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Evidence for Gut-Associated *Serratia symbiotica* in Wild Aphids and Ants Provides New Perspectives on the Evolution of Bacterial Mutualism in Insects

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Abstract

Many insects engage in symbiotic associations with diverse assemblages of bacterial symbionts that can deeply impact on their ecology and evolution. The intraspecific variation of symbionts remains poorly assessed while phenotypic effects and transmission behaviors, which are key processes for the persistence and evolution of symbioses, may differ widely depending on the symbiont strains. *Serratia symbiotica* is one of the most frequent symbiont species in aphids and a valuable model to assess this intraspecific variation since it includes both facultative and obligate symbiotic strains. Despite evidence that some facultative *S. symbiotica* strains exhibit a free-living capacity, the presence of these strains in wild aphid populations, as well as in insects with which they maintain regular contact, has never been demonstrated. Here, we examined the prevalence, diversity, and tissue tropism of *S. symbiotica* in wild aphids and associated ants. We found a high occurrence of *S. symbiotica* infection in ant populations, especially when having tended infected aphid colonies. We also found that the *S. symbiotica* diversity includes strains found located within the gut of aphids and ants. In the latter, this tissue tropism was found restricted to the proventriculus. Altogether, these findings highlight the extraordinary diversity and versatility of an insect symbiont and suggest the existence of novel routes for symbiont acquisition in insects.

Keywords Ant · Aphid · Bacterial mutualism · Gut symbiont · Horizontal transmission · *Serratia symbiotica*

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Introduction

Animals exhibit diverse relationships with different types of symbiotic bacteria [1]. Facultative symbionts are common in insects and can greatly influence their ecology and evolution [2, 3]. In contrast to the obligate symbionts that are essential for successful host growth and reproduction, facultative bacterial partners only deliver fitness benefits in the context of specific ecological conditions [2]. While the evolutionary importance of facultative symbionts in insect populations is now well established, the significance of their intraspecific variation on their associated phenotypes and their transmission behaviors remains fairly unexplored.

Among insects, aphids (Hemiptera: Aphididae) serve as excellent models for elucidating the functional importance of symbiotic interactions as they engage in a particularly wide range of mutualistic symbioses with bacteria [2]. Aside from their obligate nutritional symbiont *Buchnera aphidicola*, these sap-feeding insects may harbor a wide range of facultative symbionts that are maternally transmitted and usually occur in a fraction of the individuals within a population [2, 4, 5]. In contrast to *Buchnera*, which is hosted in specialized host cells called bacteriocytes, these facultative partners have a more flexible tissue tropism because they can inhabit different types of tissues, including bacteriocytes, sheath cells, and hemolymph [2]. Facultative symbionts can typically undergo occasional intraspecific and interspecific horizontal transfers that may lead to novel associations and, subsequently, to the acquisition of ecologically important traits by the newly infected insects [6–9].

Although the phenotypic effects of several symbiont species residing in aphids have already been depicted in many studies [2, 3, 10–12], these are most often limited to a small number of insect species and rarely consider the diversity of strains contained in the same species of symbiont. However, intraspecific variation in facultative symbionts should reflect the variety of phenotypic effects and associated costs as has been demonstrated in the case of protective symbionts [13, 14]. Another remarkable example is the case of the bacterial symbiont species *Wolbachia pipientis* whose associated phenotypes are strain-specific: while certain strains have mutualistic effects, others display deleterious effects for their host [15–17]. Strain variation may also affect tissue tropism phenotypes and transmission patterns and behaviors, which are crucial for the fixation, persistence, and evolution of the symbiosis in insect populations [18–20]. Compared to the obligate symbionts engaged in a long co-evolutionary history with their host, facultative symbionts are involved in more recent symbiotic associations and exhibit a more variable degree of dependence with their host. These features likely influence the rate of their horizontal transfers and, consequently, their ability to engage in new symbiotic associations with novel hosts [21]. Assessing the intraspecific variation of these facultative partners is therefore essential to refine our understanding of the origin of mutualism and to better appreciate how these microorganisms spread within insect populations.

Serratia symbiotica is one of the most frequent facultative symbionts found in aphids [2, 22] and a valuable candidate for assessing the significance of intraspecific variation in symbionts as it displays contrasting biological features in term of lifestyle, genome reduction, cell shape, and tissue tropism. On the one hand, *S. symbiotica* includes strains with highly reduced genomes which are associated with a nutritional co-obligate role and which have been described in a limited number of aphid species of the subfamily Lachninae [23–25]. On the other hand, *S. symbiotica* includes strains of a facultative nature whose protective effects against environmental heat stress and parasitoids were mainly studied in the pea aphid *Acyrthosiphon pisum* under laboratory conditions [26–28]. In addition, facultative strains with a free-living capacity under anaerobic conditions, whose associated biological effects are unknown, were isolated on rich medium [29–31]. This lack of a total interdependence with respect to their host as well as the genomic features of the cultivated strain CWBI-2.3^T suggest that these so-called free-living *S. symbiotica* are involved in a nascent stage of symbiosis [29, 32, 33]. The extent of these free-living strains in wild insect populations as well as their tissue tropism has never been addressed, while they may represent remarkable candidates to assess the evolutionary scenarios for the acquisition of bacteria and the origins of bacterial mutualism.

The aim of the present work was to highlight the presence of *S. symbiotica* strains with a potential free-living capacity in wild aphid populations. The lack of complete interdependence with respect to their host makes certain *S. symbiotica* strains more likely to pass from one host to another to initiate the establishment of new symbiotic associations. Through a field study, we sought to determine the prevalence, diversity, and tissue tropism of *S. symbiotica* in natural populations of aphids as well as in tending ants. Using diagnostic PCR, fluorescence in situ hybridization, and phylogenetic approaches in a complementary way, we revealed that the *S. symbiotica* diversity includes strains residing within the aphid gut. This is the first time that such tissue tropism has been observed for *S. symbiotica* in wild aphids. Our study also demonstrated that *S. symbiotica* occurs in tending ants where it was found located within the proventriculus. The discovery of gut-associated *S. symbiotica* suggests novel routes for symbiont acquisition and highlights new questions regarding the evolution of bacterial mutualism in insects.

Materials and Methods

Insect Sample Collection and Identification

Specimens representing 26 aphid species were examined (Table S1, Supporting information). Particular attention was

given to populations of *Aphis* and *Periphyllus* genera for two reasons. First, it is assumed that *S. symbiotica* is highly prevalent in these two genera [34]. Then, *S. symbiotica* strains successfully isolated and cultivated in our laboratory were initially isolated from several *Aphis* species [30, 31]. Field specimens were sampled between July and September 2015 on various host plants at several locations in Belgium. When tending ants were observed within aphid colonies, individuals were systematically collected. A total of 90 aphid colonies plus 30 pools of associated tending ants were examined. Samples dedicated to genetic analyses were preserved in 90% ethanol at 4 °C until use, while samples devoted to the microscopic observations were stored in acetone at ambient temperature.

Insect DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the instructions of the manufacturer. Each DNA extraction was performed on a pool of two to six individuals (two to three individuals in the case of ants) from the same colony rather than a single individual to reduce the risk of missing infection when *S. symbiotica* is present. For insect species identification, the primers LepF and LepR were used to amplify the target 658-bp fragment of cytochrome c oxidase subunit I (COI) gene [35, 36]. Primers are listed in Table S2 (Supporting information). The PCR assays were performed in a final volume of 15 μl, containing 1 μl of genomic DNA, 0.5 μM of each primer, 200 μM dNTPs, 1× buffer, and 0.625 unit of Taq DNA polymerase (Roche). The thermocycling profile consisted of 94 °C for 1 min; 6 cycles of 94 °C for 1 min, 45 °C for 1 min and 30 s, and 72 °C for 1 min and 15 s; followed by 36 cycles of 94 °C for 1 min, 51 °C for 1 min and 30 s, and 72 °C for 1 min and 15 s; with a final 5 min extension period of 72 °C. Amplicons were purified before sequencing (Macrogen Inc., Amsterdam). Insects were then identified into species by comparing resulting COI sequence data to GenBank nucleotide database using BLAST.

Diagnostic Screening for *S. symbiotica*

All samples were screened for the presence of *S. symbiotica* by amplifying a partial region of the 16S rRNA gene using the specific primers 16SA1 and PASScmp [37]. Primers are listed in Table S2 (Supporting information). The PCR assays were performed as previously described [38]. DNA from an infected line of the pea aphid *Acyrthosiphon pisum* was used as positive control (Tucson aphid clones provided by the Nancy Moran Lab [27]). The amplicons were then purified and sequenced in both directions (Macrogen Inc., Amsterdam). The resulting sequences were compared to sequences on GenBank using BLAST. All the sequences were deposited in GenBank (Table S3, Supporting information).

To better target subsequent microscopic observations on *S. symbiotica* strains that potentially display a free-living capacity, pairs of primers were designed to target the protein-coding genes *flgA*, *proA*, and *hisA* (Table S2, Supporting information). These genes are present in the genome of the free-living strain *S. symbiotica* CWBI-2.3^T but are absent from the other sequenced *S. symbiotica* genomes [23, 25, 39, 40]. The specificity of these primers was validated using DNAs from two other isolated free-living *S. symbiotica* strains as positive controls (24.1 and Apa8-A1 strains) and DNAs from *S. symbiotica* strain Tucson and *S. marcescens* strain Db11 as negative controls.

The PCR assays were performed under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation (95 °C, 30 s), annealing (57 °C, 1 min and 30 s), extension (72 °C, 1 min and 30 s); and a final extension at 72 °C for 7 min. The amplicons were purified and then sequenced (Macrogen Inc., Amsterdam).

To determine differences in frequency of infections by *S. symbiotica* in tending ants depending on the infectious status of the aphid colonies, we used a generalized linear model (GLM), with a Binomial error structure and a logit-link function. Analyses were conducted using a software R v. 3.3.1. [41].

Phylogenetic Analyses

Strain diversity in *S. symbiotica* was characterized by sequencing four household genes: *accD*, *gyrB*, *murE*, and *recJ* (listed in Table S2, Supporting information) [42–44]. DNA samples that were found positive for *S. symbiotica* were subjected to PCR amplification under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation (94 °C for 30 s), annealing (60–64 °C, depending on primers, cf. Table S2 [Supporting information], 30 s), extension (72 °C, 1 min); and a final extension at 72 °C for 5 min. The amplicons were purified and then sequenced in both directions (Macrogen Inc., Amsterdam). Forward and reverse sequences were assembled and edited using Geneious® v.9.1.5 [45].

Phylogenetic relationships were evaluated for 29 *S. symbiotica* strains obtained in this work, from three *S. symbiotica* strains that were isolated in our laboratory (CWBI-2.3^T, 24.1, and Apa8 A1 strains) (see Table S3, Supporting information, for voucher information and GenBank accession numbers), and from sequences available in GenBank for the five *S. symbiotica* strains whose genomes have been sequenced [23, 25, 29, 39, 40] (Table S3, Supporting information). *S. proteamaculans* 568 was used as outgroup. SeaView v4.6.1 was used to align sequences, and all ambiguously aligned regions identified by Gblocks [46] were eliminated, as were regions of incomplete data at the 3′ and 5′ ends of the targeted regions. Substitution models implementing different rates for transitions were selected for each partition according to the BIC criterion as implemented by jModeltest2 [47]. These models were first implemented in

homogeneous Bayesian analyses for each region individually to assess the congruence of the phylogenetic signal. Incongruence was operationally defined as the presence of incompatible bipartitions found in branches supported with posterior probabilities > 95% from separate analyses of two data sets. Following the threshold of 95%, which ensures that strongly conflicting signals between partitions are eliminated, no supported conflict among the partitions was identified. All partitions were therefore combined to run the final heterogeneous Bayesian analysis of the concatenated dataset. The accession URL of the TreeBase project with alignments and phylogenetic trees is indicated in the section on data accessibility.

Four Markov chain Monte Carlo (MCMC) simulations were run independently for 10,000,000 generations with MrBayes. Trees and model parameters were sampled every 10,000 generations. Convergence of the MCMCs was estimated in three ways. First, the standard deviation of split frequencies was < 0.01 after 10,000,000 generations. Second, visual inspection of the plot of the log-likelihood score at each sampling point suggested that the four chains reached stationarity. Third, the posterior probability plots of all splits for paired MCMC runs showed high correlation, which diagnoses convergence among the four chains [48]. The 100 trees of the burn-in for each run were excluded from the tree set, and the remaining trees from each run were combined to form the full sample of trees assumed to be representative of the posterior probability distribution.

Localization of *S. symbiotica*

To determine the tissue tropism of *S. symbiotica* in samples of interest, whole-mount fluorescent in situ hybridization (FISH) was performed as previously described [38, 49]. The following probes labeled with the fluorescent dye cyanine were used for in situ hybridization: Cy5-ApisP2a (5′-Cy5-CCTCTTTT GGGTAGATCC-3′) targeting 16S rRNA of *B. aphidicola* and Cy3-PASSisR (5′-Cy3-CCCGACTTTATCGCTGGC-3′) targeting 16S rRNA of *S. symbiotica*. Host insect tissues were stained with SYTOX Green. Stained samples were whole mounted and viewed under a Zeiss LSM 710 confocal microscope. Negative controls consisted of uninfected aphids and no-probe staining. Positive controls consisted of artificially and naturally infected aphids.

Data Availability DNA sequences: GenBank accessions MF185189-MF185200, MF375536-MF375631, and MF959552-MF959597. Phylogenetic data, including alignments: TreeBase accession URL http://purl.org/phylo/treebase/phylows/study/TB2:S21617?x-access-code=2aea2c9256296ccca434311fb859f339&format=html. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Results

Distribution of *S. symbiotica* Infection

The presence of *S. symbiotica* was assayed in aphid colonies from 26 species (Table S1, Supporting Information). Of the 90 colonies examined, 16S rRNA PCR assays indicated the presence of *S. symbiotica* in 38 colonies belonging to 10 species (34.4%): *A. fabae*, *A. grossulariae*, *A. pomi*, *A. frangulae*, *Chaitophorus populialbae*, *Drepanosiphum platanoides*, *Macrosiphoniella absinthii*, *Uroleucon sonchi*, *Periphyllus lyropictus*, and *P. acericola* (Table S1, Supporting information). Thirty-seven percent (13/35) of the colonies of the genus *Aphis* were found infected, with a prevalence of 30% (7/23) in *A. fabae*, the most represented *Aphis* species in our sampling. The prevalence of *S. symbiotica* was remarkably high in the genus *Periphyllus* since almost all the colonies were found infected (*P. acericola*: 17/20; *P. lyropictus*: 2/2).

S. symbiotica was also found in the pools of tending ants with a prevalence of up to 28% (8/29). The symbiont was found in *Lasius niger* (7/26), the ant species that was usually found in the aphid colonies, and in *Myrmica scabrinodis* (1/1). The prevalence of *S. symbiotica* reached 60% (6/10) in ants sampled within aphid colonies that were found positive for the symbiont. We asked whether the presence of *S. symbiotica* in tending ants was correlated with the infection status of the aphid colonies that were visited. We found that the prevalence of *S. symbiotica* in ants was significantly higher when collected in infected aphid colonies ($N = 30$; df = 1; $\chi^2 = 2414$; $p = 0{,}016$) (Fig. 1).

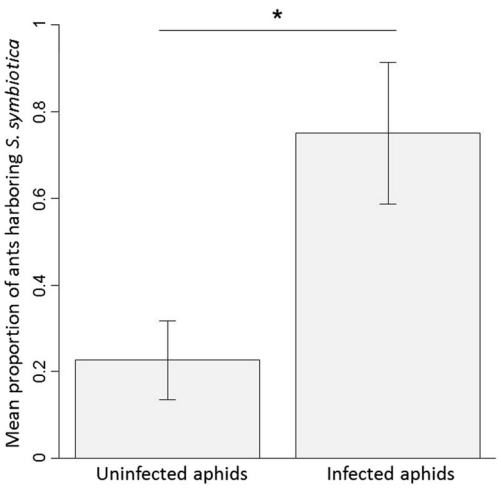

Fig. 1 Prevalence of *S. symbiotica* in ants differs depending on the infection status of the aphid colonies. Columns represent the mean proportion of ants infected with *S. symbiotica* according to uninfected aphid colonies or infected aphid colonies. Error bars depict the standard error. Asterisks show significant differences between the groups (GLM (binomial) ($N = 30$; df = 1; $\chi^2 = 2414$; $p = 0{,}016$))

Diversity of *S. symbiotica* Infection

Bacterial sequences from the *accD*, *gyrB*, *murE*, and *recJ* genes were taken from the 38 colonies that were positive for infection by *S. symbiotica*, as well as from infected ants. In 29 colonies, the sequences were easily readable without messy peaks, indicating that coinfection of different *S. symbiotica* strains in any of these colonies is unlikely. Colonies displaying polymorphic sequences, or from which only one easily readable sequence was obtained, were discarded from the phylogenetic analysis (Table S1, Supporting information).

The phylogeny of the strains of *S. symbiotica* is strongly structured by the taxonomic identity of their host (Fig. 2). Our phylogenetic analyses established the existence of five distinct clades. *S. symbiotica* strains found in *A. pisum* (subfamily Aphidinae) and *C. tujafilina* (subfamily Lachninae) and whose genomes have been sequenced [39, 40] formed the clade A (but with no support) [39, 40]. Co-obligate *S. symbiotica* strains from *C. cedri* and *T. salignus* (subfamily Lachninae) that exhibit a long-term co-evolutionary history with their host formed the clade B (posterior probability of 1.00). Strains found in *P. lyropictus* (subfamily Chaitophorinae) formed the clade C (posterior probability of 1.00). *S. symbiotica* strains from *A. fabae*, *A. pomi*, and *M. absinthii* (subfamily Aphidinae) for which the genes *flgA*, *proA*, and *hisA* were amplified formed a monophyletic clade (Clade D, posterior probability of 0.98) with strains exhibiting a free-living capacity in laboratory conditions (strains CWBI-2.3^T, 24.1, and Apa8 A1). Clade E was composed of *S. symbiotica* strains found in *P. acericola* (subfamily Chaitophorinae), *U. sonchi* (subfamily Pterocommatinae), and *D. platanoides* (subfamily Drepanosiphinae) (posterior probability of 1.00). The strains found in the aphid species *A. fabae* (subfamily Aphidinae) and *C. populialbae* (subfamily Chaitophorinae), as well as in ants (*L. niger*) formed a paraphyletic grade with clade E (posterior probability of 0.99).

Tissue Tropism of *S. symbiotica*

Several strains of *S. symbiotica* have previously been isolated during the characterization of the microbiota of several laboratory and wild aphids collected in Belgium [30, 31]. Strains 24.1 and CWBI-2.3^T were initially isolated from *A. fabae* specimens and strain Apa8-A1 from *Aphis passeriniana*. Nevertheless, although it was assumed that the strain CWBI-2.3^T was initially isolated from bacteriocytes and sheath cells [30], the tissue tropism of these strains that are able to live independently of a host has never been clearly investigated. Returning to the field, we revealed the existence of a distinct clade (clade D in our study) that pools together the three previously isolated free-living *S. symbiotica* strains with several strains identified in this survey. All the strains pooled in this clade were systematically found positive for the primers

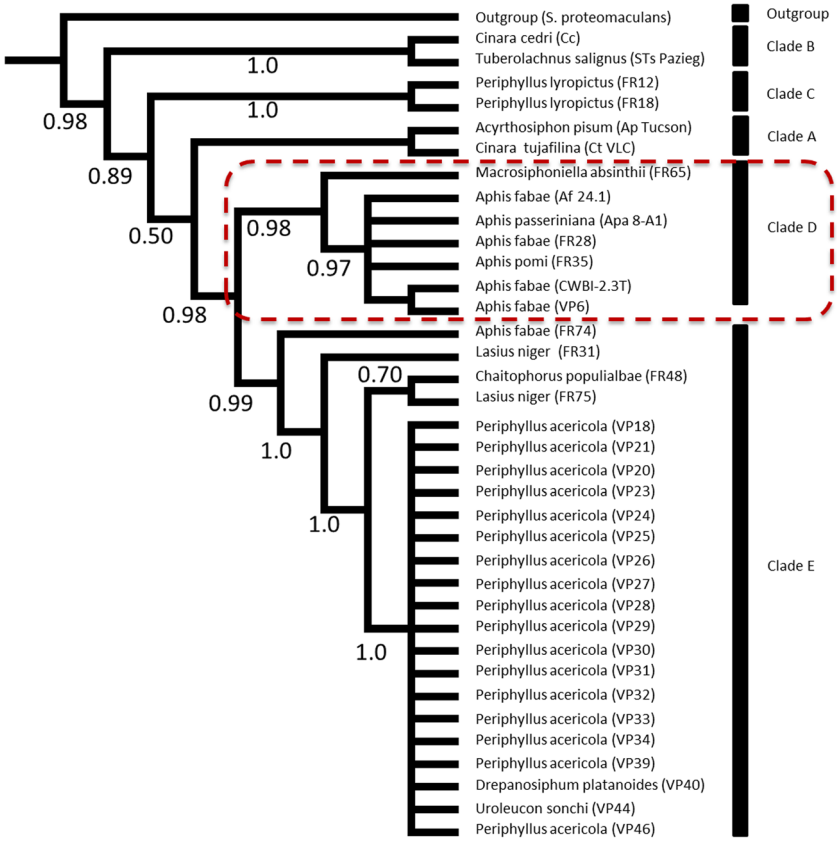

Fig. 2 Cladogram of the 50% consensus tree from the posterior distribution of threes from MrBayes analysis of *accD*, *gyrB*, *murE*, and *recJ* sequences in *S. symbiotica*. The number below the branches represents their posterior probabilities. The names given for each terminal node reflect the taxonomic identity of the host from which the *S. symbiotica* strain was sequenced. The clade grouping *S. symbiotica* strains found in the context of this study together with strains previously isolated and exhibiting a free-living capacity in laboratory conditions is highlighted by red dashed lines (color figure online)

targeting the genes *flgA*, *proA*, and *hisA* (Table S1, Supporting information). Whole-mount FISH was therefore used to specifically examine the tissue tropism of these strains in wild aphids that were collected here. Our observations revealed the existence of *S. symbiotica* strains residing in the digestive tract of aphids (Fig. 3).

These gut-associated *S. symbiotica* were found either aggregated in the midgut (Fig. 3a–d) or into the whole gut (Fig. 3e). Specimens of wild aphids of all ages have been sampled, and the degree of infection should vary with stage of development. Although we did not determine the stage of aphid development prior microscopic observations, the shape of their bacteriome made some estimates possible. In young aphids, the bacteriome forms a bilobed structure as illustrated by aphids of Fig. 3a–d, while in adults, it breaks apart for forming groups of bacteriocytes disseminated throughout the abdomen as illustrated by aphids of Fig. 3e [50, 51]. Our observations suggest that the infection begins somewhere in the midgut in young aphids before spreading to the entire digestive tract during host development.

S. symbiotica was detected in about one-third of the sampled ants. We therefore also examined the tissue tropism of the symbiont in these insects. Our micrographic evidence clearly indicates that *S. symbiotica* was found in the digestive tract of ants, a location restricted to the proventriculus (Fig. 4). In ants, this portion of the digestive tract connects the crop with the midgut and is divided into four distinctive parts: the calyx that is connected to the crop, the occlusory tract, the bulb that is made of circular muscles, and the cylinder that is connected with the midgut [52]. Our FISH images clearly suggest that the tissue tropism of *S. symbiotica* is restricted inside the bulb cavity, a structure that has a regulatory role in the flow of food that passes through the digestive tract, and which acts as a filter for blocking the entry of microorganisms and large particles into the midgut [53].

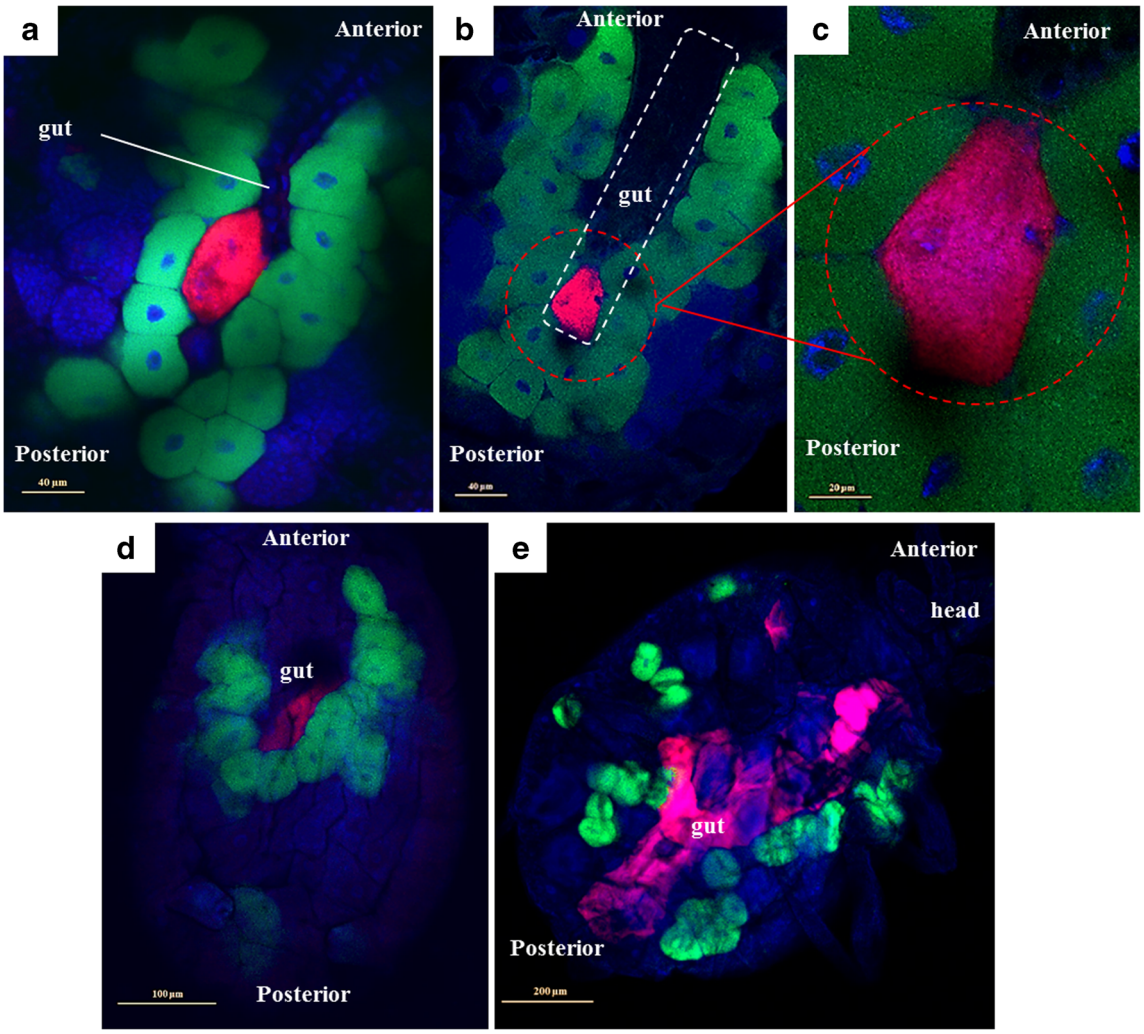

Fig. 3 Whole-mount FISH of *S. symbiotica* (clade D associates) in naturally infected *A. fabae* (ventral views). Red Cy3 signals are *S. symbiotica*, green Cy5 signals are *B. aphidicola*, and blue SYTOX Green signals are aphid tissues. **a**–**d** *S. symbiotica* found residing at the level of the midgut of young aphids. **e** *S. symbiotica* found residing in the whole tortuous digestive tract of an adult (color figure online)

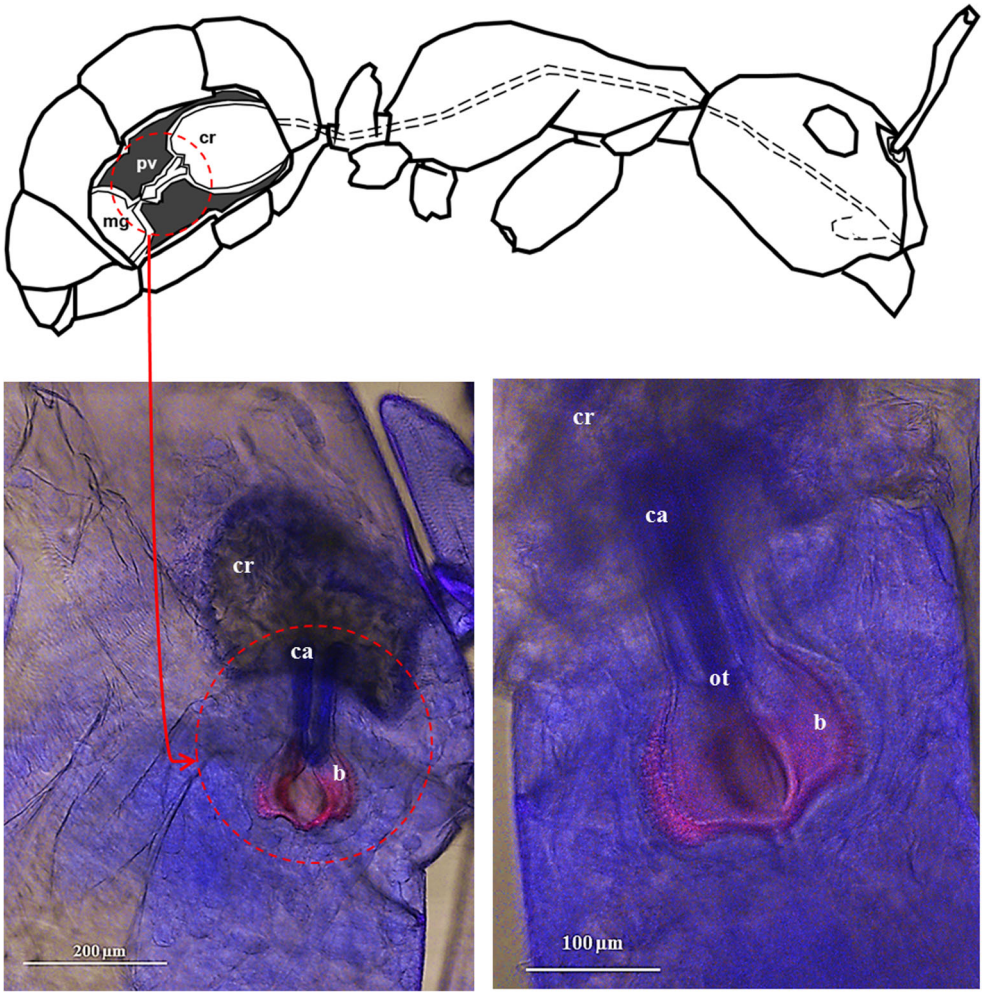

Fig. 4 Whole-mount FISH of *S. symbiotica* in ants (*L. niger*) (lateral view). Red Cy3 signals are *S. symbiotica*, and blue SYTOX Green signals are ant tissues. *S. symbiotica* is located within the bulb of the proventriculus. mg midgut, pv proventriculus, cr crop, ca calyx, b bulb, ot occlusory tract. The cylinder connecting the proventriculus to the midgut is poorly visible on our micrographs (color figure online)

Discussion

Here, we examined the presence of these strains within wild aphid populations and associated ants using a step-by-step integrated approach. Our results revealed the existence of *S. symbiotica* strains that reside naturally within the digestive tract of insects. To our knowledge, this is the first time that that such a tissue tropism is reported for *S. symbiotica* in wild aphids and ants. Regarding aphids, all previous studies reported a more intracellular tissue tropism for *S. symbiotica* as it has been found residing within bacteriocytes, sheath cells, and hemolymph [54–56]. We did not find *S. symbiotica* in other tissues such as hemolymph or bacteriocytes. But since our FISH approach was applied on whole aphid individuals, we cannot absolutely assert that they are completely absent from these tissues. The presence of gut-associated *S. symbiotica* strains in aphids and ants suggests that the intraspecific variety of this symbiont is wider than previously defined and addresses major issues regarding the transmission behaviors and the phenotypes associated with these *S. symbiotica* strains. First, this extracellular tissue location raises the question of their mobility within insect populations and their ability to initiate new symbiotic relationships. The discovery of gut-associated *S. symbiotica* also raises the question of the biological significance of such strains in insect hosts.

A bacterial tissue tropism at the gut level is not necessarily associated with a free-living capacity; dependent-culture approaches are definitively required to confirm such biological feature. *S. symbiotica* strains found positive for *flgA*, *proA*, and *hisA* genes and colonizing the aphids gut were found to be grouped in a common phylogenetic clade together with previously isolated *S. symbiotica* strains. Therefore, it is likely that cultivable *S. symbiotica* strains, including the strain CWBI-2.3^T whose genome has been sequenced, are originally extracellular strains isolated from the aphid digestive tract. This extracellular localization raises the question of the ability of these *S. symbiotica* strains to establish novel and persistent relationships with insects [1]. The genome size of strain CWBI-2.3^T is more than five times larger than the genome of the co-obligate strain associated with *Tuberolachnus salignus* (subfamily Lachninae) [29, 33] and exhibits a large range of colonization factors [32]. While the symbiotic status of this free-living strain remains unclear, its genomic features as well as its ability to rapidly infect the aphid gut in laboratory conditions suggest that it is involved in a nascent stage of

symbiosis [29, 32, 33, 38]. In addition, the discovery of gut-associated strains supports the hypothesis that mutualistic *S. symbiotica* strains have evolved from free-living precursors with the oral opening as a preferred route of entry [18, 21].

Aphid honeydew is a sugar-rich sticky liquid suitable for microorganism growth [57, 58]. The gut location of certain *S. symbiotica* strains makes them more likely to infect the honeydew that can mediate of intra- and interspecific horizontal transfers of symbionts [59, 60]. To test this hypothesis, the presence of *S. symbiotica* in wild insect populations was assessed in the context of myrmecophilous relationships. Indeed, honeydew excreted by aphids can be collected as food by various species of ants. In return, ants provide protection against predators and parasites [61, 62]. Almost no studies have examined the presence of aphid symbionts in the context of myrmecophily, whereas ant tending is observed in two thirds of aphid species present in Europe [63]. Here, we demonstrated that *S. symbiotica* infections occur not only in aphids but also in ants with about one third of our samples being infected, and an increasing probability of finding infected ants when tending aphid colonies harboring *S. symbiotica*. In these insects, *S. symbiotica* exhibited a localization that was found restricted inside the bulb cavity of the proventriculus. Unfortunately, we failed to consistently obtain sequences of sufficient quality from the *S. symbiotica* strains detected in ants to enrich our phylogenetic analysis. The sequences obtained from these insects were most of the time hardly readable or missing, and we were not able to establish an identity match between *S. symbiotica* strains found in ants and strains found in the tended aphid colonies. In addition, the few strains of *S. symbiotica* associated with ants that could be integrated into the phylogenetic analysis did not occur in the same clades as the extracellular strains of aphids. These results do not establish the existence of direct horizontal transfers between aphids and ants. It is likely that a same individual ant tends different aphid colonies and feeds on other food sources during foraging [64], with the possibility to pick up different *S. symbiotica* strains coming from environmental sources. In addition, by acting as a pump regulating the flow of food through the digestive tract, the proventriculus plays an important role in the trophallaxis behavior consisting of the transfer of food among members of the colony through mouth-to-mouth or anus-to-mouth [52, 65]. So, even if a correlation between the presence of *S. symbiotica* in ants and the infection status of the tended aphid colonies was established, ants may possibly harbor a mixture of strains coming from various sources, which may explain the difficulty, we had to obtain readable sequences for *S. symbiotica* in these insects. Previous studies have detected the presence of *S. symbiotica* in the gut of two ant species: *Formica cinerea* [66] and *Camponotus japonicus* Mayr [67, 68]. Taken together, these observations support the hypothesis that *S. symbiotica* is present in ants that are insects known to maintain mutualistic relationships with a wide variety of gut-associated bacteria [69–71]. Whether *S. symbiotica* is a well-established symbiont or a passing commensal resident in these insects remains to be elucidated. Since the gut lining of these insects is shed during the final larval molt, it is likely that these gut-associated *S. symbiotica* are regularly reacquired, not strictly maintained and susceptible to move through various routes [70]. Further studies are needed to determine the extent to which *S. symbiotica* persists in the tissues of ants and to establish the biological significance of its presence in these insects.

The existence of gut-associated *S. symbiotica* further raises the question of their biological significance. There is a series of evidence that *S. symbiotica* can be associated with a nutritional function. In Lachninae aphids, it has been clearly established that co-obligate strains of *S. symbiotica* play a key nutritional role by providing the host with amino acids and vitamins [23, 25, 40]. In the pea aphid *A. pisum*, it has been demonstrated that an intracellular *S. symbiotica* strain of a facultative nature can compensate for loss of obligate nutritional symbiont *B. aphidicola* [72]. A recent large-scale study revealed that *S. symbiotica* is found more frequently in specialist aphids compared with polyphagous species, suggesting that the symbiont could affect aphid plant use and display a nutritional role [34]. This hypothesis is supported by the presence of *S. symbiotica* forming a specific clade in almost all our investigated *Periphyllus* colonies. For their part, Henry et al., 2015 [34] found an occurrence of 79% in *P. testudinaceus* populations collected on *Acer pseudoplatanus*. *S. symbiotica* also exhibits a high prevalence in populations of the specialist aphid *Sipha maydis* [73]. The nutritive role of *S. symbiotica* has mainly been assessed from a genomic point of view on a few strains harbored by specialist aphids of the Lachninae subfamily. It is expected that a nutritive role associated with *S. symbiotica* takes place in other specialist aphid species where the symbiont occurs in almost 100% of the individuals. The nutritional role of *S. symbiotica* has mostly been assessed in the context of co-obligate mutualism. The findings of gut-associated strains bring a fresh perspective regarding the potential nutritional role of *S. symbiotica* since in insects, gut symbionts are often pointed as important contributors to food digestion, vitamins production, and toxin neutralization [69, 74]. In herbivorous insect species, gut bacteria can facilitate herbivory by enabling the digestion of plant polymers, by neutralizing plant toxins and by provisioning of essential nutrients, such as amino acids or vitamins, which have unbalanced profiles in plant tissues [75]. In comparison to the more drastically reduced *S. symbiotica* sequenced genomes, the genome of the free-living strain *S. symbiotica* CWBI-2.3^T displays intact pathways for the synthesis of most essential amino acids and vitamins [56], which could benefit the aphid in case of nutritional stress. Further experiments are required to clarify the putative nutritional role of extracellular as well as intracellular *S. symbiotica* strains. In addition to these nutritional

aspects, other putative beneficial effects associated with these gut-associated *S. symbiotica* cannot be excluded, such as protection against parasite invasion [69, 76–78], or the release of kairomones that can mediate intra- and interspecific communication in insects by attracting natural enemies or enhancing mutualistic interactions with other insects [57, 58, 69].

Conclusion

Assessing the intraspecific variability of symbiotic bacteria is crucial to finely assess their associated effects and their transmission behaviors. Facultative symbionts harbored by aphids have always been referred as intracellular endosymbionts since they have been mostly described as bacterial partners living within host cells such as bacteriocytes and sheath cells (and to some extent in the hemolymph). This intracellular tropism is generally perceived as the sign of an intimate and stabilized relationship in which the symbiont follows a linear evolutionary trajectory parallel to that of the host. Co-obligate *S. symbiotica* strains testify to such an evolutionary scenario. By establishing the existence of gut-associated *S. symbiotica* strains in insects, we show a completely different face of a symbiont species. These labile *S. symbiotica* strains are expected to exhibit a broader range of metabolic abilities compared to intracellular relatives, as well as a genome with continuous rearrangements due to an ongoing uptake and loss of genes and the integrations of mobile genetic elements. Extracellular *S. symbiotica* strains could represent a reservoir for a rapid acquisition of new beneficial traits by the host, selected according to the selection pressures of the moment. Where do these strains come from? Are they mutualistic bacteria or commensals in transit? Are they initially present in the soil and capable of transiting through plants? Are they evolving into an intracellular lifestyle with an ability to replace other symbionts? Field and laboratory studies should puzzle out answers to these questions to refine our perception of the evolution of bacterial mutualism.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interest.

References

1. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci 110:3229–3236. https://doi.org/10.1073/pnas.1218525110
2. Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol 55:247–266. https://doi.org/10.1146/annurev-ento-112408-085305
3. Feldhaar H (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36:533–543. https://doi.org/10.1111/j.1365-2311.2011.01318.x
4. Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42:165–190. https://doi.org/10.1146/annurev.genet.41.110306.130119
5. Ferrari J, Vavre F (2011) Bacterial symbionts in insects or the story of communities affecting communities. Philos Trans R Soc B Biol Sci 366:1389–1400. https://doi.org/10.1098/rstb.2010.0226
6. Sandström JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. Mol Ecol 10:217–228. https://doi.org/10.1046/j.1365-294X.2001.01189.x
7. Duron O, Wilkes TE, Hurst GDD (2010) Interspecific transmission of a male-killing bacterium on an ecological timescale. Ecol Lett 13:1139–1148. https://doi.org/10.1111/j.1461-0248.2010.01502.x
8. Gehrer L, Vorburger C (2012) Parasitoids as vectors of facultative bacterial endosymbionts in aphids. Biol Lett rsbl20120144. https://doi.org/10.1098/rsbl.2012.0144
9. Jousselin E, Cœur d'Acier A, Vanlerberghe-Masutti F, Duron O (2013) Evolution and diversity of *Arsenophonus* endosymbionts in aphids. Mol Ecol 22:260–270. https://doi.org/10.1111/mec.12092
10. Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, Simon JC, Fukatsu T (2010) Symbiotic bacterium modifies aphid body color. Science 330:1102–1104. https://doi.org/10.1126/science.1195463
11. Dion E, Polin SE, Simon J-C, Outreman Y (2011) Symbiont infection affects aphid defensive behaviours. Biol Lett 7:743–746. https://doi.org/10.1098/rsbl.2011.0249
12. Simon J-C, Boutin S, Tsuchida T, Koga R, le Gallic JF, Frantz A, Outreman Y, Fukatsu T (2011) Facultative symbiont infections affect aphid reproduction. PLoS One 6:e21831. https://doi.org/10.1371/journal.pone.0021831
13. Oliver KM, Smith AH, Russell JA (2014) Defensive symbiosis in the real world – advancing ecological studies of heritable, protective bacteria in aphids and beyond. Funct Ecol 28:341–355. https://doi.org/10.1111/1365-2435.12133
14. Leclair M, Pons I, Mahéo F, Morlière S, Simon JC, Outreman Y (2016) Diversity in symbiont consortia in the pea aphid complex is associated with large phenotypic variation in the insect host. Evol Ecol 30:925–941. https://doi.org/10.1007/s10682-016-9856-1
15. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007) From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biol 5:e114. https://doi.org/10.1371/journal.pbio.0050114

16. Teixeira L, Ferreira Á, Ashburner M (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol 6:e1000002. https://doi.org/10.1371/journal.pbio.1000002
17. Min K-T, Benzer S (1997) *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. Proc Natl Acad Sci 94:10792–10796. https://doi.org/10.1073/pnas.94.20.10792
18. Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. Nat Rev Microbiol 8:218–230. https://doi.org/10.1038/nrmicro2262
19. Egan S, Thomas T (2015) Microbial symbiosis of marine sessile hosts - diversity, function and applications. Frontiers Media SA
20. Salem H, Florez L, Gerardo N, Kaltenpoth M (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. Proc R Soc Lond B Biol Sci 282: 20142957. https://doi.org/10.1098/rspb.2014.2957
21. Clayton AL, Oakeson KF, Gutin M, Pontes A, Dunn DM, von Niederhausern AC, Weiss RB, Fisher M, Dale C (2012) A novel human-infection-derived bacterium provides insights into the evolutionary origins of mutualistic insect–bacterial symbioses. PLoS Genet 8:e1002990. https://doi.org/10.1371/journal.pgen.1002990
22. Zytynska SE, Meyer ST, Sturm S, Ullmann W, Mehrparvar M, Weisser WW (2015) Secondary bacterial symbiont community in aphids responds to plant diversity. Oecologia 180:1–13. https://doi.org/10.1007/s00442-015-3488-y
23. Lamelas A, Gosalbes MJ, Manzano-Marín A, Peretó J, Moya A, Latorre A (2011) *Serratia symbiotica* from the aphid *Cinara cedri*: a missing link from facultative to obligate insect endosymbiont. PLoS Genet 7:e1002357. https://doi.org/10.1371/journal.pgen.1002357
24. Lamelas A, Gosalbes MJ, Moya A, Latorre A (2011) The genome of *Buchnera aphidicola* from the aphid *Cinara tujafilina* provides new clues about the evolutionary history of metabolic losses in bacterial endosymbionts. Appl Environ Microbiol AEM.00141–11. https://doi.org/10.1128/AEM.00141-11
25. Manzano-Marín A, Simon J-C, Latorre A (2016) Reinventing the wheel and making it round again: evolutionary convergence in *Buchnera–Serratia* symbiotic consortia between the distantly related Lachninae aphids *Tuberolachnus salignus* and *Cinara cedri*. Genome Biol Evol 8:1440–1458. https://doi.org/10.1093/gbe/evw085
26. Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol Entomol 27:189–195. https://doi.org/10.1046/j.1365-2311.2002.00393.x
27. Burke G, Fiehn O, Moran N (2009) Effects of facultative symbionts and heat stress on the metabolome of pea aphids. ISME J 4:242–252. https://doi.org/10.1038/ismej.2009.114
28. Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci 100:1803–1807. https://doi.org/10.1073/pnas.0335320100
29. Foray V, Grigorescu AS, Sabri A, Haubruge E, Lognay G, Francis F, Fauconnier ML, Hance T, Thonart P (2014) Whole-genome sequence of *Serratia symbiotica* strain CWBI-2.3T, a free-living symbiont of the black bean aphid *Aphis fabae*. Genome Announc 2: e00767–e00714. https://doi.org/10.1128/genomeA.00767-14
30. Sabri A, Leroy P, Haubruge E, Hance T, Frere I, Destain J, Thonart P (2011) Isolation, pure culture and characterization of *Serratia symbiotica* sp. nov., the R-type of secondary endosymbiont of the black bean aphid *Aphis fabae*. Int J Syst Evol Microbiol 61:2081–2088. https://doi.org/10.1099/ijs.0.024133-0
31. Grigorescu AS, Renoz F, Sabri A, et al (2017) Accessing the hidden microbial diversity of aphids: an illustration of how culture-dependent methods can be used to decipher the insect microbiota. Microb Ecol 1–14. https://doi.org/10.1007/s00248-017-1092-x
32. Renoz F, Champagne A, Degand H, Faber AM, Morsomme P, Foray V, Hance T (2017) Toward a better understanding of the mechanisms of symbiosis: a comprehensive proteome map of a nascent insect symbiont. PeerJ 5:e3291. https://doi.org/10.7717/peerj.3291
33. Manzano-Marín A, Latorre A (2016) Snapshots of a shrinking partner: genome reduction in *Serratia symbiotica*. Sci Rep 6. https://doi.org/10.1038/srep32590
34. Henry LM, Maiden MCJ, Ferrari J, Godfray HCJ (2015) Insect life history and the evolution of bacterial mutualism. Ecol Lett 18:516–525. https://doi.org/10.1111/ele.12425
35. Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proc Natl Acad Sci U S A 103:968–971. https://doi.org/10.1073/pnas.0510466103
36. D'acier AC, Cruaud A, Artige E et al (2014) DNA barcoding and the associated PhylAphidB@se website for the identification of European aphids (Insecta: Hemiptera: Aphididae). PLoS One 9: e97620. https://doi.org/10.1371/journal.pone.0097620
37. Fukatsu T, Nikoh N, Kawai R, Koga R (2000) The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). Appl Environ Microbiol 66:2748–2758
38. Renoz F, Noël C, Errachid A, Foray V, Hance T (2015) Infection dynamic of symbiotic bacteria in the pea aphid *Acyrthosiphon pisum* gut and host immune response at the early steps in the infection process. PLoS One 10:e0122099. https://doi.org/10.1371/journal.pone.0122099
39. Burke GR, Moran NA (2011) Massive genomic decay in *Serratia symbiotica*, a recently evolved symbiont of aphids. Genome Biol Evol 3:195–208. https://doi.org/10.1093/gbe/evr002
40. Manzano-Marín A, Latorre A (2014) Settling down: the genome of *Serratia symbiotica* from the aphid *Cinara tujafilina* zooms in on the process of accommodation to a cooperative intracellular life. Genome Biol Evol 6:1683–1698. https://doi.org/10.1093/gbe/evu133
41. R Development Core Team (2006) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org. Accessed 15 Dec 2015
42. Henry LM, Peccoud J, Simon J-C, Hadfield JD, Maiden MJC, Ferrari J, Godfray HCJ (2013) Horizontally transmitted symbionts and host colonization of ecological niches. Curr Biol 23:1713–1717. https://doi.org/10.1016/j.cub.2013.07.029
43. Łukasik P, Guo H, van Asch M, Henry LM, Godfray HCJ, Ferrari J (2015) Horizontal transfer of facultative endosymbionts is limited by host relatedness. Evolution 69:2757–2766. https://doi.org/10.1111/evo.12767
44. Degnan PH, Moran NA (2008) Evolutionary genetics of a defensive facultative symbiont of insects: exchange of toxin-encoding bacteriophage. Mol Ecol 17:916–929. https://doi.org/10.1111/j.1365-294X.2007.03616.x
45. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. https://doi.org/10.1093/bioinformatics/bts199
46. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17:540–552
47. Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772–772. https://doi.org/10.1038/nmeth.2109
48. Nylander JAA, Wilgenbusch JC, Warren DL, Swofford DL (2008) AWTY (are we there yet?): a system for graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics 24:581–583. https://doi.org/10.1093/bioinformatics/btm388
49. Ryuichi Koga TT (2009) Quenching autofluorescence of insect tissues for in situ detection of endosymbionts. Appl Entomol Zool - APPL ENTOMOL ZOOL 44:281–291. https://doi.org/10.1303/aez.2009.281

50. Baumann P, Baumann L, Lai C-Y, Rouhbakhsh D, Moran NA, Clark MA (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annu Rev Microbiol 49:55–94. https://doi.org/10.1146/annurev.mi.49.100195.000415
51. Simonet P, Duport G, Gaget K, et al (2016) Direct flow cytometry measurements reveal a fine-tuning of symbiotic cell dynamics according to the host developmental needs in aphid symbiosis. Sci Rep 6:srep19967. https://doi.org/10.1038/srep19967
52. Eisner T, Wilson EO (1952) The morphology of the proventriculus of a formicine ant. Psyche J Entomol 59:47–60. https://doi.org/10.1155/1952/14806
53. Lanan MC, Rodrigues PAP, Agellon A, Jansma P, Wheeler DE (2016) A bacterial filter protects and structures the gut microbiome of an insect. ISME J 10:1866–1876. https://doi.org/10.1038/ismej.2015.264
54. Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. Appl Environ Microbiol 71:3302–3310. https://doi.org/10.1128/AEM.71.6.3302-3310.2005
55. Koga R, Meng X-Y, Tsuchida T, Fukatsu T (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. Proc Natl Acad Sci 109:E1230–E1237. https://doi.org/10.1073/pnas.1119212109
56. Manzano-Marín A, Szabó G, Simon J-C, Horn M, Latorre A (2017) Happens in the best of subfamilies: establishment and repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. Environ Microbiol 19:393–408. https://doi.org/10.1111/1462-2920.13633
57. Leroy PD, Sabri A, Heuskin S, Thonart P, Lognay G, Verheggen FJ, Francis F, Brostaux Y, Felton GW, Haubruge E (2011) Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. Nat Commun 2:348. https://doi.org/10.1038/ncomms1347
58. Fischer CY, Lognay GC, Detrain C, Heil M, Grigorescu A, Sabri A, Thonart P, Haubruge E, Verheggen FJ (2015) Bacteria may enhance species association in an ant–aphid mutualistic relationship. Chemoecology 25:223–232. https://doi.org/10.1007/s00049-015-0188-3
59. Darby AC, Douglas AE (2003) Elucidation of the transmission patterns of an insect-borne bacterium. Appl Environ Microbiol 69:4403–4407. https://doi.org/10.1128/AEM.69.8.4403-4407.2003
60. Sabri A, Vandermoten S, Leroy PD, Haubruge E, Hance T, Thonart P, de Pauw E, Francis F (2013) Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. PLoS One 8:e74656. https://doi.org/10.1371/journal.pone.0074656
61. Buckley RC (1987) Interactions involving plants, Homoptera, and ants. Annu Rev Ecol Syst 18:111–135
62. Katayama N, Suzuki N (2003) Bodyguard effects for aphids of *Aphis craccivora* Koch (Homoptera: Aphididae) as related to the activity of two ant species, *Tetramorium caespitum* Linnaeus (Hymenoptera: Formicidae) and *Lasius niger* L. (Hymenoptera: Formicidae). Appl Entomol Zool 38:427–433. https://doi.org/10.1303/aez.2003.427
63. Stadler B (1997) The relative importance of host plants, natural enemies and ants in the evolution of life-history characters in aphids. In: Dettner PDK, Bauer PDG, Völkl DW (eds) Vertical food web interactions. Springer, Berlin Heidelberg, pp 241–256
64. Portha S, Deneubourg J-L, Detrain C (2004) How food type and brood influence foraging decisions of *Lasius niger* scouts. Anim Behav 68:115–122. https://doi.org/10.1016/j.anbehav.2003.10.016
65. Liebig J, Heinze J, Hölldobler B (1997) Trophallaxis and aggression in the ponerine ant, *Ponera coarctata*: implications for the evolution of liquid food exchange in the Hymenoptera. Ethology 103:707–722. https://doi.org/10.1111/j.1439-0310.1997.tb00180.x
66. Sirviö A, Pamilo P (2010) Multiple endosymbionts in populations of the ant *Formica cinerea*. BMC Evol Biol 10:335. https://doi.org/10.1186/1471-2148-10-335
67. He H, Chen Y, Zhang Y, Wei C (2011) Bacteria associated with gut lumen of *Camponotus japonicus* Mayr. Environ Entomol 40:1405–1409. https://doi.org/10.1603/EN11157
68. Li X, Nan X, Wei C, He H (2012) The gut bacteria associated with *Camponotus japonicus* Mayr with culture-dependent and DGGE methods. Curr Microbiol 65:610–616. https://doi.org/10.1007/s00284-012-0197-1
69. Engel P, Moran NA (2013) The gut microbiota of insects—diversity in structure and function. FEMS Microbiol Rev 37:699–735. https://doi.org/10.1111/1574-6976.12025
70. Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce NE (2009) Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. Proc Natl Acad Sci 106:21236–21241. https://doi.org/10.1073/pnas.0907926106
71. Hu Y, Łukasik P, Moreau CS, Russell JA (2014) Correlates of gut community composition across an ant species (*Cephalotes varians*) elucidate causes and consequences of symbiotic variability. Mol Ecol 23:1284–1300. https://doi.org/10.1111/mec.12607
72. Koga R, Tsuchida T, Fukatsu T (2003) Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. Proc R Soc Lond B Biol Sci 270:2543–2550. https://doi.org/10.1098/rspb.2003.2537
73. Fakhour S, Ambroise J, Renoz F, Foray V, Gala JL, Hance T (2018) A large-scale field study of bacterial communities in cereal aphid populations across Morocco. FEMS Microbiol Ecol 94. https://doi.org/10.1093/femsec/fiy003
74. Dillon RJ, Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. Annu Rev Entomol 49:71–92. https://doi.org/10.1146/annurev.ento.49.061802.123416
75. Hansen AK, Moran NA (2014) The impact of microbial symbionts on host plant utilization by herbivorous insects. Mol Ecol 23:1473–1496. https://doi.org/10.1111/mec.12421
76. Meister S, Agianian B, Turlure F, Relógio A, Morlais I, Kafatos FC, Christophides GK (2009) *Anopheles gambiae* PGRPLC-mediated defense against bacteria modulates infections with malaria parasites. PLoS Pathog 5:e1000542. https://doi.org/10.1371/journal.ppat.1000542
77. Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G (2011) Natural microbe-mediated refractoriness to Plasmodium infection in *Anopheles gambiae*. Science 332:855–858. https://doi.org/10.1126/science.1201618
78. Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc Natl Acad Sci 108:19288–19292. https://doi.org/10.1073/pnas.1110474108