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Asaia symbionts interfere with infection by Flavescence dorée phytoplasma in leafhoppers

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Abstract

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

Key words

Flavescence dorée, Acetic acid bacteria, vector competence, symbiotic control
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.
**Introduction**

Many insects carry and transmit microbial pathogens to their animal and plant hosts. The transmission of such pathogens is usually carried out during the feeding process, often requiring incubation for multiplication in the salivary glands. Stable colonization of the vector by the pathogen is necessary for transmission and is successfully completed in only a subset of individuals in an insect population (Weiss and Aksoy 2011; Galetto et al. 2011; Krugner et al. 2012; Ammar et al. 2016). Imperfect vector competence is regulated by the ingested load of the pathogen, the insect-pathogen genotype interaction, and many environmental factors (Tabachnick 2015). Other factors that limit pathogen spread include the presence of organ-specific barriers in the host, insufficient host survival rates, or the immune response of the insects (Alto et al. 2005; Galetto et al. 2009; Westbrook et al. 2010; Weiss and Aksoy 2011, Mandrioli et al. 2015; Shapiro et al. 2016). Key to the transmission process are the digestive tract and the salivary glands of the insect. In the midgut, interactions with the peritrophic matrix and gut epithelia have been found to be crucial for microbial invasion of the insect’s hemocoel (Killiny et al. 2005; Lo et al. 2013; Rashidi et al. 2015; Buchon et al. 2016; Malta et al. 2016).

Insects are inhabited by a multitude of microbial symbionts capable of establishing variable beneficial (mutualist) and neutral (commensal) interactions with their hosts. The microbial community hosted by insects may influence their competence in transmitting pathogens as vectors (Weiss and Aksoy 2011; Kramer and Ciota 2015; Trivedi et al. 2016) through competitive exclusion, anti-pathogen activity or immunomodulation. For example, both negative and positive correlations between various symbiotic bacteria and the plant pathogen ‘*Candidatus Liberibacter asiaticus*’ have been documented in *Diaphorina citri* Kuwayama (Fagen et al. 2012); infection with Palm Creek virus has been shown to control the presence of the West Nile virus in *Culex annulirostris* Skuse (Hall-Mendelin et al. 2016). The manipulation of the microbial communities associated with pathogen vectors has been proposed to improve the insect’s performance related to interesting phenotypes (Mueller and Sachs 2015), possibly driving the evolution of the vector in the direction of reduced vector competence,
similarly to techniques designed for pathogen control based on genetic shifting (Powell and Tabachnick 2014).

Phytoplasmas are pathogenic bacteria in plants belonging to the class Mollicutes. They are transmitted from plant to plant by insect vectors, namely leafhoppers, planthoppers, and psyllids (Maejima et al. 2014). More than 1,000 plant diseases are activated by phytoplasmas, causing losses among economically important species worldwide (Maejima et al. 2014; Perilla-Henao et al. 2016).

One of the most bothersome phytoplasma-related diseases in Europe is Flavescence dorée (FD), which affects grapevines. The symptoms associated with this disease can be severe and even lead to the death of infected plants, causing extensive economic loss (Chuche and Thiéry, 2014). Based on the analysis of 16SrRNA gene, the FD agents are included in two subgroups of 16SrV phytoplasma group, namely subgroups –C and –D, both widespread in the mainly affected countries (Davis and Delly, 2001; Martini et al., 2002). In nature, the vector of FD phytoplasmas (FDp) is the leafhopper Scaphoideus titanus Ball. Because rearing the monovoltine S. titanus and maintaining FDp in grapevines are both challenging under laboratory conditions, the cicadellid Euscelidius variegatus Kirschbaum is commonly used as an experimental vector in the broad bean Vicia faba L. in the laboratory (Bressan et al. 2005).

Even though the microbial communities of many phytoplasma vectors have been previously described (Marzorati et al. 2006; Gonella et al. 2011; Wangkeeree et al. 2011, 2012; Iasur-Kruh et al. 2013; Ishii et al. 2013; Powell et al. 2015), little information on the influence of the symbiotic microbiome on competence of the leafhopper vector of phytoplasmas is currently available (Trivedi et al. 2016) despite the relevance to worldwide agriculture of these pathogens. Interactions between symbiotic bacteria and phytoplasma inside the insects have been proposed (Ishii et al. 2013) but not yet demonstrated. In S. titanus, symbiotic Acetic Acid Bacteria (AAB) of the genus Asaia have been identified and proposed as potential symbiotic control candidates (Crotti et al. 2009). Indeed, this symbiotic bacterium was reported to be dominant in the leafhopper and to be vertically and horizontally transmitted; moreover Asaia is easy to cultivate and transform and it efficiently colonizes
various phylogenetically distant hosts (Crotti et al. 2009). *Asaia* strains with different phenotypic
traits have been isolated from plant tissues and have been frequently found in association with
different insect orders, including Hymenoptera, Diptera and Hemiptera (Crotti et al. 2010). Some of
these strains have been shown to inhibit the diffusion of other organisms, including vector-borne
pathogenic agents (Hughes et al. 2014; Sant’Anna et al. 2014). However, the potential of insect-
associated *Asaia* to inhibit FD transmission in insect vectors has never been tested.

Here, we evaluated the potential of different *Asaia* strains to interfere with the vector competence of
leafhoppers for FDp in the laboratory. For this purpose, we used the experimental vector *E. variegatus*
and the broad bean *Vicia faba* L. (Salar et al. 2013).

**Materials and methods**

**Insect and plant material and bacterial strains**

*E. variegatus* individuals used in this work were from a laboratory line reared on oat (*Avena sativa*
L.) in growth chambers at 25°C with a photoperiod of 16:8 (L:D) at the DISAFA laboratories. Broad
beans (*Vicia faba* L.) infected by FDp were obtained by exposing seedlings to *E. variegatus*
specimens infected by FDp (strain FD-C). Healthy broad bean and oat seedlings were grown in screen
houses that were separated from the insect rearing space and other phytoplasma sources.

*Asaia* colonization experiments in *E. variegatus* were performed using spontaneous rifampicin-
resistant mutants of *Asaia* SF2.1 (Favia et al., 2007), *Asaia* AE6.5 (Crotti et al., 2009) and *Asaia*
SF15.14. The latter was isolated from a female *Anopheles stephensi* Liston mosquito derived from a
colony reared in the insectary at the University of Camerino since 1988. Briefly, following a surface
sterilization procedure consisting of three washings with 0.9% NaCl, mosquitoes were homogenated
in 200 ml 0.9% NaCl. Twenty microlitres of the homogenate were inoculated into an enrichment
medium at pH 3.5, followed by plating on a CaCO₃-rich agarized medium, as reported by Favia et al.
(2007). The 16S rRNA gene of the SF15.14 strain was amplified using the 27F and 1495R primers
(Lane 1991), as described by Mapelli et al. (2013). The nearly full-length sequence was deposited in the European Nucleotide Archive’s database under accession number LT838398. Spontaneous rifampicin-resistant mutants of *Asaia* were obtained by exposing well-grown cultures of *Asaia* strains (SF2.1, AE6.5 and SF15.14) to 100 µg/ml rifampicin overnight. This process generated the SF2.1 RifR, AE6.5 RifR and SF15.14 RifR strains, respectively.

To evaluate the capability of the bacterial strains to produce an air-liquid interface (ALI) biofilm, static cultures of the three *Asaia* strains were grown in tubes with 5 ml of modified potato dextrose broth (PDB), containing 20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, 8 g/L potato infusion, with pH 6. After overnight growth, 100 µl of bacterial cultures (diluted to OD 1) were inoculated into the tubes and incubated at 30°C for 10-12 days.

**Assessment of *Asaia* infection in *E. variegatus***

To verify whether our population of *E. variegatus* was naturally infected by *Asaia*, 30 adults were collected (15 males and 15 females) and submitted to the following molecular diagnostic analysis. After DNA extraction (according to Gonella et al. 2012), *Asaia*-specific PCR was performed with the Asafor/Asarev primer pair (Crotti et al. 2009). The sequence of the 16S rRNA of the *Asaia* strain from all positive *E. variegatus* was extended with two additional specific PCRs to amplify the flanking regions at the 5’ and 3’ ends of the fragment for *Asaia*-specific amplification. We used the *Asaia*-specific primer Asafor in combination with the universal reverse primer 1495R and Asarev with the universal forward primer 27F as described previously (Mapelli et al. 2013). The amplicons were sequenced in both directions. The final sequence, obtained by assembling the two 16S rRNA gene contigs, was used to analyse the phylogenetic position of the isolate. A phylogenetic tree was constructed based on the maximum likelihood method with the software MEGA 7. Additionally, the near full-length sequence of *Asaia* 16S rRNA gene from one specimen was deposited in the European Nucleotide Archive’s database under accession number LT838399.
Ten adult leafhoppers were used in attempts to isolate the *Asaia* bacteria. Insect surface sterilization was performed by rinsing the insects with 1% sodium hypochlorite for one minute, followed by washing with 0.9% NaCl for five minutes and a final rinse in 0.9% NaCl. Four single or two pooled (3 insects in each pool) individuals were homogenized in 200 µl of 0.9% NaCl. One-hundred µl of the homogenates and their serial dilutions were plated on mannitol agar (MAN) plates (Vacchini et al. 2017), combined with 100 µg/ml cycloheximide and incubated at 30°C. Furthermore, 20 µl of the homogenates were inoculated in TA1, YE and ABEM liquid media (Vacchini et al. 2017; Favia et al., 2007), combined with 100 µg/ml cycloheximide and incubated at 30°C under aerobic conditions with shaking, until turbidity in the medium was observed. Serial dilutions were then plated on MA (1% D-glucose, 1% glycerol, 1% bactopeptone, 0.5% yeast extract, 0.7% CaCO₃, 1% ethanol, 1.5% agar, pH 6.8) or ABEM (2% D-glucose, 0.5% ethanol, 0.8% yeast extract, 0.7% CaCO₃, 1.2% agar, pH 7) agar plates as described by Vacchini et al. (2017) and Favia et al. (2007). Based on morphology, several colonies were purified on agarized media, and pure strains were stored in 15% glycerol at -80°C. Dereplication of the isolates was performed by comparing the isolates’ Intergenic Transcribed Spacer (ITS)-PCR profiles (Vacchini et al. 2017). From bacterial representatives for each ITS profile group, a partial fragment of 16S rRNA gene was amplified (Vacchini et al., 2017); amplicons were sequenced at Eurofin Genomics (Vimodrome, Milano). The partial 16S rRNA gene sequences from representatives of each ITS group were deposited in the European Nucleotide Archive’s database under the accession numbers LT934417-LT934420 under the study ID PRJEB22871 (http://www.ebi.ac.uk/ena/data/view/LT934417-LT934420).

**Asaia colonization of *E. variegatus***

To evaluate the capability of *Asaia* isolates to colonize *E. variegatus* stably, we provided the isolates to third instar nymphs following procedures described by Crotti et al. (2009). Briefly, cells were grown at 30°C under the selection of rifampicin, harvested by centrifugation (10 min, 3000 g), washed three times with 0.9% NaCl and adjusted to 10⁸ cells/ml in 5% (w/v) sucrose solution in Tris-EDTA.
at pH 8. Cell suspensions were provided to the nymphs and they were allowed to feed for 48 hours. This artificial diet was provided to a total of 810 nymphs: 270 of them were fed with the SF2.1 Rif\textsuperscript{R} strain, another 270 with the SF15.14 Rif\textsuperscript{R} strain, and the remaining 270 with the AE6.5 Rif\textsuperscript{R} strain. An additional 270 specimens were not fed in the presence of any \textit{Asaia} strain and were used as the control. The total number of nymphs corresponding to each treatment was then divided into three groups of 90. The first 90 insects in each group were immediately fed an equivalent, cell-free sugar diet for 24 more hours to avoid possible contamination by transient bacterial cells. The remaining 180 nymphs were maintained in plastic cages (24 × 16 × 12 cm) covering FD-infected broad beans for one week. Subsequently, 90 specimens were immediately collected, and the others were reared for a latency access period (LAP) of 21 days on healthy oat plants. At the end of every experimental step, 60 individuals were taken and preserved at -20°C for \textit{Asaia}-specific qPCR (Favia et al. 2007), whereas 30 nymphs were used for reisolation trials. In the reisolation experiments, we took advantage of the rifampicin resistance trait to selectively isolate only the strains that we previously provided to the insect and avoiding possible contamination with native culturable bacterial symbionts. Insects were surface sterilized as described above and homogenized in 900 \textmu{l} of saline; these suspensions were then plated on MA medium (Vacchini et al. 2017) combined with 100 \textmu{g/ml} rifampicin. One-hunderd \textmu{l} of the last saline washing solution used for each treatment was plated in triplicate on a separate plate as a control. Plates were incubated at 30°C for two days; total DNA was extracted from colonies by sodium dodecyl sulfate-proteinase K-cetyltrimethyl ammonium bromide treatment (Sambrook et al., 1989) and stored at -20°C. Intergenic transcribed spacer (ITS)-PCR fingerprinting was performed as described above. ITS-PCR amplification patterns of all the isolates were visually compared to those produced by the pure strains of \textit{Asaia}.
FDp transmission trials were set up under laboratory conditions to compare FDp acquisition and transmission efficiencies of *E. variegatus* colonized by different *Asaia* strains (Table 1, Fig. S1). In Experiment 1, the minimum transmission efficiency of *E. variegatus* individuals exposed to the three tested *Asaia* strains was determined. Three groups of 70 third instar nymphs were fed with *Asaia* strains SF2.1 Rif\(^R\), SF15.14 Rif\(^R\), or AE6.5 Rif\(^R\), respectively, as described above; 70 specimens never fed in the presence of any *Asaia* strain were used as a control. Subsequently, insects were reared on FDp-infected broad beans for an acquisition access period (AAP) of one week and then moved to healthy oat seedlings for a LAP of 21 days. At the end of this period, leafhoppers were collected and individually reared on sterile sugar diets for 48 hours. Finally, 60 insects and their diets were collected and preserved at -20°C for molecular analyses. The remaining 10 specimens in each group were subjected to dissection of their midguts and preserved for Fluorescence In Situ Hybridization (FISH) analysis (see the FISH section below for a detailed method description).

Experiments 2-4 were carried out to specifically compare the FDp transmission efficiency by leafhoppers exposed or not exposed (control) to *Asaia* SF15.14 Rif\(^R\). Groups of 60 individuals were used in each treatment. In Experiment 2, the efficiencies in transmitting FDp to a plant were compared using healthy broad beans as infection recipients; in Experiments 3 and 4, transmission efficiencies when the leafhoppers were subjected to constant pathogen pressure were evaluated. In these three experiments, the steps involving *Asaia* acquisition (not performed on individuals in control groups) and FDp acquisition were the same as in Experiment 1. In Experiment 2, LAP was carried out under the same conditions as those of Experiment 1; conversely, Experiments 3 and 4 were conducted with a latency on FDp-infected broad beans. For the final phytoplasma inoculation, insects were singularly maintained on sterile sugar diets for 48 hours (Experiment 3) or on healthy broad bean seedlings for one week (Experiments 2 and 4), before being collected and preserved at -20°C for subsequent analyses. The sugar diets were collected along with the insects for molecular analysis. Broad bean leaf samples were collected after being grown in an insect-proof chamber (25°C, RH 70%) for three weeks after the beginning of phytoplasma inoculation.
DNA extraction and PCR-based analyses

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

Quantitative real-time PCR was performed to measure the presence and concentration of *Asaia* cells in colonized *E. variegatus*, insects, artificial diet and plants at the end of the transmission trials; the latter were tested for FDp as well. All qPCR reactions were performed on a Chromo4 real-time instrument (Bio-Rad, Milan, Italy) using the SsoFast™ EvaGreen® Supermix (Bio-Rad). In insects used in the bacterial colonization studies, specific reactions targeting *Asaia* 16S rRNA gene were carried out with the Asafor/Asarev primers according to Favia et al. (2007). On the samples used in the FDp transmission trials, 16SrV group phytoplasma-specific reactions were performed with the fAY/rEY primer pair (Marcone et al. 1996; Marzachi et al. 2001), under conditions described elsewhere (Galetto et al. 2005). The average FDp Genome Units (GU) per sample were considered to be the 16S rRNA gene copy numbers divided by two, because this gene was estimated to be in two copies in the FDp’s genome (Schneider and Seemüller 1994). *Asaia* GUs per sample were considered as 16S rRNA gene copies divided by four, assuming that four rRNA gene copies per cell were present in *Asaia*’s genome on average (Crotti et al. 2009). In addition, to normalize the absolute density of FDp and *Asaia* in insects, a qPCR targeting the insect’s 18S rRNA gene (MqFw / MqRv) was used as reported by Marzachi and Bosco (2005). Normalized FDp and *Asaia* GUs were calculated per pg of insect 18S rRNA gene. To assess the diets, we calculated the normalized FDp concentration per whole sample (expressed as ng of total DNA obtained from 300 μl of sugar solution), whereas results from the plant samples were expressed as FDp GU per 100 mg of leaves.
To construct standard curves for the real-time PCRs, the PCR-amplified 16S rRNA gene of *Asaia* and FDp were cloned using the pGEM T-easy Vector Cloning Kit (Promega). For each reaction, the detection limit was calculated as the lowest concentration of cloned amplicons used for determining the standard curves that were successfully amplified. Detection limits corresponded to $3.80 \times 10^0$ FDp GU per sample and $1.15 \times 10^0$ *Asaia* GU per sample.

**Fluorescence in Situ Hybridization**

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

**Statistical analyses**

Statistical analyses were performed with SPSS Statistics 22 (IBM Corp. Released 2013, Armonk, NY). Data of infected individuals detected in FDp transmission trials (Experiments 1-4) were analysed using a generalized linear model (GLM) with a binomial probability distribution and a
Bonferroni post hoc test (P<0.05). Quantitative PCR data relative to the concentration of FDp cells observed in positive samples within each experiment were log-transformed after adding the constant 10. Transformed data were analysed by Student’s t tests (P<0.05), or by one-way analysis of variance (ANOVA) followed by means separation by a Tukey test (P<0.05) when variance homogeneity was satisfied (Levene test, P<0.05).

Results

Asaia occurring in and colonizing E. variegatus

Laboratory individuals of E. variegatus showed an infection rate with native Asaia of 23.33%. Specifically, 33.33% and 13.33% of males and females were infected, respectively. The phylogenetic analysis of Asaia sequences obtained from positive leafhoppers showed that they clustered together with Asaia bogorensis, A. platycodi, A. prunellae, A. siamensis and A. spathodeae, as well as with other strains within this genus previously isolated from Diptera and Lepidoptera. Moreover, the native Asaia strain of E. variegatus was phylogenetically close to many other insect-associated isolates, including isolate SF2.1 used in this study. Conversely, strains SF15.14 and AE6.5 clustered together separately from other symbiotic strains (Fig.1).

However, no bacterial isolates referring to Asaia could be obtained from any of the E. variegatus specimens examined in the isolation attempts. According to the differences in colony morphologies on the plates, 14 isolates were purified and thus dereplicated on the basis of the ITS fingerprinting profiles. Four ITS profiles were obtained; the 16S rRNA gene sequencing of their representatives identified the isolates as belonging to Comamonas sp. (Betaproteobacteria), Pantoea sp. (Gammaproteobacteria), Pseudomonas sp. (Gammaproteobacteria) and Curtobacterium sp. (Actinobacteria) (Table S1).

We then assessed the capability of exogenous Asaia strains to be acquired and maintained in the insect for a time comparable to that required for phytoplasma transmission, i.e., 28 days (AAP+LAP). Specifically, we used two strains of Asaia reported in previous publications, i.e., Asaia SF2.1 and
Asaia AE6.5 (Favia et al. 2007; Crotti et al. 2009), and Asaia SF15.14, which we isolated from a female A. stephensi mosquito (Acc. Num. LT838398). These strains differed in their capacity to produce ALI biofilm, i.e., a pellicle on the surface of the liquid medium (Armitano et al. 2014). Specifically, Asaia SF2.1 did not form a thick floating pellicle when cultured under static conditions, whereas SF15.14 and AE6.5 did build a thick ALI biofilm (Fig. 2). Interestingly, it is possible to observe that the SF2.1 static culture was more turbid when compared with the SF15.14 and AE6.5 static cultures, likely suggesting that since SF2.1 cells were not entrapped in the ALI biofilm, they could swim in the medium (Fig. 2A). These phenotypes were also confirmed in the spontaneous rifampicin-resistant mutants (Fig. 2B).

Reisolation assays from insects exposed to the three isolates of Asaia, followed by ITS fingerprinting for confirming strain identity, showed that Asaia could be successfully reisolated on selective media containing rifampicin, with high reisolation rates (Table 2). The highest reisolation rate was observed for the SF2.1 Rif\(^R\) strain after 30 days, i.e. at the end of Asaia and phytoplasma AAPs (two plus seven days) and LAP (21 days). However, when recording reisolation rates after two days, immediately at the end of Asaia AAP, insects treated with the the SF2.1 Rif\(^R\) strain had the lowest percentage of reisolation. No isolation of Asaia strains was achieved with individuals from the control group nor from the saline washing solutions.

The control-group leafhoppers used for qPCR were first tested because preliminary qualitative PCR exhibited infection with a native Asaia. Some of the samples (33.33%) indicated that the density of Asaia cells exceeded the detection limit, as expected, because the primers used for qPCR amplified this strain as well. None of the samples had a concentration higher than \(2.20 \times 10^1\) Asaia GU; this value was therefore used as a blank threshold, and insects with lower Asaia concentrations were considered negative for the introduced bacteria. In samples with higher rates, this threshold value was subtracted to avoid overestimation due to native Asaia amplification. After these adjustments, the concentration of Asaia in positive leafhoppers was quite stable, with a slightly increasing trend over time, between \(6.35 \times 10^2\) and \(5.55 \times 10^5\) Asaia GU per sample (Table 2).
FDp transmission trials

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

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

FISH experiments performed on the midguts of leafhoppers from experiment 1 (Fig. 4) confirmed that bacteria were found in the guts of all specimens (Fig. 4C, G, K), as indicated by DAPI staining.
that outlined the epithelial tissues (Fig. 4D, H, L). Moreover, *Asaia* was detected in *E. variegatus* specimens fed with all strains of the bacteria (Figs. 4A, E, I, M), although individuals reared in the laboratory did not produce any *Asaia*-specific hybridization signal (Fig. S2). *Asaia* was typically found to become established close to the midgut epithelia (Fig. 4A). On the other hand, FDp could be massively detected in some midguts collected from leafhoppers treated with *Asaia* SF15.14 Rif\(^R\) as well as with other strains (Figs. 4B, F, J, N) and in control insects (Fig. S2). A specific colonization pattern was recorded in some of the insects fed with *Asaia* SF15.14 Rif\(^R\): spots of fluorescence were observed in the guts (Fig. 4M, N). These spots may correspond to bacterial clusters, and they were visible after hybridization with both the *Asaia*-specific and the 16SrV phytoplasma-specific probes. No FDp-specific hybridization was detected from mass-reared insects for which only DAPI staining and the eubacterial probe showed a positive signal (Fig. S2).

Based on results obtained from experiment 1, we designed experiments 2-4 to compare the FDp infection levels between individuals with a natural *Asaia* background with those colonized by *Asaia* SF15.14 Rif\(^R\), the only strain found to reduce infection rates in *E. variegatus*. In experiment 2, a significantly lower percentage of *Asaia* SF15.14 Rif\(^R\)-fed insects positive for FDp than in the control (binomial GLM: df = 1; \(\chi^2 = 5.198; P<0.05\)) was found; remarkably, this percentage was double that in experiment 1. Similarly, FDp infection rates of inoculated broad beans were significantly divergent (binomial GLM: df = 1; \(\chi^2 = 4.552; P<0.05\)), although they were higher than those of artificial diets recorded in experiment 1. However, no significant differences were observed between FDp densities in insects (Student’s t Test: df = 20; \(t = 0.676; P = 0.51\)) and broad beans (Student’s t Test: df = 12; \(t = 0.375; P = 0.71\)), and between recorded transmission rates (binomial GLM: df = 1; \(\chi^2 = 0.649; P = 0.42\)).

In experiment 3, the FDp infection rates of both insects and artificial diets were higher than those recorded in the previous experiments; in either case, the percentage of FDp-infected samples was significantly lower for the group treated with the SF15.14 Rif\(^R\) strain than the control, according to binomial GLM (test on leafhopper samples: df = 1; \(\chi^2 = 25.587; P<0.05\); test on diet samples: df = 1;
\( \chi^2 = 17.490; P<0.05 \). Moreover, the FDp concentration in positive insects exposed to the SF15.14 Rif\textsuperscript{R} strain was significantly lower than that of the control (Student’s t test: df = 61; t = 3.892; P<0.05). On the other hand, no differences between artificial diets provided to treated and untreated insects were observed (Student’s t test: df = 41; t = 0.339; P = 0.74); similarly, no differences were detected between FDp transmission rates (binomial GLM: df = 1; \( \chi^2 = 0.944; P = 0.33 \)).

In experiment 4, *E. variegatus* specimens fed with *Asaia* SF15.14 Rif\textsuperscript{R} had a significantly lower FDp infection rate than the control group had (binomial GLM: df = 1; \( \chi^2 = 7.202; P<0.05 \)). Conversely, FDp infection rates recorded for the respective broad beans showed no significant differences (binomial GLM: df = 1; \( \chi^2 = 2.417; P = 0.12 \)), even though the percentage of positive plants exposed to the SF15.14 Rif\textsuperscript{R} strain was lower than the control (Table 3). Likewise, the concentration of FDp in *E. variegatus* individuals fed the SF15.14 Rif\textsuperscript{R} strain was significantly lower than that of the control group (Student’s t test: df = 47; t = 1.441; P<0.05), whereas the abundances of phytoplasma cells detected in infected broad beans did not significantly diverge (Student’s t test: df = 32; t = 0.385; P = 0.70). In addition, no significant difference was found between FDp transmission rates (binomial GLM: df = 1; \( \chi^2 = 0.608; P = 0.43 \)); however, in this single experiment, the percentage of successful phytoplasma transmission for leafhoppers exposed to *Asaia* SF15.14 Rif\textsuperscript{R} was surprisingly higher than the control, but not significantly different.

Interestingly, even though none of *Asaia* strains colonized 100% of *E. variegatus* specimens, the percentages of co-infected insects (*Asaia + FDp*) recorded in each experiment was not different from infection rates of phytoplasma alone (Table S2).

**Discussion**

The presence and colonization of *Asaia* in leafhoppers has been previously reported (Crotti et al. 2009; Gonella et al. 2012) and our results indicate that *E. variegatus* hosts *Asaia* as well. Similarly to *S. titanus* (Crotti et al. 2009), native *Asaia* was detectable in *E. variegatus* by molecular methods only but could not be isolated in pure cultures. In our attempts to isolate *Asaia*, we employed several media
that were previously effective for isolating *Asaia* or other AAB from insects (Crotti et al. 2009; Vacchini et al. 2017). It is possible that growing *Asaia* living in *E. variegatus* requires specific factors produced by the host or by other microorganisms associated with the gut of *E. variegatus*, such as those we were able to isolate (Table S1). Moreover, we observed a relative phylogenetic divergence among the native *Asaia* strain and two of the exogenous isolates that were provided to the leafhopper, specifically the ALI biofilm-producer strains SF15.14 RifR and AE6.5 RifR (Fig. 1), suggesting that they could differentially interact with the host. The gut of *E. variegatus* turned out to be a suitable environment for the establishment of exogenous *Asaia* strains isolated from other insects. The three rifampicin-resistant *Asaia* strains we have studied, i.e., SF2.1 RifR, AE6.5 RifR and SF15.14 RifR, successfully colonized the bodies of leafhoppers as indicated by their detection and reisolation from the host in high numbers up to four weeks after the uptake of the bacterial cells. This capability was observed for all the tested strains, in agreement with the high colonization versatility of different insect hosts previously observed in *Asaia* (Crotti et al. 2009). Cultivation-dependent and independent analyses testing *Asaia* infection and the concentration in *E. variegatus* indicated that infection levels do not decrease over time and that the concentration of exogenous *Asaia* cells in the colonized *E. variegatus* individuals was quite stable over time as well, even though it could not reach the value of 1-2×10^6, which was previously detected in *S. titanus* (Gonella et al. 2012).

The capability of *Asaia* strains with different phenotypes to affect FDp transmission was assessed as well, since some AAB have been described as having probiotic activity by inhibiting pathogens (Haghshenas et al. 2015). A main mechanism of antagonism identified in these bacteria is related to the production of acetic acid, although most of *Asaia* isolates do not produce it (Yamada et al. 2000). Interestingly, a pre-feeding treatment of phlebotomine flies with an *Asaia* strain (isolated from a wild-caught *Lutzomyia longipalpis* sand fly) reduced the size of the flies harbouring *Leishmania* parasites, likely enhancing or contributing to the mechanisms of colonization resistance against the parasite (Sant’Anna et al. 2014). Moreover, it can be expected that different *Asaia* strains can coexist in the same insects, possibly determining different types of effects on the host and the pathogens they
transmit. Indeed, multiple *Asaia* infections with different strains inhabiting individual insects have been found in mosquitoes (Chouaia et al. 2010), with each infection playing a different role in its host. Moreover, it has been shown that elimination of *Asaia* from the gut by way of antibiotic treatment delays larval development in *A. stephensi* (Chouaia et al. 2012). *Asaia* has been shown to block the transmission of the reproductive manipulator *Wolbachia* in *Anopheles* mosquitoes (Hughes et al. 2014) and the negative interference between these two bacteria has been observed to occur in the mosquitoes’ gonads (Rossi et al. 2014). Here, we confirmed the occurrence of different effects exerted by different *Asaia* strains in leafhoppers as well. Particularly, one out of the three strains we tested affected the suitability of *E. variegatus* to be colonized by FDp. The SF15.14 Rif\(^R\) strain was capable of producing a thick ALI biofilm. We speculate that the reduced FDp acquisition rate observed in leafhoppers exposed to the SF15.14 Rif\(^R\) strain of *Asaia* could be related to competitive physical exclusion, i.e., obstruction by the biofilm of midgut attachment sites needed by FDp to recognize and cross the gut barrier. Indeed, *Asaia* SF15.14 Rif\(^R\), besides being located close to the midgut epithelium, which is a key tissue through which FDp reaches the haemolymph and colonizes the insect (Rashidi et al. 2015), was found to build bacterial masses, which apparently may entrap phytoplasma cells, possibly preventing efficient establishment in the host’s body. However, these considerations are not sufficient to justify the reduced number of FDp-infected leafhoppers. Indeed, the individuals exposed to the other ALI biofilm-producing strain included in this study (AE6.5 Rif\(^R\)) were not less infected by FDp than the control, although the provided isolate produced a thick ALI biofilm similar to that produced by the SF15.14 Rif\(^R\) strain. Gram-negative bacteria, including AAB such as *Komagataeibacter xylinus*, produce pellicles mainly comprising exopolysaccharides, among which cellulose is generally one of the main components (Armitano et al. 2014). *Asaia* strains have been observed to be able to produce cellulose (Kumagai et al. 2011), but further studies are necessary to investigate the composition of the pellicles produced by the strains we have investigated and to determine if cellulose, as well as other exopolysaccharides (together with or as an alternative to cellulose) are produced. The biofilms produced by SF15.14 Rif\(^R\) and AE6.5 Rif\(^R\) could have different
compositions, and this could be important in their different effect on FDP transmission. This possibility is supported by the fact that, even though *Asaia* and FDP were detected by FISH analyses in the guts of leafhoppers treated with all strains of studied bacteria, bacterial clusters (which could contribute to the reduction of phytoplasma transmission) were observed by FISH only in the SF15.14 RifR strain. However, we must point out that we examined a limited number of insects by FISH. We cannot therefore exclude the possibility that the AE6.5 RifR strain is able to form bacterial masses as well. In summary, the possibility that interference with FDP infection of *E. variegatus* determined by *Asaia* SF15.14 RifR is not related to biofilm production, but to other phenotypic traits, cannot be ruled out by the data presented here. Further investigations are necessary to ascertain if biofilm production capacity of *Asaia* is implicated in interference of FDP transmission.

Other antagonistic functions against pathogens played by AAB are related to the production of extracellular polysaccharides that can provide protective immunity (Li et al. 2004). Polymers produced by AAB might determine if immunostimulation occurs in the insect host. Previous studies on Diptera indicated that *Asaia* performed immune modulation (Capone et al. 2013); however, whether or not this activity is due to polysaccharides has not been determined. The immunity of *E. variegatus* has recently been explored (Tedeschi et al. 2017) and further work could evaluate the role played by the SF15.14 RifR *Asaia* strain in activating immune responses.

Although the SF15.14 RifR strain was found to reduce FDP infection in *E. variegatus*, the inhibition of phytoplasma colonization was imperfect given that the pathogens were not totally eliminated from the insects fed bacteria; moreover, in some cases, their inoculation substrates were infected as well. Also, the concentration of FDP cells in the individuals treated with *Asaia* SF15.14 RifR was not lower than in control insects, suggesting that if the pathogen succeeds in establishing itself in the insect, its growth is not controlled by the presence of *Asaia*. Moreover, the percentage of infected leafhoppers that successfully transmitted the phytoplasma was not reduced, and in one case it was even higher than the control (experiment 4, see Table 3). Whatever the machinery limiting the pathogen infection 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 Asaia infection, even when considering the experiments with the SF15.14 RifR strain. This evidence supports the lack of the anti-phytoplasma effect observed in experiments involving Asaia SF2.1 RifR and AE6.5 RifR. On the other hand, when considering insects treated with Asaia SF15.14 RifR, 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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lotta contro la flavescenza dorata della vite) project. D.D. thanks King Abdullah University of Science
and Technology for support through baseline research funds. The authors thank Prof. Guido Favia
for providing *A. stephensi* samples.

Compliance with ethical standards

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

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

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

References

Abbà S, Galetto L, Vallino M, Rossi M, Turina M, Sicard A, Marzachì C (2017) Genome sequence,
prevalence and quantification of the first iflavirus identified in a phytoplasm insect vector.


Ammar E-D, Ramos JE, Hall DG, Dawson WO, Shatters RG, Jr (2016) Acquisition, replication and
inoculation of *Candidatus* Liberibacter asiaticus following various acquisition periods on


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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Asaia strains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LAP conditions</th>
<th>IAP medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>SF2.1 Rif&lt;sup&gt;R&lt;/sup&gt;, SF15.14, Rif&lt;sup&gt;R&lt;/sup&gt; &lt;br&gt; AE6.5 Rif&lt;sup&gt;R&lt;/sup&gt;, control (no Asaia)</td>
<td>Healthy oat</td>
<td>Artificial diet</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>SF15.14 Rif&lt;sup&gt;R&lt;/sup&gt;, control (no Asaia)</td>
<td>Healthy oat</td>
<td>Broad bean</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>SF15.14 Rif&lt;sup&gt;R&lt;/sup&gt;, control (no Asaia)</td>
<td>FDp-infected broad bean</td>
<td>Artificial diet</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>SF15.14 Rif&lt;sup&gt;R&lt;/sup&gt;, control (no Asaia)</td>
<td>FDp-infected broad bean</td>
<td>Broad bean</td>
</tr>
</tbody>
</table>

<sup>a</sup>The experiments with each strain was run separately
**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 (2.20×10¹) 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.


<table>
<thead>
<tr>
<th>Strain</th>
<th>Collection time</th>
<th><em>Asaia</em> reisolation rate (%) ± SE</th>
<th><em>Asaia</em> density (transformed value ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF2.1 Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>End of <em>Asaia</em> AAP</td>
<td>53.33 ± 0.01 (16/30)</td>
<td>1.00×10⁴ (12.81± 0.14) [N=16]</td>
</tr>
<tr>
<td></td>
<td>End of FDp AAP</td>
<td>86.67 ± 0.01 (26/30)</td>
<td>6.35×10² (12.64± 0.06) [N=26]</td>
</tr>
<tr>
<td></td>
<td>End of AAPs + LAP</td>
<td>93.33 ± 0.02 (28/30)</td>
<td>5.55×10⁴ (13.50± 0.22) [N=28]</td>
</tr>
<tr>
<td>SF15.14 Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>End of <em>Asaia</em> AAP</td>
<td>80.00 ± 0.07 (24/30)</td>
<td>5.17×10⁴ (13.05± 0.12) [N=24]</td>
</tr>
<tr>
<td></td>
<td>End of FDp AAP</td>
<td>76.67 ± 0.04 (23/30)</td>
<td>1.39×10³ (12.58± 0.13) [N=23]</td>
</tr>
<tr>
<td></td>
<td>End of AAPs + LAP</td>
<td>76.67± 0.02 (23/30)</td>
<td>5.08×10⁴ (13.52± 0.19) [N=23]</td>
</tr>
<tr>
<td>AE6.5 Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>End of <em>Asaia</em> AAP</td>
<td>80.00 ± 0.02 (24/30)</td>
<td>2.59×10⁴ (11.70± 0.23) [N=24]</td>
</tr>
<tr>
<td></td>
<td>End of FDp AAP</td>
<td>80.00 ± 0.02 (24/30)</td>
<td>1.27×10³ (12.60± 0.10) [N=24]</td>
</tr>
<tr>
<td></td>
<td>End of AAPs + LAP</td>
<td>80.00 ± 0.02 (24/30)</td>
<td>5.86×10⁴ (13.33± 0.23) [N=24]</td>
</tr>
<tr>
<td>Control (no <em>Asaia</em>)</td>
<td>End of FDp AAP</td>
<td>0.00% (0/30)</td>
<td>&lt;2.20×10¹</td>
</tr>
<tr>
<td></td>
<td>End of AAP + LAP</td>
<td>0.00% (0/30)</td>
<td>&lt;2.20×10¹</td>
</tr>
</tbody>
</table>
Table 3. Phytoplasma infection rates from FDP transmission trials. The percentage of positive samples according to qPCR analyses on leafhoppers and sugar diets or broad beans during experiments 1–4 (see Table 1) are presented. For experiment 1, different letters indicate significantly different values according to ANOVA or binomial GLM ($P < 0.05$). For experiments 2–4, asterisks indicate significantly different values according to Student’s t tests or binomial GLM ($P < 0.05$). 2Percentage of 16SrV phytoplasma-positive individuals related to the total tested samples (N=60). 3Percentage of phytoplasma-positive leafhoppers that actually transmitted FDP to their feeding substrate, related to the total infected specimens. The number of positive samples is indicated in parentheses. SE: Standard Error; ns: not significant.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Asaia strain</th>
<th>FDP infection rate (%) ± SE in E. variegatus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FDP infection rate (%) ± SE in feeding substrates&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FDP transmission rate (%) ± SE&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SF2.1 Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.33 ± 0.22 a (14/60)</td>
<td>8.33 ± 0.06 ns (5/60)</td>
<td>35.71 ±0.13 ns (5/14)</td>
</tr>
<tr>
<td></td>
<td>SF15.14 Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.00 ± 0.09 b (3/60)</td>
<td>1.67 ± 0.18 ns (1/60)</td>
<td>33.33 ± 0.33 ns (1/3)</td>
</tr>
<tr>
<td></td>
<td>AE6.5 Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.67 ± 0.22 a (13/60)</td>
<td>10.00 ± 0.16 ns (6/60)</td>
<td>46.15 ± 0.14 ns (6/13)</td>
</tr>
<tr>
<td>Control (no Asaia)</td>
<td></td>
<td>25.00 ± 0.19 a (15/60)</td>
<td>10.00 ± 0.13 ns (6/60)</td>
<td>40.00 ± 0.13 ns (6/15)</td>
</tr>
<tr>
<td>2</td>
<td>SF15.14 Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.00 ± 0.04 * (6/60)</td>
<td>5.00 ± 0.05 * (3/60)</td>
<td>50.00 ± 0.22 ns (3/6)</td>
</tr>
<tr>
<td>Control (no Asaia)</td>
<td></td>
<td>26.67 ± 0.06 * (16/60)</td>
<td>18.33 ± 0.03 * (11/60)</td>
<td>68.75 ± 0.12 ns (11/16)</td>
</tr>
<tr>
<td>3</td>
<td>SF15.14 Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.33 ± 0.06 * (17/60)</td>
<td>16.67 ± 0.07 * (10/60)</td>
<td>59.12 ± 0.12 ns (10/17)</td>
</tr>
<tr>
<td>Control (no Asaia)</td>
<td></td>
<td>76.67 ± 0.06 * (46/60)</td>
<td>55.00 ± 0.08 * (33/60)</td>
<td>71.74 ± 0.07 ns (33/46)</td>
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<tr>
<td>4</td>
<td>SF15.14 Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.33 ± 0.06 * (17/60)</td>
<td>21.67 ± 0.05 ns (13/60)</td>
<td>76.47 ± 0.10 ns (13/17)</td>
</tr>
<tr>
<td>Control (no Asaia)</td>
<td></td>
<td>53.33 ± 0.06 * (32/60)</td>
<td>35.00 ± 0.06 ns (21/60)</td>
<td>65.62 ± 0.09 ns (21/32)</td>
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</table>
Figure 1. Phylogenetic position of the *Asaia* strain recorded in *E. variegatus*. Other *Asaia* strains isolated from insect hosts are included along with *A. astibilis*, *A. bogorensis*, *A. krungthepensis*, *A. platycodi*, *A. prunellae*, *A. siamensis* and *A. spathodeae*. The phylogenetic tree was constructed based on the almost-complete 16S rRNA gene by the maximum likelihood method with the MEGA 7 software. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. The scale bar represents the sequence divergence. *Acetobacter persici* (AB665070), belonging to the AAB group, was used as an outgroup.
Figure 2. Growth behaviours in static cultures of *Asaia* SF2.1, AE6.5 and SF15.14 strains (A) and their spontaneous rifampicin-resistant mutants (B). The strains were inoculated in tubes containing 5 ml of modified PDB and cultivated under static conditions for 10-12 days. CN: negative controls containing PDB medium with (B) or without (A) 100 µg/ml rifampicin; no bacterial inoculum was added.
Figure 3. Phytoplasma infection rates and densities recorded in FDp transmission trials. Data obtained from experiment 1, comparing specimens exposed to all tested Asaia strains, are shown in A (percentage of FDp-infected samples) and C (mean phytoplasma concentration), respectively. Different letters indicate significantly different values according to binomial GLM or ANOVA (P < 0.05). In C, the FDp density recorded in substrate provided to leafhoppers fed with the SF15.14RifR strain was not included, because only one positive diet was found. The FDp infection rates and densities obtained in experiments 2-4, comparing individuals fed with Asaia SF15.14RifR and the control, are shown in B and D, respectively. Asterisks indicate significantly different values according to binomial GLM or Student’s t tests (P< 0.05). Regarding the percentage of infected samples (A and B), N=60. Average densities (C and D) were calculated as the number of 16SrV phytoplasma cells per sample: single insect, single diet unit (300 μl sugar solution), 100 mg of broad bean leaf. Values
below the detection limit ($3.80 \times 10^0$) were considered negative. All positive samples were used, their number is indicated below each column.
**Figure 4.** FISH experiments on midguts of *E. variegatus* individuals from experiment 1. Micrographs showing results from FISH of the midgut of: a specimen fed with *Asaia* SF 15.14 Rif<sup>r</sup> (A-D; M-O), a leafhopper treated with *Asaia* SF 2.1 Rif<sup>r</sup> (E-H), an insect exposed to *Asaia* AE6.5 Rif<sup>r</sup> (I-L). Hybridizations with *Asaia* (cyan signal), 16SrV phytoplasma (red signal), and eubacterial (green signal) probes are shown. DAPI stains are presented in blue. Arrows in A indicate the specific localization of *Asaia* close to the midgut epithelia; asterisks in M and N indicate signal spots referable to *Asaia* and phytoplasma cell masses observed in insects treated with the SF 15.14 Rif<sup>r</sup> strain. Bars = 75 μm.
Table S1. Identification of cultivable bacteria associated with *E. variegatus*.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>ITS group</th>
<th>No. isolates per ITS group</th>
<th>Identity (Acc. Num.)</th>
<th>% identity (bp)</th>
<th>RDP classification (family: genus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV5</td>
<td>1</td>
<td>8</td>
<td><em>Pantoea agglomerans</em> (JX089401)</td>
<td>97 (534/549)</td>
<td>Enterobacteriaceae: <em>Pantoea</em></td>
</tr>
<tr>
<td>EV9</td>
<td>2</td>
<td>2</td>
<td><em>Pseudomonas</em> sp. (KX450447)</td>
<td>100 (593/603)</td>
<td>Pseudomonadaceae: <em>Pseudomonas</em></td>
</tr>
<tr>
<td>EV10</td>
<td>3</td>
<td>1</td>
<td><em>Comamonas</em> sp. (KR055003)</td>
<td>99 (858/858)</td>
<td>Comamonadaceae: <em>Comamonas</em></td>
</tr>
<tr>
<td>EV12</td>
<td>4</td>
<td>3</td>
<td><em>Curtobacterium flaccumfaciens</em> (KY970145)</td>
<td>100 (776/778)</td>
<td>Microbacteriaceae: <em>Curtobacterium</em></td>
</tr>
</tbody>
</table>

\(^a\)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.
Table S2. Colonization levels of *Asaia* strains in *E. variegatus* specimens used for FDp transmission experiments. *Asaia* concentrations obtained by qPCR analyses on leafhoppers during experiments 1-4 (see Table 1) are presented, along with the percentages of individuals showing both *Asaia* and FDp infection. *Asaia* cells per positive insect. Values below the blank threshold (2.20×10³) were considered negative. Transformed values (10+log) used for statistical analyses are indicated in parentheses together with their standard errors (SE). All *Asaia*-positive insects were used to calculate average densities; their number is indicated in square brackets. *Percentage of FDp-infected specimens (see Table 2), considering only those samples that tested positive in *Asaia*-specific qPCR. The number of co-infected insects is indicated in parentheses.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th><em>Asaia</em> strain</th>
<th><em>Asaia</em> density (transformed value± SE)(^a)</th>
<th><em>Asaia</em>-FDp co-infection rate (%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SF2.1 Rif(^R)</td>
<td>5.42×10⁵ (13.48±0.22) [N=41]</td>
<td>21.95±0.07 (9/41)</td>
</tr>
<tr>
<td></td>
<td>SF15.14 Rif(^R)</td>
<td>1.32×10⁵ (12.38±0.31) [N=36]</td>
<td>8.33±0.05 (3/36)</td>
</tr>
<tr>
<td></td>
<td>AE6.5 Rif(^R)</td>
<td>5.86×10⁴ (13.33±0.22) [N=39]</td>
<td>20.51±0.07 (8/39)</td>
</tr>
<tr>
<td></td>
<td>Control (no <em>Asaia</em>)</td>
<td>&lt;2.20×10¹</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>SF15.14 Rif(^R)</td>
<td>1.03×10⁵ (13.31±0.19) [N=39]</td>
<td>12.82±0.05 (5/39)</td>
</tr>
<tr>
<td></td>
<td>Control (no <em>Asaia</em>)</td>
<td>&lt;2.20×10¹</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>SF15.14 Rif(^R)</td>
<td>5.22×10⁴ (13.33±0.22) [N=35]</td>
<td>20.00±0.08 (7/35)</td>
</tr>
<tr>
<td></td>
<td>Control (no <em>Asaia</em>)</td>
<td>&lt;2.20×10¹</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>SF15.14 Rif(^R)</td>
<td>6.50×10¹ (12.98±0.13) [N=36]</td>
<td>22.22±0.10 (8/36)</td>
</tr>
<tr>
<td></td>
<td>Control (no <em>Asaia</em>)</td>
<td>&lt;2.20×10¹</td>
<td>ND</td>
</tr>
</tbody>
</table>
**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.
**Figure S2.** Negative controls of FISH experiments. The FISH results are shown for the midguts of an *E. variegatus* specimen belonging to the control group of experiment 1 (never fed with *Asaia* and exposed to FDp AAP + LAP) in A-D; an adult from the mass rearing (never fed in the presence of *Asaia* or FDp) in E and F; an individual treated with no probe in G-I. Hybridizations with *Asaia* (cyan signal), 16SrV phytoplasma (red signal), and eubacterial (green signal) probes are shown. DAPI stains are presented in blue. A consistent signal was detected for eubacteria (B) and phytoplasma (C) in the insect used in experiment 1. Conversely, hybridization with the *Asaia*-specific probe (D, F, I) failed in all trials; similarly, no signal related to the phytoplasma-specific probe was detected in the no probe trial (H). Indeed, in these panels, only a diffused background signal is visible. Bars = 75μm.
### Figure 2

<table>
<thead>
<tr>
<th></th>
<th>CN</th>
<th>SF2.1</th>
<th>SF15.14</th>
<th>AE6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>![ CN ]</td>
<td>![ SF2.1 ]</td>
<td>![ SF15.14 ]</td>
<td>![ AE6.5 ]</td>
</tr>
<tr>
<td>B</td>
<td>![ CN ]</td>
<td>![ SF2.1 Rif^R ]</td>
<td>![ SF15.14 Rif^R ]</td>
<td>![ AE6.5 Rif^R ]</td>
</tr>
</tbody>
</table>
Figure 3
<table>
<thead>
<tr>
<th></th>
<th>Asaia strains\textsuperscript{a}</th>
<th>LAP conditions</th>
<th>IAP medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td>SF2.1 Rif\textsuperscript{R}, SF15.14, Rif\textsuperscript{R} AE6.5 Rif\textsuperscript{R}, control (no Asaia)</td>
<td>Healthy oat</td>
<td>Artificial diet</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td>SF15.14 Rif\textsuperscript{R}, control (no Asaia)</td>
<td>Healthy oat</td>
<td>Broad bean</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td>SF15.14 Rif\textsuperscript{R}, control (no Asaia)</td>
<td>FDp-infected broad bean</td>
<td>Artificial diet</td>
</tr>
<tr>
<td><strong>Experiment 4</strong></td>
<td>SF15.14 Rif\textsuperscript{R}, control (no Asaia)</td>
<td>FDp-infected broad bean</td>
<td>Broad bean</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The experiments with each strain was run separately.
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 (2.20×10¹) 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.


<table>
<thead>
<tr>
<th>Strain</th>
<th>Collection time</th>
<th><em>Asaia</em> reisolation rate (± SE)*</th>
<th><em>Asaia</em> density (transformed value± SE)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF2.1 RifR</td>
<td>End of <em>Asaia</em> AAP</td>
<td>53.33 ± 0.01 (16/30)</td>
<td>1.00×10⁴ (12.81± 0.14) [N=16]</td>
</tr>
<tr>
<td></td>
<td>End of FDp AAP</td>
<td>86.67 ± 0.01 (26/30)</td>
<td>6.35×10³ (12.64± 0.06) [N=26]</td>
</tr>
<tr>
<td></td>
<td>End of AAPs + LAP</td>
<td>93.33 ± 0.02 (28/30)</td>
<td>5.55×10³ (13.50± 0.22) [N=28]</td>
</tr>
<tr>
<td>SF15.14 RifR</td>
<td>End of <em>Asaia</em> AAP</td>
<td>80.00 ± 0.07 (24/30)</td>
<td>5.17×10³ (13.05± 0.12) [N=24]</td>
</tr>
<tr>
<td></td>
<td>End of FDp AAP</td>
<td>76.67 ± 0.04 (23/30)</td>
<td>1.39×10³ (12.58± 0.13) [N=23]</td>
</tr>
<tr>
<td></td>
<td>End of AAPs + LAP</td>
<td>76.67± 0.02 (23/30)</td>
<td>5.08×10³ (13.52± 0.19) [N=23]</td>
</tr>
<tr>
<td>AE6.5 RifR</td>
<td>End of <em>Asaia</em> AAP</td>
<td>80.00 ± 0.02 (24/30)</td>
<td>2.59×10³ (11.70± 0.23) [N=24]</td>
</tr>
<tr>
<td></td>
<td>End of FDp AAP</td>
<td>80.00 ± 0.02 (24/30)</td>
<td>1.27×10³ (12.60± 0.10) [N=24]</td>
</tr>
<tr>
<td></td>
<td>End of AAPs + LAP</td>
<td>80.00 ± 0.02 (24/30)</td>
<td>5.86×10³ (13.33± 0.23) [N=24]</td>
</tr>
<tr>
<td>Control (no <em>Asaia</em>)</td>
<td>End of FDp AAP</td>
<td>0.00% (0/30)</td>
<td>&lt;2.20×10¹</td>
</tr>
<tr>
<td></td>
<td>End of AAP + LAP</td>
<td>0.00% (0/30)</td>
<td>&lt;2.20×10¹</td>
</tr>
</tbody>
</table>
**Table 3.** Phytoplasma infection rates from FDP transmission trials. The percentage of positive samples according to qPCR analyses on leafhoppers and sugar diets or broad beans during experiments 1-4 (see Table 1) are presented. For experiment 1, different letters indicate significantly different values according to ANOVA or binomial GLM (P < 0.05). For experiments 2-4, asterisks indicate significantly different values according to Student’s t tests or binomial GLM (P< 0.05). 2Percentage of 16SrV phytoplasma-positive individuals related to the total tested samples (N=60). 3Percentage of phytoplasma-positive leafhoppers that actually transmitted FDP to their feeding substrate, related to the total infected specimens.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Asaia strain</th>
<th>FDP infection rate (%) ± SE in E. variegatusa</th>
<th>FDP infection rate (%) ± SE in feeding substratesb</th>
<th>FDP transmission rate (%) ± SEc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SF2.1 RifR</td>
<td>23.33 ± 0.22 a (14/60)</td>
<td>8.33 ± 0.06 ns (5/60)</td>
<td>35.71 ±0.13 ns (5/14)</td>
</tr>
<tr>
<td></td>
<td>SF15.14 RifR</td>
<td>5.00 ± 0.09 b (3/60)</td>
<td>1.67 ± 0.18 ns (1/60)</td>
<td>33.33 ± 0.33 ns (1/3)</td>
</tr>
<tr>
<td></td>
<td>AE6.5 RifR</td>
<td>21.67 ± 0.22 a (13/60)</td>
<td>10.00 ± 0.16 ns (6/60)</td>
<td>46.15 ± 0.14 ns (6/13)</td>
</tr>
<tr>
<td></td>
<td>Control (no Asaia)</td>
<td>25.00 ± 0.19 a (15/60)</td>
<td>10.00 ± 0.13 ns (6/60)</td>
<td>40.00 ± 0.13 ns (6/15)</td>
</tr>
<tr>
<td>2</td>
<td>SF15.14 RifR</td>
<td>10.00 ± 0.04 * (6/60)</td>
<td>5.00 ± 0.05 * (3/60)</td>
<td>50.00 ± 0.22 ms (3/6)</td>
</tr>
<tr>
<td></td>
<td>Control (no Asaia)</td>
<td>26.67 ± 0.06 * (16/60)</td>
<td>18.33 ± 0.03 * (11/60)</td>
<td>68.75 ± 0.12 ms (11/16)</td>
</tr>
<tr>
<td>3</td>
<td>SF15.14 RifR</td>
<td>28.33 ± 0.06 * (17/60)</td>
<td>16.67 ± 0.07 * (10/60)</td>
<td>59.12 ± 0.12 ms (10/17)</td>
</tr>
<tr>
<td></td>
<td>Control (no Asaia)</td>
<td>76.67 ± 0.06 * (46/60)</td>
<td>55.00 ± 0.08 * (33/60)</td>
<td>71.74 ± 0.07 ms (33/46)</td>
</tr>
<tr>
<td>4</td>
<td>SF15.14 RifR</td>
<td>28.33 ± 0.06 * (17/60)</td>
<td>21.67 ± 0.05 ns (13/60)</td>
<td>76.47 ± 0.10 ms (13/17)</td>
</tr>
<tr>
<td></td>
<td>Control (no Asaia)</td>
<td>53.33 ± 0.06 * (32/60)</td>
<td>35.00 ± 0.06 ns (21/60)</td>
<td>65.62 ± 0.09 ms (21/32)</td>
</tr>
</tbody>
</table>
Table S1. Identification of cultivable bacteria associated with *E. variegatus*.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>ITS group</th>
<th>No. isolates per ITS group</th>
<th>Identity (Acc. Num.)</th>
<th>% identity (bp)</th>
<th>RDP classification (family: genus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV5</td>
<td>1</td>
<td>8</td>
<td><em>Pantoea agglomerans</em> (JX089401)</td>
<td>97 (534/549)</td>
<td>Enterobacteriaceae: <em>Pantoea</em></td>
</tr>
<tr>
<td>EV9</td>
<td>2</td>
<td>2</td>
<td><em>Pseudomonas</em> sp. (KX450447)</td>
<td>100 (593/603)</td>
<td>Pseudomonadaceae: <em>Pseudomonas</em></td>
</tr>
<tr>
<td>EV10</td>
<td>3</td>
<td>1</td>
<td><em>Comamonas</em> sp. (KR055003)</td>
<td>99 (858/858)</td>
<td>Comamonadaceae: <em>Comamonas</em></td>
</tr>
<tr>
<td>EV12</td>
<td>4</td>
<td>3</td>
<td><em>Curtobacterium flaccumfaciens</em> (KY970145)</td>
<td>100 (776/778)</td>
<td>Microbacteriaceae: <em>Curtobacterium</em></td>
</tr>
</tbody>
</table>

aIsolate code indicates the code assigned to the ITS-group representative isolate.
bITS group indicates the number of the different ITS groups obtained after dereplication by ITS-PCR.
cNo. Isolates per ITS group refers to the number of isolates included in the indicated ITS group.