Acetic Acid Bacteria, Newly Emerging Symbionts of Insects

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Recent research in microbe-insect symbiosis has shown that acetic acid bacteria (AAB) establish symbiotic relationships with several insects of the orders Diptera, Hymenoptera, Homoptera, and Hemiptera, all relying on sugar-based diets, such as nectars, fruit sugars, or phloem sap. To date, the fruit flies Drosophila melanogaster and Bactrocera oleae, mosquitoes of the genera Anopheles and Aedes, the honey bee Apis mellifera, the leafhopper Scaphoideus titanus, and the mealybug Saccharicoccus sacchari have been found to be associated with the bacterial genera Acetobacter, Gluconacetobacter, Gluconobacter, Asaia, and Saccharibacter and the novel genus Commensalibacter. AAB establish symbiotic associations with the insect midgut, a niche characterized by the availability of diet-derived carbohydrates and oxygen and by an acidic pH, selective factors that support AAB growth. AAB have been shown to actively colonize different insect tissues and organs, such as the epithelia of male and female reproductive organs, the Malpighian tubules, and the salivary glands. This complex topology of the symbiosis indicates that AAB possess the keys for passing through body barriers, allowing them to migrate to different organs of the host. Recently, AAB involvement in the regulation of innate immune system homeostasis of Drosophila has been shown, indicating a functional role in host survival. All of these lines of evidence indicate that AAB can play different roles in insect biology, not being restricted to the feeding habit of the host. The close association of AAB and their insect hosts has been confirmed by the demonstration of multiple modes of transmission between individuals and to their progeny that include vertical and horizontal transmission routes, comprising a venereal one. Taken together, the data indicate that AAB represent novel secondary symbionts of insects.

Acetic acid bacteria (AAB), especially members of the genera Acetobacter and Gluconacetobacter, have a significant historical role in human activities, having been used over millennia for the production of vinegar for consumption and for medicinal purposes. AAB of the family Acetobacteraceae are ubiquitous and are known to be adapted to various sugar- and ethanol-rich environments. AAB are obligate aerobes, and most of them are unable to oxidize ethanol, sugars, and polyalcohols completely, accumulating large amounts of the corresponding oxidation products in their culture medium. Their oxidative capacity is commercially exploited not only for vinegar production but also in the manufacture of foods and chemical compounds, i.e., kombucha tea, cocoa, sorbose, gluconic acid, etc. (40).

AAB can be isolated from a variety of substrates and natural environments like plants, flowers, herbs, fruits, and fermented foods and beverages. Great attention has recently been directed toward symbiotic associations between AAB and insect hosts, which are emerging as a novel niche for AAB isolation. Interestingly, these prokaryotes have been reported to be associated with insects that rely on sugar-based diets, in particular those belonging to the orders Diptera, Hymenoptera, and Hemiptera (13).

Symbiotic associations can profoundly affect the evolutionary history, lifestyle, and physiology of organisms. Consequently, in recent years, the interactions between insects and microorganisms have received considerable attention (53). The remarkable adaptability of insects to very diverse terrestrial habitats, comprising those that are nutritionally limited or unbalanced, has contributed to their evolutionary success in different ecological environments. In many cases, microbial partners allow their insect hosts to specialize in nutrient-deficient food resources, giving them competitive advantages (24). For example, insects that feed exclusively on nutritionally poor...
diets such as plant sap, vertebrate blood, or woody material possess microbial endosymbionts. Arthropod-associated microorganisms play numerous different roles besides the nutritional aspects, influencing development, reproduction and speciation, defense against natural enemies, and immunity (14). For example, the aphid facultative endosymbiont Hamiltonella defensa increases the protection of its host from parasitoid wasps (56).

In this minireview, a description of the currently available information about AAB recovered from insects is presented. The main insect sources so far for AAB are bees, mosquitoes, fruit flies, and sugarcane mealybugs; it is, however, likely that the number of insect species found to harbor AAB will significantly increase in the near future. Since the beginning of the 20th century, it was well known that gluconobacters, environmental sugar-loving microbes, represent a significant component of the honeybee microflora (65), while only more recently AAB were found to also inhabit mosquitoes, fruit flies, and leafhoppers.

The family Acetobacteraceae comprises a large variety of species. Since the description of Acetobacter Beijerinck (7) and Gluconobacter Asai (1), the taxonomy of AAB has undergone many changes, especially in the last 30 years. These changes are due to the new species ascribed to the family, the reclassification of strains (67), and the development and introduction of novel technologies for microbial taxonomy. Early classification systems were mainly based on the analyses of morphological and biochemical characteristics. Nowadays, taxonomic classification is based on what is called “polyphasic taxonomy,” in which independent approaches such as phenotypic, chemotaxonomic, and genotyping analyses are combined (10). According to this classification approach, the AAB of the family Acetobacteraceae are classified into 13 genera: Acetobacter, Gluconobacter, Gluconacetobacter, Acidomonas, Asaia, Koza-kia (40), Swaminathania (45), Saccharibacter (37), Neosasaia (69), Granulibacter (33, 34), Commensalibacter (60), Tanticharoenia (68), and Ameyamaea (70).

WHICH INSECT HOSTS DO AAB COLONIZE?

Bees were the first insects from which AAB were recovered (65). Bees and the products of beekeeping have been extensively studied over the years, taking into account the microbiological perspective (31). Among the great variety of microorganisms that were identified and isolated, Gluconobacter, Gluconacetobacter, and Acetobacter are some of the predominant bacterial groups of the bee microbiota (4, 36, 50, 51). Another hymenopteran, the endoparasitoid wasp Ameyamaea (An.) stephensi, An. maculipennis, An. gambiae, and Aedes aegypti (13, 22). Asaia sp. was found as a dominant bacterium within the insect microbiota community by the use of several techniques. Asaia sp. was also identified in the bacterial community of the leafhopper Scaphoideus titanus, the vector of flavescence dorée in grapes (13, 46). Assessed by quantitative PCR, 16S rRNA genes of Asaia sp. constituted 4.9% of the total bacterial 16S rRNA gene copies per leafhopper. Ashbolt and Inkerman (2) detected the presence of AAB in another leafhopper, Perkinsiella saccharicida, for which an AAB community of $5 \times 10^{3}$ cells per individual was estimated.

Other insects in which Asaia sp. symbionts were detected by cultivation-independent techniques are the hymenopteran Marietta leopoldina, a hyperparasitoid (47), and the lepidopteran Pieris rapae, the cabbage white butterfly (59).

The pink sugarcane mealybug Saccharicoccus sacchari is a common insect found in sugarcane-growing countries which is able to host several members of the family Acetobacteraceae (2). At least $10^{6}$ AAB are typically carried by an adult female mealybug actively feeding on aerial storage, whereas fewer than $10^{5}$ bacteria per insect are maintained in less active adults and individuals from underground mealybug populations. AAB isolates from S. sacchari were identified as A. aceti, Glu- conacetobacter (Ga.) diazotrophicus, Ga. liquefaciens, and Ga. sacchari by Franke et al. (26). Further experiments, though, led the authors to the conclusion that AAB are only a relatively small proportion of the microbial community in S. sacchari (25, 27, 28). The presence of AAB in other mealybugs like Plano- coccus sp. and Dysmicoccus brevis was also demonstrated (2). A list of AAB that colonize insect hosts is given in Table 1. It should be emphasized that all of these insects rely on sugar-based diets such as nectar, fruit sugars, or phloem sap.

WHICH INSECT ORGANS DO AAB INHABIT?

A major habitat of insect-associated microorganisms is the digestive system, due to the availability of nutrients that are degraded by both host enzymes and microbial activity (17). The microbiota in an insect gut is influenced by structural and physiological factors, as well as by the quality of the ingested food. However, AAB symbionts have been found associated not only with the insect interior but also with the insect surface, as in the case of D. melanogaster (58), underlining their ability to survive in unfavorable environments. Growing on the surface of Drosophila, Acetobacter sp. can have a smaller-than-normal size, as shown by scanning electron microscopy analysis (58). AAB are able to survive other harsh conditions represented, for instance, by starvation or an adverse environment, entering into a viable but not culturable (VBNC) state and reducing the size of the microbial...
Asaia Gluconacetobacter sp.
ability of partially digested food coming from the midgut, as well

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AAB and insect host(s) | Reference(s)
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**Acetobacter sp.**
*D. melanogaster* (Diptera: Drosophilidae) | 11, 12, 58, 61
*A. mellifera* (Hymenoptera: Apidae) | 4, 50
*B. oleae* (Diptera: Tephritidae) | 42
*A. tabida* (Hymenoptera: Braconidae) | 71
*S. sacchari* (Hymenoptera: Pseudococcidae) | 2

C. intestini
*D. melanogaster* (Diptera: Drosophilidae) | 60, 61

Gluconobacter sp.
*A. mellifera* (Hymenoptera: Apidae) | 4, 51
*D. melanogaster* (Diptera: Drosophilidae) | 11, 12, 58, 61, 61
*S. sacchari* (Hymenoptera: Pseudococcidae) | 2

Gluconacetobacter sp.
*D. melanogaster* (Diptera: Drosophilidae) | 11, 12, 61
*A. mellifera* (Hymenoptera: Apidae) | 4, 36, 50
*S. sacchari* (Hymenoptera: Pseudococcidae) | 2

Asaia sp.
*Anopheles* sp. (Diptera: Culicidae) | 13, 15, 22
*A. aegypti* (Diptera: Culicidae) | 13
*S. titanus* (Hemiptera: Cicadellidae) | 13, 46
*M. leopardana* (Hymenoptera: Aphelinidae) | 47
*P. rapae* (Lepidoptera: Pieridae) | 59

Saccharibacter floricola
*A. mellifera* (Hymenoptera: Apidae) | 51

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cells (49). Consequently, AAB populations from various substrates can be underestimated when the evaluation is performed only by a culture-based approach. The application of culture-independent techniques such as epifluorescence staining and real-time PCR overcomes this problem (6).

Simple or complex microbial consortia can inhabit the insect gut. For instance, in *D. melanogaster*, 2S phylogenotypes with just a few dominant bacterial species, among which are AAB, are associated with the insect gut (12). The anterior hindgut region is the most densely inhabited part of the digestive system, due to the availability of partially digested food coming from the midgut, as well as the products excreted by the Malpighian tubules. It has been estimated that 10^8 to 10^9 bacterial cells per g of gut content are present in the honeybee gut (39, 57), while >10^8 bacteria are typically recovered from an entire old *Drosophila* fly (58).

The insect gastrointestinal tract (GIT), more structured in the adult stage than in the larval one, is organized similarly in bees, mosquitoes, and fruit flies. The mouth is followed by the esophagus, the ventriculus (stomach), the rectum, and the anus. *Drosophila* flies also possess an acidic crop, together with an alkaline ventriculus and a slightly acidic hindgut, while mosquitoes have three diverticula that arise near the terminal end of the esophagus. The ventral one, also called a crop, is acidic and able to enlarge into the abdomen. A sugar meal, such as floral nectar, is stored in the diverticula and passes slowly to the midgut for the digestion step (63). Bees possess a honey stomach, an enlargement of the esophagus in which nectar is stored during flight and which ends with the proventriculus. This structure avoids contamination of the nectar with the contents of the stomach, which follows the proventriculus (66). The adult bee gut is normally acidic in comparison to the larval one. The pH is reported to vary from 4.8 to 7.0, depending on the acidity of the ingested pollen. Mohr and Tebbe (50) reported pH values of approximately 4.0 for larvae fed with worker jelly, as well as with nectar and honey.

In the insect digestive system, AAB find a suitable environment in which they flourish and reproduce, mainly due to the aerobic environment, the acidic pH, and the presence of diet-derived sugars. Moreover, AAB establish a tight association with insect epithelial cells. AAB are known to produce polysaccharide matrices that are involved in microbial interactions. One of the polysaccharides produced by bacteria is cellulose, which, in animal pathogens, is implicated in biofilm formation, in multicellular behavior, in adhesion to animal cells, or in stress tolerance (5). Several members of the family *Acetobacteraceae* possess the ability to produce cellulose. *Ga. xylinus* is the best-known cellulose producer, but other examples can be found, such as *Ga. kombuchae*, *Ga. swingsii*, *Ga. rhaeticus*, *Ga. nataicola*, and some strains of *Ga. hansenii*, *Ga. europeus*, and *Ga. oboedens* (21, 44). The presence of a cellulose operon has also been reported for a strain of *Asaia bogorensis* isolated from tropical flowers (NCBI accession no. AB355706).

Investigations by transmission electron microscopy (TEM) of the microbiome associated with the epithelia of mosquitoes and other insects showed an extensive mass of extracellular polysaccharide matrix around the AAB symbionts (13, 22, 42, 62). This matrix establishes tight contact between the microbial cells and the host epithelium, implying a role in microbial interaction with host surfaces. Crotti et al. (13) also discussed the possibility that this extracellular matrix could protect the bacterial cell from adverse conditions such as alkaline or acidic pH or high osmolarity. Indeed, several members of the family
Acetobacteraceae (Tanticharoenia, Acidomonas, Asaia, Saccharibacter, Swaminathania, and Neoasaia spp.) show the ability to grow at high osmolarity (30% glucose), whereas only weak growth under this condition is reported for Kozakia sp. (69, 70).

Asaia sp., a dominating bacterium in the microbiota associated with Anopheles sp. and Aedes aegypti mosquitoes and with the leafhopper S. titanus, was analyzed by TEM and in situ hybridization (ISH), proving its ability to produce a gelatinous matrix around the bacterial cells in contact with the host gut epithelium (13, 22). The capability of this strain to colonize and lodge in the gut system was also demonstrated using strains labeled with fluorescent proteins (13, 22). It was shown that the symbiont produces a thick pellicle when grown in glass tubes without shaking (Fig. 1B), a feature typically found in AAB. For instance, when AAB occur as spoilers in bottled wine, a distinctive ring of bacterial biomass is deposited in the neck of the bottle at the interface between the wine and the air headspace (6).

Transmission experiments performed with Asaia sp. strains tagged with fluorescent proteins (Fig. 1C) (13). During colonization of the gut, salivary glands, and reproductive organs, Asaia titanus showed a horizontal transmission route with rapid colonization of the gut, salivary glands, and reproductive organs. Asaia sp. can also be passed horizontally through feeding between insects that belong to phylogenetically distant species. For instance, it was shown that Asaia sp. from Anopheles spp. is able to cross colonize other sugar-feeding insects like Aedes aegypti and the hemipteran Stinkbug (13).

These investigations showed that several routes occur simultaneously (15). Environmental acquisition appears to be an important way of transmission, aided by the ubiquitous nature of the symbiont, but transmission from the mother to the offspring and from the father to the mother and then to the offspring also occurs (15). An egg-smearing mode of vertical transmission of Asaia sp. in S. titanus was proven by fluorescence ISH (FISH) and colonization experiments with strains labeled with fluorescent proteins (Fig. 1C) (13). During
ing ovarian egg development, an increasing ordered disposition of the superficial bacterial biomass is observable: from a more disperse pattern at the initial developmental stages, the bacterial cells are finally confined to the apical egg regions.

Transfer of Asaia sp. from larvae to pupae and from pupae to adults was also demonstrated (15). It is likely that during insect metamorphosis, Asaia sp. can be transtadially transmitted and escapes the reduction/removal of midgut bacteria that normally occurs during development, perhaps residing at other sites (and thus not being included in the meconium), or being resistant to the antimicrobial exuvial fluid that is ingested as part of the ecdisial process (52).

THE INSECT BODY CAN BE AN APPROPRIATE NICHE FOR AAB

The presence of AAB in the insect digestive system and in other organs reflects the physiochemical properties of these habitats (such as the type of food ingested, redox conditions, and pH) and the metabolic responses and capabilities of these microorganisms (17).

The distribution of arthropod-associated AAB in the different insect species reflects their diverse preference of carbon sources acquired by insect nutrition. Gluconobacter sp., preferring sugars, is generally isolated from honeybees, while ethanol-loving Acetobacter sp. is mainly recovered from Drosophila and Bactrocera flies. Nectar and honey are basically sugar solutions composed of sucrose, glucose, and fructose (9, 64), thus being favorable substrates for Gluconobacter sp. On the other hand, Drosophila flies are attracted by fermenting fruits that are a more suitable environment for acetobacters. Drosophila flies are mostly fungivores, and their association with fruit is indirect in that they primarily eat yeasts that live in rotting fruit. Drosophila flies are also called vinegar flies because they are attracted to the acidic odor of fermentation. Moreover, insects from which AAB are usually recovered have sugar-rich diets that are unbalanced for nitrogen content, so they have to fulfill the nitrogen requirement from other sources, such as the atmosphere (55). Among AAB, several examples of N2-fixing bacteria have been reported, such as Ga. diazotrophicus (32, 66), Ga. johannae, Ga. azotocaptans (30), Ga. kombuchae (21), A. peroxydans (54), A. nitrogenifigens (20), and Swaminathania salitolerans (45). However, further investigations are needed to evaluate if insect-associated AAB could contribute to insect nitrogen metabolism or recycling. The ability of several AAB strains to grow on media devoid of vitamins (16) also suggests the possibility that AAB in insects could synthesize vitamins or cofactors utilized by the host. A. pasteurianus, A. peroxydans, A. estunensis; Ga. liquefaciens, As. bogorensis (43), and Asaia sp. isolated from An. stephensi have been showed to be prototrophic with respect to vitamins. On the other hand, experimental removal of AAB from their insect hosts (as in the case of the Asaia-Anopheles system) did not appear to be detrimental to the host, underlining the secondary symbiotic status of these bacteria.

The GIT and circulatory system of AAB-associated insects lack anoxic niches (8, 12). Studies on the gut microbiota associated with Drosophila or Apis spp. confirmed the absence of obligate anaerobic bacteria and the presence of aerobic, facultatively aerobic, or acrotolerant bacteria (12, 50). AAB have a respiratory metabolism that requires oxygen as the final electron acceptor, but they are also able to survive in environments with low oxygen availability (19, 38, 49). Hence, they can find a favorable environment in the insect gut.

Within the insect, AAB, as well as other symbiotic microorganisms, have to face the host innate immune system (55). According to the results obtained by Ryu et al. (61), the AAB microbiome is involved in modulating Drosophila immunity. The authors described a delicate equilibrium between the gut commensals and the fly innate immune system. The normal flora in the fly gut was sufficient to suppress the growth of pathogenic bacteria, maintaining the pathogenic commensal Gluconobacter (G.) morbifer at a low level. When the system was perturbed, an increased number of G. morbifer bacteria led to gut apoptosis. Hence, in healthy individuals, the immune system allows the dominance of two AAB strains (A. pomorum and Commensalibacter intestini), which suppress the proliferation of G. morbifer by competition. Another study gained insight into the interaction between the acetic acid bacterium Asaia sp. and the innate immune system of An. gambiae (18). When the expression of a host gene involved in the innate immune response (AgDscam) gene was silenced, Asaia sp., whose favorable site of colonization is the gut (22), was no longer controlled by the innate immune system and proliferated massively in the host hemolymph (18).

AAB are considered fastidious microorganisms due to their difficult isolation and cultivation on synthetic media, regardless of the great number of media proposed for their growth (6). Moreover, they have been shown to be capable of entering the VBNC state (6, 49). It was not possible to isolate Asaia sp. from S. titanus leafhoppers, in spite of the well-documented presence of the symbiont in the insect as assayed with several cultivation-independent techniques (13). The authors speculated that the failure to isolate Asaia sp. might have been due to the unfavorable environment represented by S. titanus in a not actively feeding state or due to the development of unknown nutritional requirements by the bacteria.

CONCLUDING REMARKS AND PERSPECTIVES

For a long time, AAB have been considered environmental and ubiquitous bacteria. However, an increasing number of reports of stable associations of AAB with insects and the fact that they follow several transmission routes for their propagation in the next insect generation indicate that insect-associated AAB cannot be considered just environmental microorganisms but are also symbionts of the insect body, where they occupy a specific favorable niche. Phylogenetic studies of insect-associated AAB and those recovered from the environment do not show phylogenetic congruence of the insect-associated AAB (Fig. 2). This suggests that AAB have been acquired from the environment by their insect hosts recently (14). Moreover, not being essential for host survival, AAB have to be considered secondary symbionts of insects.

Clarification of the function(s) exerted by the bacteria in and for their hosts will be a major step toward understanding the bacterium-insect association. AAB are probably involved in many aspects of insect biology, such as (i) the host
metabolism by supplying nutrients or by oxidizing certain substrates, (ii) defense against harmful microorganisms by decreasing the gut pH or by competitive exclusion, (iii) contributing to the maintenance of host gut homeostasis (61), (iv) interference with cell-to-cell communication through the production of volatile compounds, and (v) maintaining an equilibrium of the microbial consortia by supplying metabolites to other microbes beneficial to the

FIG. 2. Phylogenetic positions of insect-associated AAB among the most representative members of family Acetobacteraceae. The tree shown is based on bacterial 16S rRNA gene sequences. AAB recovered from insects are in bold. Shaded species belong to clusters including environmental AAB, as well as those recovered from insects. Values at nodes are percentages of bootstrap replications calculated from 2,000 replicate trees. Accession numbers of reference sequences are in parentheses. The species used as the outgroup belongs to the Gammaproteobacteria taxon. The scale bar represents 10% sequence divergence.
host. Genome sequencing of the insect-associated AAB will be a particularly valuable tool in this context.

Not only are further studies needed to clarify the role of insect-associated AAB, but efforts should also be directed toward understanding AAB biological diversity and behavior in the host. An interesting topic could be the investigation of body invasion mechanisms adopted by AAB, whether they follow tissue tropism, as shown for the alphaproteobacterial symbiont Wolbachia sp. (29).

Symbiotic biology receives increasing attention since insect symbionts can potentially be used to control vector-borne diseases or suppress insect pests. AAB could be used as agents for natural or paratransgenicsymbiotic control. Bacteria like Asaia spp. or Acetobacterium tropicalis possess features interesting in this context, like easy cultivability, preservability, transformability, and dominance and prevalence in the insect microbiome. Moreover, vertical and horizontal transmission routes and cross-colonizing capability could ensure the spread of these biological agents through host populations (23).

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