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Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders

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Summary

Bacterial symbionts of insects have been proposed for blocking transmission of vector-borne pathogens. However, in many vector models the ecology of symbionts and their capability of cross-colonizing different hosts, an important feature in the symbiotic control approach, is poorly known. Here we show that the acetic acid bacterium *Asaia*, previously found in the malaria mosquito vector *Anopheles stephensi*, is also present in, and capable of cross-colonizing other sugar-feeding insects of phylogenetically distant genera and orders. PCR, real-time PCR and *in situ* hybridization experiments showed *Asaia* in the body of the mosquito *Aedes aegypti* and the leafhopper *Scaphoideus titanus*, vectors of human viruses and a grapevine phytoplasma respectively. Cross-colonization patterns of the body of *Ae. aegypti*, *An. stephensi* and *S. titanus* have been documented with *Asaia* strains isolated from *An. stephensi* or *Ae. aegypti*, and labelled with plasmid- or chromosome-encoded fluorescent proteins (Gfp and DsRed respectively). Fluorescence and confocal microscopy showed that *Asaia*, administered with the sugar meal, efficiently colonized guts, male and female reproductive systems and the salivary glands. The ability in cross-colonizing insects of phylogenetically distant orders indicated that *Asaia* adopts body invasion mechanisms independent from host-specific biological characteristics. This versatility is an important property for the development of symbiont-based control of different vector-borne diseases.

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Introduction

Microorganisms play crucial roles in the biology and life cycle of most arthropod species, affecting nutrition, development, reproduction, immunity, defence against natural enemies and speciation (Dale and Moran, 2006; Moran, 2006; Feldhaar and Gross, 2009). Animal symbioses are categorized according to the extent of dependence between the host and the symbiont, which generally depends on evolutionary antiquity of the symbiosis. While obligate primary symbionts are essential for the host survival and/or reproduction, secondary symbionts are facultative and thought to be of more recent acquisition, even though they can contribute to the fitness of the host, e.g. conferring resistance to parasites (Dale and Moran, 2006). Most primary symbionts are vertically transmitted to the progeny with a process starting at early stages of oogenesis or embryogenesis. Vertical transmission is common also in secondary symbionts, but they can also colonize novel hosts through horizontal transmission

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among host individuals belonging to the same or different species (Dale and Moran, 2006).

Representatives from the group of the acetic acid bacteria (AAB), e.g. *Acetobacter*, *Gluconacetobacter* and *Gluconobacter*, have recently been demonstrated to be naturally associated, not only with plants (Kommanee *et al*., 2008), but also with insects, such as the fruit fly *Drosophila melanogaster* (Corby-Harris *et al*., 2007; Cox and Gilmore, 2007; Ren *et al*., 2007; Ryu *et al*., 2008), the olive fly *Bactrocera oleae* (Kounatidis *et al*., 2009), the honeybee *Apis mellifera* (Jeyaprakash *et al*., 2003; Mohr and Tebbe, 2006; Babendreier *et al*., 2007) and the pink sugarcane mealybug *Saccharococcus sacchari* (Ashbolt and Inkerman, 1990). Cox and Gilmore (2007) found that in *D. melanogaster*, *Acetobacter* was one of the most abundant genera accounting for 29% of all phylotypes. The detection of AAB in the above insects, all having plant materials and sugar-rich matrices as food sources, suggests that these bacteria might play roles in food exploitation.

Among AAB, the genus *Asaia* differentiates because it does not (or weakly) oxidize ethanol to acetic acid. Besides tropical plants, where it was originally isolated (Yamada *et al*., 2000; Katsura *et al*., 2001; Yukphan *et al*., 2004; Malimas *et al*., 2008), *Asaia* has thus far been found associated with few insect species. These include the hemipteran *Scaphoideus titanus*, the leafhopper vector of the phytoplasma causing Flavescence Dorée, a severe disease of grapevine (Marzorati *et al*., 2006), and three mosquito vectors of malaria, *Anopheles stephensi*, *Anopheles maculipennis* and *Anopheles gambiae*. *Asaia* has been found stably associated with larvae and adults of *An. stephensi*, dominating the microbiota of the mosquito (Favia *et al*., 2007). The distribution of *Asaia* in the body of *An. stephensi* has been investigated by the use of a strain, previously isolated from the mosquito, after genetic modification to express a green fluorescent protein (Gfp). The Gfp-tagged strain efficiently colonized the gut, salivary glands and male and female reproductive organs. It is noteworthy that *Asaia*, after assumption with a sugar-based diet by females, was detected in the gut and then in the salivary glands of the insect, crucial organs for the development of the cycle of the malaria parasites *Plasmodium* spp. (Favia *et al*., 2007). By using fluorescent strains it was shown that, in *An. stephensi*, *Asaia* is vertically transmitted from the mother to offspring (Favia *et al*., 2007), but also undergoes paternal transmission to the progeny, by the way of venereal transfer from male to female during mating (Damiani *et al*., 2008).

The efficient capacity of *Asaia* of colonizing adults and larvae of *An. stephensi* by the way of multiple transmission routes (horizontal acquisition through feeding, venereal transmission from male to female and maternal transmission to offspring), and the discovery of this bacterium in other insect vectors (i.e. other *Anopheles* species and *S. titanus*) raise the question of whether, and to which extent, this bacterium can cross-colonize different insect hosts. This capacity would allow investigating the basis of host–symbiont specific interaction and could be very promising towards the development of *Asaia*-based symbiotic control approaches to block parasite transmission by insect vectors. A high specificity of the host–symbiont interaction characterizes primary symbionts that are vertically transmitted to the progeny but are not cultivable. This hampers their use for symbiotic control. On the other hand, secondary symbionts include culturable microorganisms that are transmitted both vertically and horizontally. For symbiotic control applications, multiple transmission routes and broad host range carry some potential risks regarding biosafety of the biocontrol symbiotic microorganism, as well as opportunities for a successful disease transmission control. The more the transmission routes, the higher the chances of success are. In addition, if a symbiont is present in many different hosts, the chances of environmental spread and horizontal transmission to the target species are higher. In this work, the capacity of *Asaia* symbionts to cross-colonize insects of phylogenetically distant orders, like *Diptera* and *Hemiptera*, has been evaluated by using as models *An. stephensi* and *Aedes aegypti* (*Diptera*) and *S. titanus* (*Hemiptera*). We chose these species according to their evolutionary distance, one, *Ae. aegypty*, phylogenetically close to *An. stephensi*, the other, *S. titanus*, much more distant. The presence of *Asaia* and the ability to cross-colonize organs and tissues of these insects were assessed by *in situ* hybridization, quantitative real-time PCR and cultivation, and by *in vivo* studies with *Asaia* strains labelled with Gfp or DsRed fluorescent proteins after isolation from *An. stephensi* (Favia *et al*., 2007) and *Ae. aegypti*.

Results

Detection of Asaia *in* Ae. aegypti *and* S. titanus

Electron microscopy observations on midgut sections of *Ae. aegypti* and *S. titanus* individuals (Fig. 1) allowed us to observe bacterial cells resembling *Asaia* in both insects. Figure 1A shows a portion of the midgut of *Ae. aegypti* filled with bacteria, embedded in an extracellular matrix, whose morphology is very similar to that already reported for *Asaia* in *An. stephensi* (Favia *et al*., 2007). Two morphological signatures, i.e. a bright filamentous nucleoid region and electron-dense cytoplasm micro-inclusions resembling enterosomes, organelles rarely described in bacterial cells (Schaechter *et al*., 2006), were previously recognized and described for *Asaia* cells (Favia *et al*., 2007). The finding of these signatures in bacterial cells of midgut sections of *Ae. aegypti* and

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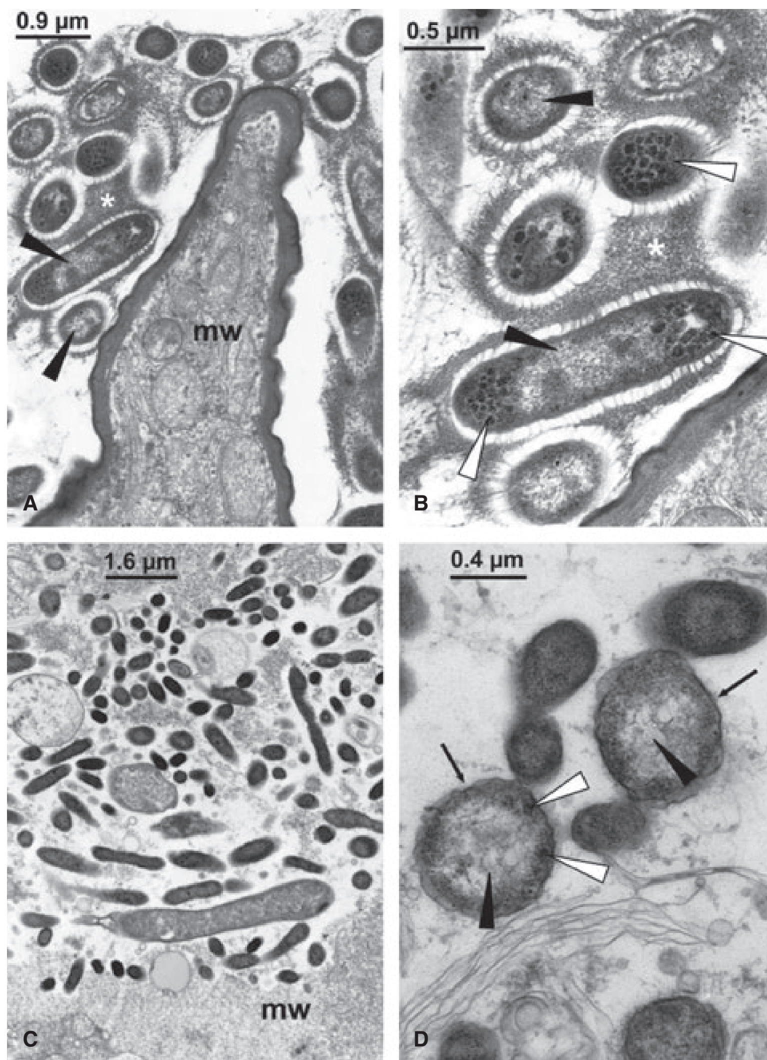

Fig. 1. Transmission electron microscopy micrographs of the midgut of *Ae. aegypti* and *S. titanus*.
A. Midgut of *Ae. aegypti* filled with bacteria that can be interpreted as belonging to the genus *Asaia*; the bacteria are embedded within an extracellular matrix (asterisk). Bright filamentous nucleoid regions are indicated by black arrowheads; mw: midgut wall.
B. Detail of (A); note the presence of the nucleoid region (black arrowheads) and the enterosomes in the bacterial cytoplasm (white arrowheads) and extracellular matrix (asterisk).
C. Midgut of *S. titanus* filled with a highly polymorphic bacterial flora; mw: midgut wall.
D. Detail of the midgut lumen of *S. titanus* showing two bacteria (indicated with arrows) that can be interpreted as belonging to the genus *Asaia*. Bright nucleoid regions are indicated by black arrowheads, while enterosomes are indicated with white arrowheads.

S. titanus individuals suggested the presence of *Asaia*. Thus, according to their ultrastructure, the bacteria in *Ae. aegypti* could be attributed to the genus *Asaia* (Fig. 1B). In *S. titanus*, the midgut hosts a polymorphic bacterial flora (Fig. 1C). Some of the bacteria observed in the gut of *S. titanus* also presented a bright filamentous nucleoid region (Fig. 1D), resembling that described for *Asaia*.

In order to verify the presence of *Asaia* sp. in *Ae. aegypti*, DNA was extracted from individual mosquitoes at different life cycle stages and PCR was performed using *Asaia*-specific primers. A total of 150 individuals, 30 for each of five successive generations, were analysed. The expected bands were detected in all the tested individuals, both in the preimago stages, i.e. larvae and pupae (40 individuals each, respectively), and adults (35 males and 35 females). To confirm the identity of the amplified products, 35 were randomly chosen for sequencing. All of them showed nucleotide identity higher than 99% with *Asaia bogorensis* and *Asaia siamensis*. *Asaia*-specific PCRs were also carried out on mosquito male and female guts (respectively from 10 and 15 individuals), male and female reproductive organs and salivary glands (from 20 individuals each). All of the samples were positive for *Asaia* and the identity of the amplified 16S rRNA gene fragments was confirmed as *Asaia* by sequencing 12 randomly chosen amplicons.

Forty adults of *S. titanus* (males and females, 20 each) were analysed for the presence of *Asaia* in the body following the same approach used for *Ae. aegypti*, resulting in 30 positive samples (14 males and 16 females respectively). Among the positive samples, 11 individuals (5 males and 6 females) were analyed by quantitative PCR in order to measure the relative abundance of *Asaia* within the microbiota in the body of *S. titanus*. Real-time quantitative PCRs targeting specifically *Asaia* sp. or total bacteria were used to determine the *Asaia* and the total bacterial 16S rRNA gene copies. The two values were then used to calculate the *Asaia* to total bacteria 16S

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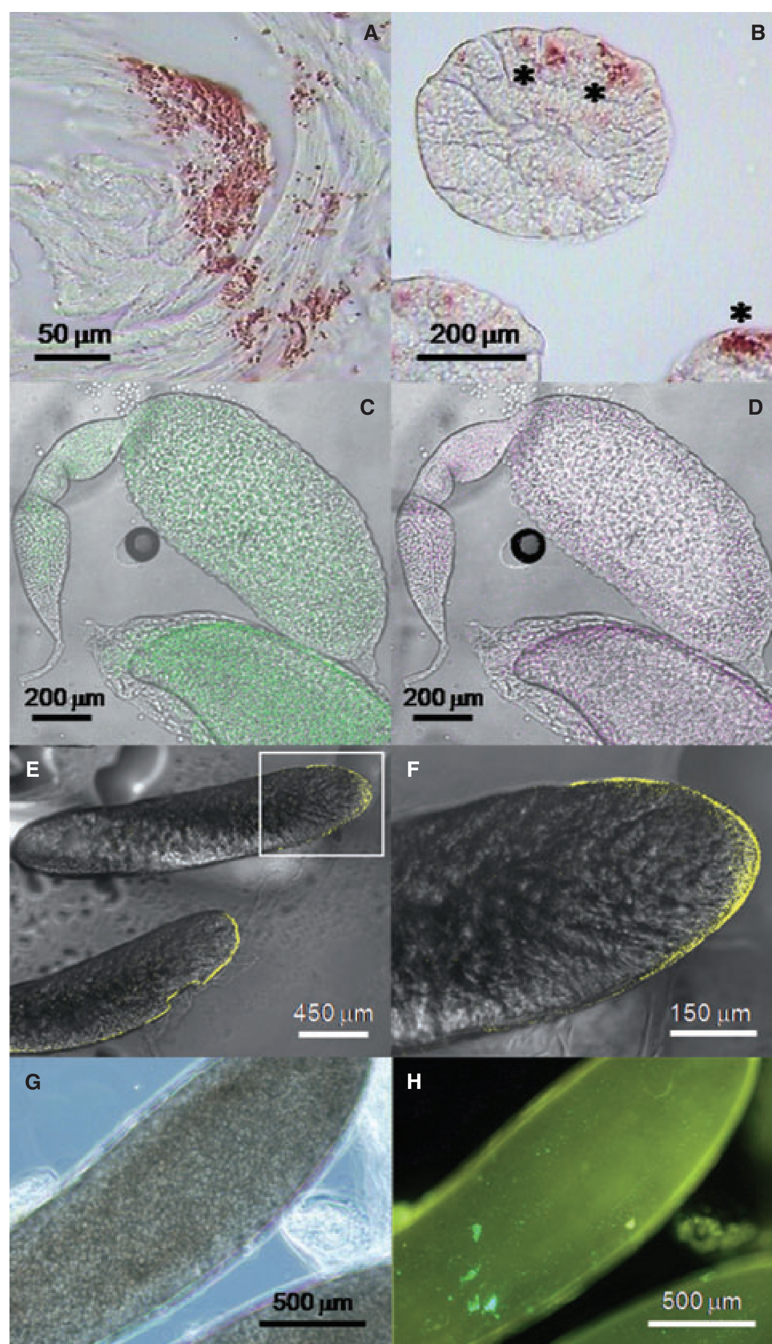

Fig. 2. Detection of naturally occurring *Asaia* sp. in *S. titanus* by ISH and FISH and colonization of the ovarian eggs of *S. titanus* by *Asaia* SF2.1(Gfp). Signals obtained by ISH with *Asaia*-specific probes (Asaia1 and Asaia2, 5′-labelled with digoxigenin) were observed in the spermatic bundles (A) and in the Malpighian tubules (asterisks) (B). Ovarian eggs, investigated by FISH, are shown in (C–F). The universal bacterial probe EUB388, labelled with FITC (green colour), was used for total bacteria visualization in (C). Specific *Asaia*-probes, Asaia1 and Asaia2 probes 5′ end labelled with the fluorochrome Cy5, showed the presence of *Asaia* cells all around the immature eggs (D). Ovarian eggs in a more advanced stage of maturation hybridized with the Cy3-labelled *Asaia*-specific probes Asaia1 and Asaia2 show high concentrations of *Asaia* cells around the egg apical regions (E and F). Single cells and microcolonies of Gfp-labelled *Asaia* can be observed on the surface of mature ovarian eggs (H). The correspondent phase contrast image is presented in (G).

rRNA gene copy ratio within the insects. The number of *Asaia* 16S rRNA gene copies per individual covered a range from 1.0×10^3 to 7.3×10^8, with an average value of $9.4\ (\pm\ 6.9) \times 10^7$. The number of total bacteria 16S rRNA gene copies per individual covered a range from 1.4×10^6 to 1.9×10^9, with an average value of $8.3\ (\pm\ 3.9) \times 10^8$. Assuming that about four rRNA gene copies per cell are present in *Asaia*, as observed in other AABs (http://ribosome.mmg.msu.edu/rrndb/index.php, Klappenbach *et al*., 2001), we could estimate an average of 2.3×10^7 *Asaia* cells associated with an individual of *S. titanus*. The average *Asaia* to *Bacteria* 16S rRNA gene copy ratio (ABR) was 0.049, indicating that *Asaia* 16S rRNA gene copies constituted a mean of 4.9% of the total bacterial 16S rRNA gene copies.

In order to investigate the presence and the localization of *Asaia* in different leafhopper organs, *in situ* hybridization (ISH) and fluorescent ISH (FISH) were performed. *Asaia* was detected by ISH in testicles, intermixed within the spermatic bundles (Fig. 2A) and in the Malpighian

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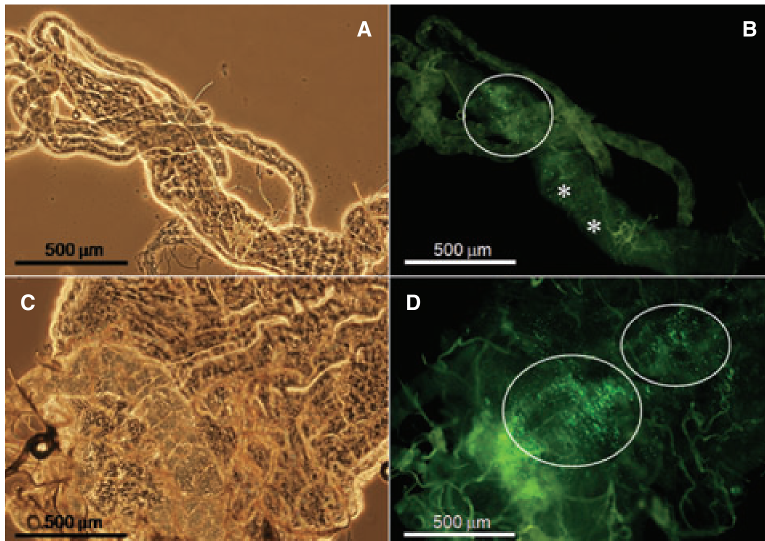

Fig. 3. Colonization of the adult gut of *Ae. aegypti* by *Asaia* SF2.1(Gfp). Phase contrast (A and C) and fluorescence (B and D) microscope images of guts of a male (A and B) and a female (C, D), fed with a sucrose solution containing *Asaia* SF2.1(Gfp). Clusters (circles) and spots (asterisks) of bright fluorescent recombinant *Asaia* cells are visible by fluorescence microscopy.

tubules (Fig. 2B). Leafhopper ovarian eggs were investigated by FISH. Figure 2C–D show ovarioles and an immature egg hybridized with the universal bacterial probe EUB338 (green colour) or with the *Asaia*-specific probes (pink colour) respectively. The immature eggs were covered by bacteria most of which corresponded to *Asaia*. *Asaia*-specific probe was also used on more mature ovarian eggs, detecting bright signals all around the egg with particular concentration on the egg edge reads apical regions (Fig. 2E–F). Hence, during the egg maturation *Asaia* appeared to pass from a dispersed localization scattered on the entire egg surface to a more peripheral localization with an apparent higher cell concentration in the egg apical regions. In all the ISH and FISH experiments no signals were observed in tissues treated with RNase or in the absence of the probe.

Isolation of Asaia *from* Ae. aegypti

Asaia sp. from *Ae. aegypti* individuals was successfully isolated in pure culture by using a liquid enrichment medium at pH 3.5 followed by plating in a carbonate-containing solid medium (Yamada *et al.*, 2000). The enrichment medium allowed the elimination of those microorganisms not tolerating low pH, while the following plating on $CaCO_3$-containing medium permitted the isolation of acid-producing bacteria capable of creating $CaCO_3$ dissolution haloes around the colonies. Twenty-three pink-pigmented, shiny and smooth colonies isolated from four adult mosquitoes, two females and two males, were selected for further analyses. All the collected strains were positive when subjected to *Asaia*-specific PCR. 16S rRNA gene sequencing of three randomly selected colonies, named AE5.2, AE6.5 and AE10.8, confirmed their identification as *Asaia* sp. with a nucleotide identity higher than 99% with *A. bogorensis* or *A. siamensis*.

The same approach was adopted for isolation of *Asaia* sp. from nymphs and adults of *S. titanus.* However, no *Asaia* strains could be isolated, neither when sorbitol was substituted with glucose, sucrose or mannitol as carbon source.

Colonization patterns of Asaia *in mosquitoes and leafhoppers*

The pattern of colonization of the host body by *Asaia* was investigated in *Ae. aegypti*, *An. stephensi* and *S. titanus* individuals. Mosquitoes and leafhoppers were fed with sucrose solutions containing fluorescent *Asaia* cells: both species of mosquitoes were fed with *Asaia* cells expressing plasmid-encoded Gfp while leafhoppers were fed with *Asaia* cells expressing either plasmid-encoded Gfp or chromosome-encoded DsRed. *Asaia* strains SF2.1(Gfp) and AE10.8(Gfp), originally isolated from *An. stephensi* (Favia *et al.*, 2007) and *Ae. aegypti*, respectively, were provided to mosquitoes together with kanamycin in order to avoid the rapid loss of the plasmid carrying the Gfp. Indeed, experiments conducted *in vitro* showed that, without antibiotic supplementation, the percentage of *Asaia* cells retaining plasmid pHM2-Gfp halved after 24 generations, dropping to 21% after 36 generations. To overcome the biasing effect of the antibiotic selection, *Asaia* strain SF2.1(DsRed), chromosomally tagged with a DsRed coding cassette and thus indefinitely retaining fluorescence, was prepared. This strain was provided to insects in the same conditions of strains SF2.1(Gfp) or AE10.8(Gfp) but without antibiotic in the medium.

The colonization patterns of *Asaia* labelled with optical markers in the body parts of mosquitoes and leafhopper

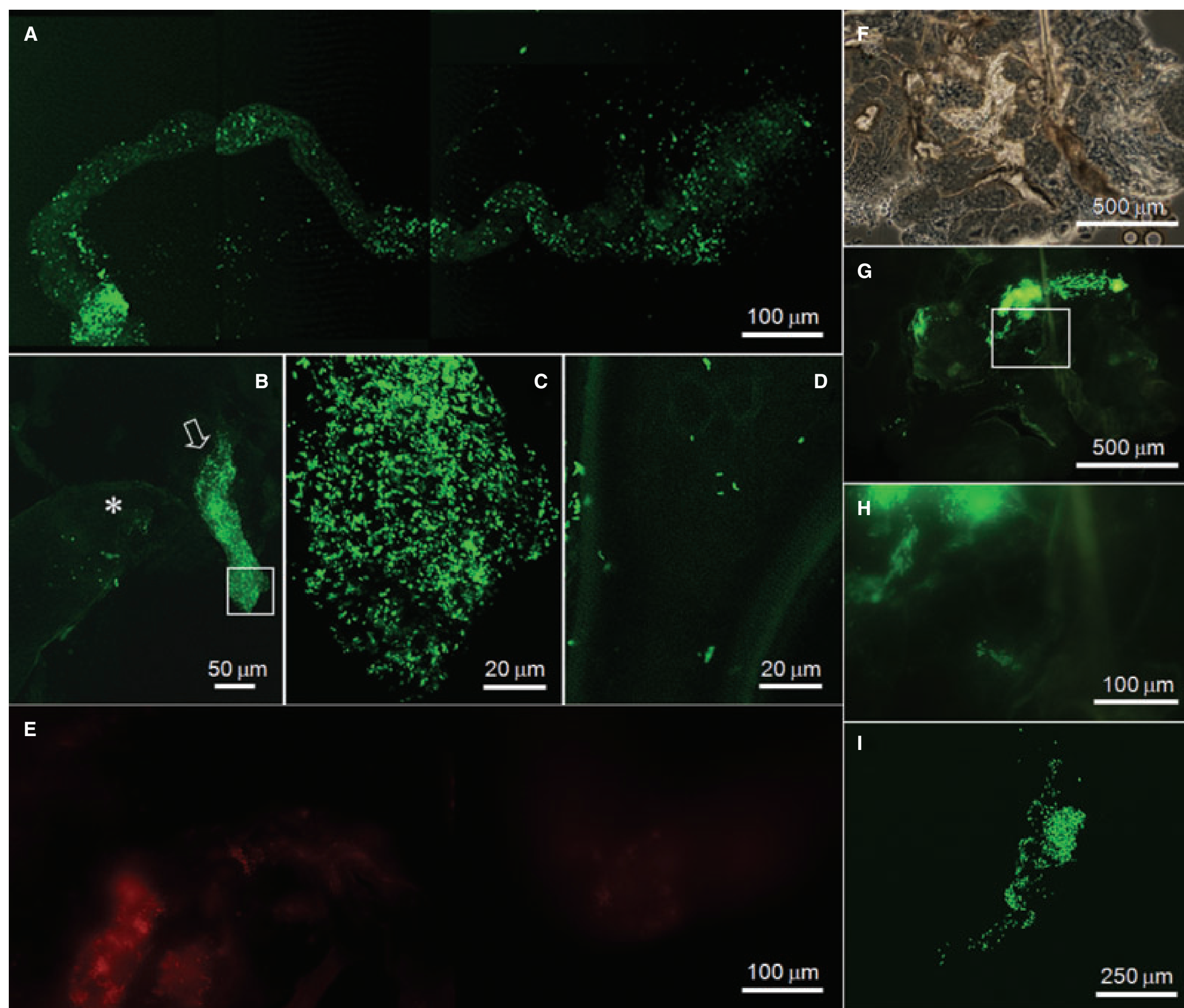

Fig. 4. Colonization of *S. titanus* by *Asaia* SF2.1(Gfp) (A–D and F–I) and SF2.1(DsRed) (E), documented by confocal laser scanning (A–D, I) and fluorescence (E, G, H) microscopy.
A. Reconstructed image of a female leafhopper gut obtained by overlapping four successive sections.
B. View of the ovary duct (arrow) next to an egg (asterisk). The ovary duct is fully colonized by the recombinant strain.
C. Magnification of a portion of the ovary duct (inset in panel B) in which large microcolonies of *Asaia* can be observed.
D. Observation of *Asaia* SF2.1(Gfp) on the surface of an immature egg.
E. Colonization of *S. titanus* gut by *Asaia* SF2.1(DsRed).
G. Salivary glands showing a massive colonization of *Asaia* SF2.1(Gfp). The corresponding phase contrast image is shown in (F).
H. Magnification of a portion of the salivary gland (inset in panel G) in which large microcolonies of *Asaia* can be observed.
I. Colonization of the deferent duct in the male reproductive system of *S. titanus* by *Asaia* SF2.1(Gfp).

are shown in Figs 3 and 4. The Gfp-tagged strains SF2.1(Gfp) and AE10.8(Gfp) were used for the experiments of cross-colonization of *Ae. aegypti* (Fig. 3) and *An. stephensi* (data not shown; Favia *et al*., 2007) respectively. Both strains were able to efficiently cross-colonize the guts of males and females of both mosquitoes. Most of the individuals examined (45 out of 77, 58.4% in *Ae. aegypti* and 36 out of 36, 100% in *An. stephensi*) showed fluorescent cells and microcolonies in the gut. In both males (Fig. 3A and B) and females (Fig. 3C and D), detectable colonization first appeared already after 12 h from feeding and was recorded after 24, 48 or 72 h from the bacterial exposure. Indeed, massive colonization of the gut, intended as the detection of large microcolonies in different portions of the entire gut, was recorded in *Ae. aegypti* as well as in *An. stephensi* already after 24 or 48 h post exposure. Similarly, the analysis was also extended to the reproductive organs and salivary glands of individuals of the two mosquito species. *Asaia* colonization in the male genital system was achieved in 3 out of

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37 (8.1%) *Ae. aegypti* specimens and in 6 out of 12 (50%) *An. stephensi* specimens. Concerning female organs, colonization was detected in 1 out of 39 (2.6%) individuals of *Ae. aegypti* and 7 out of 19 (36.8%) *An. stephensi*. In salivary glands, colonization was revealed in 3 out of 27 (11.1%) *Ae. aegypti* specimens and in 5 out of 17 (29.4%) *An. stephensi* specimens.

Colonization of the body of adult *S. titanus* by *Asaia* supplemented through the diet was studied by using Gfp and DsRed fluorescent derivatives of strain SF2.1. In Fig. 4A, a reconstruction of *S. titanus* intestine by overlapping four sections of the organ acquired by confocal laser scanning microscopy (CLSM) shows a massive colonization of *Asaia* along all the gut. Colonization of the gut was observed in 74% and 62% of individuals, respectively, exposed to Gfp- or DsRed-labelled *Asaia* cells (39 of 53 and 34 of 55 individuals respectively). In Fig. 4F–H, a typical colonization pattern of the salivary glands of *S. titanus* is shown. In total, 32% and 29% of individuals exposed, respectively, to Gfp- or DsRed-labelled *Asaia* cells (12 of 38 and 10 of 34 individuals respectively) showed colonization of the salivary glands by the bacterium. Massive colonization with fluorescent bacterial cells was detected in the female gonoduct (Fig. 4B). Higher magnification of Gfp signals made possible to appreciate the homogeneous distribution of *Asaia* SF2.1(Gfp) in the ovary duct and accumulations of Gfp-positive microcolonies (Fig. 4C). The presence of structured microcolonies, with cells undergoing division, indicates an active bacterial growth. A similar colonization pattern was observed when DsRed-labelled bacteria were used in the diet. In total, 60% and 57% of individuals exposed, respectively, to Gfp- or DsRed-labelled *Asaia* cells (25 out of 42 and 28 out of 49 respectively) resulted positive to colonization of the female reproductive system. Fluorescent cells were also able to reach the immature eggs contained in the ovaries. In Fig. 4D and in Fig 2G and H fluorescent cells are observed all around an immature egg in an ovary of *S. titanus*. Figure 4E shows a leafhopper gut recolonized by DsRed-tagged *Asaia*. Similarly to *Ae. aegypti*, *Asaia* SF2.1(Gfp) demonstrated to be capable of colonizing the male reproductive apparatus of *S. titanus*. Figure 4I shows the massive colonization of the male deferent ducts.

The specificity of the colonization of the insect body by *Asaia* was assessed by feeding the insects with a DsRed-labelled *Escherichia coli* or a Gfp-labelled *Pseudomonas putida* alone or in mixture with *Asaia* labelled with the Gfp or the DsRed fluorescent proteins. In particular, the mosquitoes were fed with mixtures of Gfp-labelled *Asaia* and a DH5α pKan(DsRed) *E. coli.* After mosquito dissection only green signals of *Asaia* cells were detected, while red signals were never found in the different organs and tissues examined. In the case of *S. titanus*, experiments for assessing the specificity of colonization of the host body were performed by using: (i) a mixture of *P. putida* KT2442 (*gfp* Rif^r), carrying the gene encoding for the fluorescent protein on the bacterial chromosome, with *Asaia* SF2.1(DsRed); (ii) a mixture of *E. coli* DH5α pKan(DsRed) with *Asaia* SF2.1(Gfp); (iii) *P. putida* KT2442 (*gfp* Rif^r) alone; and (iv) *E. coli* DH5α pKan(DsRed) alone. The green or red signals of *P. putida* KT2442 (*gfp* Rif^r) or *E. coli* DH5α pKan(DsRed) were never recorded by fluorescence or CLS microscopy of the insect dissections. These experiments confirmed a specific ability of *Asaia* to colonize the bodies of the two insect hosts.

Discussion

Symbiotic control strategies rely on the exploitation of symbiotic microorganisms with the aim of interfering with the transmission of diseases vectored by insects or to impair the insect biological cycle. Ideally, primary symbionts would be the most interesting for developing a symbiotic control approach since they are obligate symbionts and established a very strict dependence on the host. They are generally restricted to a given specific host. The efficient vertical transmission of these symbionts would guarantee their rapid spread. However, due to a long co-evolution with the host they present several characteristics, including genome reduction, the loss of some essential cellular functions that determine uncultivability. As a consequence, they are not tractable for developing an effective symbiotic control approach. On the contrary, secondary or facultative symbionts can retain the ability to grow in cell-free media, being tractable for microbiological and genetic characterization and manipulation, essential steps for the development of symbiotic control approaches. The capability of growing outside the insect host allows these microorganisms to grow in environmental niches other than the host body, and to be horizontally acquired by the host from the environment. The capability of colonizing different hosts is also interesting, since it would allow to the symbiont multiple ways of environmental release. These capabilities are important ecological traits for the efficacy of symbiotic control, since they would increase the chances of spread of the symbiont in the target host populations.

To verify the capability of *Asaia* to move from one host to another and efficiently colonize different body systems, we used three insect models having as common trait diets based on sugar-rich matrices. Two of the insect models are phylogenetically closely related, the mosquitoes *An. stephensi* and *Ae. aegypti* (*Diptera*). The third, the leafhopper *S. titanus* (*Hemiptera*), is phylogenetically distant from the other two. Indeed, *Diptera* and *Hemiptera* represent lineages that likely separated over 300 million

years ago (the holometabolous and heteromethabolous lineages respectively).

We successfully detected and isolated *Asaia* sp. from *Ae. aegypti*. This finding represents an original result if compared with previous studies investigating the microbiota of this insect (Luxananil *et al*., 2001; Gusmão *et al*., 2007). We speculate that the failure of previous studies in detecting and isolating this AAB possibly derives from the primers used in PCR and the lack of a suitable cultivation method. Indeed, *Asaia* is a relatively slow growing bacterium, having a duplication time of about 2 h. Hence, it can be easily overgrown by fast growing bacteria unless selection factors are used. To overcome this inconvenience we used a pre-enrichment step in a liquid medium at pH 3.5 that has already been used for isolating *Asaia* from plant material (Yamada *et al*., 2000). Such a selection allows *Asaia* to overgrow acid-sensitive bacteria.

In *S. titanus*, we were able to detect *Asaia* in 30 of 40 individuals analysed. However, we cannot actually define if *Asaia* is fixed in the population of *S. titanus* since a higher number of individuals should be tested. We could not isolate *Asaia* in pure culture from any of the *S. titanus* individuals tested. The lower prevalence of *Asaia* in *S. titanus* than in mosquitoes as detected by PCR, suggests that *S. titanus* could be a niche supporting *Asaia* growth, however, less favourable than mosquitoes of the *Anopheles* or *Aedes* genera. This is in agreement with Ashbolt and Inkerman (1990), who found that the presence of AAB associated with leafhoppers of the species *Perkinsiella saccharicida* was low (10^3 AAB per individual) compared with that determined on the pink sugar cane mealybug *Saccharococcus sacchari* (10^6 AAB per individual). On the other hand, the high number of *Asaia* cells detected by quantitative PCR in some *S. titanus* individuals could be due to an actively feeding state of the insect, as they were maintained for some days on sucrose solution. In our experiments, we did not attempt the isolation of *Asaia* from individuals fed with artificial sucrose solution in the laboratory. Another explanation of the failure in isolating *Asaia* from *S. titanus* is that the symbiont of the leafhopper could have different nutritional requirements from those of mosquitoes, driven by the different type of diet of the host. To verify this hypothesis further media and cultivation conditions different from those we adopted should be used.

The presence of AAB in insects has been documented since 1981 (Lambert *et al*., 1981). In the last few years an increased number of studies reported AAB as a remarkable component of the microbial community associated to arthropods, raising the question of the role of these microorganisms in the animal host. There is an apparent correlation between AAB and the insect nutritional behaviour. Acetic acid bacteria have been found associated with insects with a sugar-based diet. Indeed, AAB are well known to grow on a wide range of sugars with differences according to their metabolisms, e.g. *Gluconobacter* spp. prefer sugar-rich niches, while *Acetobacter* spp. predominate in ethanol-containing environment. Among AAB, *Asaia* spp. show unique characteristics: no or scarce production of acetic acid from ethanol, inhibition of growth in presence of 0.35% acetic acid and weak capability of oxidizing acetate to carbon dioxide and water (Kersters *et al*., 2006; Ano *et al*., 2008). We identified *Asaia* in *Ae. aegypti* adults, which feed on nectar and other sugar solutions. According to plant species, nectar contains sucrose, glucose and fructose. The leafhopper *S. titanus* feeds on the phloem sap that contains sucrose as a main carbon substrate. The relationship between a sugar-feeding habit of the insect host and the presence of AAB in the body has been found in mosquitoes and leafhoppers, but also in other insects feeding on sugar-rich matrices, like *A. mellifera*, *D. melanogaster* and *S. sacchari* (Ashbolt and Inkerman, 1990; Jeyaprakash *et al*., 2003; Mohr and Tebbe, 2006; Babendreier *et al*., 2007; Corby-Harris *et al*., 2007; Cox and Gilmore, 2007; Ren *et al*., 2007; Ryu *et al*., 2008).

The characteristics of the feeding material combined with those of the external environment are also important in supporting AAB presence and favouring their diffusion. Nectar has a low pH (3.9) (Mohr and Tebbe, 2006 and references therein), the exudate of mealybugs is acidic (pH 3) with fructose as the major sugar present (Ashbolt and Inkerman, 1990) and the surface of *Drosophila* spp. can be contaminated by ethanol (Ren *et al*., 2007). Similarly to the external surface, the insect gut represents a unique environment that favours the survival of specific bacterial species (Ren *et al*., 2007), as it occurs in vertebrates. It is generally assumed that the survival of microorganisms in the gut requires resistance to pH, redox potential and ionic strength of the midgut and to the digestive enzymes. For example, the acidity of the crop and the hindgut (Dow and Harvey, 1988; Moffett and Koch, 1992; Clark, 1999), like the low pH in the gut of *A. mellifera* (Mohr and Tebbe, 2006 and references therein), likely contributes to support the proliferation of AAB in these insects. In *Anopheles* spp., the presence of a polysaccharidic extracellular matrix around the cells of *Asaia* (Favia *et al*., 2007) could protect the bacterium from adverse conditions, such as alkaline or acidic pH, like that of larvae (Dadd, 1975; Corena *et al*., 2005) or of adults (Gusmão *et al*., 2007), respectively, or high osmolarity (Yukphan *et al*., 2005).

Whatever it is the role of *Asaia* in the biology of the insects investigated (the documented presence in various species of mosquitoes, i.e. *An. stephensi*, *An. maculipennis*, *An. gambiae*, *Ae. aegypti*, would suggest a key role) its identification in species of different orders, like *Diptera*

and *Hemiptera*, suggests that it may be a widespread symbiont of insects. The efficiency of interspecies transfection of bacterial symbionts has been previously demonstrated with horizontal colonization experiments of tsetse flies with bacterial cells of the genus *Sodalis* (Weiss *et al*., 2006). The data of colonization experiments with fluorescent proteins-expressing *Asaia* cells reported in the present study proved the capability of this bacterium to cross-colonize insects belonging to phylogenetically distant orders. In two phylogenetically related mosquito species, like *An. stephensi* and *Ae. aegypti*, a different body colonization prevalence by a bacterial strain isolated from the other mosquito species was observed. In all the organs examined *Ae. aegypti* colonized by *Asaia* strain SF2.1(Gfp) showed a remarkably lower percentage of infected individuals than *An. stephensi* colonized with *Asaia* strain AE10.8(Gfp). However, we cannot deduce from the actual experimental design if these differences were to be attributed to the insect host or the *Asaia* strain used. We speculate that different bacterial phenotypes, like cell surface properties, could remarkably affect the cross-colonization host range and capability. Indeed, the different colonization efficiency observed with the different host–symbiont combinations indicates that further research work should be done to understand the drivers of colonization specificity.

After acquisition with the diet, *Asaia* reaches the gut lumen and, crossing the numerous physical and biochemical barriers that prevent the entrance in the haemocoel of undesired microorganisms (Vallet-Gely *et al*., 2008), it is able to colonize different body parts. In this regard, when experiments of gene silencing were carried out on AgDscam, an essential factor of *An. gambiae* immune defence against bacteria, a remarkable proliferation of bacteria, including *A. bogorensis*, occurred within the mosquito haemolymph (Dong *et al*., 2006). Besides the guts of *Ae. aegypti*, *An. stephensi* and *S. titanus*, the Gfp-labelled strain showed also the ability to efficiently colonize the salivary glands and the male and female reproductive organs of *Ae. aegypti* and *S. titanus*, highlighting a colonization pattern similar to that previously reported for *An. stephensi* (Favia *et al*., 2007). As already proved for *An. stephensi*, the abundance of *Asaia* in different tissues of the reproductive organs may indicate a possible transmission of the bacterium from the mother (Favia *et al*., 2007) or the father (Damiani *et al*., 2008) to the offspring. It is noteworthy the detection of *Asaia* by FISH all around ovarian eggs of *S. titanus* (Fig. 2D–F) as well as the finding of Gfp-labelled *Asaia* around immature eggs of this insect (Figs 2H and 4D). We could thus hypothesize that vertical transmission of *Asaia* in *S. titanus* occurs through egg smearing and/or by the penetration of the bacteria across the egg membrane during the egg development.

Although the mechanism of the vertical transmission route remains to be elucidated, the capability of the bacterium to be widespread in natural insect populations represents an important property for its application in potential symbiotic control strategies both as a natural and as a paratransgenic delivery system (Riehle and Jacobs-Lorena, 2005). Understanding the nature of the symbiosis supported by *Asaia* in the different hosts, its role for the insect biology and some details of the vertical transmission are important issues that remain to be answered. However, the capability of the bacterium to be widespread in different and phylogenetically distant insect species, together with the easy culturability, transformability and the efficiency in colonizing different hosts, makes this bacterium an interesting candidate for symbiotic control of human diseases, such as malaria or dengue fever, as well as plant diseases like the Flavescence Dorée of grapevine (Marzorati *et al*., 2006).

Experimental procedures

Insect collection

Aedes aegypti individuals came from a lab strain kindly provided by the Suisse Tropical Institute (Basilea) and reared in the laboratory at the University of Camerino (Italy) since 2006. *Anopheles stephensi* samples came from a colony reared since 1988 at the University of Camerino. Mosquitoes were kept in cages ($25 \times 25 \times 25$ cm) made of a steel frame covered with nettings and maintained at 30 ± 1°C temperature, 70 ± 5% humidity, with a dark/light period of 12 h. *Scaphoideus titanus* individuals were collected in vineyards with heavy symptoms of Flavescence Dorée from various areas of the Piedmont region during the summer season. Field-collected individuals were immediately used for *Asaia* isolation. For quantitative real-time PCR experiments, *S. titanus* individuals were kept for a week in laboratory cages at the University of Turin (Italy) and maintained on standard, sterilized sucrose solution at room temperature (25–30°C), before killing for the subsequent analyses.

Transmission electron microscopy

Laboratory-reared *Ae. aegypti* and field-collected *S. titanus* insects were dissected with sterile scalpels and little pliers in a sterile saline solution to separate the salivary glands, the gut, the fat bodies and the ovaries. Samples were fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 3 h at 4°C. The samples were then washed in the same buffer and post-fixed in 1% OsO_4 in the same buffer for 1.5 h at 4°C. Successively, all samples were dehydrated in ethanol and embedded in Epon 812. Thin sections (80 nm) were stained with uranil acetate and lead citrate and examined under a Zeiss EM900 transmission electron microscope.

DNA extraction and PCR-based analyses

DNA extraction from whole *S. titanus* and *Ae. aegypti* individuals and from dissected organs of *Ae. aegypti* was

performed as previously described (Favia *et al.*, 1994). Extracted DNA was used as template in conventional PCRs with *Asaia*-specific primers, Asafor (5′-GCG CGT AGG CGG TTT ACA C-3′) and Asarev (5′-AGC GTC AGT AAT GAG CCA GGT T-3′), targeted on the 16S rRNA gene of this bacterium. Reaction mixtures were carried out in a final volume of 25 μl, using 0.2 units of Taq Gold DNA polymerase (Roche), 1× PCR buffer, 2.5 mM of each dNTP, 25 pmol of each primer, 2 mM $MgCl_2$ and 50 ng of DNA. Reactions were run for 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C and then a final extension of 10 min at 72°C. The 180 bp PCR products were randomly chosen and sequenced with Asafor primer. Sequences were compared with the sequence database at the National Centre for Biotechnology Information by using BLASTN (Altschul *et al.*, 1990).

Quantitative real-time PCRs were performed on the I-Cycler (Bio-Rad) using *Asaia*-specific primer set Asafor/Asarev or eubacterial universal primers 357F (5′-CTA CGG GAG GCA GCA G-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) according to Favia and colleagues (2007). For realizing standard curves, the 16S rRNA gene of *Asaia* amplified by PCR was cloned using pGEM T-easy Vector Cloning Kit (Promega). Following calculation of 16S rRNA gene copies of *Bacteria* and *Asaia*, the *Asaia* to *Bacteria* 16S rRNA gene copy ratio (ABR) was calculated and used as an estimate of the relative abundance of *Asaia* sp. in the bacterial community associated with different individuals of *S. titanus* (Favia *et al.*, 2007).

In situ *hybridization and whole mount fluorescent* in situ *hybridization*

Field-collected *S. titanus* insects were dissected in sterile saline. ISH on paraffin-embedded sections of organs and tissues was performed as described previously (Beninati *et al.*, 2004; Favia *et al.*, 2007). Briefly, formamide concentration was adjusted at 30% and hybridization temperature was set at 46°C. For specific detection of *Asaia* cells, two probes, Asaia1 (5′-AGC ACC AGT TTC CCG ATG TTA T-3′) and Asaia2 (5′-GAA ATA CCC ATC TCT GGA TA-3′), were used as described previously (Favia *et al.*, 2007). Probe EUB338, routinely used as a universal bacterial probe even though several phyla are not completely covered (Daims *et al.*, 1999), was used as a bacterial positive control. Probes Asaia1 and Asaia2 have the following mismatches with the next non-target AAB (in parentheses are reported the 16S rRNA gene accession number and the numbers of mismatches for probe Asaia1 and Asaia2 respectively): *Halobharath swaminathanianus* (AF459454, 1,1); *Kozakia baliensis* (AB056321, 2,2); *Gluconacetobacter hansenii* (X75620, 4,2); *Gluconacetobacter kombuchae* (AY688433, 4,2); *Gluconobacter oxydans* (AB308275, 3,6); *Gluconobacter japonicus* (AB470922, 4,3); *Acetobacter tropicalis* (AB470916, 4,2); *Acetobacter syzygii* (AB264094, 4,2). The probes were 5′-labelled with digoxigenin in order to be recognized by an antidigoxigenin antibody coupled with a horseradish peroxidase. Staining was performed with 3-amino-9-ethylcarbazole and observation was with a light microscope (Favia *et al.*, 2007). Alternatively, for fluorescent *in situ* hybridization (FISH) probes Asaia1 and Asaia2 were labelled at the 5′ end with the fluorochrome Cy5 (indodicarbocyanine, absorption/emission at 650/670 nm) or with the fluorochrome Cy3 (indocarbocyanine, absorption/emission at 550/570 nm). The probe EUB338 were labelled with fluorescein isothiocyanate (FITC, absorption/emission at 494/520 nm). Insect dissections were fixed in a 4% solution of paraformaldehyde in PBS 1× for 30 min and then washed in PBS 1×. Samples were incubated for 1 min at the temperature of 37°C with a 100 μg ml^{-1} pepsin solution and washed again in PBS 1×. Successively, an overnight hybridization was carried out in dark conditions with 50 μl of hybridization buffer (2× SSC, 50% formamide, 1 μg ml^{-1} probes). Dissections were then washed in 500 μl of washing buffer (2× SSC and 50% formamide) and subsequently two washes in 0.1× SSC at 37°C were performed, followed by two washes in 1× PBS at 37°C. Then 50 ng of propidium iodide or DAPI were added, and incubated for 5 min at room temperature. After two washes in 1× PBS at 37°C, samples were mounted in antifading medium, then observed in a laser-scanning confocal microscope SP2-AOBS (Leica).

Both for ISH and FISH, control experiments involved either treatment of slides with RNase prior to probe hybridization step or the absence of the probe. Positive control experiments with the universal bacterial probe EUB338 (Fuchs *et al.*, 1998) were also performed applying the same conditions.

Asaia *sp. isolation*

Asaia strains were isolated from four adult mosquitoes using an enrichment medium (pH 3.5) as described by Yamada and colleagues (2000). Mosquitoes were washed three times with 0.9% NaCl and homogenated in 200 μl 0.9% NaCl. Twenty microlitres of homogenate were inoculated into the enrichment medium and let to grow at 30°C for 2 days with shaking (160 r.p.m.). When microbial growth occurred, the microorganisms were plated on calcium carbonate agar plates. Circular, pink-pigmented colonies capable of causing clearing of the calcium carbonate were selected. The identification as *Asaia* sp. was confirmed by specific *Asaia* PCR amplification with Asafor and Asarev primers and by sequencing of the almost entire 16S rRNA gene after amplification with universal bacterial primers 27F (5′-TCG ACA TCG TTT ACG GCG TG-3′) and 1495R (5′-CTA CGG CTA CCT TGT TAC GA-3′).

For isolation of *Asaia* from *S. titanus* the same procedure for isolation from *Ae. aegypti* was adopted, except that, besides sorbitol, other different carbon sources, including glucose, sucrose, mannitol, were used in single or in mixture in the pre-enrichment step. A total of 40 individuals recovered from different vineyard in Piedmont were homogenated and used for isolation.

Construction of Asaia *tagged with fluorescent proteins*

Two transformants of *Asaia* strain SF2.1, isolated from *An. stephensi*, and one transformant of strain AE10.8, isolated from *Ae. aegypti*, were used in colonization experiments of the insects. Strain SF2.1(Gfp) was prepared as previously described (Favia *et al.*, 2007). This strain was tagged with plasmid pHM2-Gfp and used in feeding

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experiments in presence of 100 μl ml^{-1} kanamycin. Strain AE10.8(Gfp) was prepared by the same procedure used for strain SF2.1.

Strain SF2.1(DsRed) (Damiani *et al.*, 2008) was constructed by insertion of a mini-Tn5 gene cassette containing the *dsRed* gene expressed from *E. coli* ribosomal promoter *rrnBP1* into the chromosome of *Asaia* SF2.1 by conjugation and transposition (Mølbak *et al.*, 2007). Briefly, the delivery plasmid TTN151 was mobilized from *E. coli* Mv1190λ*pir* to the recipient using the helper strain *E. coli* HB101(pRK600). Transconiugants that underwent transposition events were selected by plating on GLY medium at pH 4.0 (where *E. coli* cannot grow) supplemented with kanamycin (200 μg ml^{-1}). DsRed fluorescence of colonies was observed using a Leica DM4000B equipped with Leica filter set G/R (excitation filter, BP 490/20 nm; dichromatic mirror, 505; emission filter, LP 525/20 and excitation filter, BP 575/30 nm; dichromatic mirror, 600; emission filter, LP 635/40). Among the *Asaia* transconjugants, a clone which grew at the same rate as SF2.1, named strain SF2.1(DsRed), was selected for performing recolonization experiments in *A. aegypti* and *S. titanus* without the presence of antibiotics in the feeding solution.

Evaluation of stability of Asaia *strains tagged with fluorescence proteins*

Asaia SF2.1(Gfp) was grown overnight in GLY medium with kanamycin (100 μg ml^{-1}) and diluted the next day into fresh GLY medium without antibiotic. The culture was allowed to grow to an OD_{600} of ~1 and then diluted. This was repeated three consecutive times over the course of experiment in order to follow the bacterium for a total of approximately 36 generations. The proportion of kanamycin-resistant bacterial cells was determined by plating on GLY agar plates added with kanamycin every time that the culture reached an OD_{600} of 1. The total cells were in parallel counted by plating on GLY plates without the antibiotic. The same procedure was used for strains AE10.8(Gfp) and SF2.1(DsRed).

Colonization of insects by Asaia *expressing Gfp and DsRed*

Asaia strains SF2.1(Gfp), AE 10.8(Gfp) and SF2.1(DsRed) were used in colonization experiments of *An. stephensi*, *Ae. aegypti* and *S. titanus*. The first two strains were always used under the selection of kanamycin, while the third strain was used without antibiotic. Strain SF2.1(Gfp) was used in colonization experiments of *Ae. aegypti*, while strain AE10.8(Gfp) was used for the colonization of *An. stephensi*. In the case of *S. titanus* leafhoppers, they were fed with either SF2.1(DsRed) or strain SF2.1(Gfp). After growing at 30°C cells were harvested by centrifugation (10 min, 3000 *g*), washed three times with 0.9% NaCl and adjusted to 10^8 cells ml^{-1} in 30 ml of 5% (w/v) sucrose aqueous solution for *An. stephensi* and *Ae. aegypti*, or 5% sucrose solution in Tris-EDTA pH 8 for *S. titanus*. Cell suspensions, supplemented with 100 μg ml^{-1} of kanamycin for strains SF2.1(Gfp) and AE10.8(Gfp), were administrated to the insects allowing them to feed for periods of 2 days after which the bacterial feeding solutions were substituted with equivalent, cell-free solutions with the addition of the kanamycin when foreseen. After 12, 24, 48 and 72 h after exposure to the cells suspensions, the insects were withdrawn and gut, salivary glands and reproductive organs were dissected in PBS. The organs were fixed with 4% paraformaldeyde for 10 min at 4°C with the exception of the salivary glands. The slides were then mounted in glycerol-PBS for the microscopy analyses. The samples were analysed with IX71 fluorescence microscope (Olympus, Melville, NY, USA) and a MRC600 confocal laser scanning microscope (Bio-Rad).

As colonization controls, two bacteria never associated to insects has been used. The first was a strain of *P. putida*, a typical inhabitant of soil. The second was *E. coli* a typical symbiont of the gut of mammals. For the mosquitoes, *An. stephensi* and *Ae. aegypti*, *Asaia* SF2.1(Gfp) and *E. coli* DH5α pKan(DsRed) were used for cocolonization experiments. Both bacteria carried a plasmid with a gene cassette encoding a fluorescent protein (Gfp in the case of *Asaia*, DsRed in the case of *E. coli*). In the case of *S. titanus*, *Pseudomonas putida* KT2442 (*gfp* Rif^r) and *E. coli* DH5α pKan(DsRed) were independently used alone, for colonization experiments, or in combination with *Asaia* SF2.1(DsRed) and *Asaia* SF2.1(Gfp), respectively, for cocolonization experiments. *P. putida* KT2442 (*gfp* Rif^r) carried a Gfp cassette in the chromosome. Bacterial suspensions of 10^8 cells ml^{-1} for each strain were appropriately prepared and administered to the insects as described above. The samples were then processed, as already indicated.

Sequence accession numbers

Sequences of *Asaia* strains AE5.2, AE6.5 and AE10.8 isolated from *Ae. aegypti* and of two *Asaia*-specific PCR fragments amplified from the metagenome of *Ae. aegypti* and *S. titanus* (named AE4 and ST2 respectively) have been deposited in the DDBJ-EMBL-GenBank databases under the accession numbers FN297840–FN297844.

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