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INVERTEBRATE MICROBIOLOGY

Accessing the Hidden Microbial Diversity of Aphids: an Illustration of How Culture-Dependent Methods Can Be Used to Decipher the Insect Microbiota

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Abstract Microorganism communities that live inside insects can play critical roles in host development, nutrition, immunity, physiology, and behavior. Over the past decade, high-throughput sequencing reveals the extraordinary microbial diversity associated with various insect species and provides information independent of our ability to culture these microbes. However, their cultivation in the laboratory remains crucial for a deep understanding of their physiology and the roles they play in host insects. Aphids are insects that received specific attention because of their ability to form symbiotic associations with a wide range of endosymbionts that are considered as the core microbiome of these sap-feeding insects. But, if the functional diversity of obligate and facultative endosymbionts has been extensively studied in aphids, the diversity of gut symbionts and other associated microorganisms received limited consideration. Herein, we present a culture-dependent method that allowed us to successfully isolate microorganisms from several aphid species. The isolated microorganisms were assigned to 24 bacterial genera from the Actinobacteria, Firmicutes, and Proteobacteria phyla and three fungal genera from the Ascomycota and Basidiomycota phyla. In our study, we succeeded in isolating already described bacteria found associated to aphids (e.g., the facultative symbiont *Serratia symbiotica*), as well as microorganisms that have never been described in aphids before. By unraveling a microbial community that so far has been ignored, our study expands our current knowledge on the microbial diversity associated with aphids and illustrates how fast and simple culture-dependent approaches can be applied to insects in order to capture their diverse microbiota members.

Keywords Insect · Aphid microbiota · Culture-dependent method · Molecular phylogeny · Symbiotic bacteria

Alina S. Grigorescu and François Renoz contributed equally to this work.

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Introduction

Insects owe to a great extent their adaptation to different environments to the relationships they developed with various microorganisms [1–4]. Aphids (Hemiptera: Aphididae) are phloem sap-feeding insects that represent a relevant model to study such insect-bacteria relationships [5]. Indeed, they have established associations with (i) obligate (or primary) endosymbionts, which are harbored in special host cells called bacteriocytes and fundamentally supply nutritional roles [6–8], and (ii) facultative (or secondary) symbionts, which can be localized in a variety of host tissues and can enhance the adaptation of aphids to specific environmental conditions.

For example, facultative symbionts have shown their ability to improve host heat tolerance [9, 10], host resistance to natural enemies [11–14], host plant specialization [15, 16], and host survival [17] or to interfere with predation by inducing aphid body color modification [18]. Yet, culture-dependent studies performed about 20 years ago showed that the aphid microbiota is not only limited to the obligate and facultative symbionts but also included microorganisms isolated from the gut lumen [19–21]. These studies, however, remained obscured by the importance given to the known endosymbionts and by the fact that even after those initial findings on the gut microbiota of aphids, these insects were still thought to have an almost-sterile digestive tube [19]. Thus, no major efforts were put afterwards to unravel the rare members of the aphid microbiota, leaving this category of microorganisms associated with aphids largely unknown.

Culture-dependent techniques were the first approach that scientists used for studying microbial diversity but lost power with the rise of metagenomic strategies. These new powerful approaches provided additional exploration venues for aphid microbiota, given that most of the known associated endosymbionts are non-culturable due to their adaptation to a host-dependent lifestyle [22, 23]. Thus, some culture-independent studies concluded that the known obligate and facultative symbionts represent the major flora associated with aphids [24–27]. Nevertheless, a few additional bacterial strains, similar to *Pseudomonas* sp., *Acinetobacter* sp., *Pelomonas* sp., and *Burkholderia* sp. [28]; *Staphylococcus* sp. [26]; and *Erwinia* sp. and *Pantoea* sp. [27], were also detected, suggesting that the aphid microbiota is more diverse than previously thought. At the same time, several microbial culture-dependent approaches have succeeded to isolate some aphid microbial partners, suggesting that these approaches are able to detect microorganisms that may be present in such low concentrations that are not detected by metagenomic approaches [29]. If metagenomic sequencing provides some information independent of our ability to culture bacteria associated to insects, their cultivation in the laboratory is required for better valuing their physiology and the role they play in host insects. For example, Leroy et al. [30] showed that *Staphylococcus sciuri*, a bacterium isolated from *Acyrthosiphon pisum* honeydew, is responsible for the attraction of the aphid natural enemies, and Fischer et al. [31] demonstrated the existence of *Staphylococcus sciuri* strains which release semiochemical factors promoting the attraction of ants in myrmecophilous relationships. The isolation and culturing of different members of the aphid microbiota also represents a great advantage for performing high-throughput analyses, such as the genome sequencing of *Serratia symbiotica* strain CWBI-2.3^T, a facultative symbiont that was isolated from the black bean aphid *Aphis fabae* [32].

Given the many advantages that a culture-dependent approach has in terms of future possible studies and complementing metagenomic studies [29, 33] and the fact that no recent culture-dependent studies were done on aphids, we chose this *old-fashioned* approach in order to assess the diversity of microorganisms that can be found associated with different aphid species and to try to define the core bacterial taxa that can be found in aphids beyond their obligate and facultative symbiotic partners. In our study, we considered both aphid species that were laboratory reared as well as specimens found in wild populations. The culturing method previously developed by Sabri et al. [34] was chosen because it is the only one that proved successful for isolating a free-living strain of *S. symbiotica*, a facultative symbiont which was otherwise considered non-culturable, as well as other bacterial isolates from the aphid honeydew [30, 35]. In the present study, our culture-dependent approach succeeded to isolate more than 20 bacterial genera and three fungal genera. The results obtained here illustrate the power that culture-dependent methods can have to reveal the hidden part of the microbiota of an insect.

Materials and Methods

Aphid Strains

Fifteen aphid species and strains (seven laboratory reared and eight from wild populations) were used in this study as listed in Table S1. The laboratory-reared aphid clones were maintained in continuous parthenogenetic culture on *Vicia faba* or *Cucumis sativus* plants at 20 ± 2 °C and long-day conditions (16 h of light and 8 h of darkness). From time to time, several adult individuals of different ages were removed from the host plants for dissections, as described below. Most of the aphids from wild populations used here came from a sampling campaign carried on in May 2013 in Tunisia. The aphid species were identified according to the morphological criteria and the host plant [36]. *Aphis fabae* came from a previous collection campaign carried on in Belgium in May 2011. After collection, the aphids were placed in small humid plastic containers with few leaves from the host plants to keep them alive. For each collected aphid sample, adult individuals were dissected within 10 days after their collection.

Isolation of Microorganisms from Wild and Laboratory Aphids

In order to isolate microorganisms from the body cavity of aphids, individuals (i.e., between 10 and 90 as listed in Table S1) of each aphid strain were first surface sterilized by sequentially dipping them into 96% denatured ethanol for 3–4 min and into 4% bleach for about 1 min and then washed with sterile water. Following this disinfection step, two

methods were used to isolate microorganisms. The first method consisted in aseptically placing one individual aphid into a 100 μL drop of sterile culture medium 863 [34] deposited inside a sterile Petri dish. Each aphid was then either stabbed with a sterile needle on its dorsal side to release hemolymph into the medium or crushed with flame-sterilized forceps. The drop of medium containing aphid hemolymph or crushed aphid extract was then plated on 868 medium agar plates (the same as the 863 medium but additionally having 17 g of agar per L). The second method consisted in aseptically placing a few disinfected aphid individuals in an Eppendorf tube containing 1 mL of 863 medium and crushing the aphids with a small pestle. The crushed aphid extract was used to prepare several serial dilutions. One hundred microliters of each dilution, as well as the non-diluted extract, was plated on 868 agar plates. This second method was applied only to the laboratory-reared strain of *Aphis gossypii* and the wild strains of *Aphis fabae* and *Brachycaudus cardui* collected from artichoke plants. All plates, regardless of the methods, were incubated at 20 °C up to 3 weeks. No specific negative controls were performed as many of the plates resulted in no growth. When growth was obtained, candidate colonies with different morphologies were restreaked several times on fresh plates for strain purification and further analyses. For long-term preservation, biomass was resuspended in 863 medium supplied with 15% glycerol and congealed at − 80 °C.

Total DNA Extraction

All DNA extractions were performed using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's protocols for Gram-positive bacteria or fungi. Total DNA of the isolated bacterial and fungal strains was extracted from biomass grown at 20 °C for 2 to 5 days on 868 agar plates.

Identification of Isolates

In order to identify the isolates and to discriminate between similar isolates, a multilocus gene sequencing approach was attempted, consisting in amplifying and sequencing fragments of the 16S ribosomal RNA (rRNA), *gyrB*, and *recA* genes for the bacterial isolates and the nearly complete 18S rRNA gene and the internal transcribed spacer (ITS) region for the fungal isolates. All primers used in this study are listed in Table S2.

All PCR reactions contained 1× ReadyMix Taq PCR Reagent Mix (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μM of each of the forward and reverse primers, and ~ 50 ng of genomic DNA as template. The obtained PCR products were further purified using the GFX PCR DNA and Gel Band Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's indications and sequenced at the GIGA Center at the University of Liège using the Big Dye v3.1 Kit and an ABI 3730 DNA Analyzer (Applied Biosystems/Life Technologies, Carlsbad, CA, USA).

For all bacterial isolates, the nearly complete 16S rRNA gene was PCR amplified using the universal primers 16SP0 and 16SP6. The PCR program included a 5-min initial denaturation step at 95 °C, followed by 26 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and a final extension for 10 min at 72 °C. A fragment of the 16S rRNA gene containing the V3–V5 hypervariable regions was sequenced with primers 338F and 907R. For some of the isolated strains (i.e., those for which a 16S rRNA gene sequence length higher than 1000 bp is provided in Table S3), the nearly complete 16S rRNA gene was sequenced using the additional primers F1, F2, F3, F4, R1, R2, R3, and R4 as well as the primers 16SP0 and 16SP6 used for the PCR amplification.

In addition to the 16S rRNA gene, a fragment of approximately 950 bp of the *gyrB* gene of some gammaproteobacterial isolates (i.e., *S. symbiotica* AfA3.1, *S. symbiotica* ApaA8.1, *Pantoea* sp. Gossypii A4, *Pseudomonas* sp. AgA28.2, *Pantoea* sp. AnA5.1, *Pseudomonas* sp. Artichoke A2, and *Pseudomonas* sp. Artichoke A4.1) was amplified using primers gyr-320 and rgyr-1260 and a 212-bp fragment of the *recA* gene was amplified from 36 bacterial isolates using primers recAF and recAR. The PCR programs were similar to those used for the amplification of the 16S rRNA gene, except that 35 cycles were performed and the elongation time was 1 min. For the amplification of *recA*, annealing was performed at 52 °C for 1 min. The same PCR primers were then used for sequencing the amplified fragments.

For all fungal isolates, the nearly complete 18S rRNA gene was amplified with primers PriA and PriB and using the same PCR program as for the 16S rRNA amplification, except that 30 PCR cycles were performed instead of 26. The 18S rRNA genes were then sequenced using the PCR primers and primers SR2, SR7, SR7R, and SR10R. In addition to the 18S rRNA gene, the ITS region was amplified with the universal primers ITS1 and ITS4 for the Ascomycota isolates and with specific primers ITS1-F and ITS4-B for the Basidiomycota isolates. The PCR program was identical to the one used for the amplification of the bacterial *gyrB* gene. The same PCR primers were then used for sequencing the amplified regions.

The electropherograms corresponding to the bidirectional sequencing of each gene were assembled and edited with the CodonCode Aligner software (version 4.2.7; CodonCode Corporation, Centerville, MA, USA). The resulting 16S, 18S, ITS, *gyrB*, and *recA* sequences were compared against the nucleotide collection (nr/nt) and the reference RNA sequence (refseq_ma) databases of GenBank by using the MegaBlast option in the BLASTN program [37], with default settings. The inferred amino acid sequences of the *gyrB* and

recA fragments were compared against the protein database using the BLASTP program. The identification of our isolates was carried out at the genus level.

Diagnostic PCR

For the isolates identified as potentially *Staphylococcus saprophyticus* or *Staphylococcus xylosus* based on the 16S rRNA sequencing, a diagnostic PCR was performed in order to discriminate between the two species. Three PCR primer sets were used: one set (i.e., primer 1 and primer 2) that was specific for *S. saprophyticus* and yielding a 210-bp fragment and two sets that were specific for *S. xylosus*: XYL-F and XYL-R, yielding a 539-bp fragment, and xylBF and xylBR, yielding an 899-bp fragment. These primer sequences are provided in Table S2. The PCR reactions were set up as described above, and the PCR program was the same as for the amplification of the 16S rRNA gene, except that the elongation time was 1 min for the 899-bp fragment and 40 s for the two smaller fragments. The PCR products were resolved by electrophoresis on 1% (*w*/*v*) agarose gels stained with 1 μg/mL ethidium bromide.

Phylogenetic Trees

The 16S and 18S rRNA gene sequeences obtained here for the bacterial and fungal isolates were optimally aligned with DNAMAN (version 7; Lynnon Corp., Quebec, Canada) using ClustalW [38]. Phylogenetic trees were constructed with the neighbor-joining method [39], as implemented in ClustalW, with the Jukes-Cantor substitution model. The tree topologies were tested by bootstrap analysis with 500 resamplings. In order to determine the phylogenetic relationship of the newly obtained sequences, additional sequences from the GenBank database were added to the DNA matrix.

Statistical Analyses

Statistical analyses were performed using the software R version 3.0.1 (R Development Core Team, 2014).

Nucleotide Sequence Accession Numbers

The 16S and 18S sequences determined in this study were deposited in GenBank under accession numbers KM586937–KM587009 and KM579597. The other sequences were deposited under the following accession numbers: KP172000–KP172035 for *recA* sequences, KP125322–KP125327 and KM579602 for *gyrB* sequences, and KP100166–KP100171 for ITS sequences. The accession numbers for other sequences used in this study which were obtained from GenBank are indicated in the phylogenetic tree diagrams (Figs. 1, 2, and 3).

Fig. 1 Phylogenetic relationships between the bacterial isolates obtained in this study based on the 16S rRNA gene sequences corresponding to the V3–V5 hypervariable regions. A few additional bacterial species were included for comparison, shown in bold. The GenBank accession numbers for the 16S rRNA gene sequences of the bacteria that were not isolated in this study are provided in parenthesis. Bootstrap values are shown for nodes with > 70% probability of 500 replicates. The scale bar indicates an estimated change of 1%

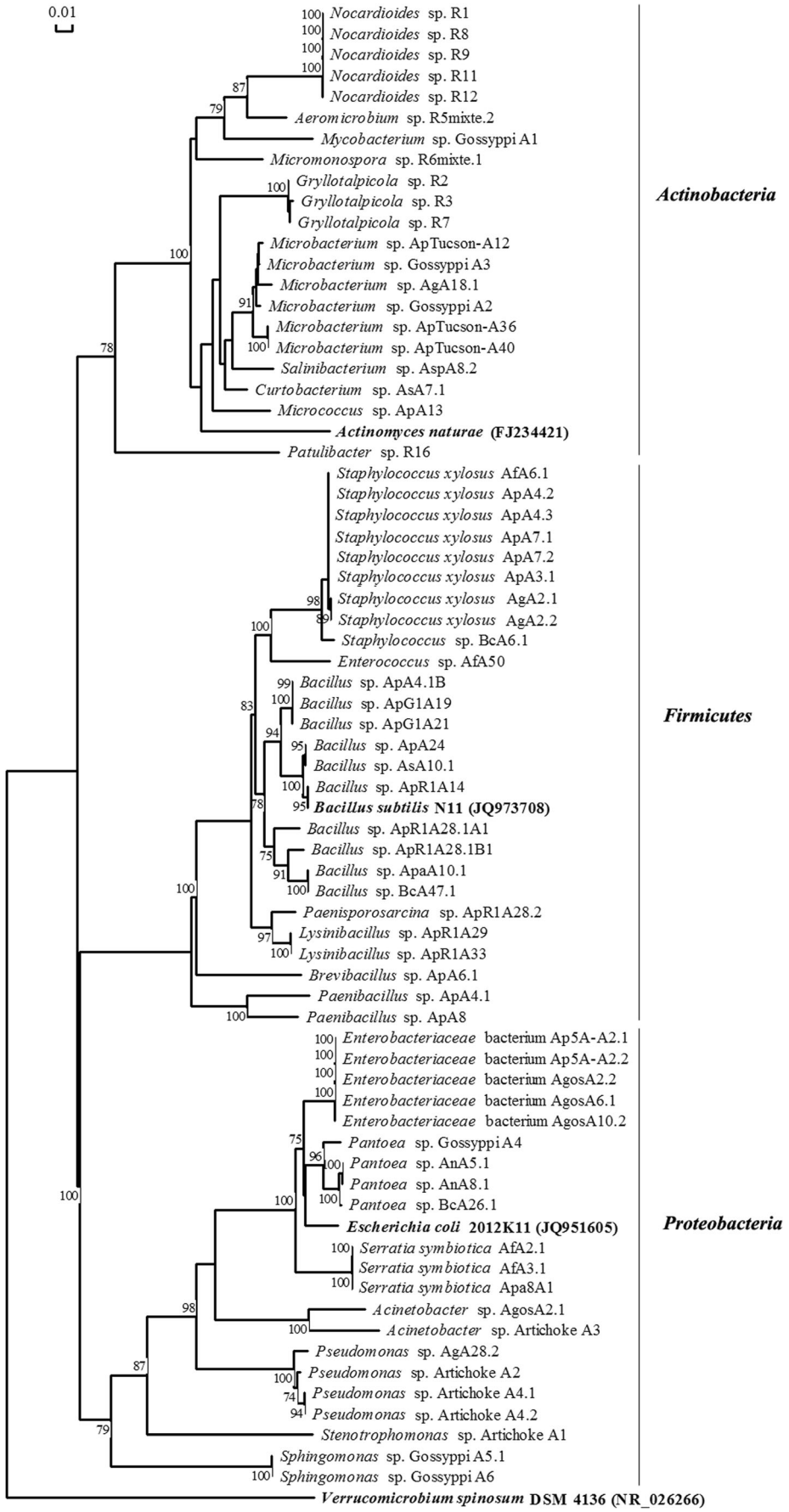
Results

Identification Strategy and Taxonomic Assignment

In total, 74 candidate colonies with different morphologies (e.g., color and shape) which were obtained from the dissection of a total of 474 aphids were selected for identification. The dissected aphids belonged to three laboratory-reared species and six natural species, originating from different regions of the world (i.e., USA, Tunisia, and Belgium) (Table S1). Based on initial microscopic observations, the candidate isolates included both bacterial (68 isolates) and fungal (six isolates).

All bacterial isolates were initially identified at the genus level by sequencing a 570–590-bp fragment of the 16S rRNA gene spanning the V3–V5 hypervariable regions (Table S2). But, in order to verify whether that fragment provided enough information for identification, the nearly complete 16S rRNA gene of 11 isolates (i.e., those for which a 16S rRNA gene sequence length higher than 1000 bp was reported) was also sequenced.

We obtained the same results with both fragment lengths, proving that the small 16S fragment was enough for a rapid identification at the genus level. Only two isolates could not be well identified at the genus level: isolate Gossypii A4 (*Pantoea*/*Kluyvera*/*Enterobacter*) and isolate AspA8.2 (*Salinibacterium*/*Galbitalea*/*Homoserinimonas*) (Table S3). Furthermore, a few isolates (Ap5A-A2.1, Ap5A-A2.2, AgosA2.2, AgosA6.1, and AgosA10.1), which could belong to the genus *Rosenbergiella*, were broadly identified as *Enterobacteriaceae* bacterium due to the high similarity with poorly identified microorganisms isolated from other insects and organisms (Table S3, Fig. 3). Furthermore, sequencing an approximately 950-bp fragment of the *gyrB* gene and a 212-bp fragment of the *recA* gene provided some additional sequence resolution. Thus, the isolate Gossypii A4 was assigned to the genus *Pantoea* when the highest basic local alignment search tool (BLAST) scores obtained with *gyrB* and *recA* nucleotide sequences provided respectively 94 and 92% sequence identity with *Pantoea rwandensis*. Moreover, the BLAST analysis performed with the inferred amino acid sequence of *gyrB* provided 98% identity with *Pantoea rodasii*. Furthermore, the discrimination between some isolates with identical 16S rRNA gene sequences was improved based on


0.01
Nocardioides sp. R1
Nocardioides sp. R8
Nocardioides sp. R9
Nocardioides sp. R11
Nocardioides sp. R12
Aeromicrobium sp. R5mixte.2
Mycobacterium sp. Gossyppi A1
Micromonospora sp. R6mixte.1
Gryllotalpicola sp. R2
Gryllotalpicola sp. R3
Gryllotalpicola sp. R7
Microbacterium sp. ApTucson-A12
Microbacterium sp. Gossyppi A3
Microbacterium sp. AgA18.1
Microbacterium sp. Gossyppi A2
Microbacterium sp. ApTucson-A36
Microbacterium sp. ApTucson-A40
Salinibacterium sp. AspA8.2
Curtobacterium sp. AsA7.1
Micrococcus sp. ApA13
Actinomyces naturae (FJ234421)
Patulibacter sp. R16
Actinobacteria
Staphylococcus xylosus AfA6.1
Staphylococcus xylosus ApA4.2
Staphylococcus xylosus ApA4.3
Staphylococcus xylosus ApA7.1
Staphylococcus xylosus ApA7.2
Staphylococcus xylosus ApA3.1
Staphylococcus xylosus AgA2.1
Staphylococcus xylosus AgA2.2
Staphylococcus sp. BcA6.1
Enterococcus sp. AfA50
Bacillus sp. ApA4.1B
Bacillus sp. ApG1A19
Bacillus sp. ApG1A21
Bacillus sp. ApA24
Bacillus sp. AsA10.1
Bacillus sp. ApR1A14
Bacillus subtilis N11 (JQ973708)
Bacillus sp. ApR1A28.1A1
Bacillus sp. ApR1A28.1B1
Bacillus sp. ApaA10.1
Bacillus sp. BcA47.1
Paenisporosarcina sp. ApR1A28.2
Lysinibacillus sp. ApR1A29
Lysinibacillus sp. ApR1A33
Brevibacillus sp. ApA6.1
Paenibacillus sp. ApA4.1
Paenibacillus sp. ApA8
Firmicutes
Enterobacteriaceae bacterium Ap5A-A2.1
Enterobacteriaceae bacterium Ap5A-A2.2
Enterobacteriaceae bacterium AgosA2.2
Enterobacteriaceae bacterium AgosA6.1
Enterobacteriaceae bacterium AgosA10.2
Pantoea sp. Gossyppi A4
Pantoea sp. AnA5.1
Pantoea sp. AnA8.1
Pantoea sp. BcA26.1
Escherichia coli 2012K11 (JQ951605)
Serratia symbiotica AfA2.1
Serratia symbiotica AfA3.1
Serratia symbiotica Apa8A1
Acinetobacter sp. AgosA2.1
Acinetobacter sp. Artichoke A3
Pseudomonas sp. AgA28.2
Pseudomonas sp. Artichoke A2
Pseudomonas sp. Artichoke A4.1
Pseudomonas sp. Artichoke A4.2
Stenotrophomonas sp. Artichoke A1
Sphingomonas sp. Gossyppi A5.1
Sphingomonas sp. Gossyppi A6
Proteobacteria
Verrucomicrobium spinosum DSM 4136 (NR_026266)

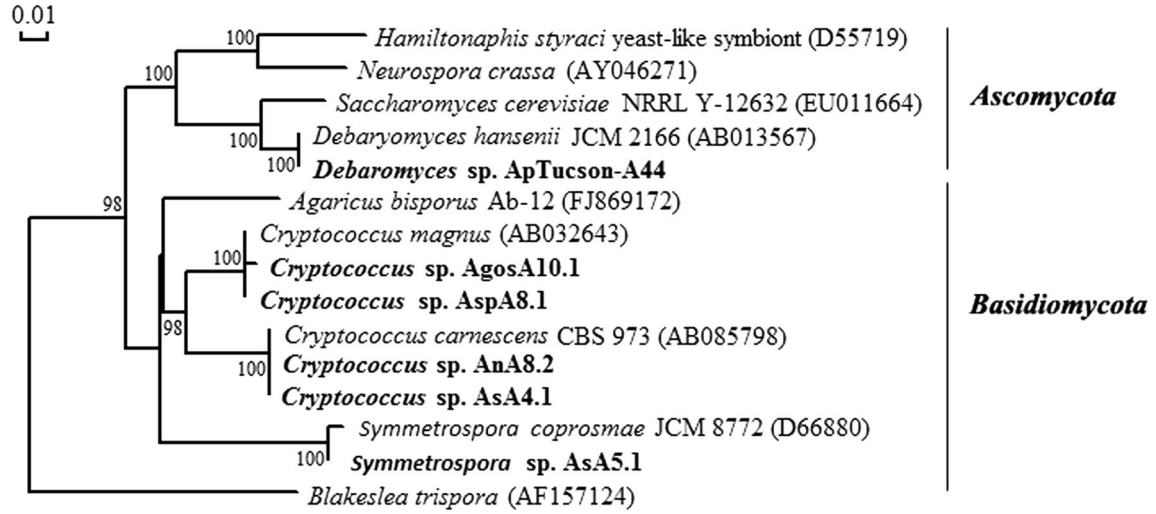


Fig. 2 Phylogenetic relationships between the fungal isolates obtained in this study, shown in bold, based on the partial 18S ribosomal RNA (rRNA) gene sequeences. Other ascomycete and basidiomycete species were included for comparison, as well as a yeast-like symbiont found in the aphid *Hamiltonaphis styraci*. The GenBank accession numbers for the 18S rRNA gene sequeences of the organisms that were not isolated in this study are provided in parenthesis. Bootstrap values are shown for nodes with > 80% probability of 500 replicates. The scale bar indicates an estimated change of 1%

the *recA* sequencing. Thus, the *Enterobacteriaceae* bacterium Ap5A-A2.1 is a different strain than the *Enterobacteriaceae* bacterium AgosA2.2 (86.32% *recA* sequence identity) and the *Enterobacteriaceae* bacterium AgosA6.1 (86.79% *recA* sequence identity), but the two last isolates could be closely related strains because they have identical sequences of 16S rRNA gene sequences and present 99.53% sequence identity for the *recA* gene fragments. Similarly, the isolates ApA4.1B, ApG1A19, and ApG1A21 could be identical strains of *Bacillus* sp. and the isolates ApTucson-A36 and ApTucson-A40 could be identical strains of *Microbacterium* sp. because they have identical sequences of 16S rRNA and *recA* gene fragments. The isolates AnA5.1 and AnA8.1 could be closely related strains of *Pantoea* sp., having identical sequences of 16S rRNA and 99.53% sequence identity for the *recA* gene fragments.

For a better discrimination between two potential *Staphylococcus* species (i.e., *S. saprophyticus* and *S. xylosus*), a diagnostic PCR was carried on, which consisted in amplifying DNA fragments with primers that were specific for *S. saprophyticus* and *S. xylosus*, respectively. All tested isolates (i.e., ApA3.1, ApA4.2, ApA4.3, ApA7.1, ApA7.2, AfA6.1 AgA2.1, and AgA2.2) gave positive amplification only with the primers that were specific for *Staphylococcus xylosus*.

The six fungal isolates were initially assigned to the phyla of Ascomycota or Basidiomycota based on sequencing the nearly complete 18S rRNA gene. The identification at the genus level was determined based on sequencing the ITS region using universal primers for Ascomycota isolates and basidiomycete-specific primers for the Basidiomycota isolates (Table S3).

After identification, the 68 bacterial isolates were assigned to 24 bacterial genera belonging to three different phyla (i.e., Actinobacteria, Firmicutes, and Proteobacteria), while the six fungal isolates were assigned to three fungal genera belonging to two different phyla (i.e., Ascomycota and Basidiomycota). The identification of each isolate based on the 16S (bacteria) and ITS (fungi) BLASTs can be seen in Table S3. Table 1 summarizes the microbial taxa that were identified in the different host species. The identified taxa were distributed across five different categories after an extensive review of the literature: the "aphid symbionts" category includes taxa described as mutualistic symbionts specifically found in aphids, the "aphid gut associates" category includes taxa that have been previously described in the aphid gut, the "insect gut associates" category brings together taxa that have been previously described in the digestive tract of insects other than aphids, the "plant associates" category includes taxa that have been found to be associated to plants, and the "environmental microorganisms" category includes all identified taxa whose effects on associated hosts are unclear or totally unknown.

Global Microbial Community Composition

The global microbial community associated to aphids and represented by 24 bacterial genera and three fungal genera reveals an unexpectedly high diversity obtained with our culture-dependent approach. Bacteria represent the overwhelming majority of our isolates. Firmicutes, Actinobacteria, and Proteobacteria represent respectively 38, 31, and 31% of the bacterial isolates. Sixty-two percent of the Firmicutes are cocci, and 38% are bacilli. With 90%, the Gammaproteobacteria represent the most common taxa within

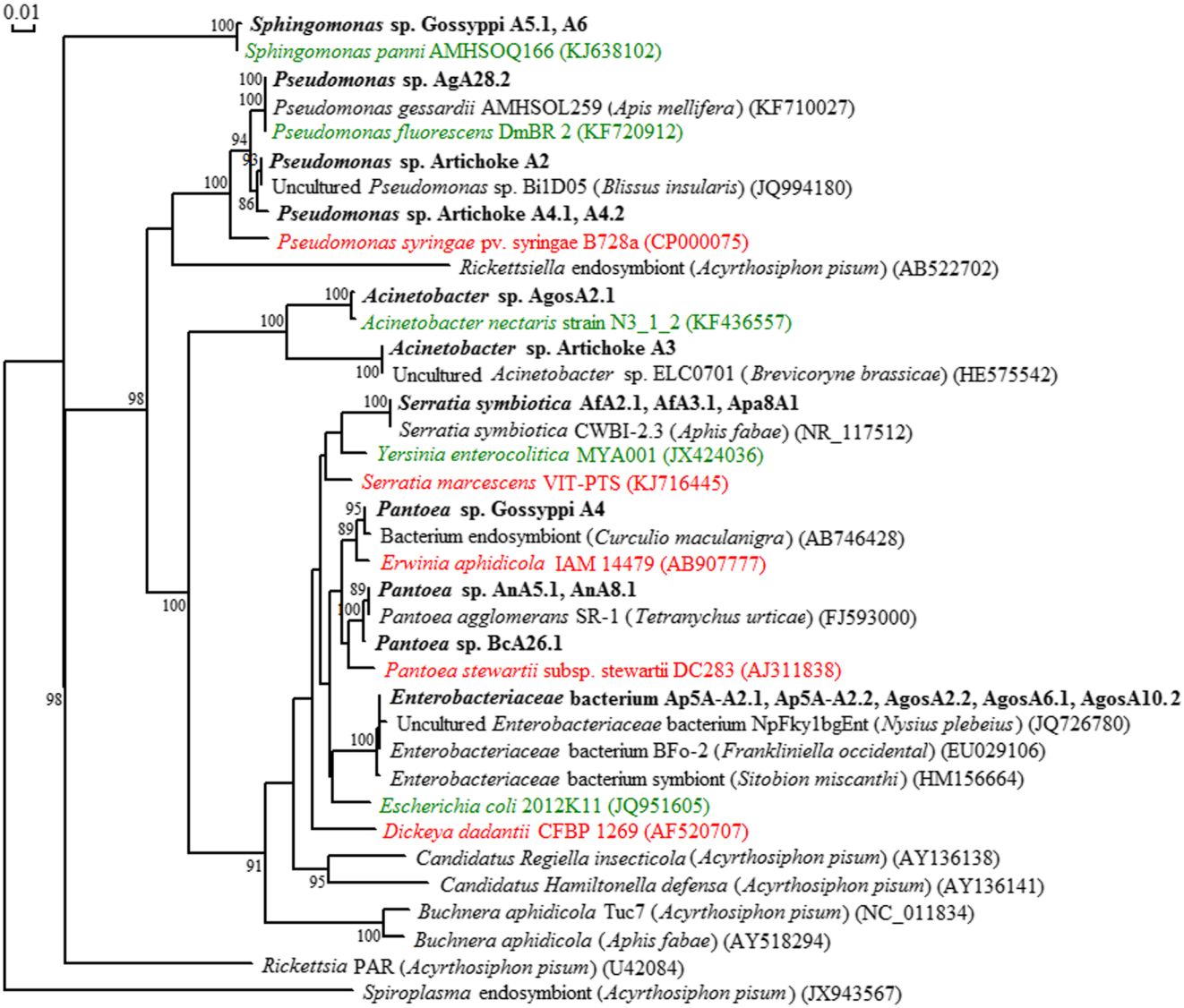


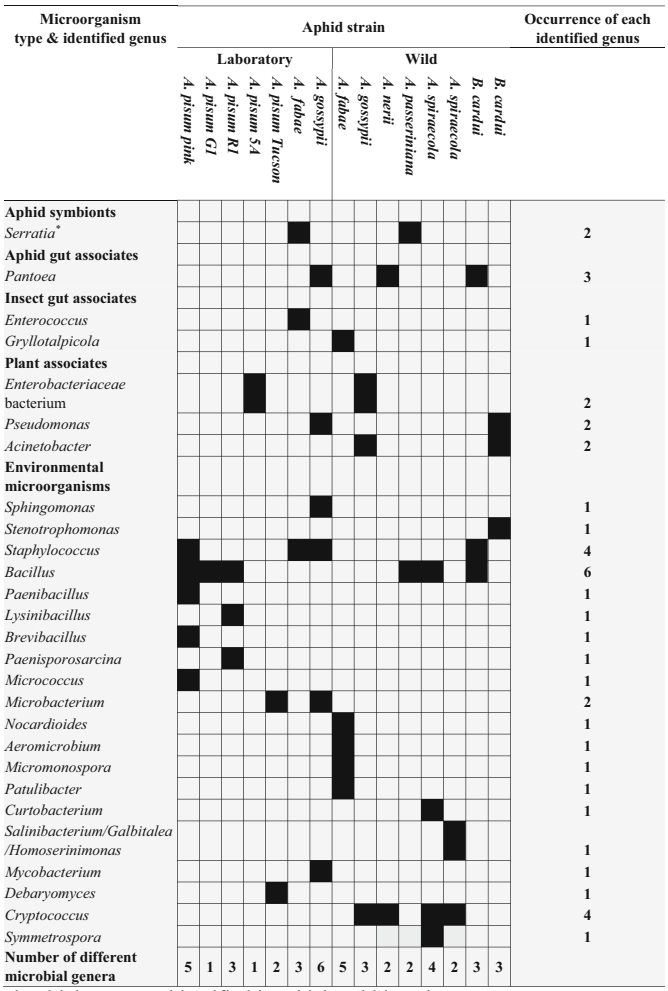
Fig. 3 Phylogenetic relationships between the proteobacterial isolates obtained in this study (bold) and different symbionts from aphids and other insects (regular) as well as free-living relatives (green) and known phytopathogens and entomopathogens that can use aphids as hosts (red). The phylogenetic tree was constructed based on the 16S rRNA gene sequences corresponding to the V3–V5 hypervariable regions. The GenBank accession numbers for the 16S rRNA gene sequences of the bacteria that were not isolated in this study are provided in parenthesis. Bootstrap values are shown for nodes with > 80% probability of 500 replicates. The scale bar indicates an estimated change of 1%

the Proteobacteria. The microbial diversity per aphid strain is, however, relatively low since one to six different microbial genera were detected in each aphid strain and each of these microbial genera was detected in up to six different aphid strains (Table 1). On average, three different microbial genera were detected per aphid strain, but many of these microbial genera were generally detected in only one aphid strain.

The majority of the identified genera were assigned to environmental microorganisms (Table 1). The most prevalent microbial taxa were *Bacillus* and *Staphylococcus*. *Bacillus* was isolated from six aphid strains belonging to the species *A. pisum*, *Aphis passeriniana*, *Aphis spiraecola*, and *B. cardui*. *Staphylococcus* was also found in four species: *A. pisum*, *A. fabae*, *A. gossypii*, and *B. cardui*. But, the majority of environmental microorganisms correspond to microbial genera that were generally detected in only one aphid strain. Several plant associates were also detected like *Enterobacteriaceae* bacterium, *Pseudomonas*, and *Acinetobacter*. We also isolated three putative insect gut associates: *Enterococcus* and *Gryllotalpicola* that were only found in *A. fabae*, and *Pantoea* that was isolated from *A. gossypii*, *Aphis nerii*, and *B. cardui*. Finally, we also succeeded to isolate the facultative symbiont *S. symbiotica* from *A. fabae* and *A. passeriniana*.

The comparison of isolated microorganisms according to aphid lifestyle (from wild populations versus laboratory

Table 1 Distribution of the 24 bacterial and three fungal genera across the different wild and laboratory-reared aphid strains considered in this study. Features of aphid strains are detailed in Table S1



The black highlights indicate which microbial taxa was identified in which aphid strain

*This genus was included in the "aphid symbionts" category because all the *Serratia* isolates obtained here were clearly identified as *S. symbiotica*

reared) tends to reveal some similarities and differences. Similarities consist in the fact that both the wild and the laboratory-reared aphids had an average of about three microbial genera per aphid strain and that we detected members of Firmicutes, Actinobacteria, Proteobacteria, and fungi in both types of aphids. Moreover, the proteobacterial isolates were found in a relatively similar proportion (31% in the laboratory-reared aphids and 35% in the natural aphids) and included four-core genera that were isolated from both wild and laboratory-reared aphids (i.e., *Serratia*, represented by the facultative symbiont *S. symbiotica*, a new genus represented by an *Enterobacteriaceae* bacterium, *Pantoea*, and *Pseudomonas*). The Firmicutes isolates also included two-core genera that were isolated from both wild and laboratory-reared aphids (i.e., *Bacillus* and *Staphylococcus*). The differences related to the aphid lifestyle are mainly represented by the fact that the Actinobacteria and the fungi isolated from the wild aphids belong to different genera than those isolated from the laboratory-reared aphids (Table S3, Table 1). In addition, the proportions of Actinobacteria versus Firmicutes are substantially different in the wild and the laboratory-reared aphids (Fisher's exact test, $P = 0.002$). There are 3.4 times more actinobacterial than Firmicutes genera in the natural aphids, whereas there are 2.3 times more Firmicutes than actinobacterial genera in the laboratory-reared aphids. This pattern is conserved even if we restrict the analysis to *A. gossypii* and *A. fabae* for which we have samples reared under both natural and laboratory conditions (Fisher's exact test, $P = 0.018$).

Diversity Among Isolates

Among our bacterial isolates, the highest phylogenetic diversity was observed for the Actinobacteria, with ten genera detected in the laboratory-reared and the wild aphids together, followed by Firmicutes and

Proteobacteria, each with seven genera (Fig. 1), and fungi, with only three genera (Fig. 2). Most of the actinobacterial genera isolated here have not been previously reported in aphids, except for *Microbacterium*, a genus that was detected by Gauthier et al. [27] in the pea aphid. Here, it was isolated from two laboratory-reared aphid strains (i.e., *A. pisum* Tucson and *A. gossypii*). Some of our actinobacterial isolates, however, are closely related to species found in other insects. Thus, *Microbacterium* sp. Gossypii A2 presents 100% sequence identity with a strain of *Microbacterium testaceum* that was found in cattle ticks (Table S3) [40]. Two other actinobacterial isolates, *Gryllotalpicola* sp. R2 and R3, present 99% sequence identity with *Gryllotalpicola kribbensis* strain PU-02 that was found in the guts of the African mole cricket *Gryllotalpa africana* (Table S3) [41]. As evidenced in Fig. 3, many of our proteobacterial isolates present over 99% sequence identity with Proteobacteria isolated or detected in other aphid strains and insects. Thus, the isolate *Acinetobacter* sp. Artichoke A3 is closely related to the uncultured *Acinetobacter* sp. isolate ELC0701 detected in the cabbage aphid *Brevicoryne brassicae* [42]. The isolate *Pseudomonas* sp. AgA28.2 is closely related to *Pseudomonas gessardii* strain AMHSOL259 isolated from the honey bee *Apis mellifera* (GenBank: KF710027, unpublished). Furthermore, the isolate *Pseudomonas* sp. Artichoke A2 is closely related to the uncultured *Pseudomonas* sp. clone Bi1D05 detected in the southern chinch bug *Blissus insularis* [43], the isolates *Pantoea* sp. AnA5.1 and AnA8.1 are closely related to *Pantoea agglomerans* strain SR-1 found in the two-spotted spider mites *Tetranychus urticae* (GenBank: FJ593000, unpublished), and the isolates *Enterobacteriaceae* bacterium Ap5A-A2.1, Ap5A-A2.2, AgosA2.2, AgosA6.1, and AgosA10.2 are closely related to the uncultured *Enterobacteriaceae* bacterium clone NpFky1bgEnt found in the stinkbug *Nysius plebeius* [44] as well as the *Enterobacteriaceae* bacterium BFo-2 found in the western flower thrip *Frankliniella occidentalis* [45] and the *Enterobacteriaceae* bacterium symbiont detected in the aphid *Sitobion miscanthi* [46]. Nonetheless, except for three *S. symbiotica* strains isolated here (i.e., strains AfA2.1, AfA3.1, and Apa8A1), none of the other proteobacterial isolates are phylogenetically related to any known aphid endosymbiont (Fig. 3). All Firmicutes isolates from this study, except for *Enterococcus* sp. AfA50, are environmental bacteria, among which, the genera *Bacillus*, *Staphylococcus*, and *Paenibacillus* have already been reported in aphids (Table 1) [19, 20, 26, 27]. The fungal isolates obtained in this study have not been previously reported in aphids and are not phylogenetically related to the yeast-like symbiont described for the aphid *Hamiltonaphis styraci* (Fig. 2) [47].

Discussion

It is now well established that insects may be engaged in a wide range of relationships with microorganisms [48–50]. Because they harbor various symbiotic bacteria in their tissues, aphids represent a valuable model to study the functional diversity of microorganisms associated with insects as well as the evolution of such partnerships [48]. Nevertheless, the global microbial diversity that is harbored by these sap-feeding insects remains fairly unappreciated. Indeed, major research efforts have been paid on their obligate and facultative symbionts, eluding the diversity of gut associates and environmental microorganisms that these insects may gain from their environment. Yet, gut associates and acquired environmental bacteria are now regarded with an increasing attention in insects and other animals since these microbial communities can deliver various beneficial services to their host, including host nutrition, resistance against parasite and pathogens, and resistance against insecticides [49, 51–53]. In addition to these beneficial effects, some microbes can also have deleterious effects for their host, influence their vectorial capabilities in the transmission of pathogens [54–56], or mediate intraspecific and interspecific communication [30, 31, 57].

Here, we used a culture-dependent approach in order to assess the diversity of microorganisms that can be found associated with different aphid species. Our results are in agreement with recent molecular studies on aphid microbiota that were able to detect other microbial species in addition to the known endosymbionts [26–28]. With the identification of 24 bacterial genera and three fungal genera, our findings reveal that the global microbial community associated to aphids is more diverse than previously thought. By considering several wild and laboratory-reared aphid species, we successfully isolated microorganisms that include both members that have been previously reported in aphids as well as newly reported microorganisms found in these insects. While microbial genera such as *Bacillus*, *Staphylococcus*, *Micrococcus*, *Pantoea*, *Pseudomonas*, and *Acinetobacter* are systematically being isolated or detected in many insects, including aphids [19–21, 26–28], the fungal genera isolated by our approach (i.e., *Debaryomyces*, *Cryptococcus*, and *Symmetrospora*) as well as some of the actinobacterial genera detected (e.g., *Nocardioides*, *Patulibacter*, *Gryllotalpicola*, and *Salinibacterium*/*Galbitalea*/*Homoserinimonas*) are reported here for the first time in association with aphids, to our knowledge. Despite this obvious microbial diversity, both the wild and the laboratory-reared aphids exhibit a quite low diversity in comparison to other insect groups [26]. Nevertheless, this relatively low microbial diversity may also reflect the limitations of our approach since we only used one microbial cultivation condition and not all the colonies developed on the plates were analyzed. For these reasons, the obtained results

most probably underestimate the microbial diversity associated with the analyzed aphids. In addition, given the limitations of molecular techniques, the microbial diversity could not be well evaluated to the species level. This is because the identification based on the 16S rRNA and ITS sequencing is suitable only up to the genus level. We tried to improve the initial results by sequencing the nearly complete *gyrB* gene from a few gammaproteobacterial isolates and a 212-bp fragment of the *recA* gene from 36 isolates, but no additional resolution was obtained at the species level. Given that the *recA* gene does not have hypervariable regions like the 16S rRNA gene and that sequence variability rather occurs throughout the entire gene [58], it remains a big challenge to find universal primers that could amplify the entire *recA* gene from all isolates.

Regarding the core bacterial community found in wild as well as laboratory-reared aphids, we did not observe strong differences in terms of microbial species diversity, and we show that the proteobacterial and the Firmicutes isolates included respectively four-core genera (i.e., *Serratia*, represented by the facultative symbiont *S. symbiotica*, a new genus represented by an *Enterobacteriaceae* bacterium, *Pantoea*, and *Pseudomonas*) and two-core genera (i.e., *Bacillus* and *Staphylococcus*). The microorganisms isolated here can be classified as symbionts, gut associates, plant associates, and environmental microorganisms (Table 1).

The microbial communities present in the gut of aphids have received limited consideration. Yet, scattered observations suggest that their digestive tract may constitute a suitable environment for bacterial associates which either cause the death of the infected individuals or have no deleterious effects on the aphid host [59–63]. Although our isolation approach focuses on entire hosts, the majority of the isolates we found are expected to be part of the aphid gut microbiota since the oral route is one of the most widespread entries of microorganisms in animals [64] and many of the taxa identified here have already been found in the digestive tract of other groups of insects. Several of our isolates are putative phytopathogens that may also exhibit entomopathogenic effects such as *Erwinia aphidicola*, *Dickeya dadantii*, *Pseudomonas syringae*, and *Pantoea stewartii* [19, 59–63]. The genus *Pantoea*, which has been isolated from several aphid species, may include *P. stewartii*, which has been depicted as an aphid pathogen [61], and *P. agglomerans*, a plant pathogen which has been identified with a high prevalence in phylloxera-inducing species [65, 66] and in locust where the bacterium produces components of an aggregation pheromone [57]. We also identified several putative plant associates from the genus *Pseudomonas* that comprise plant growth-promoting species and phytopathogens [67], and *Acinetobacter* isolates that are known to include growth-promoting bacteria [68]. We also isolated for the first time from aphids two bacterial gut associates belonging to the genera that can be found in other insect species: *Enterococcus* which is frequently found in the digestive tract of many animal species [69] and *Gryllotalpicola* which has been identified as gut resident in crickets [41] and termites [70]. If *enterococci* are considered as commensal and putative pathogens in insects [71], the effects associated with *Gryllotalpicola* remain totally unknown.

While previous metagenomic studies detected known aphid endosymbionts and only a few additional microorganisms [27], our study detected various microorganisms, including gut associates and environmental microorganisms. The apparent low bacterial diversity found in the aphid gut is generally attributed to the quasi absence of microorganisms in the plant phloem on which these insects feed, therefore limiting the acquisition of environmental bacteria from food resources [26]. However, a wide bacterial diversity has been detected in a pear phloem sample (Fakhour et al., unpublished data), and several studies indicate that bacteria can circulate through sap, including bacterial symbionts [60, 72, 73]. It is likely that while feeding, aphids come into contact with microorganisms present in the phloem and epiphytic microbes, which are ingested and then transit through the insect digestive tract and potentially invade other tissues as well. The phloem may therefore constitute a reservoir of microorganisms that are picked up by sap-feeding insects. But, without a precise identification at the species level, it is difficult to define the exact influence that may have each isolated microorganism obtained here on their aphid host. It is also important to bear in mind that some microorganisms found in insects might be commensals or transient partners that do not exhibit any kind of beneficial or antagonistic effects [49]. In addition, aphids exhibit a reduced immune system in comparison to other insects [63, 74], a characteristic expected to confer them a greater tolerance toward the commensal microbiota. Future laboratory experiments, as well as the genome sequencing of certain strains, should clarify the tissue tropism of these microorganisms as well as their potential role in aphids.

Regarding putative beneficial effects, some of these isolates could be important to aphids by providing them with some advantages such as antimicrobial protection or helping them to interact with the host plants and other insects. For example, Actinobacteria are known to provide antimicrobial benefits [75] and non-pathogenic rhizobacteria such as *Pseudomonas fluorescens* make aphids less attractive to parasitoids [76]. With many Actinobacteria isolated here, among which one isolate, ApA13, potentially being *Micrococcus luteus*, which is known for strong inhibitory activity against antagonistic fungi [75], and other isolates, AgA28.2, Artichoke A4.1, and Artichoke A4.2, potentially being strains of *P. fluorescens* (Table S3), there is a strong indication that aphids often benefit from the interaction with associated microorganisms. Another bacterium, *Staphylococcus xylosus*, which, in this study, was isolated from different laboratory-reared aphid strains (Table S3), was shown to contribute to the

emission of volatile organic compounds that attract beneficial insects for aphids such as tending ants [77]. An unprecedented discovery made by Sabri et al. [35] showed that the aphid honeydew constitutes a reservoir of bacterial proteins with potential nutritional role, most probably helping aphids interact with their host plants. A fraction of these proteins were attributed to the known obligate and facultative symbionts (i.e., *Buchnera aphidicola* and *S. symbiotica*), and the rest of these proteins were associated with free-living bacterial species like *Staphylococcus sciuri* and *S. saprophyticus*, *Acinetobacter calcoaceticus*, *Escherichia coli*, and *Serratia marcescens*, which most probably colonize the digestive tract of aphids. *S. symbiotica* as well as *Staphylococcus* and *Acinetobacter* were isolated in this study, reinforcing the supposition that some of our isolates could have beneficial roles for aphids. It is also possible that some of the microorganisms isolated here may have negative effects on aphids such as providing septicemia and death or attracting natural enemies like the *Staphylococcus sciuri* isolate described by Leroy et al. [30]. With most of our isolates being non-pathogenic environmental and plant-associated bacteria (Table 1), among which some are close to bacteria recently isolated from floral nectar such as *Rosenbergiella* sp. (isolates Ap5A-A2.1, Ap5A-A2.2, AgosA2.2, AgosA6.1, and AgosA10.2 in Table S3) [78, 79] and *Acinetobacter nectaris* (isolate AGosA2.1 in Table S3) [80], our results suggest that aphids can ingest different microorganisms from their environment, which probably either have a neutral effect on aphids or might become involved in mutualistic relationships with the aphids. However, it is important to keep in mind here that bacteria are labile organisms which can rapidly evolve and whose associated effects (neutral versus beneficial versus antagonistic) can be defined only for a specific environment and under particular conditions, which are under continuous transformation [81]. There are plenty examples of reversions and transitions regarding the symbiotic lifestyle of bacteria [82–84]. Nevertheless, an interesting question that rises from this study is whether aphids could be vectors for disseminating microorganisms with plant growth-promoting potential rather than just phytopathogens and plant viruses as previously demonstrated [60]. Some of our isolates (e.g., AgA28.2, Artichoke A4.1, Artichoke A4.2, ApA4.1B, ApG1A19, ApG1A21, ApA24, AsA10.1, and ApR1A14) have high 16S rRNA sequence similarity with strains of *P. fluorescens*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, and *Bacillus subtilis*, which are known plant growth-promoting rhizobacteria (Table S3) [85]. The great advantage of this study is that all strains isolated here are culturable and have been preserved for future investigations on their precise influence on aphids as well as in more complete systems such as plant-aphid or plant-aphid and other insects (i.e., predators or mutualists).

Interestingly, our approach succeeded in isolating novel strains of *S. symbiotica*. The case of this symbiont is particularly interesting because it illustrates how strains belonging to the same bacterial species can exhibit very different beneficial effects. Indeed, while some strains have shown their aptitude to bring resistance to parasitoids [11], others exhibit an ability to protect the host from the harmful effects of extremely high temperatures [10]. In addition, other strains appear to have a nutritive function [86]. Moreover, it is known that the degree of dependency of symbiotic species to their host can greatly differ according to the strains: if certain strains appear uncultivable because of the long co-evolutionary history with their host, others, on the contrary, are regarded as nascent symbionts which have retained free-living capacities [63, 87]. Culture-dependent approaches are therefore relevant to assess not only the functional diversity of the microbial communities present in insects but also the evolutionary origin of symbiotic systems in these animals.

Conclusion

Microbial communities are recognized as playing an essential role in various aspects of insect biology such as reproduction, nutrition, host development, or defense against natural enemies. Microbial cultures have long been the first method for studying the microbiota of many organisms. But, this method has been considered outdated by many researchers since metagenomic approaches have emerged to depict the wide diversity of microorganisms which can be associated with insects and other eukaryotes. Yet, their cultivation in laboratory is required to better appreciate their physiology and the role they can play in insects through genome sequencing and infection experiments. Our results clearly underline the potential of culture-dependent methods for describing minority bacterial populations which cannot be detected by metagenomic approaches. Our study also represents an attempt to isolate a maximum of different microorganisms from aphids by utilizing a sugar-rich growth medium chosen to reflect the high sugar content in aphids' food [88]. In the view of the results obtained here as well as previous results [30, 31, 34], the medium that we used represents a remarkable tool to isolate microorganisms associated to insects. This investigation could be considered as the incipient step of further research that should investigate more thoroughly the potentiality of a persistent aphid microflora that is not limited to known endosymbionts. To conclude, we believe that this type of approach should be applied to other invertebrates considered as large reservoirs of symbiotic and pathogenic bacteria.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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