An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects

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Across animals and plants, numerous metabolic and defensive adaptations are a direct consequence of symbiotic associations with beneficial microbes. Explaining how these partnerships are maintained through evolutionary time remains one of the central challenges within the field of symbiosis research. While genome erosion and co-cladogenesis with the host are well-established features of symbionts exhibiting intracellular localization and transmission, the ecological and evolutionary consequences of an extracellular lifestyle have received little attention, despite a demonstrated prevalence and functional importance across many host taxa. Using insect–bacteria symbioses as a model, we highlight the diverse routes of extracellular symbiont transfer. Extracellular transmission routes are unified by the common ability of the bacterial partners to survive outside their hosts, thereby imposing different genomic, metabolic and morphological constraints than would be expected from a strictly intracellular lifestyle. We emphasize that the evolutionary implications of symbiont transmission routes (intracellular versus extracellular) do not necessarily correspond to those of the transmission mode (vertical versus horizontal), a distinction of vital significance when addressing the genomic and physiological consequences for both host and symbiont.

1. Introduction

Through a variety of interactions, resident microorganisms have played a significant role in the origin and evolution of animals [1]. Among animals, insects serve as excellent models to elucidate the functional importance of these interactions, because they engage in a particularly wide range of mutualisms with bacteria and fungi [2].

The remarkable diversity in form and function of insect-microbial interactions, however, can only be rivaled by the variety of symbiont transmission structures and behaviours that contribute towards the fixation, persistence and evolution of such partnerships [2]. These adaptations ensure that beneficial microbes can be transferred across host generations directly from parent to offspring (vertical mode), indirectly from conspecific host individuals as well as from the environment (horizontal mode), or through a combination of transmission mechanisms (mixed-mode) [3].

Some of the best-studied insect-bacterial mutualisms (e.g. aphids and Buchnera, carpenter ants and Blochmannia, tsetse flies and Wigglesworthia) have yielded extensive knowledge on the transmission ecology and the ensuing evolutionary consequences of mutualistic interactions between insects and intracellularly localized symbionts [4–12]. These obligate mutualists can be transmitted in a number of ways during oogenesis or embryogenesis [4]. For example, in carpenter ants, Blochmannia is vertically transmitted via an acute intracellular infection of the ovaries and subsequent incorporation into the eggs [9]. Similarly, in aphids, Buchnera is transovarially transferred to the developing eggs via a highly selective mechanism...
at the ovariole tips [10]. As for tsetse flies, their B vitamin-supple-
menting symbiont Wigglesworthia is transmitted via milk gland 
secretions as the larva develops in utero [11]. While many intra-
cellular symbionts are maternally transmitted to the offspring 
[4], a recent study [12] has demonstrated both maternal and 
paternal vertical transmission of Rickettsia in leafhoppers.

The evolutionary implications of an intracellular lifestyle 
coupled with the strict vertical transmission mode of many 
insect symbionts have been the focus of considerable atten-
tion [5–8]. Nonetheless, research efforts of the past few 
decades have resulted in a steadily increasing body of knowl-
edge on the diversity, function and evolutionary history of 
extracellularly localized and transmitted symbionts in insects 
(electronic supplementary material, table S1). Given this 
wealth of recent data, it is now feasible to assess the funda-
mental ecological and evolutionary implications of these 
types of transmission routes for both host and microbe.

In addition to providing an overview of extracellularly 
transmitted bacterial symbioses in insects, we discuss the evol-
utionary origin of such associations and the factors influencing 
their persistence. We also emphasize the impact of symbiont 
transmission on the coevolutionary trajectory of the symbioses,
and on the genomic and metabolic signatures of the bacterial 
partners. By illustrating that some extracellularly localized 
and transmitted bacterial symbionts can exhibit similar pat-
terns of metabolic integration and host-microbe coevolution 
as strictly intracellular mutualisms, we stress that the mode 
of symbiont transmission (vertical versus horizontal) is a 
more accurate indicator of mutualism stability and integrative 
potential than the stage at which microbes are transmitted 
(prenatal versus postnatal).

2. Overview of extracellular transmission routes 
of insect symbionts

Despite broad functional and taxonomic diversity (figure 1; 
electronic supplementary material, table S1), extracellularly 
transmitted symbionts can be unified by the ability to survive 
outside of their host for part, or all, of their lifetime. This 
feature markedly differentiates them from the majority of 
intracellular symbionts, where survival outside the host is 
no longer possible [5].
In insects, extracellular transmission routes for bacterial symbionts include environmental determination, coprophagy, smearing of brood cell or egg surface, social acquisition, capsule transmission or infection via jelly-like secretions (figure 2).

(a) Environmental determination
In animals, the acquisition of specific beneficial microbes from the environment is particularly prevalent in marine invertebrates including tubeworms and luminescent squids [17,18]. However, recent studies examining the microbial symbionts of several broad-headed bug species and whiteflies demonstrate that terrestrial environments can also be a suitable source for the acquisition of beneficial microbes by insect hosts [13,19].

Bean bugs (Riptortus pedestris), as well as many other species within the Lygaeoidea and Coreoidea superfamilies, harbour environmentally acquired Burkholderia symbionts that localize primarily within crypts along their posterior midgut section [20]. The environmental dimension of the symbionts' transmission route was first established following the inadvertent generation of developmentally regressed, aposymbiotic (symbiont-free) R. pedestris when the bugs were reared in sterile bottles. In fact, eggs laid in sterile laboratory settings by Burkholderia-infected individuals were also completely devoid of symbionts, strongly suggesting that the bugs acquired their free-living symbionts every generation from the environment, particularly the soil [19]—not unlike well-established plant–microbe partnerships involving rhizobia [21].

Environmental symbiont acquisition can also occur in associations with predominantly vertical transmission. In addition to their primary endosymbiont Portiera, whiteflies (Bemisia tabaci) also harbour a number of secondary symbionts, including a widely occurring Rickettsia sp. [22]. While Rickettsia has been demonstrated to be primarily transmitted vertically via the eggs during embryogenesis [22], significant inconsistencies were nonetheless observed between the phylogenies of hosts and symbionts, suggesting that the microbe probably undergoes substantial horizontal exchange between whiteflies [23]. Caspi-Fluger et al. [13] confirmed this by demonstrating that the symbiont can be transmitted among B. tabaci via the host plant, as demonstrated by the detection of Rickettsia in the phloem of cotton, basil and black nightshade plants following feeding by an infected whitefly. Additionally, Rickettsia-free individuals were successfully re-infected with the symbionts when allowed to feed on the same leaf (despite physical separation) as Rickettsia-infected B. tabaci [13]. However, the subsequent vertical transmission of horizontally acquired Rickettsia to the whitefly progeny has not yet been demonstrated. While the predominant route for symbiont acquisition in this system is vertical (via the egg), such findings suggest that plants may also serve as sources and sinks for symbiont inoculants in herbivorous insects.

(b) Coprophagy
Acquisition of beneficial bacteria through conspecific probing of faeces has been described as a predominant route of symbiont transmission for several insect groups, including Hemiptera (true bugs), Blattaria (cockroaches) and Isoptera (termites) (electronic supplementary material, table S1). The symbionts usually reside in the insect gut, where they are shed alongside the gut lumen and excreted in faeces [24].

Symbiont acquisition by aposymbiotic individuals then requires direct contact with faeces during or after excretion. Interestingly, coprophagic symbiont transfer has been suggested to provide the opportunity for biological control of the reduviid bug Rhodnius prolixus, an important insect vector of the Chagas disease-causing parasite, Trypanosoma cruzi [25]. Despite near ubiquitous infection of adult R. prolixus with the actinobacterial symbiont Rhodococcus rhodnii in
natural populations, newly hatched nymphs are aposymbiotic until they acquire the symbiont by probing conspecific faeces [25]. The route of symbiont transfer, coupled with the bacterium’s amenability for genetic transformation, could facilitate biological control via paratransgenesis, i.e. the introduction and expression of exogenous trypanocidal genes via the symbionts [25,26]. Such findings highlight the potential for manipulation of extracellularly transmitted symbioses to control vector-borne diseases.

**(c) Social acquisition**

Advanced social behaviour in insects imposes different parameters for the transmission of microbial partners. A central feature of many social and subsocial insects is the intimate interaction of conspecifics through behaviours such as trophallaxis, the transfer of food or other fluids through mouth-to-mouth (stomodeal) or anus-to-mouth (proctodeal) feeding [27]. These behaviours can facilitate exchange of microbes among nest members, thereby contributing to maintenance of a beneficial microbiota, as has been demonstrated in ants [16,28,29], termites [30] and bees [31,32]. In fact, it has been speculated that the evolution of complex social forms could be reinforced, among other factors, by the convenience of acquiring beneficial microbes through recurring contact with conspecifics [33].

Recent examination of the gut microbiota of different bee species suggests that sociality plays an integral role in maintenance of the distinctive microbial communities within the Apoidea superfamily [31,34]. While the majority of solitary bee microbiota examined by Martinson *et al.* [35] seem to be indiscriminately dominated by *Burkholderia* or *Wobbachia*, the social caribiculate clade (including *Bombus* and *Apis*) carry a largely conserved microbiota that may have co-evolved with the hosts as a byproduct of eusocial behaviour. In honeybees (*Apis mellifera*), workers lack this distinctive microbial community upon eclosion, and recent findings demonstrate that they acquire the most dominant members of the microbiota either through social contact with nestmates (trophallaxis), specifically nurses, or via contact with the hive components (e.g. combs and honey) [32,36]. As for bumblebees, molecular analyses carried out across three host species (*B. sonorus*, *B. impatiens* and *Bombus* sp.) show that two of the most dominant bacterial strains (*Stoolgrassella atei* and *Gilliamella apicola*) are transmitted vertically from the mother colony to daughter queens, and that social contact among nest-mates following pupal emergence is required for intra-colony transmission [31].

Social transmission of beneficial symbionts has also been described for fungus-farming ants (*Atinni: Formicidae*) in their association with the defensive mutualist *Pseudonocardia* [28]. Most attine ant genera extracellularly harbour the symbiont in specialized cuticular crypts [16] (figure 2i and j), and the presence of *Pseudonocardia* on foundress queens during their mating flight suggests a vertical transmission route linking parent and offspring colonies [16,28]. The singular association of each nest to individual *Pseudonocardia* strains further implies that the symbionts proliferate among nest members via social behaviour [37,38], which was confirmed in a recent study by Marsh *et al.* [29]. Here, the ants were found to only acquire *Pseudonocardia* following contact with nest-mates within the first 2 h after emerging from their pupal cases.

***(d) Egg and oviposition site inoculation***

Smearing bacteria over the surface of newly deposited eggs is one of the most commonly described routes of extracellular symbiont transfer and has been reported for various insect orders, including Diptera, Coleoptera, Hymenoptera and Hemiptera (figure 1; electronic supplementary material, table S1). Successful infection primarily depends on the ability of nymphs or larvae to acquire their bacterial symbionts shortly after hatching, usually through active probing of egg or brood cell surfaces.

Within the Hemiptera, numerous studies have reported that egg smearing by the mother leads to successful transmission of beneficial microbes, particularly among bugs of the Pentatomomorpha infraclass (e.g. firebugs, stinkbugs, shield bugs, etc.) (electronic supplementary material, table S1). For example, firebugs (*Pyrrhocoridae*) deposit excretion droplets that are later taken up from the egg surface by young nymphs, ensuring successful transfer of the two co-occurring actinobacterial symbionts *Coryobacterium glomerans* and *Gordonibacter* sp. [14] (figure 2c–e). Similarly, in shield bugs of the family Acanthosomatidae, a γ-proteobacterial symbiont is harboured in cavities that are sealed off from the midgut main tract, as well as in a pair of lubricating organs associated with the female ovipositor [39]. It is through these specialized organs that the symbionts are vertically transmitted via egg surface contamination. Across both insect groups, disruption of the symbiont transmission route through the surface sterilization of newly laid eggs results in aposymbiotic individuals that suffer retarded growth, higher mortality and lower reproductive success [39,40].

By contrast, transmission of defensive *Streptomyces* symbionts of solitary digger wasps (*Philanthus, Trachypus* and *Philanthinus* spp.) relies not on surface contamination of eggs but rather of brood cells where eggs are deposited [15] (figure 2f–h). Prior to oviposition, female wasps secrete a symbiont-containing white substance from their antennal glands and onto the ceiling of brood cells [15]. During cocoon spinning, larvae then take up the bacteria, which confer protection against pathogenic fungi in the brood through production of antibiotic substances on the cocoon [41].

**(e) Capsule and jelly transmission**

Plataspid and urostylidid bugs use two of the most specialized mechanisms for extracellular symbiont transmission at the oviposition site [42–44]. In Plataspididae, adult females produce symbiont-enclosing ‘capsules’, which they deposit among their newly laid egg masses to ensure the successful vertical transmission of their γ-proteobacterial midgut mutualist *Candidatus Ishikawaella capsulata* (figure 2k–m) [42]. Infection of newly hatched nymphs is associated with capsule feeding [42]. In addition to the adverse fitness effects associated with aposymbiosis (e.g. high juvenile mortality and slow development), capsule removal causes bugs to wander from the egg masses rather than rest in aggregation—the typical behaviour associated with capsule feeding [45]. This suggests that insect behaviour may be linked to ensuring successful symbiont acquisition, as has been demonstrated in social insects.

In *Urostylis westwoodii* (*Urostylidae*), jelly-like secretions deposited by mothers over newly laid egg masses represent a remarkable adaptation with versatile biological roles [44]. While the sugar- and amino acid-rich jelly allows the newly hatched nymphs to withstand the nutritional burdens of overwintering underground (in the absence of their natural food source of plant sap), Kaiwa *et al.* [44] have also
implicated the gelatinous structure in ensuring the successful transmission of the gut symbiont *Candidatus* Tachikawaea gelatinosa following ingestion (figure 2n,o).

3. Transition from a free-living state to symbiosis

Symbiont transmission mechanisms represent adaptations to ensure the maintenance of mutualisms. It is imperative to understand the factors that initially contributed towards the emergence of these mutualisms, and the evolutionary transitions that have shaped their history. Insects that rely on extracellular routes for transmission of their beneficial microbes present us with excellent systems to address such topics, given the diversity in evolutionary states of the symbioses, ranging from facultative and horizontally acquired, to obligate and vertically transmitted.

The origin of bacterial mutualisms, however, remains one of the most elusive questions within the field of symbiosis [46]. Traditionally, several hypotheses have been suggested for the initial evolution of microbial symbioses [47–49]. Recent phylogenetic analyses demonstrate the plausibility and occurrence of all these scenarios and suggest that the evolution of bacterial mutualists from environmental strains may represent a particularly common occurrence [50].

For extracellularly transmitted symbionts, there are several systems consistent with environmentally acquired bacteria providing immediate benefits to their hosts upon establishment. In leguminous plants, the nitrogen-fixing ability of soil rhizobia coupled with the gain of a core set of symbiosis loci as selected for by access to a metabolically stable environment (i.e. the host), enabled the establishment and maintenance of a cosmopolitan mutualistic partnership [21]. Analogous mutualisms that exemplify this dynamic have also been described for insects. Within the bean bug’s (*R. pedestris*) association with *Burkholderia*, recent findings by Kikuchi et al. [51] demonstrate that under certain conditions, the environment can select for the optimal symbiont; which, in turn, can inadvertently align the bilateral benefits of the host and symbiont without the prerequisite of strict vertical transmission.

However, for such mutualisms to be evolutionarily stable, the benefits of investing in a vertical or horizontal symbiotic transmission mechanism by the host must outweigh a couple of significant costs: the risk of not acquiring the symbiont and/or acquiring an antagonistic partner (e.g. pathogens, parasites or cheaters). In other words, insects that rely on environmentally acquired symbionts [19,20] probably run a higher risk of aposymbiosis as well as pathogen exposure than mutualisms featuring adaptations that ensure faithful vertical transmission of the microbial partner [43,44].

For example, for *R. pedestris* and several other bug species, failure to pick up specific bacterial symbionts during a short development window significantly affects fitness [52]. Similarly, the uptake of a suboptimal symbiont also poses risks for the host in terms of competitively subsisting on resources in a specific niche [51]. Interestingly, across the true bugs, there are multiple independent origins of *Burkholderia* symbioses, many, and possibly all, of which are presumed to be dependent on environmental acquisition [20]. These bugs – *Burkholderia* partnerships are interspersed among other systems that use vertical transmission through both internal and external mechanisms. One possibility is that environmental acquisition is selected for in fluctuating environments when the symbiont genotype that is most optimal varies significantly across space or time. Thus, being able to switch partners, and possibly to actively select the optimal partner or to allow potential partners to outcompete one another, may supersede the costs of risking the failure to obtain an optimal partner.

This raises interesting questions relating to the initial stages of symbiont colonization; specifically, what are the mechanisms that mediate the recognition and uptake of the right symbiont and how does the host select for these microbes while eliminating less beneficial ones?

Mechanisms could be behavioural, in which insects actively seek out symbionts with certain traits, or physiological, in which insects take in a diversity of microbes and then actively winnow down associations to a narrow few. There has been little exploration of the former, but there is increasing evidence for the latter. As previously discussed, *R. pedestris* possesses remarkably efficient symbiont detection and uptake mechanisms, where a mere 80 *Burkholderia* cells in a gram of soil are sufficient for successful infection [52] during a highly specific developmental window of acquisition [53]. The efficient establishment of only a small subset of the diverse microbes that these bugs encounter in soil is mediated, at least in part, through complex immunological processes that prevent the growth of other bacteria and may tightly regulate proliferation of the symbionts [54–57]. Antimicrobial peptides isolated from the haemolymph of the *Burkholderia*-harbouring coreoid *Alydus calcatus*, for example, can suppress growth of some soil-dwelling Gram-negative bacteria (e.g. *Escherichia coli*) but not *Burkholderia* symbionts [57]. Comparative transcriptomic analyses of the midguts of symbiont-containing and aposymbiotic *R. pedestris* revealed an upregulation of cysteine-rich antimicrobial peptides, which could inhibit growth of some bacteria and also regulate symbiont populations [56]. These findings are complemented by evidence suggesting that *Burkholderia* symbiont establishment and proliferation requires the bacteria to have several genes necessary for combating host-induced stress [54,55]. This suggests that the host’s physiology and immune system may be under selection to suppress proliferation of non-symbionts while allowing for regulated growth of symbiont populations. *Burkholderia* may have become the primary symbionts because they possessed the necessary features to facilitate establishment (e.g. motility, resistance to host antimicrobial activity) prior to, and independent of, any host-mediated selection.

Another factor that could favour the evolution of environmental acquisition would be the ubiquity of the microbe in the environment. Common occurrence of beneficial symbionts in the environment could relax selection to maintain vertical transmission mechanisms. To date, we have little data on the prevalence of beneficial symbionts in environmental reservoirs in systems where environmental acquisition is known (but see [13,19,57,58]). Further insight into factors favouring the evolution of environmentally acquired mutualisms will require such environmental sampling as well as characterization of symbiosis mechanisms across groups of insects, such as has been started, to some extent, for the true bugs [20].

4. Evolutionary transitions among transmission routes

Given the diverse mechanisms of extracellular transmission and their varying ecological and evolutionary implications,
it is tempting to speculate on likely scenarios of evolutionary transitions between transmission routes. Owing to the high selective pressures of endowing beneficial symbionts efficiently to offspring, the majority of specific mutualistic insect–bacteria interactions appear to transition to vertical or mixed-mode transmission routes over the course of evolution.

Extracellular nutritional symbionts are usually localized within the gut, and significant numbers of cells are often shed and excreted along with faecal matter [14,24]. Thus, transmission via faeces (i.e. coprophagy and proctodeal trrophallaxis) constitutes a simple transitory step from environmental acquisition to vertical transmission, as it does not require any specialized morphological adaptations of the host [2]. In taxa with social interactions between parents and offspring or within groups of related or unrelated conspecifics, direct transfer of faeces by proctodeal trophallaxis also ensures symbiont transmission along with the provisioning of enzymes that may facilitate digestive processes in immature individuals [30]. Non-social insects without direct contact between symbiotic and aposymbiotic individuals, on the other hand, can increase the probability of successful transmission to the offspring by applying symbiont-containing faeces to locations that have high chances of being frequented and probed by the hatching larvae [2]. The egg surface is the most commonly used and reliable place of symbiont application and uptake (e.g. [2,39,59]), but in special cases with locally confined developmental conditions, the brood cell surface can be equally suitable [15].

Coincident with, or subsequent to, an increased fidelity in vertical symbiont transmission, several insects with extracellular symbionts have evolved specialized structures to house and transmit symbionts to the offspring (e.g. [42,44]). As discussed for the capsule and jelly transmitted symbionts of plataspid and urostylidid bugs, these structures can serve to protect the symbionts from abiotic stresses during egg development [44], as well as allow for enhanced control over the identity and number of symbiont cells allocated to offspring, thereby ensuring that the progeny are endowed with sufficient numbers of viable symbionts for successful colonization [60]. While speculative, such adaptations may also mitigate the chances of co-transmitting potentially detrimental microbes, which is consistent with theoretical predictions implicating the restriction of symbiont migration as a mechanism adopted by the host in order to reduce virulent tendencies arising from competition between heterospecific symbiont lineages [61].

Finally, the highest level of integration between host and symbiont occurs when symbiont transmission is internalized within the host’s body. Starting out with an extracellular symbiosis, however, this can be achieved through a shift in the symbiont’s lifestyle to intracellular maintenance and transmission to the developing oocyte or embryo (e.g. aphids–Buchnera, carpenter ants–Blochmannia) [4].

5. Implications of transmission ecology for symbiont genome evolution

Obligate mutualisms can have a strong effect on the evolution of the bacterial partner’s genome. As exemplified by Buchnera and Sulcia—the primary intracellular endosymbionts of pea aphids and sharpshooters, respectively—as well as many others, symbiont genomes can undergo strikingly convergent patterns of degradation and reduction [5]. These small genomes exhibit extensive AT nucleotide enrichment and undergo accelerated molecular evolution [6,7]. Such features are presumed to be driven by gene loss resulting from a combination of strong genetic drift in small populations undergoing severe bottlenecks during transmission, and relaxed selection to no longer maintain genes necessary for an extracellular lifestyle [7].

As a result, many of the aforementioned genomic features became consequential hallmarks of an intracellular lifestyle within animal hosts [8]. Thus, it seemed unlikely that extracellularly transmitted and localized symbionts would undergo similar patterns of reductive genome evolution, considering that these microbes reside outside of the insect host for part, or all, of their life cycle, where they can readily undergo recombination to offset gene loss due to genetic drift, and generally need to retain a larger set of genes to survive in less stable environmental conditions and to move between various habitats (e.g. different host tissues and outside of hosts).

Nonetheless, studies examining genomic features of some extracellularly transmitted symbionts indicate that similar evolutionary processes can occur in these symbionts as those restricted to a strict intracellular lifestyle (figure 3 and electronic supplementary material, table S4).

Specifically, extracellularly transmitted symbionts of plataspid (capsule transmission), acanthosomatid (egg smuggling) and urostylidid (jelly transmission) bugs possess reduced genomes (estimated sizes around 0.7–0.9 Mb) [39,43,44,62]. Additionally, Ishikawaella symbionts isolated from the plataspid M. punctatissima exhibit other genomic features reminiscent of intracellular symbiotic bacteria (i.e. AT nucleotide bias and few mobile elements) [62]. Examination of Ishikawaella’s metabolic potential reveals that despite significant gene loss, the bacterium retains the ability to synthesize almost all essential amino acids, in addition to some vitamins and cofactors [62]. This is consistent with the suggested benefit of the symbiont for M. punctatissima, whose plant diet is poor in essential amino acids and certain vitamins.

Despite exhibiting similar patterns of reductive genome evolution, pairwise comparisons of gene profiles between Ishikawaella and a range of intracellularly localized and transmitted symbionts revealed a number of important discrepancies that may reflect their different ecologies [62]. Most prominent was the complete retention of genes involved in the Krebs cycle, as well as many other genes underlying energy production and conversion. This was attributed to the more stringent metabolic requirements of an extracellular lifestyle, where access to metabolic intermediates in the host cytoplasm is not an option, unlike for many obligate intracellular symbionts. Additionally, Ishikawaella possesses a greater number of genes involved in the synthesis of amino acids and cofactors than Buchnera (aphids), Blochmannia (ants) or Wigglesworthia (tsetse flies), a condition that implies a broader metabolic repertoire for supplementation, and/or a younger coevolutionary history with its host [62].

The genomic features of these symbionts provide insight into the evolutionary forces driving genome reduction in obligate microbial mutualists [39,43,44,62] by demonstrating that these convergent traits are not strictly a consequence of an intracellular lifestyle but rather are more likely due to...
increased impact of genetic drift associated with a host-restricted lifestyle. This highlights small population sizes and strong bottlenecks promoted by spatial isolation, prior to and/or during transmission, as important factors for genome evolutionary patterns in heritable symbionts.

These findings, coupled with analyses of a broad range of bacterial genomes demonstrating a clear inverse correlation linking genome size and the incidence of genetic drift [63] further support the concept that reductive genome evolution can be associated with intracellularity but is not necessarily derived from it.

6. Host–symbiont coevolutionary dynamics

Acquisition of complex traits and adaptations by insects to ensure that their progeny are endowed with beneficial microbes often results in symbiotic systems that are evolutionarily stable and mutually obligatory. The high fidelity exhibited by these partnerships can be quantified (and visualized) through the congruent branching patterns of host and symbiont phylogenies in what is commonly referred to as co-cladogenesis [5]. Strict co-cladogenesis has been demonstrated in a number of insect-bacterial mutualisms featuring intracellular symbionts, for example, among sap-feedings insects (e.g. aphids, sharpshooters, etc.) [4].

With co-cladogenesis as a measure for mutualism fidelity, inferring phylogenetic relationships between symbiotic bacteria relative to their insect hosts can provide insights into the evolutionary implications of different routes of symbiont transmission. Bugs of the Pentatomomorpha infraorder—whose bacterial partners colonize similar gut regions but use different transmission routes and modes to initiate infection—provide a point of comparison for consequences of transmission routes on host–symbiont evolution (figure 4). In members of this group that acquire beneficial bacteria via the environment (e.g. soil), there are numerous discrepancies between symbiont and host phylogenies [20] (figure 4a). Some host species harbour different symbiont genotypes, while others share a single identical symbiont [20,57]. This lack of fidelity is driven by the bugs acquiring symbionts from a potentially diverse, shared environmental reservoir.
7. Conclusion and future perspectives

In addition to providing insights into evolutionary aspects of symbiosis, insect-bacterial mutualisms that rely on extracellular mechanisms for symbiont transmission present excellent opportunities to elucidate functional aspects of these partnerships. Given a transiently aposymbiotic phase during the early stages of insect development, alongside the ability of the microbe to survive outside of the host’s body for part of its lifetime—two conditions universally shared across the aforementioned systems—it is in many cases experimentally feasible to physically separate both partners by disrupting the transmission cycle (e.g. [31,39,40,43,59]). Such experiments have been successfully employed to elucidate symbiont contributions towards host fitness, to assess host-symbiont specificity, and to detail the effects of symbiont replacement on host ecology (e.g. [66,67]).

Furthermore, the extracellular nature of the symbionts contributes to the likelihood that they can be cultured and genetically manipulated, which is not possible for most intracellular symbionts. In vitro cultivation and manipulation can facilitate introduction of genetically modified symbionts into their insect hosts. Thereby, the importance of candidate symbiont genes for establishment or maintenance of a mutualistic association, as well as for the fitness benefits conferred to the host, can be directly assessed [54,55]. Additionally, this strategy may prove valuable to manage agricultural pest species or disease vectors by modification of their symbionts [25]. Combining symbiont manipulation with targeted knock-down of host genes potentially involved in mediating symbiosis will undoubtedly provide unprecedented opportunities to study host-symbiont molecular interactions and investigate the genomic and physiological underpinnings of these associations.

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