
254 in *Asaia*'s genome on average (Crotti et al. 2009). In addition, to normalize the absolute density of
255 FDp and *Asaia* in insects, a qPCR targeting the insect's 18S rRNA gene (MqFw / MqRv) was used
256 as reported by Marzachì and Bosco (2005). Normalized FDp and *Asaia* GUs were calculated per pg
257 of insect 18S rRNA gene. To assess the diets, we calculated the normalized FDp concentration per
258 whole sample (expressed as ng of total DNA obtained from 300 μl of sugar solution), whereas results
259 from the plant samples were expressed as FDp GU per 100 mg of leaves.

260 To construct standard curves for the real-time PCRs, the PCR-amplified 16S rRNA gene of *Asaia*
261 and FDp were cloned using the pGEM T-easy Vector Cloning Kit (Promega). For each reaction, the
262 detection limit was calculated as the lowest concentration of cloned amplicons used for determining
263 the standard curves that were successfully amplified. Detection limits corresponded to 3.80×10^{0} FDp
264 GU per sample and 1.15×10^{0} *Asaia* GU per sample.
265

266 **Fluorescence *in Situ* Hybridization**

267 FISH experiments were performed on insect intestines, which are a key colonization niche for *Asaia*
268 (Crotti et al. 2010). Guts obtained from Experiment 1 were analysed together with dissected organs
269 from mass-reared adults collected as controls. Hybridization was carried out with *Asaia*-specific
270 probes (Favia et al. 2007) labelled with Cy3 (indocarbocyanine, absorption and emission at 550 nm
271 and 570 nm, respectively), along with the 16SrV phytoplasma-specific probe ph1298 (Lessio et al.
272 2016), labelled with Texas Red (absorption and emission at 595 nm and 620 nm, respectively), and
273 the universal bacterial probe EUB388 (Fuchs et al., 1998), labelled with fluorescein isothiocyanate
274 (FITC, absorption and emission at 494 nm and 520 nm, respectively), which was used as a positive
275 control for the hybridization experiment. Negative controls with no probe were set up too. Whole-
276 mount FISH experiments were performed on midguts dissected in sterile saline, fixed for 2 min at
277 4°C in 4% paraformaldehyde and then washed in PBS. All hybridization experimental steps were
278 performed as previously described (Crotti et al. 2009); gut cell nuclei were counterstained with DAPI.
279 After hybridization, the samples were mounted in anti-fading medium and then observed using a SP2-
280 AOBS laser scanning confocal microscope (Leica).
281

282 **Statistical analyses**

283 Statistical analyses were performed with SPSS Statistics 22 (IBM Corp. Released 2013, Armonk,
284 NY). Data of infected individuals detected in FDp transmission trials (Experiments 1-4) were
285 analysed using a generalized linear model (GLM) with a binomial probability distribution and a

286 Bonferroni post hoc test (P<0.05). Quantitative PCR data relative to the concentration of FDp cells
287 observed in positive samples within each experiment were log-transformed after adding the constant
288 10. Transformed data were analysed by Student's t tests (P<0.05), or by one-way analysis of variance
289 (ANOVA) followed by means separation by a Tukey test (P<0.05) when variance homogeneity was
290 satisfied (Levene test, P<0.05).
291

292 Results

293 *Asaia* occurring in and colonizing *E. variegatus*

294 Laboratory individuals of *E. variegatus* showed an infection rate with native *Asaia* of 23.33%.
295 Specifically, 33.33% and 13.33% of males and females were infected, respectively. The phylogenetic
296 analysis of *Asaia* sequences obtained from positive leafhoppers showed that they clustered together
297 with *Asaia bogorensis*, *A. platycodi*, *A. prunellae*, *A. siamensis* and *A. spathodeae*, as well as with
298 other strains within this genus previously isolated from Diptera and Lepidoptera. Moreover, the native
299 *Asaia* strain of *E. variegatus* was phylogenetically close to many other insect-associated isolates,
300 including isolate SF2.1 used in this study. Conversely, strains SF15.14 and AE6.5 clustered together
301 separately from other symbiotic strains (Fig.1).
302 However, no bacterial isolates referring to *Asaia* could be obtained from any of the *E. variegatus*
303 specimens examined in the isolation attempts. According to the differences in colony morphologies
304 on the plates, 14 isolates were purified and thus dereplicated on the basis of the ITS fingerprinting
305 profiles. Four ITS profiles were obtained; the 16S rRNA gene sequencing of their representatives
306 identified the isolates as belonging to *Comamonas* sp. (Betaproteobacteria), *Pantoea* sp.
307 (Gammaproteobacteria), *Pseudomonas* sp. (Gammaproteobacteria) and *Curtobacterium* sp.
308 (Actinobacteria) (Table S1).
309 We then assessed the capability of exogenous *Asaia* strains to be acquired and maintained in the insect
310 for a time comparable to that required for phytoplasma transmission, i.e., 28 days (AAP+LAP).
311 Specifically, we used two strains of *Asaia* reported in previous publications, i.e., *Asaia* SF2.1 and

312 *Asaia* AE6.5 (Favia et al. 2007; Crotti et al. 2009), and *Asaia* SF15.14, which we isolated from a
313 female *A. stephensi* mosquito (Acc. Num. LT838398). These strains differed in their capacity to
314 produce ALI biofilm, i.e., a pellicle on the surface of the liquid medium (Armitano et al. 2014).
315 Specifically, *Asaia* SF2.1 did not form a thick floating pellicle when cultured under static conditions,
316 whereas SF15.14 and AE6.5 did build a thick ALI biofilm (Fig. 2). Interestingly, it is possible to
317 observe that the SF2.1 static culture was more turbid when compared with the SF15.14 and AE6.5
318 static cultures, likely suggesting that since SF2.1 cells were not entrapped in the ALI biofilm, they
319 could swim in the medium (Fig. 2A). These phenotypes were also confirmed in the spontaneous
320 rifampicin-resistant mutants (Fig. 2B).
321 Reisolation assays from insects exposed to the three isolates of *Asaia,* followed by ITS fingerprinting
322 for confirming strain identity, showed that *Asaia* could be successfully reisolated on selective media
323 containing rifampicin, with high reisolation rates (Table 2). The highest reisolation rate was observed
324 for the SF2.1 Rif^R strain after 30 days, i.e. at the end of *Asaia* and phytoplasma AAPs (two plus seven
325 days) and LAP (21 days). However, when recording reisolation rates after two days, immediately at
326 the end of *Asaia* AAP, insects treated with the the SF2.1 Rif^R strain had the lowest percentage of
327 reisolation. No isolation of *Asaia* strains was achieved with individuals from the control group nor
328 from the saline washing solutions.
329 The control-group leafhoppers used for qPCR were first tested because preliminary qualitative PCR
330 exhibited infection with a native *Asaia*. Some of the samples (33.33%) indicated that the density of
331 *Asaia* cells exceeded the detection limit, as expected, because the primers used for qPCR amplified
332 this strain as well. None of the samples had a concentration higher than 2.20×10^1 *Asaia* GU; this
333 value was therefore used as a blank threshold, and insects with lower *Asaia* concentrations were
334 considered negative for the introduced bacteria. In samples with higher rates, this threshold value was
335 subtracted to avoid overestimation due to native *Asaia* amplification. After these adjustments, the
336 concentration of *Asaia* in positive leafhoppers was quite stable, with a slightly increasing trend over
337 time, between 6.35×10^2 and 5.55×10^5 *Asaia* GU per sample (Table 2).

338

339 **FDp transmission trials**

340 Results from FDp transmission experiments performed on *Asaia*-infected and uninfected *E.*
341 *variegatus* specimens are presented in Table 3 and Figure 3. All of the strains provided to leafhoppers
342 were found to colonize the insects, with infection rates of 58% to 70% according to *Asaia*-specific
343 qPCR (Table S2). The average symbiont concentration in positive samples ranged between 6.52×10^3
344 and 5.42×10^5, which was not dissimilar to the average symbiont concentration recorded in the
345 colonization experiments (Table 2).

346 The first of four experiments aimed at comparing the FDp transmission levels of *E. variegatus*
347 individuals fed with different strains of *Asaia* (experiment 1) revealed that leafhoppers exposed to the
348 SF2.1 Rif^R and AE6.5 Rif^R strains did not significantly diverge from the control group in FDp
349 infection rates, whereas percentage of FDP-infected insects fed the SF15.14 Rif^R strain was
350 significantly lower (binomial GLM: df = 3; $\chi^2 = 8.263$; $P<0.05$). On the other hand, no significant
351 differences were observed among FDp infection rates from the artificial diets provided to the insects
352 in experiment 1 (binomial GLM: df = 3; $\chi^2 = 3.193$; P = 0.36), even though the percentage of
353 phytoplasma-positive diet recorded for samples treated with *Asaia* SF15.14 Rif^R was about five times
354 lower than those of the other treatments. Furthermore, no differences were found among the
355 concentrations of FDp detected in positive leafhopper samples (ANOVA: df = 3, 41; F = 0.675; P =
356 0.57). The concentration of FDp recorded in artificial diets provided to insects fed with the AE6.5
357 Rif^R strain was the highest and significantly diverged from the control (ANOVA: df = 2, 14; F =
358 5.052; $P<0.05$); however, in this case, the concentration of FDp in the diets provided to individuals
359 exposed to the *Asaia* SF15.14 Rif^R strain was not included in the statistical analysis (Fig. 3C) because
360 only one positive diet was observed. The FDp transmission rate from infected insects to diets was
361 also not significantly divergent among treatments (binomial GLM: df = 3; $\chi^2 = 0.395$; P = 0.94).

362 FISH experiments performed on the midguts of leafhoppers from experiment 1 (Fig. 4) confirmed
363 that bacteria were found in the guts of all specimens (Fig. 4C, G, K), as indicated by DAPI staining

364 that outlined the epithelial tissues (Fig. 4D, H, L). Moreover, *Asaia* was detected in *E. variegatus*
365 specimens fed with all strains of the bacteria (Figs. 4A, E, I, M), although individuals reared in the
366 laboratory did not produce any *Asaia*-specific hybridization signal (Fig. S2). *Asaia* was typically
367 found to become established close to the midgut epithelia (Fig. 4A). On the other hand, FDp could
368 be massively detected in some midguts collected from leafhoppers treated with *Asaia* SF15.14 Rif^R
369 as well as with other strains (Figs. 4B, F, J, N) and in control insects (Fig. S2). A specific colonization
370 pattern was recorded in some of the insects fed with *Asaia* SF15.14 Rif^R: spots of fluorescence were
371 observed in the guts (Fig. 4M, N). These spots may correspond to bacterial clusters, and they were
372 visible after hybridization with both the *Asaia*-specific and the 16SrV phytoplasma-specific probes.
373 No FDp-specific hybridization was detected from mass-reared insects for which only DAPI staining
374 and the eubacterial probe showed a positive signal (Fig. S2).
375 Based on results obtained from experiment 1, we designed experiments 2-4 to compare the FDp
376 infection levels between individuals with a natural *Asaia* background with those colonized by *Asaia*
377 SF15.14 Rif^R, the only strain found to reduce infection rates in *E. variegatus*. In experiment 2, a
378 significantly lower percentage of *Asaia* SF15.14 Rif^R-fed insects positive for FDp than in the control
379 (binomial GLM: df = 1; $\chi^2 = 5.198$; $P<0.05$) was found; remarkably, this percentage was double that
380 in experiment 1. Similarly, FDp infection rates of inoculated broad beans were significantly divergent
381 (binomial GLM: df = 1; $\chi^2 = 4.552$; $P<0.05$), although they were higher than those of artificial diets
382 recorded in experiment 1. However, no significant differences were observed between FDp densities
383 in insects (Student's t Test: df = 20; t = 0.676; P = 0.51) and broad beans (Student's t Test: df = 12; t
384 = 0.375; P = 0.71), and between recorded transmission rates (binomial GLM: df = 1; $\chi^2 = 0.649$; P =
385 0.42).
386 In experiment 3, the FDp infection rates of both insects and artificial diets were higher than those
387 recorded in the previous experiments; in either case, the percentage of FDp-infected samples was
388 significantly lower for the group treated with the SF15.14 Rif^R strain than the control, according to
389 binomial GLM (test on leafhopper samples: df = 1; $\chi^2 = 25.587$; $P<0.05$; test on diet samples: df = 1;

390 χ^2 = 17.490; P<0.05). Moreover, the FDp concentration in positive insects exposed to the SF15.14
391 Rif^R strain was significantly lower than that of the control (Student's t test: df = 61; t = 3.892; P<0.05).
392 On the other hand, no differences between artificial diets provided to treated and untreated insects
393 were observed (Student's t test: df = 41; t = 0.339; P = 0.74); similarly, no differences were detected
394 between FDp transmission rates (binomial GLM: df = 1; χ^2 = 0.944; P = 0.33).
395 In experiment 4, *E. variegatus* specimens fed with *Asaia* SF15.14 Rif^R had a significantly lower FDp
396 infection rate than the control group had (binomial GLM: df = 1; χ^2 = 7.202; P<0.05). Conversely,
397 FDp infection rates recorded for the respective broad beans showed no significant differences
398 (binomial GLM: df = 1; χ^2 = 2.417; P = 0.12), even though the percentage of positive plants exposed
399 to the SF15.14 Rif^R strain was lower than the control (Table 3). Likewise, the concentration of FDp
400 in *E. variegatus* individuals fed the SF15.14 Rif^R strain was significantly lower than that of the control
401 group (Student's t test: df = 47; t = 1.441; P<0.05), whereas the abundances of phytoplasma cells
402 detected in infected broad beans did not significantly diverge (Student's t test: df = 32; t = 0.385; P =
403 0.70). In addition, no significant difference was found between FDp transmission rates (binomial
404 GLM: df = 1; χ^2 = 0.608; P = 0.43); however, in this single experiment, the percentage of successful
405 phytoplasma transmission for leafhoppers exposed to *Asaia* SF15.14 Rif^R was surprisingly higher
406 than the control, but not significantly different.
407 Interestingly, even though none of *Asaia* strains colonized 100% of *E. variegatus* specimens, the
408 percentages of co-infected insects (*Asaia* + FDp) recorded in each experiment was not different from
409 infection rates of phytoplasma alone (Table S2).
410

411 **Discussion**

412 The presence and colonization of *Asaia* in leafhoppers has been previously reported (Crotti et al.
413 2009; Gonella et al. 2012) and our results indicate that *E. variegatus* hosts *Asaia* as well. Similarly to
414 *S. titanus* (Crotti et al. 2009), native *Asaia* was detectable in *E. variegatus* by molecular methods only
415 but could not be isolated in pure cultures. In our attempts to isolate *Asaia*, we employed several media

416 that were previously effective for isolating *Asaia* or other AAB from insects (Crotti et al. 2009;
417 Vacchini et al. 2017). It is possible that growing *Asaia* living in *E. variegatus* requires specific factors
418 produced by the host or by other microorganisms associated with the gut of *E. variegatus*, such as
419 those we were able to isolate (Table S1). Moreover, we observed a relative phylogenetic divergence
420 among the native *Asaia* strain and two of the exogenous isolates that were provided to the leafhopper,
421 specifically the ALI biofilm-producer strains SF15.14 RifR and AE6.5 RifR (Fig. 1), suggesting that
422 they could differentially interact with the host. The gut of *E. variegatus* turned out to be a suitable
423 environment for the establishment of exogenous *Asaia* strains isolated from other insects. The three
424 rifampicin-resistant *Asaia* strains we have studied, i.e., SF2.1 Rif^R, AE6.5 Rif^R and SF15.14 RifR,
425 successfully colonized the bodies of leafhoppers as indicated by their detection and reisolation from
426 the host in high numbers up to four weeks after the uptake of the bacterial cells. This capability was
427 observed for all the tested strains, in agreement with the high colonization versatility of different
428 insect hosts previously observed in *Asaia* (Crotti et al. 2009). Cultivation-dependent and independent
429 analyses testing *Asaia* infection and the concentration in *E. variegatus* indicated that infection levels
430 do not decrease over time and that the concentration of exogenous *Asaia* cells in the colonized *E.*
431 *variegatus* individuals was quite stable over time as well, even though it could not reach the value of
432 $1\text{-}2\times10^6$, which was previously detected in *S. titanus* (Gonella et al. 2012).
433 The capability of *Asaia* strains with different phenotypes to affect FDp transmission was assessed as
434 well, since some AAB have been described as having probiotic activity by inhibiting pathogens
435 (Haghshenas et al. 2015). A main mechanism of antagonism identified in these bacteria is related to
436 the production of acetic acid, although most of *Asaia* isolates do not produce it (Yamada et al. 2000).
437 Interestingly, a pre-feeding treatment of phlebotomine flies with an *Asaia* strain (isolated from a wild-
438 caught *Lutzomyia longipalpis* sand fly) reduced the size of the flies harbouring *Leishmania* parasites,
439 likely enhancing or contributing to the mechanisms of colonization resistance against the parasite
440 (Sant'Anna et al. 2014). Moreover, it can be expected that different *Asaia* strains can coexist in the
441 same insects, possibly determining different types of effects on the host and the pathogens they

442 transmit. Indeed, multiple *Asaia* infections with different strains inhabiting individual insects have
443 been found in mosquitoes (Chouaia et al. 2010), with each infection playing a different role in its
444 host. Moreover, it has been shown that elimination of *Asaia* from the gut by way of antibiotic
445 treatment delays larval development in *A. stephensi* (Chouaia et al. 2012). *Asaia* has been shown to
446 block the transmission of the reproductive manipulator *Wolbachia* in *Anopheles* mosquitoes (Hughes
447 et al. 2014) and the negative interference between these two bacteria has been observed to occur in
448 the mosquitoes' gonads (Rossi et al. 2014). Here, we confirmed the occurrence of different effects
449 exerted by different *Asaia* strains in leafhoppers as well. Particularly, one out of the three strains we
450 tested affected the suitability of *E. variegatus* to be colonized by FDp. The SF15.14 Rif^R strain was
451 capable of producing a thick ALI biofilm. We speculate that the reduced FDp acquisition rate
452 observed in leafhoppers exposed to the SF15.14 Rif^R strain of *Asaia* could be related to competitive
453 physical exclusion, i.e., obstruction by the biofilm of midgut attachment sites needed by FDp to
454 recognize and cross the gut barrier. Indeed, *Asaia* SF15.14 Rif^R, besides being located close to the
455 midgut epithelium, which is a key tissue through which FDp reaches the haemolymph and colonizes
456 the insect (Rashidi et al. 2015), was found to build bacterial masses, which apparently may entrap
457 phytoplasma cells, possibly preventing efficient establishment in the host's body. However, these
458 considerations are not sufficient to justify the reduced number of FDp-infected leafhoppers. Indeed,
459 the individuals exposed to the other ALI biofilm-producing strain included in this study (AE6.5 Rif^R)
460 were not less infected by FDp than the control, although the provided isolate produced a thick ALI
461 biofilm similar to that produced by the SF15.14 Rif^R strain. Gram-negative bacteria, including AAB
462 such as *Komagataeibacter xylinus*, produce pellicles mainly comprising exopolysaccharides, among
463 which cellulose is generally one of the main components (Armitano et al. 2014). *Asaia* strains have
464 been observed to be able to produce cellulose (Kumagai et al. 2011), but further studies are necessary
465 to investigate the composition of the pellicles produced by the strains we have investigated and to
466 determine if cellulose, as well as other exopolysaccharides (together with or as an alternative to
467 cellulose) are produced. The biofilms produced by SF15.14 Rif^R and AE6.5 Rif^R could have different

468 compositions, and this could be important in their different effect on FDp transmission. This
469 possibility is supported by the fact that, even though *Asaia* and FDp were detected by FISH analyses
470 in the guts of leafhoppers treated with all strains of studied bacteria, bacterial clusters (which could
471 contribute to the reduction of phytoplasma transmission) were observed by FISH only in the SF15.14
472 Rif^R strain. However, we must point out that we examined a limited number of insects by FISH. We
473 cannot therefore exclude the possibility that the AE6.5 Rif^R strain is able to form bacterial masses as
474 well. In summary, the possibility that interference with FDp infection of *E. variegatus* determined by
475 *Asaia* SF15.14 Rif^R is not related to biofilm production, but to other phenotypic traits, cannot be ruled
476 out by the data presented here. Further investigations are necessary to ascertain if biofilm production
477 capacity of *Asaia* is implicated in interference of FDp transmission.
478 Other antagonistic functions against pathogens played by AAB are related to the production of
479 extracellular polysaccharides that can provide protective immunity (Li et al. 2004). Polymers
480 produced by AAB might determine if immunostimulation occurs in the insect host. Previous studies
481 on Diptera indicated that *Asaia* performed immune modulation (Capone et al. 2013); however,
482 whether or not this activity is due to polysaccharides has not been determined. The immunity of *E.*
483 *variegatus* has recently been explored (Tedeschi et al. 2017) and further work could evaluate the role
484 played by the SF15.14 Rif^R *Asaia* strain in activating immune responses.
485 Although the SF15.14 Rif^R strain was found to reduce FDp infection in *E. variegatus*, the inhibition
486 of phytoplasma colonization was imperfect given that the pathogens were not totally eliminated from
487 the insects fed bacteria; moreover, in some cases, their inoculation substrates were infected as well.
488 Also, the concentration of FDp cells in the individuals treated with *Asaia* SF15.14 Rif^R was not lower
489 than in control insects, suggesting that if the pathogen succeeds in establishing itself in the insect, its
490 growth is not controlled by the presence of *Asaia*. Moreover, the percentage of infected leafhoppers
491 that successfully transmitted the phytoplasma was not reduced, and in one case it was even higher
492 than the control (experiment 4, see Table 3). Whatever the machinery limiting the pathogen infection
493 is, the interference is most likely to occur in the midgut, probably reducing the capability of the

phytoplasma to cross epithelial cells and to reach the haemolymph. Instead, when pathogenic FDp succeed in crossing the midgut barrier, they effectively multiply and reach the insect's salivary glands, finally being passed on when the leafhoppers feed, suggesting that no *Asaia*-induced immune protection is triggered in the haemolymph. However, even though the vector competence was not affected in specimens where phytoplasma infection did succeed, the lower number of infected leafhoppers resulted in a lower number of inoculated substrates.

Besides comparing FDp infection levels in insects exposed to *Asaia* strains with the control group, we considered the percentage of individuals with double infections (*Asaia* + FDp) (Table S2). Such co-infection rates were not divergent from FDp infection rates recorded in samples belonging to the same experiment without taking into account *Asai*a infection, even when considering the experiments with the SF15.14 Rif^{R} strain. This evidence supports the lack of the anti-phytoplasma effect observed in experiments involving *Asaia* SF2.1 Rif^{R} and AE6.5 Rif^{R,}. On the other hand, when considering insects treated with *Asaia* SF15.14 Rif^{R}, we can alternatively speculate that in some individuals, the symbiont infected the host's gut in the early days of the experiment, preventing early colonization of FDp, which was then not retained in the leafhopper, or that qPCR results may lead to misinterpretation of data by underestimating infection percentages.

In conclusion, the capacity of *Asaia* to interfere with FDp colonization in the laboratory vector *E. variegatus* indicates that this AAB could potentially be exploited as a symbiotic control tool complementary to sustainable IPM strategies for the containment of Flavescence dorée. However, it must be pointed out that our results do not involve the natural hosts of FDp (*S. titanus* and grapevine). More work is hence needed before development of *Asaia*-derived products for field usage. Necessary steps remaining to be elucidated include i) understanding the mechanistic aspects of the interference, ii) validating the results of the *E. variegatus* model presented here on *S. titanus,* the vector of the disease in the field, and iii) identifying an appropriate method for establishing *Asaia* into leafhopper populations in the field.

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526

527 ## Compliance with ethical standards

528 **Conflict of interest**

529 All authors declare that they have no conflict of interest.

530 **Ethical approval**

531 All applicable international, national, and/or institutional guidelines for the care and use of animals
532 were followed.

533

534 This research described in this paper did not include any studies with human subjects.

535

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724 Int J Syst Evol Microbiol 50(2):823-829.

725 **Table 1.** Experimental design for FDp transmission trials. LAP: Latency Access Period; IAP:

726 Inoculation Access Period.

	Asaia strains[a]	LAP conditions	IAP medium
Experiment 1	SF2.1 Rif^R, SF15.14, Rif^R AE6.5 Rif^R, control (no *Asaia*)	Healthy oat	Artificial diet
Experiment 2	SF15.14 Rif^R, control (no *Asaia*)	Healthy oat	Broad bean
Experiment 3	SF15.14 Rif^R, control (no *Asaia*)	FDp-infected broad bean	Artificial diet
Experiment 4	SF15.14 Rif^R, control (no *Asaia*)	FDp-infected broad bean	Broad bean

727 [a] The experiments with each strain was run separately

728

729 **Table 2.** Colonization efficiency of different *Asaia* strains in *E. variegatus.*

730 [#]Percentage of specimens from which the rifampicin-resistance strains of *Asaia* (confirmed by ITS-
731 PCR fingerprinting) were reisolated on MA medium containing 100 µg/ml rifampicin (N=30). The
732 number of samples showing successful reisolation is indicated in parentheses. [¶]*Asaia* cells per
733 positive insect. Values below the blank threshold (2.20×10^1) were considered negative. Transformed
734 values (10+log) used for statistical analyses are indicated in parentheses together with their standard
735 errors. All *Asaia*-positive insects were used to calculate average densities; their number is indicated
736 in square brackets.

737 AAP: Acquisition Access Period; LAP: Latency Access Period; SE: Standard Error; ND: Not
738 Detectable (*Asaia* below blank threshold).

Strain	**Collection time**	***Asaia* reisolation rate (%) ± SE[#]**	***Asaia* density (transformed value± SE)[¶]**
SF2.1 Rif[R]	End of *Asaia* AAP	53.33 ± 0.01 (16/30)	1.00×10^4 (12.81± 0.14) [N=16]
	End of FDp AAP	86.67 ± 0.01 (26/30)	6.35×10^2 (12.64± 0.06) [N=26]
	End of AAPs + LAP	93.33 ± 0.02 (28/30)	5.55×10^5 (13.50± 0.22) [N=28]
SF15.14 Rif[R]	End of *Asaia* AAP	80.00 ± 0.07 (24/30)	5.17×10^3 (13.05± 0.12) [N=24]
	End of FDp AAP	76.67 ± 0.04 (23/30)	1.39×10^3 (12.58± 0.13) [N=23]
	End of AAPs + LAP	76.67± 0.02 (23/30)	5.08×10^4 (13.52± 0.19) [N=23]
AE6.5 Rif[R]	End of *Asaia* AAP	80.00 ± 0.02 (24/30)	2.59×10^3 (11.70± 0.23) [N=24]
	End of FDp AAP	80.00 ± 0.02 (24/30)	1.27×10^3 (12.60± 0.10) [N=24]
	End of AAPs + LAP	80.00 ± 0.02 (24/30)	5.86×10^4 (13.33± 0.23) [N=24]
Control (no *Asaia*)	End of FDp AAP	0.00% (0/30)	$<2.20\times10^1$
	End of AAP + LAP	0.00% (0/30)	$<2.20\times10^1$

739

740 **Table 3.** Phytoplasma infection rates from FDp transmission trials. The percentage of positive samples according to qPCR analyses on leafhoppers
741 and sugar diets or broad beans during experiments 1-4 (see Table 1) are presented. For experiment 1, different letters indicate significantly different
742 values according to ANOVA or binomial GLM ($P < 0.05$). For experiments 2-4, asterisks indicate significantly different values according to Student's
743 t tests or binomial GLM ($P< 0.05$). [#]Percentage of 16SrV phytoplasma-positive individuals related to the total tested samples (N=60). [¶]Percentage of
744 phytoplasma-positive leafhoppers that actually transmitted FDp to their feeding substrate, related to the total infected specimens.
745 The number of positive samples is indicated in parentheses. SE: Standard Error; ns: not significant.

Experiment no.	***Asaia* strain**	**FDp infection rate (%) ± SE in *E. variegatus*[#]**	**FDp infection rate (%) ± SE in feeding substrates[#]**	**FDp transmission rate (%) ± SE[¶]**
1	SF2.1 Rif^R	23.33 ± 0.22 a (14/60)	8.33 ± 0.06 ns (5/60)	35.71 ±0.13 ns (5/14)
	SF15.14 Rif^R	5.00 ± 0.09 b (3/60)	1.67 ± 0.18 ns (1/60)	33.33 ± 0.33 ns (1/3)
	AE6.5 Rif^R	21.67 ± 0.22 a (13/60)	10.00 ± 0.16 ns (6/60)	46.15 ± 0.14 ns (6/13)
	Control (no *Asaia*)	25.00 ± 0.19 a (15/60)	10.00 ± 0.13 ns (6/60)	40.00 ± 0.13 ns (6/15)
2	SF15.14 Rif^R	10.00 ± 0.04 * (6/60)	5.00 ± 0.05 * (3/60)	50.00 ± 0.22 ns (3/6)
	Control (no *Asaia*)	26.67± 0.06 * (16/60)	18.33 ± 0.03 *(11/60)	68.75 ± 0.12 ns (11/16)
3	SF15.14 Rif^R	28.33 ± 0.06 * (17/60)	16.67 ± 0.07 * (10/60)	59.12 ± 0.12 ns (10/17)
	Control (no *Asaia*)	76.67 ± 0.06 * (46/60)	55.00 ± 0.08 *(33/60)	71.74 ± 0.07 ns (33/46)
4	SF15.14 Rif^R	28.33 ± 0.06 * (17/60)	21.67 ± 0.05 ns (13/60)	76.47 ± 0.10 ns (13/17)
	Control (no *Asaia*)	53.33 ± 0.06 * (32/60)	35.00 ± 0.06 ns (21/60)	65.62 ± 0.09 ns (21/32)

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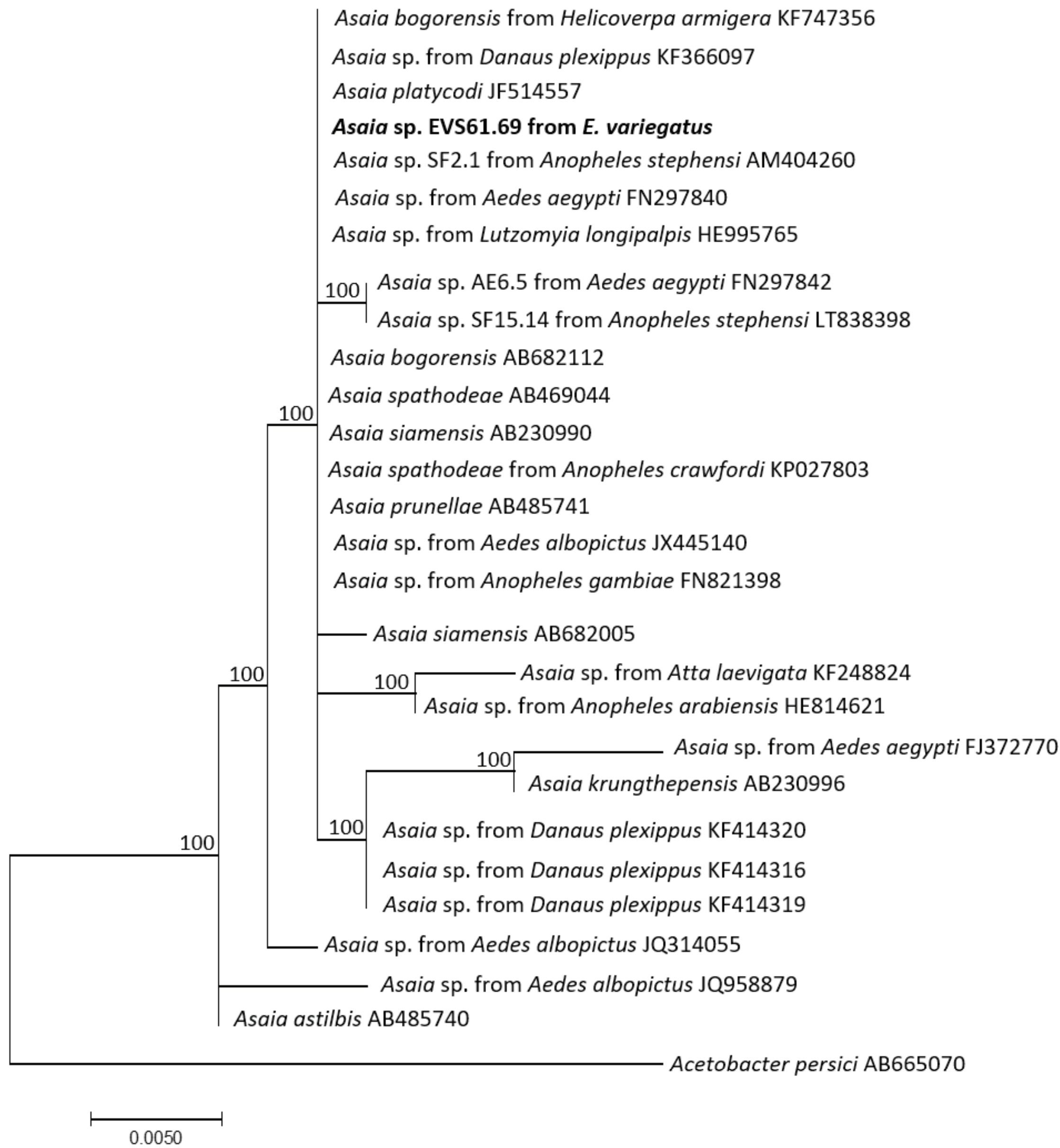

748 **Figure 1.** Phylogenetic position of the *Asaia* strain recorded in *E. variegatus*. Other *Asaia* strains
749 isolated from insect hosts are included along with *A. astibilis, A. bogorensis*, *A. krungthepensis*, *A.*
750 *platycodi*, *A. prunellae*, *A. siamensis* and *A. spathodeae*. The phylogenetic tree was constructed based
751 on the almost-complete 16S rRNA gene by the maximum likelihood method with the MEGA 7
752 software. Numbers at each node represent percentages of bootstrap replications calculated from 1,000
753 replicate trees. The scale bar represents the sequence divergence. *Acetobacter persici* (AB665070),
754 belonging to the AAB group, was used as an outgroup.

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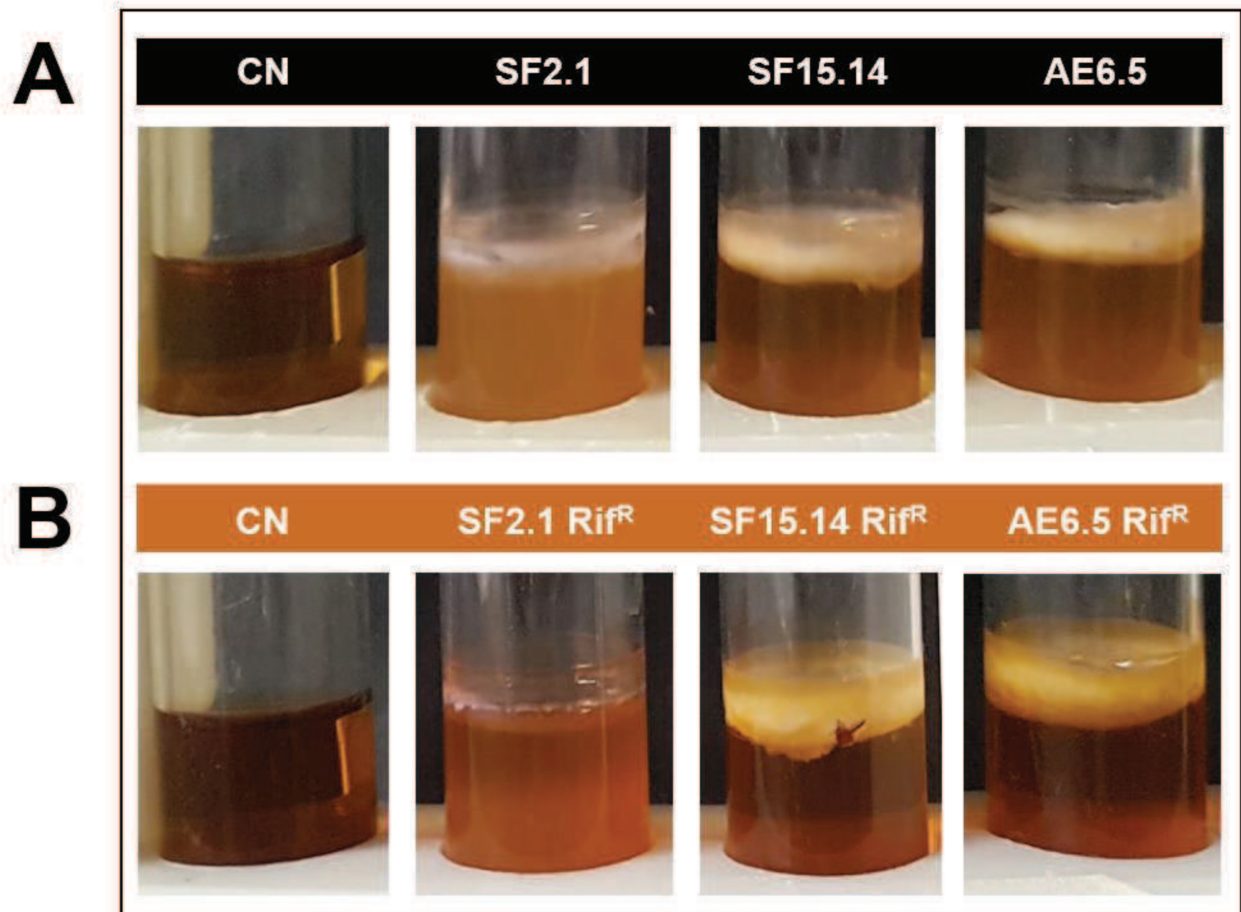

757

758 **Figure 2.** Growth behaviours in static cultures of *Asaia* SF2.1, AE6.5 and SF15.14 strains (A) and
759 their spontaneous rifampicin-resistant mutants (B). The strains were inoculated in tubes containing 5
760 ml of modified PDB and cultivated under static conditions for 10-12 days. CN: negative controls
761 containing PDB medium with (B) or without (A) 100 µg/ml rifampicin; no bacterial inoculum was
762 added.

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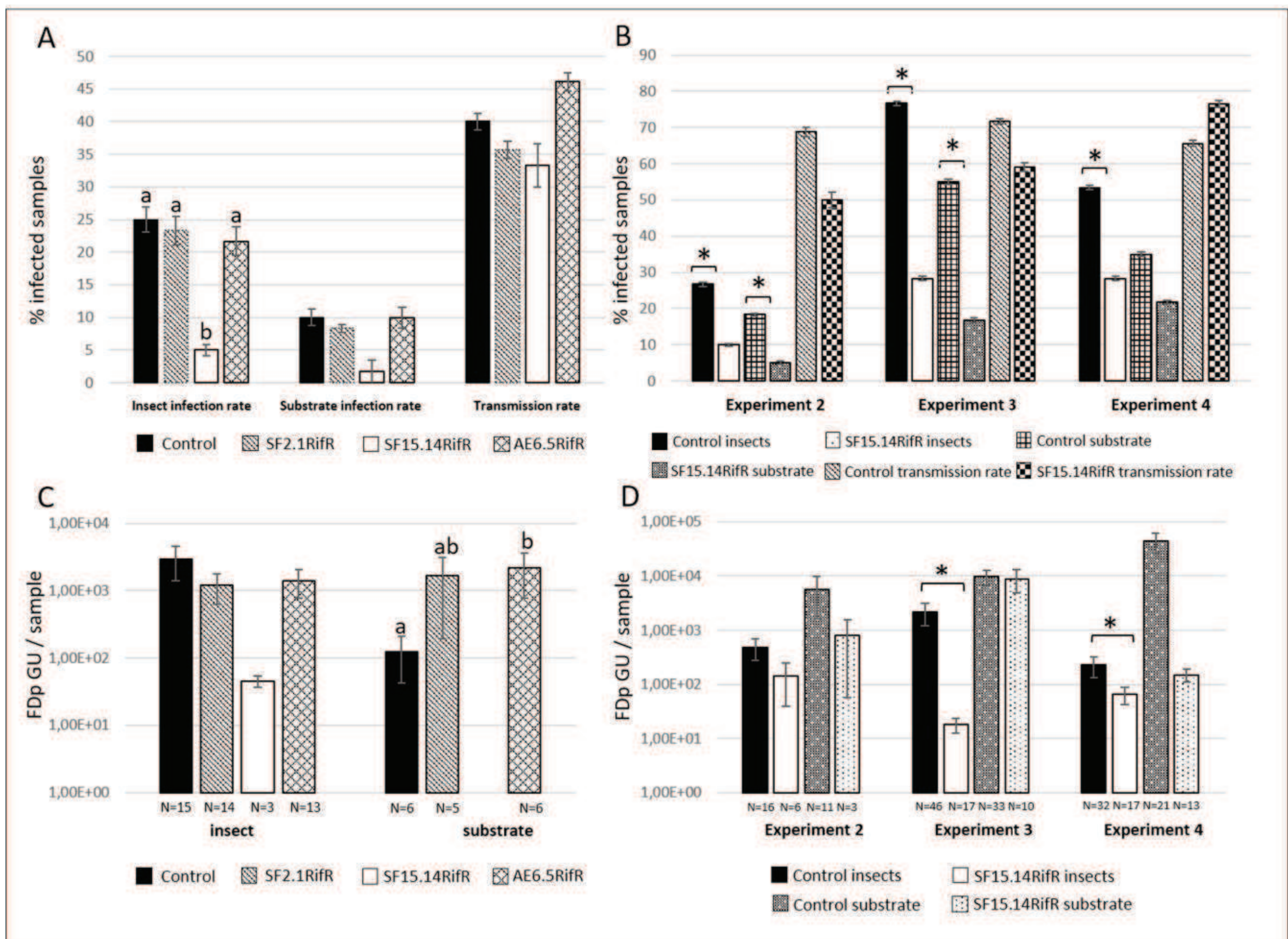

764

765 **Figure 3.** Phytoplasma infection rates and densities recorded in FDp transmission trials. Data
766 obtained from experiment 1, comparing specimens exposed to all tested *Asaia* strains, are shown in
767 A (percentage of FDp-infected samples) and C (mean phytoplasma concentration), respectively.
768 Different letters indicate significantly different values according to binomial GLM or ANOVA ($P < 0.05$).
769 In C, the FDp density recorded in substrate provided to leafhoppers fed with the SF15.14Rif^R
770 strain was not included, because only one positive diet was found. The FDp infection rates and
771 densities obtained in experiments 2-4, comparing individuals fed with *Asaia* SF15.14Rif^R and the
772 control, are shown in B and D, respectively. Asterisks indicate significantly different values according
773 to binomial GLM or Student's t tests ($P< 0.05$). Regarding the percentage of infected samples (A and
774 B), N=60. Average densities (C and D) were calculated as the number of 16SrV phytoplasma cells
775 per sample: single insect, single diet unit (300 µl sugar solution), 100 mg of broad bean leaf. Values

776 below the detection limit (3.80×10^{0}) were considered negative. All positive samples were used, their

777 number is indicated below each column.

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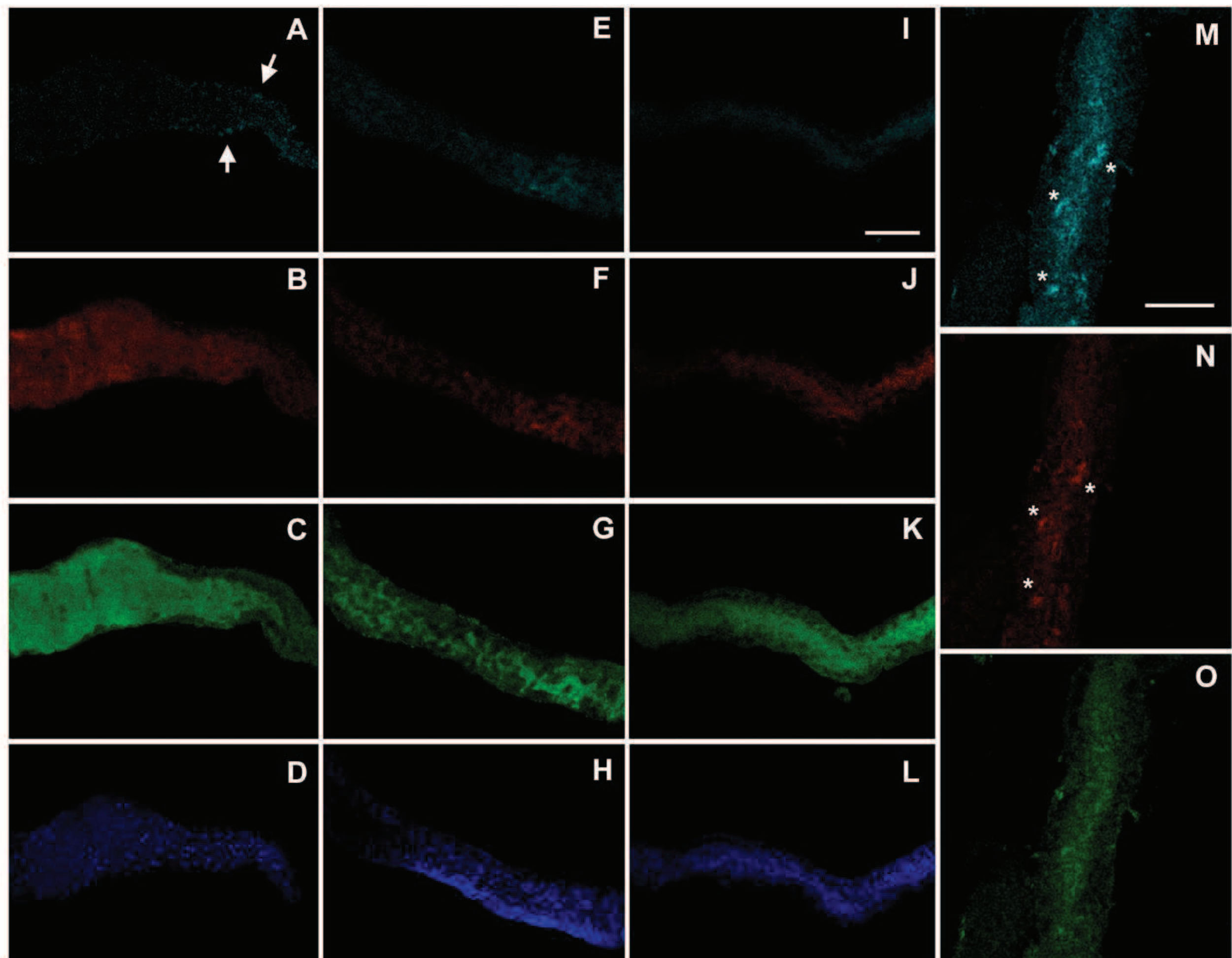

779

780 **Figure 4.** FISH experiments on midguts of *E. variegatus* individuals from experiment 1. Micrographs
781 showing results from FISH of the midgut of: a specimen fed with *Asaia* SF 15.14 Rif^R (A-D; M-O),
782 a leafhopper treated with *Asaia* SF 2.1 Rif^R (E-H), an insect exposed to *Asaia* AE6.5 Rif^R (I-L).
783 Hybridizations with *Asaia* (cyan signal), 16SrV phytoplasma (red signal), and eubacterial (green
784 signal) probes are shown. DAPI stains are presented in blue. Arrows in A indicate the specific
785 localization of *Asaia* close to the midgut epithelia; asterisks in M and N indicate signal spots referable
786 to *Asaia* and phytoplasma cell masses observed in insects treated with the SF 15.14 Rif^R strain. Bars
787 = 75μm.

788

789 **Table S1.** Identification of cultivable bacteria associated with *E. variegatus*.

Isolate code[a]	ITS group[b]	No. isolates per ITS group[c]	Identity (Acc. Num.)	% identity (bp)	RDP classification (family: genus)
EV5	1	8	*Pantoea agglomerans* (JX089401)	97 (534/549)	Enterobacteriaceae: *Pantoea*
EV9	2	2	*Pseudomonas* sp. (KX450447)	100 (593/603)	Pseudomonadaceae: *Pseudomonas*
EV10	3	1	*Comamonas* sp. (KR055003)	99 (858/858)	Comamonadaceae: *Comamonas*
EV12	4	3	*Curtobacterium flaccumfaciens* (KY970145)	100 (776/778)	Microbacteriaceae: *Curtobacterium*

790 [a]Isolate code indicates the code assigned to the ITS-group representative isolate.
791 [b]ITS group indicates the number of the different ITS groups obtained after dereplication by ITS-PCR
792 [c]No. Isolates per ITS group refers to the number of isolates included in the indicated ITS group.
793

794

795 **Table S2.** Colonization levels of *Asaia* strains in *E. variegatus* specimens used for FDp transmission
796 experiments. *Asaia* concentrations obtained by qPCR analyses on leafhoppers during experiments 1-
797 4 (see Table 1) are presented, along with the percentages of individuals showing both *Asaia* and FDp
798 infection. #*Asaia* cells per positive insect. Values below the blank threshold (2.20×10^1) were
799 considered negative. Transformed values (10+log) used for statistical analyses are indicated in
800 parentheses together with their standard errors (SE). All *Asaia*-positive insects were used to calculate
801 average densities; their number is indicated in square brackets. ¶Percentage of FDp-infected
802 specimens (see Table 2), considering only those samples that tested positive in *Asaia*-specific qPCR.
803 The number of co-infected insects is indicated in parentheses.

804

Experiment no.	***Asaia* strain**	***Asaia* density (transformed value± SE)#**	***Asaia*-FDp co-infection rate (%)¶**
1	SF2.1 Rif^R	5.42×10^5 (13.48±0.22) [N=41]	21.95±0.07 (9/41)
	SF15.14 Rif^R	1.32×10^5 (12.38±0.31) [N=36]	8.33±0.05 (3/36)
	AE6.5 Rif^R	5.86×10^4 (13.33±0.22) [N=39]	20.51 ±0.07 (8/39)
	Control (no *Asaia*)	$<2.20\times10^1$	ND
2	SF15.14 Rif^R	1.03×10^5 (13.31±0.19) [N=39]	12.82± 0.05 (5/39)
	Control (no *Asaia*)	$<2.20\times10^1$	ND
3	SF15.14 Rif^R	5.22×10^4 (13.33±0.22) [N=35]	20.00± 0.08 (7/35)
	Control (no *Asaia*)	$<2.20\times10^1$	ND
4	SF15.14 Rif^R	6.50×10^3 (12.98±0.13) [N=36]	22.22 ± 0.10 (8/36)
	Control (no *Asaia*)	$<2.20\times10^1$	ND

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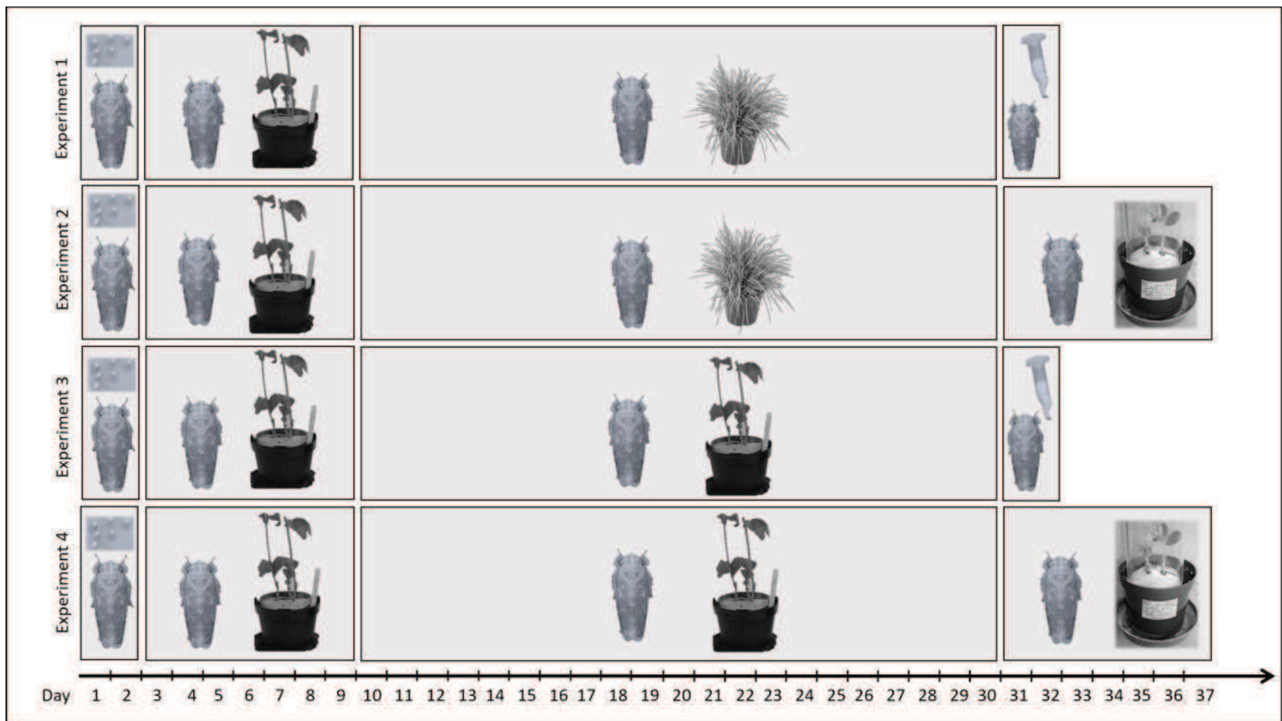

Figure S1. Graphical structure of FDp transmission trials. In Experiment 1, four treatments (strains SF2.1 Rif^{R}, SF15.14, Rif^{R}, AE6.5 Rif^{R}, control) were tested, with 70 insects each, whereas in Experiments 2-4 only strain SF15.14, Rif^{R} and the control were compared (N=60). All experiments included two days of artificial feeding with *Asaia* (no bacteria for the control) (*Asaia* AAP) and seven days of exposure to a FDp-infected broad bean (FDp AAP). Then, *E. variegatus* individuals were fed for 21 days on healthy oat plants (Experiment 1-2) or FDp-infected broad bean plants (Experiment 3-4) (LAP). Finally, the leafhoppers were maintained for two days on an artificial diet (Experiment 1, 3) or for seven days on healthy broad bean plants (Experiment 2, 4) for phytoplasma inoculation.

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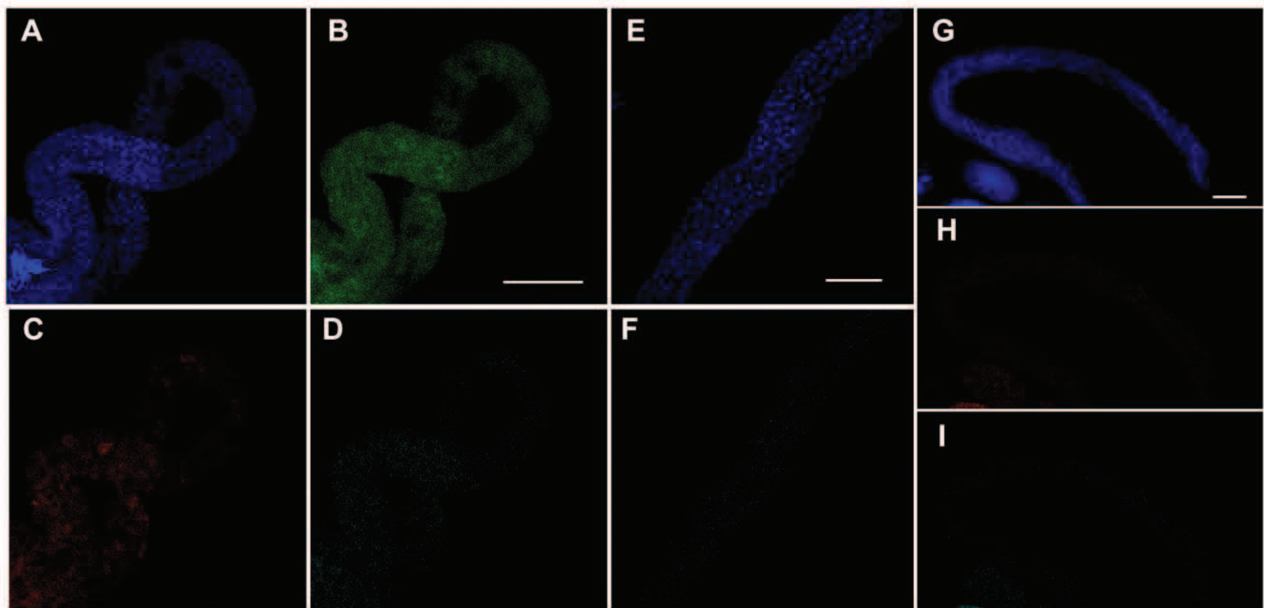

818 **Figure S2.** Negative controls of FISH experiments. The FISH results are shown for the midguts of a
819 *E. variegatus* specimen belonging to the control group of experiment 1 (never fed with *Asaia* and
820 exposed to FDp AAP + LAP) in A-D; an adult from the mass rearing (never fed in the presence of
821 *Asaia* or FDp) in E and F; an individual treated with no probe in G-I. Hybridizations with *Asaia* (cyan
822 signal), 16SrV phytoplasma (red signal), and eubacterial (green signal) probes are shown. DAPI
823 stains are presented in blue. A consistent signal was detected for eubacteria (B) and phytoplasma (C)
824 in the insect used in experiment 1. Conversely, hybridization with the *Asaia*-specific probe (D, F, I)
825 failed in all trials; similarly, no signal related to the phytoplasma-specific probe was detected in the
826 no probe trial (H). Indeed, in these panels, only a diffused background signal is visible. Bars = 75µm.

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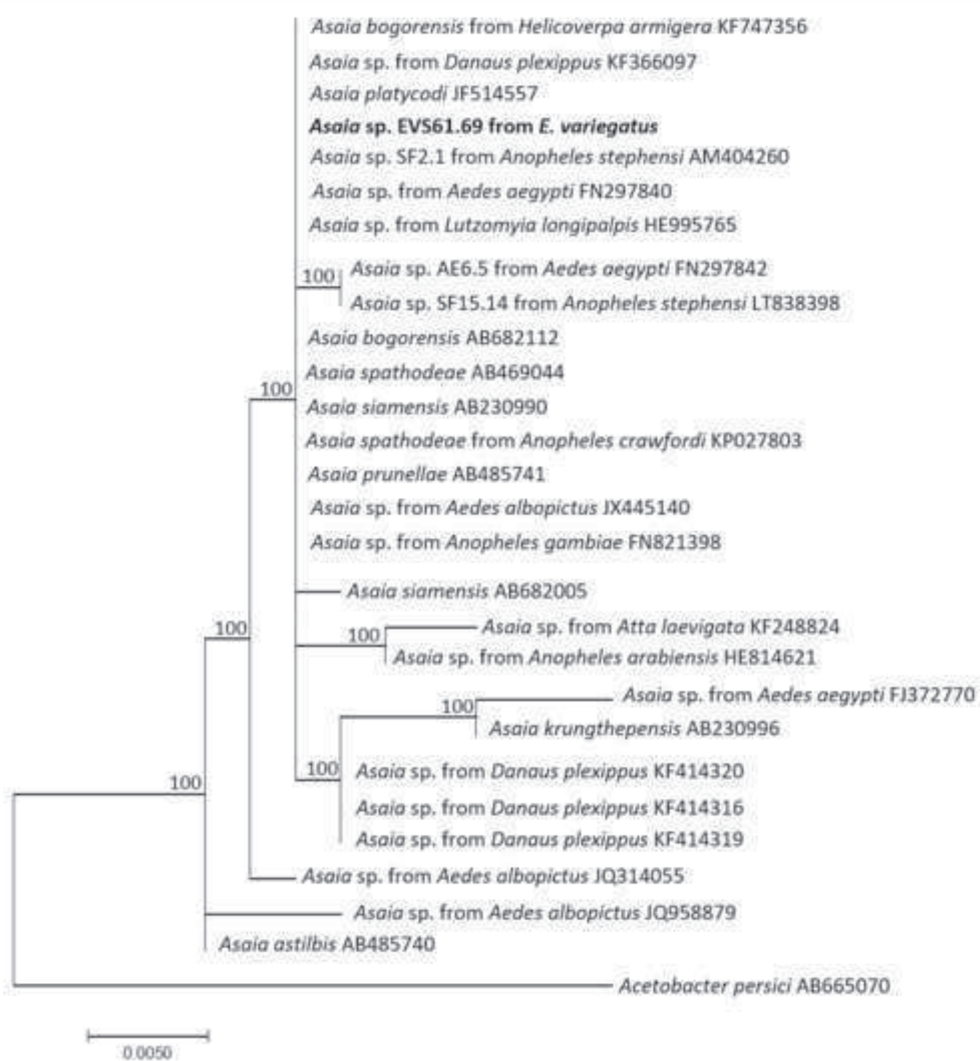

Figure 2 Click here to download Figure Figure 2.tif

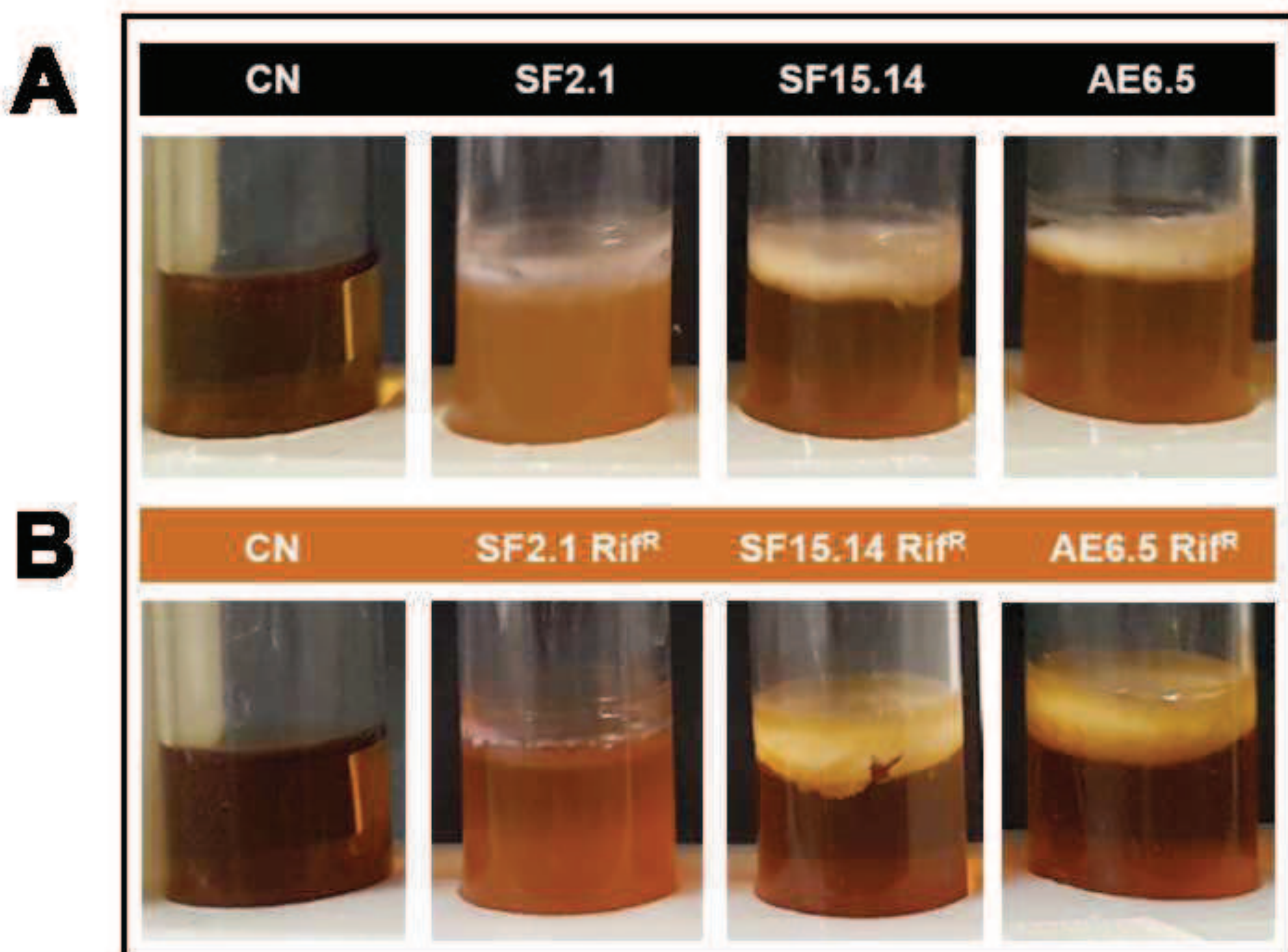

Figure 3

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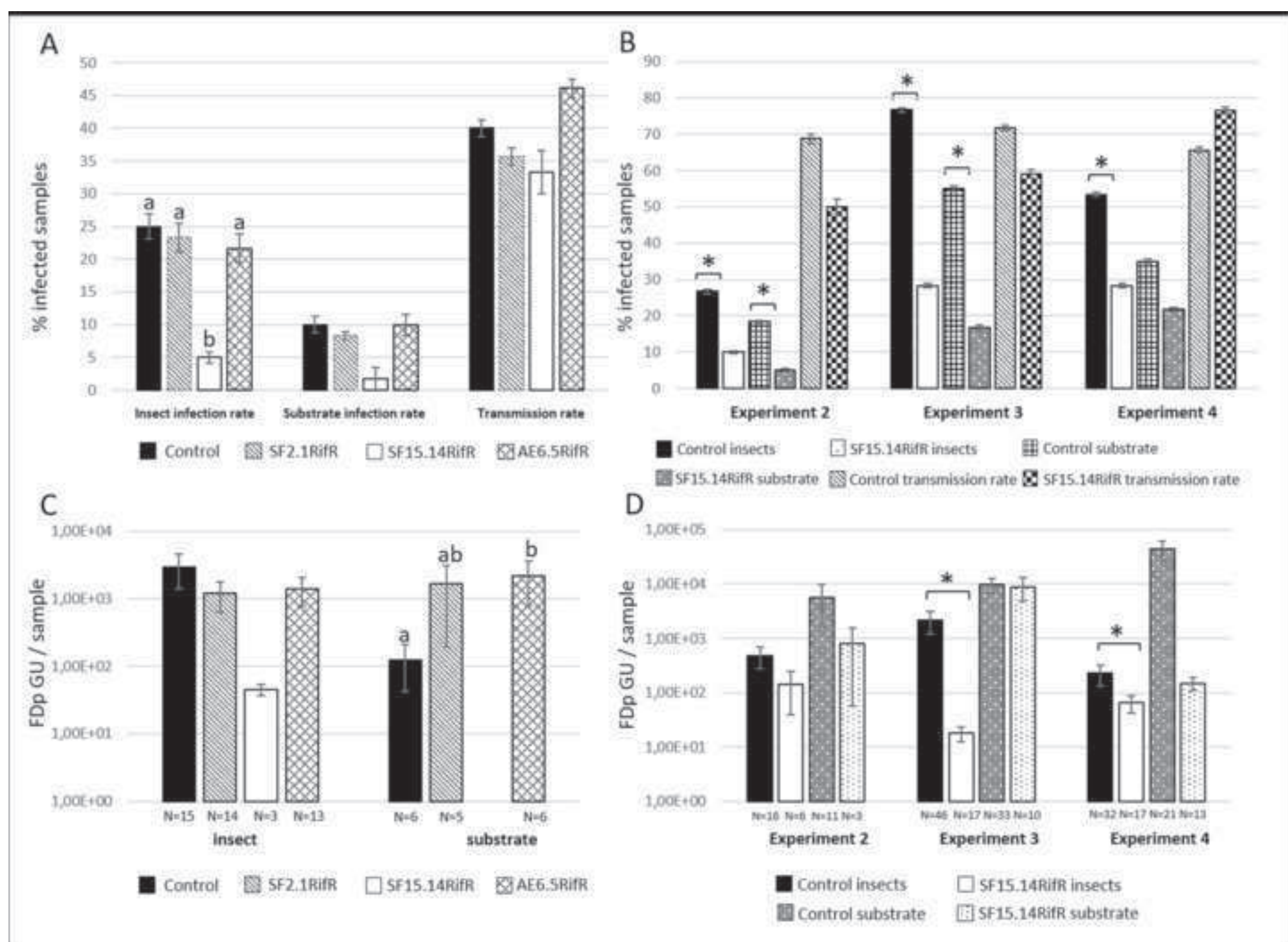

Figure 4

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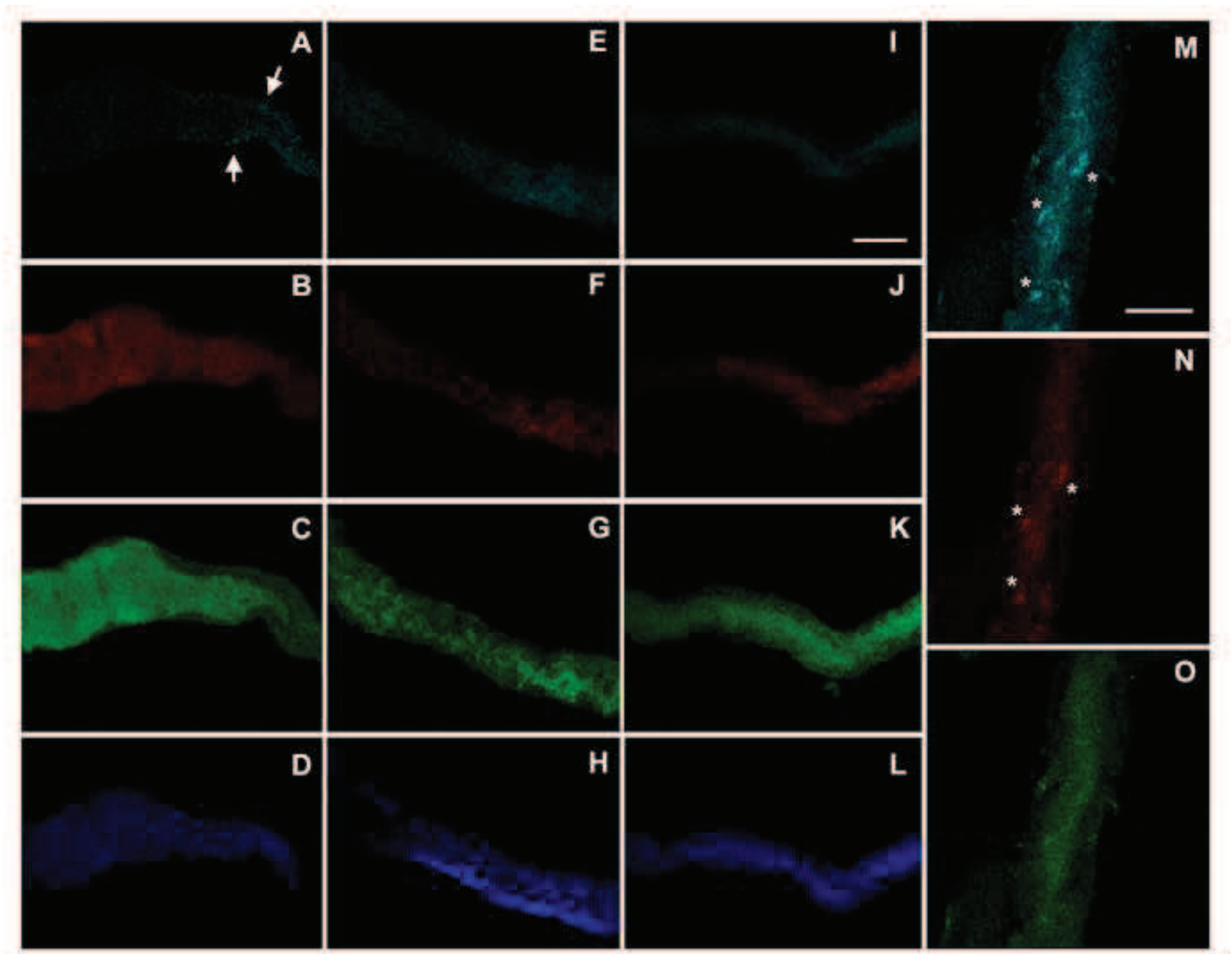

Figure S1

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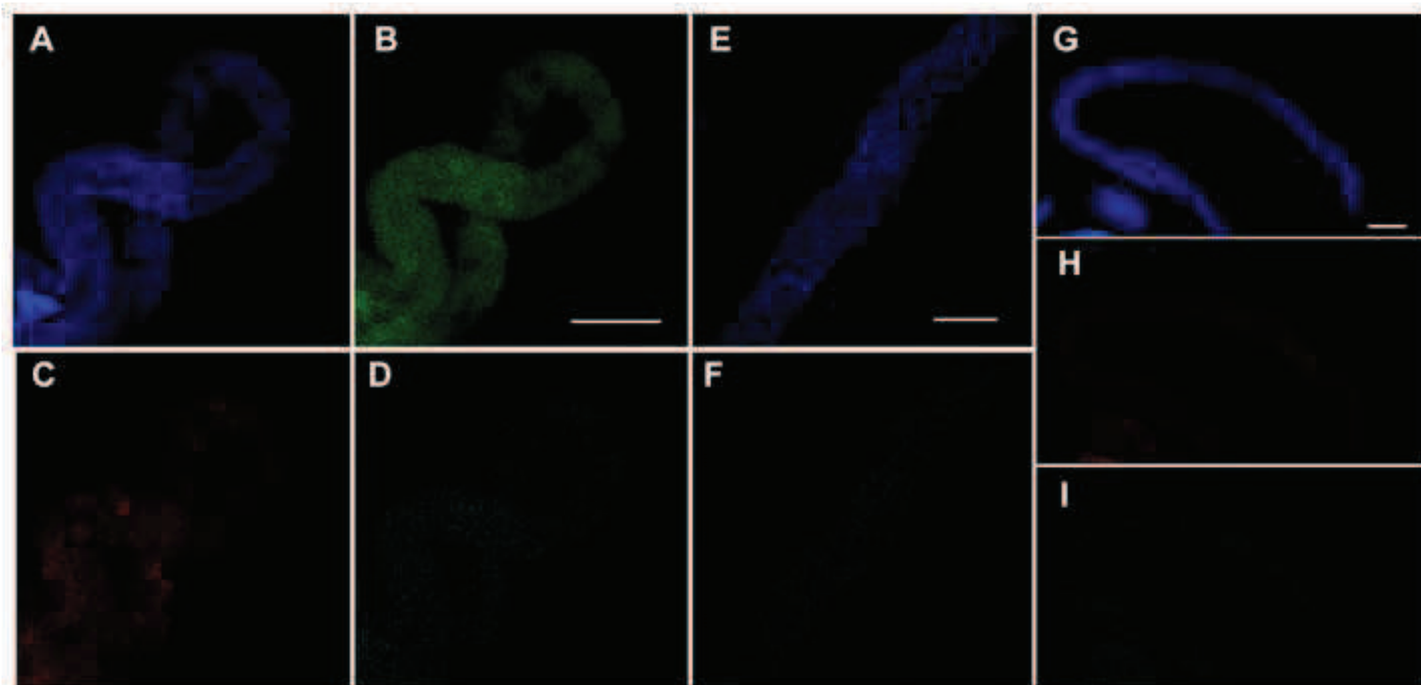

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2 Inoculation Access Period.

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Experiment 2	SF15.14 Rif^R, control (no *Asaia*)	Healthy oat	Broad bean
Experiment 3	SF15.14 Rif^R, control (no *Asaia*)	FDp-infected broad bean	Artificial diet
Experiment 4	SF15.14 Rif^R, control (no *Asaia*)	FDp-infected broad bean	Broad bean

3 [a] The experiments with each strain was run separately

4

Table 2. Colonization efficiency of different *Asaia* strains in *E. variegatus.*

[#]Percentage of specimens from which the rifampicin-resistance strains of *Asaia* (confirmed by ITS-PCR fingerprinting) were reisolated on MA medium containing 100 µg/ml rifampicin (N=30). The number of samples showing successful reisolation is indicated in parentheses. [¶]*Asaia* cells per positive insect. Values below the blank threshold (2.20×10^{1}) were considered negative. Transformed values (10+log) used for statistical analyses are indicated in parentheses together with their standard errors. All *Asaia*-positive insects were used to calculate average densities; their number is indicated in square brackets.

AAP: Acquisition Access Period; LAP: Latency Access Period; SE: Standard Error; ND: Not Detectable (*Asaia* below blank threshold).

Strain	Collection time	*Asaia* reisolation rate (%) ± SE[#]	*Asaia* density (transformed value± SE)[¶]
SF2.1 Rif^{R}	End of *Asaia* AAP	53.33 ± 0.01 (16/30)	1.00×10^{4} (12.81± 0.14) [N=16]
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	End of AAPs + LAP	93.33 ± 0.02 (28/30)	5.55×10^{5} (13.50± 0.22) [N=28]
SF15.14 Rif^{R}	End of *Asaia* AAP	80.00 ± 0.07 (24/30)	5.17×10^{3} (13.05± 0.12) [N=24]
	End of FDp AAP	76.67 ± 0.04 (23/30)	1.39×10^{3} (12.58± 0.13) [N=23]
	End of AAPs + LAP	76.67± 0.02 (23/30)	5.08×10^{4} (13.52± 0.19) [N=23]
AE6.5 Rif^{R}	End of *Asaia* AAP	80.00 ± 0.02 (24/30)	2.59×10^{3} (11.70± 0.23) [N=24]
	End of FDp AAP	80.00 ± 0.02 (24/30)	1.27×10^{3} (12.60± 0.10) [N=24]
	End of AAPs + LAP	80.00 ± 0.02 (24/30)	5.86×10^{4} (13.33± 0.23) [N=24]
Control (no *Asaia*)	End of FDp AAP	0.00% (0/30)	$<2.20\times10^{1}$
	End of AAP + LAP	0.00% (0/30)	$<2.20\times10^{1}$

16 **Table 3.** Phytoplasma infection rates from FDp transmission trials. The percentage of positive samples according to qPCR analyses on leafhoppers
17 and sugar diets or broad beans during experiments 1-4 (see Table 1) are presented. For experiment 1, different letters indicate significantly different
18 values according to ANOVA or binomial GLM (P < 0.05). For experiments 2-4, asterisks indicate significantly different values according to Student's
19 t tests or binomial GLM (P< 0.05). [#]Percentage of 16SrV phytoplasma-positive individuals related to the total tested samples (N=60). [¶]Percentage of
20 phytoplasma-positive leafhoppers that actually transmitted FDp to their feeding substrate, related to the total infected specimens.
21 The number of positive samples is indicated in parentheses. SE: Standard Error; ns: not significant.

Experiment no.	*Asaia* strain	FDp infection rate (%) ± SE in *E. variegatus*[#]	FDp infection rate (%) ± SE in feeding substrates[#]	FDp transmission rate (%) ± SE[¶]
1	SF2.1 Rif[R]	23.33 ± 0.22 a (14/60)	8.33 ± 0.06 ns (5/60)	35.71 ±0.13 ns (5/14)
	SF15.14 Rif[R]	5.00 ± 0.09 b (3/60)	1.67 ± 0.18 ns (1/60)	33.33 ± 0.33 ns (1/3)
	AE6.5 Rif[R]	21.67 ± 0.22 a (13/60)	10.00 ± 0.16 ns (6/60)	46.15 ± 0.14 ns (6/13)
	Control (no *Asaia*)	25.00 ± 0.19 a (15/60)	10.00 ± 0.13 ns (6/60)	40.00 ± 0.13 ns (6/15)
2	SF15.14 Rif[R]	10.00 ± 0.04 * (6/60)	5.00 ± 0.05 * (3/60)	50.00 ± 0.22 ns (3/6)
	Control (no *Asaia*)	26.67± 0.06 * (16/60)	18.33 ± 0.03 *(11/60)	68.75 ± 0.12 ns (11/16)
3	SF15.14 Rif[R]	28.33 ± 0.06 * (17/60)	16.67 ± 0.07 * (10/60)	59.12 ± 0.12 ns (10/17)
	Control (no *Asaia*)	76.67 ± 0.06 * (46/60)	55.00 ± 0.08 *(33/60)	71.74 ± 0.07 ns (33/46)
4	SF15.14 Rif[R]	28.33 ± 0.06 * (17/60)	21.67 ± 0.05 ns (13/60)	76.47 ± 0.10 ns (13/17)
	Control (no *Asaia*)	53.33 ± 0.06 * (32/60)	35.00 ± 0.06 ns (21/60)	65.62 ± 0.09 ns (21/32)

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Table S1. Identification of cultivable bacteria associated with *E. variegatus*.

Isolate code[a]	ITS group[b]	No. isolates per ITS group[c]	Identity (Acc. Num.)	% identity (bp)	RDP classification (family: genus)
EV5	1	8	*Pantoea agglomerans* (JX089401)	97 (534/549)	Enterobacteriaceae: *Pantoea*
EV9	2	2	*Pseudomonas* sp. (KX450447)	100 (593/603)	Pseudomonadaceae: *Pseudomonas*
EV10	3	1	*Comamonas* sp. (KR055003)	99 (858/858)	Comamonadaceae: *Comamonas*
EV12	4	3	*Curtobacterium flaccumfaciens* (KY970145)	100 (776/778)	Microbacteriaceae: *Curtobacterium*

[a]Isolate code indicates the code assigned to the ITS-group representative isolate.
[b]ITS group indicates the number of the different ITS groups obtained after dereplication by ITS-PCR
[c]No. Isolates per ITS group refers to the number of isolates included in the indicated ITS group.