Genetic innovations in animal–microbe symbioses

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Abstract | Animal hosts have initiated myriad symbiotic associations with microorganisms and often have maintained these symbioses for millions of years, spanning drastic changes in ecological conditions and lifestyles. The establishment and persistence of these relationships require genetic innovations on the parts of both symbionts and hosts. The nature of symbiont innovations depends on their genetic population structure, categorized here as open, closed or mixed. These categories reflect modes of inter-host transmission that result in distinct genomic features, or genomic syndromes, in symbionts. Although less studied, hosts also innovate in order to preserve and control symbiotic partnerships. New capabilities to sequence host-associated microbial communities and to experimentally manipulate both hosts and symbionts are providing unprecedented insights into how genetic innovations arise under different symbiont population structures and how these innovations function to support symbiotic relationships.

Symbiont transmission mode
The route by which symbionts are acquired each generation, ranging from strictly vertical (parent-to-offspring) to strictly horizontal (between non-parent–offspring pairs of hosts or between hosts and non-host sources). Mixed-mode transmission combines vertical and horizontal modes.

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Symbiotic associations with microbes have shaped animal evolution and contributed to the immense diversity in development, morphology and lifestyles seen across animal phyla[1]. Many of these symbioses are ancient, dating to the origin of major animal clades, and have had to adapt to shifts in dietary resources, the emergence of new pathogens and other changing selective pressures.

Appreciation of the dominant role of symbiosis in animal biology and human health has been relatively recent, spurred by the introduction of affordable sequencing methods about 15 years ago. Since then, the genomic and metagenomic sequencing of hosts and symbionts has given a picture of capacities, variability and evolution of symbiotic systems[2–8]. More recently, genetic tools that enable the validation of genes and pathways underlying specific symbiont functions have been developed, despite the challenges of culturing and experimentally manipulating symbiotic organisms[9,10]. As illustrated in this Review, these approaches have revealed a number of surprising mechanisms through which beneficial symbioses have been successfully maintained over long periods or have completely transformed themselves through changes in symbionts and hosts. These mechanisms can seem bewilderingly diverse, as symbioses evolve through different routes. We argue that this variation is more comprehensible by recognizing that it is largely dictated by the genetic population structure imposed on the symbionts (FIG. 1).

The rapid expansion in complete sequences of bacterial genomes has revealed distinct sets of correlated genomic characteristics that arise from differences in evolutionary forces acting on particular lineages[11]. Here, we define three categories of 'genomic syndromes' in bacterial symbionts that correspond to different modes of symbiont evolution. We refer to these as 'open', 'closed' and 'mixed' symbioses. Distinguishing these categories allows us to appreciate why symbiotic relationships innovate in strikingly different ways (FIG. 1). In open symbiotic communities, exemplified by most gut microbiomes, microbes repeatedly colonize hosts from external, environmental niches and innovation occurs through the turnover of lineages or the exchange of genetic material within and between microbial species. At the other extreme, in closed symbioses, symbionts are intimately incorporated into host development and reliably maternally transmitted along with the hosts' own genes. This symbiont transmission mode enforces strict clonality, causing genomic erosion where symbiont lineages lose rather than gain genes and limiting symbiont responses to novel selective pressures[6,12,13]. Instead, innovations in closed symbioses often involve changes in hosts such as the adoption of entirely new microbial partners[14] or the acquisition of novel host genes from bacterial sources[15]. Mixed symbiotic systems regularly rely on vertical transmission from mother to progeny but also undergo occasional horizontal transmission between host individuals or species. These mixed symbiotic systems exhibit features of both open symbioses, such as frequent gene exchange, and of closed symbioses, including extensive gene loss and rapid sequence evolution.

In this Review, we first summarize the features of different genomic syndromes as revealed by the growing availability of genomic sequences from bacterial

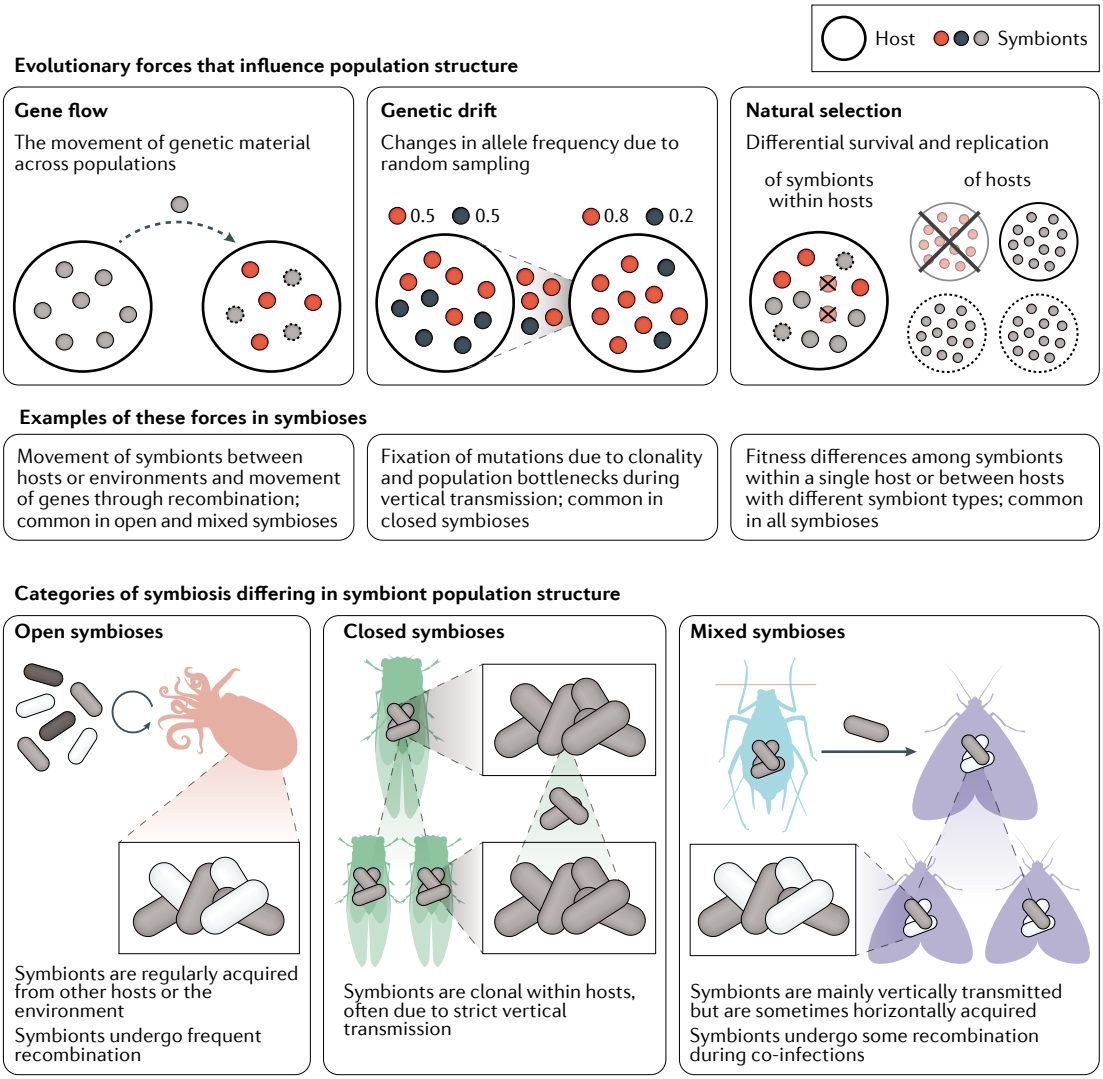

Fig. 1 | **Symbiont genetic population structure.** Genetic population structure refers to the organization of genetic variation (alleles) in a population as a consequence of evolutionary processes, including gene flow, genetic drift and natural selection. The genetic population structure of symbionts is shaped by features of the symbiotic relationship, including the symbiont transmission mode and population bottlenecks during host colonization. These features influence the diversity of strains found within hosts, the amount of genetic recombination that symbionts undergo, and the ability of purifying selection to purge the deleterious mutations that arise. Differences in genetic population structure result in different evolutionary patterns that can be categorized as open, closed and mixed symbioses, illustrated in the lower panel. In open symbioses, such as between the bobtail squid *Euprymna scolopes* (red) and strains of symbiotic *Aliivibrio* from the seawater, horizontal transmission and recombination are frequent. In closed symbioses, such as between *Hodgkinia cicadicola* and the cicada *Magicicada tredecim* (green), symbionts are vertically transmitted and clonal. In mixed symbioses, such as between *Hamiltonella defensa* and the aphid *Acyrthosiphon pisum* (blue), transmission is mostly vertical but occasionally horizontal across divergent hosts as *H. defensa* is present and vertically transmitted in the whitefly *Bemisia tabaci* (purple).

symbionts of diverse animal hosts and we link these to different evolutionary modes associated with population structure. We then highlight recent discoveries that reveal how genetic and functional innovations arise under each mode of symbiosis evolution. We focus on innovations specifically involved in maintaining the symbiosis itself while noting that symbiosis can launch hosts into novel niches and lifestyles, resulting in further adaptations. We also emphasize symbiont innovations, largely because these are currently better studied than those of hosts. We do not review the literature on the functions of animal microbiomes as these topics are covered elsewhere[1]. Likewise, we refer readers to previous reviews for specific aspects of symbiosis, including transmission mechanisms[16,17], genome reduction[2,18], how symbioses evolve[6,12,13,19] and horizontal gene transfer into host genomes[15]. We cite examples from recent studies on a variety of symbioses rather than covering particular systems in depth.

Symbiosis and genomic syndromes

The classification of a symbiotic relationship as open, closed or mixed is largely determined by inter-host transmission routes and their consequences for the

Genetic recombination
The exchange of genetic material between organisms. Recombination can be roughly classified as homologous recombination, which involves the exchange of related sequences, and non-homologous recombination, in which unrelated sequences are inserted into the genome as in the case of horizontal gene transfer.

genetic population structure of the symbionts (FIG. 1). These different symbioses exhibit some commonalities; for example, all animal symbionts must contend with host immune systems. However, the expansion in the set of sequenced symbiont genomes has revealed that their modes of evolution and their resulting genomic features differ strikingly. Notably, these categories do not neatly fit with a function-based classification; for example, all three types can be involved in the nutrition or defence of hosts. Likewise, they do not correspond to locations in the host body or tissues as all three types can be intracellular or extracellular or be associated with the gut or the bacteriome. Most gut symbioses are open and many bacteriome symbioses are closed but exceptions occur in both cases.

Open symbioses. Open symbioses vary in structure and complexity but share a common feature: symbionts are readily exchanged among host individuals or species and, in some cases, acquired from non-host environmental niches[20–22]. Crucially, the ability of symbionts to come into contact with conspecific strains or with other bacterial species, either within or outside of their host, allows symbionts to acquire genetic material through genetic recombination, either via homologous recombination or horizontal gene transfer (HGT). As a consequence, symbionts in these relationships possess genomes similar to those of widespread environmental bacteria as reflected in typical genome sizes and gene numbers, typical GC content, high coding density and strain-specific differences in gene content (FIG. 2a)[11]. As for

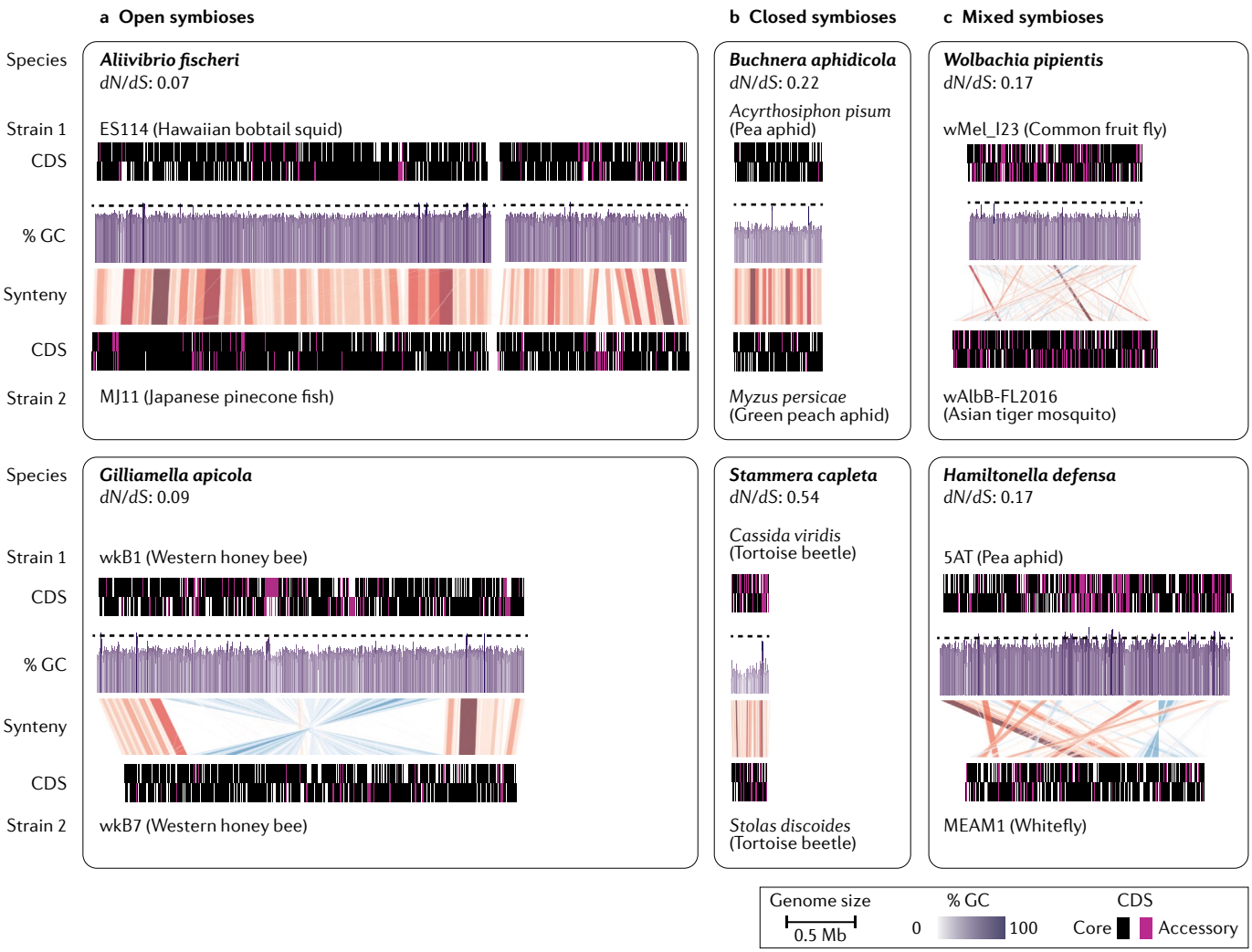

Fig. 2 | **Genomic features of bacterial species that have evolved under open, closed and mixed symbioses.** For each bacterial species, two strains were selected to identify core (shared) and accessory (unique) genes, to calculate a pairwise ratio of the non-synonymous to synonymous substitution rate (dN/dS) for core genes, and to visualize intergenomic synteny. Coding sequences (CDS) are displayed for both strains and GC content is displayed for the upper strain only (the dashed lines represent 50% GC content). Host names are given in parentheses. Symbionts in open communities retain large genomes mainly composed of protein-coding genes under strong purifying selection (dN/dS <0.1) (part **a**). They possess an average or high GC content (>30%). Their genomes are mostly syntenic, although a large inversion has occurred in *G. apicola*. Symbionts in closed communities possess reduced genomes and few genes, which are under very relaxed purifying selection (dN/dS >0.2) (part **b**). Their GC content is low (20–27% GC for *Buchnera aphidicola* strains[42], 11–17% for *Stammera capleta* strains[98]). Their genomes are highly syntenic. Symbionts in mixed communities possess genomes with varying levels of reduction, many accessory genes and weak purifying selection (dN/dS >0.1) (part **c**). They possess an average or high GC content (>30%) and their genomes possess many rearrangements and inversions.

Purifying selection
The removal of deleterious alleles by natural selection. Also referred to as negative selection. This is the most common form of selection, as mutations are more often deleterious than beneficial.

d*N*/d*S*
The ratio of non-synonymous substitutions (that is, those that change the amino acid sequence) per non-synonymous site (d*N*) to the number of synonymous substitutions (that is, those that do not change the amino acid sequence) per synonymous site (d*S*), used to determine the mode and strength of selection that has acted on genetic sequences.

Bacteriocytes
Host cells that are specialized for housing bacterial symbionts.

most free-living bacteria, their genomes are under effective purifying selection to eliminate deleterious mutations as indicated by low rates of protein evolution relative to DNA sequence evolution (d*N*/d*S* values <0.1) (FIG. 2a)[11,21].

Open systems can include many symbiont species, as in human and termite guts, or few species, as in honeybee guts, or even a single species, as for *Aliivibrio fischeri* in light organs of bobtail squid and *Burkholderia insecticola* in midgut crypts of the bean bug[5,23]. Most open symbioses involve extracellular symbionts that are exposed to the outside environment, such as symbionts associated with guts, and with surfaces of corals and sponges[24]. However, some involve intracellular symbionts, including the sulfur-oxidizing and methane-oxidizing bacteria that live as multiple strains within bacteriocytes of *Bathymodiolus* deep-sea mussels[25,26].

Closed symbioses. In closed symbioses, symbiont lineages are clonal, often due to strict maternal transmission. Clonality and population bottlenecks impose small effective population sizes and genetic drift, which leads to the degradation and diminution of genomes and loss of functions; these features have been documented repeatedly through genome sequencing of symbiotic bacteria in insects and other invertebrates[6,18,27] (FIG. 2b). Closed symbioses are often millions of years old as evidenced for the symbioses of many insects[27] and of gutless marine flatworms[28] by matching molecular phylogenetic trees of symbionts and hosts calibrated for host lineage age using fossil evidence (FIG. 3a). Commonly, such symbionts provide crucial services to hosts, such as the provisioning of essential amino acids and vitamins. As a consequence, these are usually mutually obligate associations, required for host development and reproduction. In long-established closed symbioses, symbionts are effectively fused with hosts, approaching the status of organelles[13]. Prime examples include bacterial clades that are restricted to living only in a given group of insect hosts: *Buchnera aphidicola* in aphids, *Blochmannia* spp. in carpenter ants, *Blattabacterium* spp. in cockroaches, and *Sulcia muelleri* in leafhoppers and related insects[27]. However, strict uniparental transmission is not required; closed symbioses include any cases where exclusive colonization by a single symbiont strain eliminates the opportunity for inter-strain recombination. Thus, recent analyses of genome sequences of light organ symbionts of anglerfish show that they occupy host organs as single clones and exhibit genome reduction, even though they are acquired environmentally and do not show codiversification with host matrilines[29]. In many closed symbioses, bacteria live within specialized host cells (for example, REF.[30]) but they may be extracellular, as in the pectinase-producing *Stammera* symbiont of tortoise beetles[31] and midgut crypt symbionts of urostylidid, parastrachiid and plataspid stinkbugs[32–34]. Although closed symbiotic systems have been documented most extensively for insect hosts[27], parallel cases are known from other groups, including anglerfish[29], tunicates[35], clams[36], marine flatworms[8] and protists[13,37].

Mixed symbioses. Some symbioses involve host-restricted bacteria that are routinely transmitted maternally but that occasionally jump between host matrilines within and, sometimes, between species. Examples of mixed systems include *Wolbachia* spp. in arthropods and *Hamiltonella defensa* in aphids; these are predominantly transmitted through direct infection of progeny within the mother but phylogenetic analyses show that they occasionally undergo horizontal transfer to novel hosts[38,39]. Mixed symbioses share features with both open and closed symbioses, depending on their potential for recombination. Symbiont genomes may recombine and acquire genes within co-infected hosts but they undergo loss of ancestral genes, genome shrinkage and accelerated sequence evolution as a result of clonality and genetic drift. Rates of mutation and genome rearrangement can be extremely high; for example, experimental evolution studies revealed that *Spiroplasma* symbionts within laboratory stocks of *Drosophila* spp. undergo rapid changes as evident both from genomic sequencing and observation of symbiont-based host phenotypes[40]. Outbreaks of transposable elements, large deletions and rearrangements are typical in symbiont genomes of mixed systems (FIG. 2c)[41]; these are largely absent from genomes of closed symbioses, which lack mobile elements and exhibit gene order conservation[42]. Horizontal transmission, even if infrequent, erases signatures of co-cladogenesis with hosts (FIG. 3b) and generates occasional co-infections, thereby creating arenas for genetic exchange and the acquisition of novel genes via bacteriophage or other mobile units[43]. Genomic signatures of mixed systems depend both on the frequency of horizontal transmission and on the age of the symbiosis[2,6,17,36,44]. Symbionts in mixed systems can be deleterious and/or beneficial to hosts. For example, *Wolbachia* is often a reproductive parasite that lowers the fitness of male hosts but also protects hosts against pathogens or contributes to nutrition[45–47].

Insights into symbiont evolutionary routes from genomic sequencing. The onslaught of genomic sequencing of symbionts is the main basis for recognizing these different symbiotic categories as the same genomic syndromes have emerged across bacterial phyla and across various animal hosts. For example, symbiont genomic features, such as large size and evidence of ongoing HGT, are similar across open symbioses within guts of mammals[7], termites[48] and honeybees[49], in *A. fischeri* within the bobtail squid light organ[50], and in *Curvibacter* species within the glycocalyx of *Hydra* species[51]. In closed symbioses, the shared features of genome reduction and lack of HGT are repeatedly observed for Bacteroidetes and Proteobacterial symbionts of various insect orders[18].

Caveats in categorizing symbioses. Assigning symbioses as open, closed or mixed is often clear-cut but not always. Closed systems can be readily categorized when they are ancient and exhibit pronounced genome reduction and divergent sequences[27]. However, some younger symbioses that are strictly clonal have not reached these extreme states as distinguishing genomic features emerge

slowly. In the early stages of a closed symbiosis, genomes typically accumulate recently inactivated pseudogenes but the initial genome shrinkage is not abrupt[6,52]. This point is illustrated by a study comparing genomic features of symbiotic *Burkholderia gladioli* strains in the beetle *Lagria villosa*. Some have normal-sized genomes with few pseudogenes, are cultivable and able to infect plants, from which they are newly acquired by

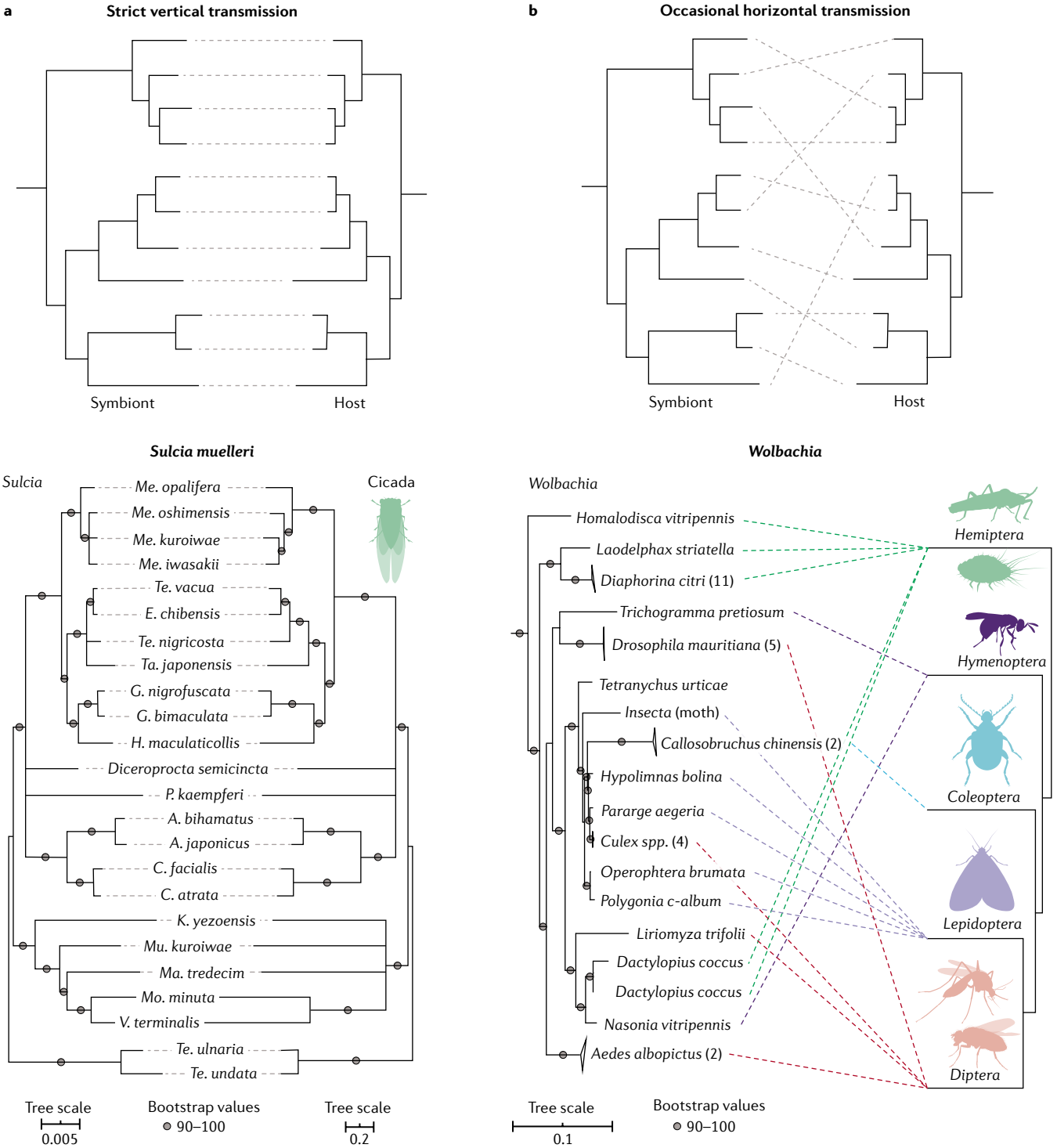

Fig. 3 | **Symbiont phylogenetic patterns depend on the frequency of horizontal transmission. a** | Symbionts that are strictly vertically transmitted, as in closed symbioses, exhibit co-cladogenesis with their host after long timescales (top). For example, *Sulcia* has co-diversified with insects in the suborder Auchenorrhyncha, including cicadas[122] (bottom). **b** | In mixed symbioses, symbionts are predominantly vertically transmitted but occasional horizontal transmission results in a mismatch of host and symbiont phylogenies over long timescales (top). For example, *Wolbachia* undergoes vertical transmission in many arthropods but shows little signal of codiversification with hosts[135] (bottom). A simplified insect phylogeny (based on data from REF.[184]) is provided for reference. Part **a** is adapted with permission from REF.[122], National Academy of Sciences. Part **b** is adapted with permission from REF.[135], CC BY 4.0 (https://creativecommons.org/licences/by/4.0/).

each beetle generation. However, one strain (*B. gladioli* Lv-StB) has a somewhat reduced genome, abundant pseudogenes (1,149 pseudogenes and only 744 intact and non-hypothetical genes) and accelerated sequence evolution, pointing to a lifestyle shift to host restriction and clonality[53]. Similarly, genomic analyses of marine bivalve symbionts reveal widely varying levels of genome reduction corresponding to the extent to which transmission is vertical versus horizontal[36] and open, mixed, and closed lineages occur in the genus *Sodalis*, with radical consequences for genome size and architecture[6]. Despite uncertainties in categorizing every system, recognizing these categories allows insight into why symbiotic systems display different genomic features and routes to innovation.

Innovations in open symbioses

Open symbiotic systems enjoy many avenues for innovation as well as for deterioration. Strains can be lost and gained and persisting residents can evolve through mutation, drift, selection and recombination (FIGS 1,4). Lineage evolution often features HGT, whereby a symbiont gains genetic material and associated functions from unrelated bacteria, potentially with consequences for hosts. Co-resident, conspecific strains can undergo homologous recombination, preventing or slowing mutation accumulation and avoiding the clonal interference that otherwise slows adaptive evolution. The relative contributions of these processes to innovation at the community level varies and each process has the potential to either benefit or harm hosts.

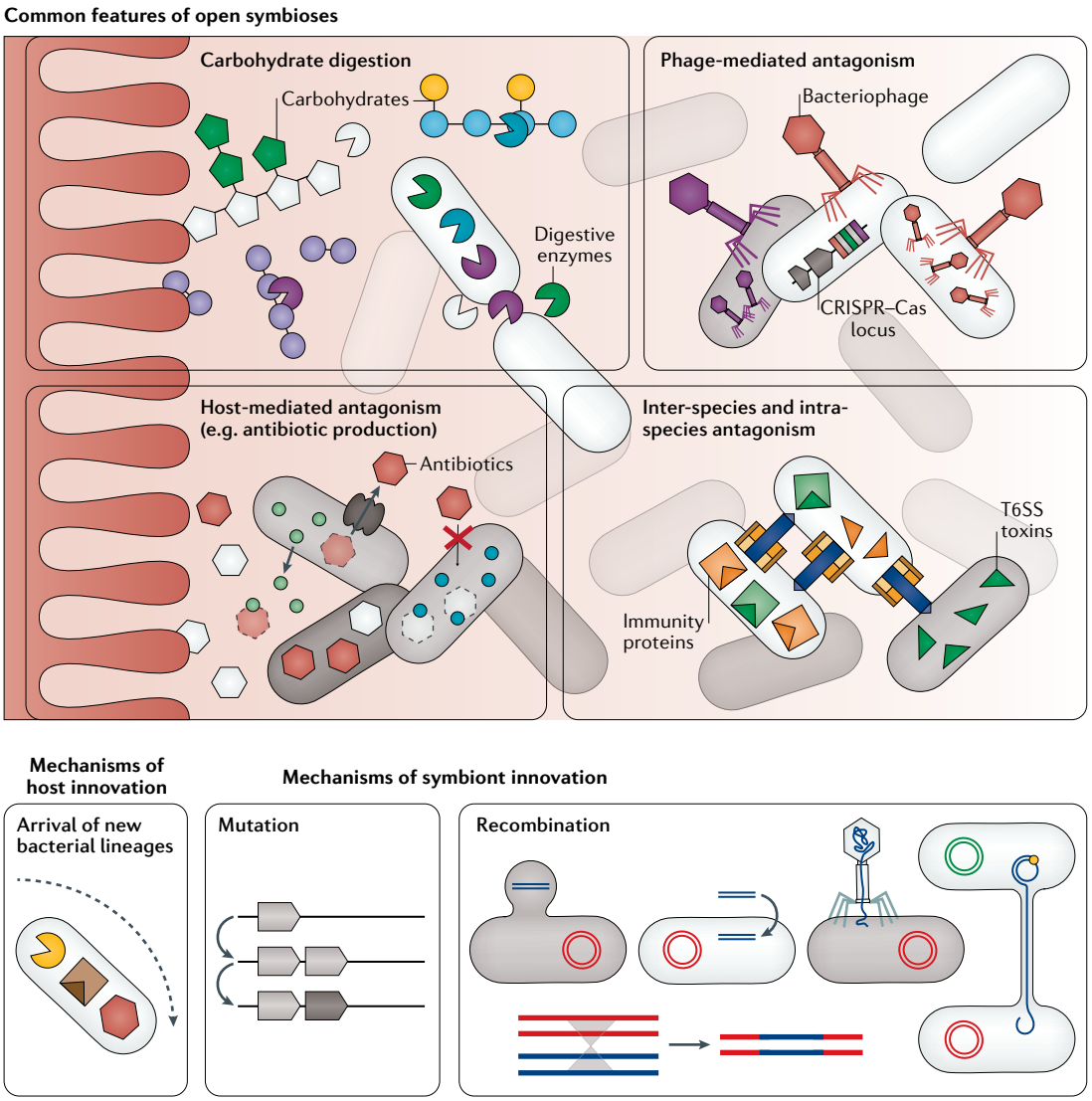

Fig. 4 | **Common features and mechanisms of innovation in open symbiotic communities.** Bacteria in open symbiotic communities face diverse selection pressures shaped by abiotic perturbations, such as changes to diet (top panel, top left), antibiotics (top panel, bottom left) and temperature, interactions between co-residing microbes such as phage-mediated antagonism (top panel, top right) and T6SS-mediated antagonism (top panel, bottom right), and host social behaviours and immune response. In these communities, innovation to maintain the symbiosis or to adapt to changing conditions is commonly accomplished through the introduction of new strains (bottom panel, left), through mutation (bottom panel, centre) and through recombination (including horizontal gene transfer), which can be mediated by extracellular vesicles, transformation, transfection or conjugation (bottom panel, right); strong natural selection acts on the resulting variants.

Strain recruitment and loss. In open symbioses, effects on host biology depend on the composition of the symbiotic community. Sometimes, many species or strains contribute to emergent phenotypes such as polysaccharide digestion or protection from pathogens. The disturbance of complex communities of symbionts can have long-term consequences for host health. For example, mice that are experimentally fed a diet deficient in complex polysaccharides experience shifts in gut microbiome composition and functionality, including irreversible losses of certain polysaccharide-digesting strains[54]. These changes can leave hosts unable to digest complex polysaccharides, even if polysaccharides are later re-introduced to the diet[54]. More generally, widespread antibiotic exposure is hypothesized to lead to disrupted gut microbiomes in populations of humans and honeybees, potentially impairing host health[55,56].

Hosts that depend on services of open microbial communities possess innovations to ensure that symbiotic strains are recruited and maintained. For both complex and simple communities, this can include behavioural adaptations. Transmission of gut microbial communities to other hosts through familial contact is common in hominids[57,58], termites[59], and social bees[60] and can produce signatures of co-evolution over long time periods[57,61,62]. In relationships where colonization occurs every generation, hosts possess innovations that allow them to filter potential colonizers so as to bar non-symbionts. The stinkbug *Riptortus pedestris* acquires bacterial symbionts from the soil at every generation. Although various bacteria enter the foregut, a specific constricted midgut region filters for motile *Burkholderia* symbionts and close relatives, then strain competition within the symbiotic organ results in an exclusive partnership[63,64]. Similarly, bobtail squids restrict colonization of the symbiotic organ by *A. fischeri* strains, in part by selecting strains on the basis of their beneficial activity of light production[65–68]. Strains use several strategies to compete within symbiotic crypts, sometimes forming stable strain mixtures in hosts[5,50,68]. In some cases, host adaptations may control symbiont proliferation as appears to be the case for the *Hydra–Curvibacter* symbiosis. The 4.37 Mb genome of *Curvibacter* includes two quorum-sensing operons and *Curvibacter* symbionts produce signalling molecules that are subsequently modified by host-encoded enzymes, resulting in dramatic shifts in symbiont gene expression and phenotype[51]. These shifts enable the robust colonization of host tissues and modulation of the *Hydra* innate immune system through the reduced production of flagellin, a trigger for host Toll-like receptors.

The reliable colonization of hosts also depends on microbial adaptations. A survey of symbiont genomes from systems in which symbionts are recruited from the environment showed the consistent presence of genes enabling both flagellum-based motility and chemotaxis; both functions are typically lost from most maternally transmitted symbionts[69]. In zebrafish, gut bacteria colonize from the surrounding water and, in experimental populations selected for host colonization ability, enhanced motility was the dominant adaptation[70,71]. Another challenge for symbionts is the need to modulate immune responses triggered by bacterial cell envelope components; for example, *Bacteroides* in the human gut microbiota dampens inflammation by modifying cell-surface molecules[72].

In open symbiotic communities, recruitment is governed not only by whether symbionts reach the symbiotic organ but also by interactions within the microbial community, both antagonistic and cooperative. Symbionts in open communities harbour extensive machinery devoted to the competition for nutrients[73,74] and to weaponry for inter-strain and inter-species warfare[75]. For example, type VI secretion systems, used to kill competing Gram-negative bacteria, are abundant and diverse in *Bacteroidales* in the human gut[75] and in *Proteobacteria* in the bee gut[76] and are used by competing *A. fischeri* strains within host crypts[77]. Other mechanisms also mediate bacterial antagonism, with varying levels of target specificity. *Escherichia coli* and other *Enterobacteriaceae* within the gut compete using microcins, which are peptides with potent toxicity for a restricted range of competing bacterial strains[78].

Evolution of resident strains. Strain turnover is not the only mechanism for change in open systems. Persisting strains can evolve within hosts, sometimes over short timescales. Analyses of genomic data for 40 dominant species in the human gut revealed that, in just a few months, strains underwent sequence evolution of existing genes, gene acquisition via HGT, and gene loss and that certain novel variants spread rapidly, implying strong positive selection[20]. Strain evolution can be fast enough to be captured by laboratory experimental evolution approaches: commensal *E. coli* strains introduced into mouse guts experience bursts of adaptive evolution over very short timescales (months)[79,80]. Strains were found to rapidly evolve enormous variation in mutation rates due to mutations in repair genes, which accelerated strain divergence[80]. Beyond mutations in existing genes, experimental evolution studies in which multiple strains were present in mouse guts showed that strong selective sweeps can be seeded by phage-mediated HGT conferring adaptive traits such as the ability to metabolize a new carbon source or resist antibiotics[79]. Other mutations, including deleterious ones, can hitchhike on positively selected haplotypes and then persist[80]. In the long-term, negative selection (against new deleterious mutations) is effective in preserving functions of ancestral genes as shown by the relative frequencies of changes at non-synonymous and synonymous sites within protein-coding genes (dN/dS ratios)[20]. Furthermore, over long timescales, strain turnover in gut communities may limit the extent of within-host adaptation of strains[20].

Whole-genome sequencing of multiple bacterial isolates from particular symbiont species has revealed that HGT, often involving bacteriophage or other mobile units, is the most potent source of novelty, generating distinct gene sets for individual strains[11,81]. Such strain-specific 'accessory' genes often confer new capabilities. One large-scale analysis of multiple strain genomes from species of *Bacteroides* (dominant and well-studied members of human gut microbiomes)

Heterologous expression
Expression of a gene in an alternative, genetically tractable host.

Trophallaxis
The exchange of food through an oral-to-oral or faecal-to-oral transmission route, commonly performed by members of the same community.

revealed that strains share only several hundred core (universal) genes but that pooled gene sets of all strains for a species (pan-genomes) can contain over 70,000 accessory genes[82]. *Bacteroides dorei* strains that are nearly identical for sequences of shared genes contain hundreds of strain-specific genes, often associated with bacteriophage[83]. Similarly, 48 *Gilliamella apicola* genomes from honeybee guts encoded 1,480 core genes but 4,408 accessory genes[84,85]. Even single-symbiont systems, exemplified by *A. fischeri*, include many strains differing in accessory gene sets[5,50] (FIG. 2a).

Repercussions of gene gain and loss. Genomic sequencing, which enables the specification of complete gene repertoires, has shown that HGT is implicated in every kind of symbiont adaptation in open symbioses, including changes related to colonizing hosts. For example, metagenomic analyses of the open symbiont communities of sponges revealed numerous phage-associated genes encoding ankyrin proteins, which are known to modulate cellular immune responses of diverse animal phyla[86]. Using both synthesized proteins and heterologous expression in *E. coli*, researchers showed that these phage-encoded ankyrins increase bacterial persistence when exposed to mouse macrophages and dampen transcriptomic signatures of immune responses that are widely conserved across animals. Based on analyses of genomes of bacterial gut symbionts, genes underlying toxin and secretion systems, which function in strain competition, are exchanged frequently among community members and are among the most dynamic genomic elements[76,87,88]. Such antagonistic interactions can result in the exclusion of invaders, potentially protecting hosts from pathogenic infection.

HGT also introduces a regular influx of new enzymatic capability into open communities, permitting symbionts to better adapt to host ecology while potentially benefiting hosts. Gut bacteria of herbivorous or omnivorous hosts often secrete carbohydrate-active enzymes (CAZymes) that degrade complex polysaccharides, providing access to the energy stored in plant cell wall components. In *Bacteroidales* spp. of human guts, strains have distinct repertoires of CAZyme loci[89,90] and similar variation occurs among *Gilliamella* and *Bifidobacterium* strains of honeybee guts[85,91]. HGT between *Spirochetes* species in termite gut communities enables the digestion of complex plant polysaccharides[48]. A combination of genomic sequencing, strain isolation and protein biochemistry was used to show that the specific polysaccharide utilization locus for digestion of the algal polysaccharide porphyran was transferred from marine bacteria to the human gut *Bacteroides* in populations whose diets regularly include seaweed[92].

Although metagenomic sequencing can provide insights into the functional capabilities of bacterial communities as a whole, the typical short-read sequences do not resolve the frequency and range of gene movement among strains through HGT. However, new sequencing methods using long-reads or proximity ligation methods are beginning to give a clearer picture of HGT in open symbioses as exemplified by a study showing the rapid transfer of antibiotic resistance genes across species within gut communities of humans both in the presence and absence of antibiotic treatment[93].

Differences in gene sets among symbionts reflect strain-specific gene loss in addition to HGT. In densely packed, host-associated communities, strains may lose genes that become superfluous due to the metabolic contributions of other community members. A result is that strains or species rely on one another for essential metabolites, a relationship referred to as syntrophy or cross-feeding[94,95]. The co-dependence that arises from complementary gene losses, termed 'Black Queen' evolution[96], may help to stabilize community composition. Based on the reconstruction of genomes from metagenomic data for as-yet uncultivated strains in the human gut microbiome, individual strains often lack widely conserved biosynthetic pathways for vitamins, amino acids and essential fatty acid components of membranes, suggesting their uptake from other community members[7]. Such co-dependent symbiotic communities are vulnerable to invasion by strains that reap benefits but do not contribute to the cost of biosynthesis, making cooperative communities less stable than competitive ones[94,95]. Potentially, host adaptations might stabilize such communities by supporting persistent spatial clustering of cooperating cell lineages: this possibility is supported by recent experimental evolution studies of reciprocally dependent *E. coli* strains demonstrating that such clustering promoted cooperation[97]. Spatial clustering could be enhanced by host anatomical features or behaviours (such as trophallaxis) that promote co-transmission.

Innovations in closed symbioses

Transmission modes that enforce clonality, such as strict maternal inheritance, result in the long-term degradation of symbiont genomes with ongoing gene loss and little or no HGT (FIG. 5). The extent of symbiont genome reduction can be drastic, even for extracellular symbionts, as illustrated by a recent analysis of the tiny genomes (215–310 kb) of the maternally transmitted, extracellular *Stammera* symbionts of tortoise beetles that provide pectin-digesting enzymes used for digesting dietary plant fibre[31,98]. Ongoing gene losses mean that, from the symbiont perspective, the association is a one-way street: established symbionts cannot revert to free-living lifestyles and cannot even switch to different host lineages. These ancient closed symbioses present a conundrum: how are these deteriorating symbioses maintained and how do they respond to changing ecological conditions?

Limitations to genomic decay. Genome decay, with different levels of severity, repeats itself in many closed symbioses; the constellation of changes, called the 'symbiosis rabbit hole', is perhaps the most distinctive genomic syndrome in prokaryotes[12]. These characteristic genomic features, reviewed previously, include tiny genomes with few genes and accelerated sequence evolution[3,6,18,27]. The functional losses include decay of central cellular functions as exemplified by thermally unstable gene products, loss of DNA repair capabilities, minimal sets of tRNA synthetases, impaired tRNA processing[99,100] and lowered translational efficiency[101]. Genome degradation often seems to be accelerated by the loss of DNA

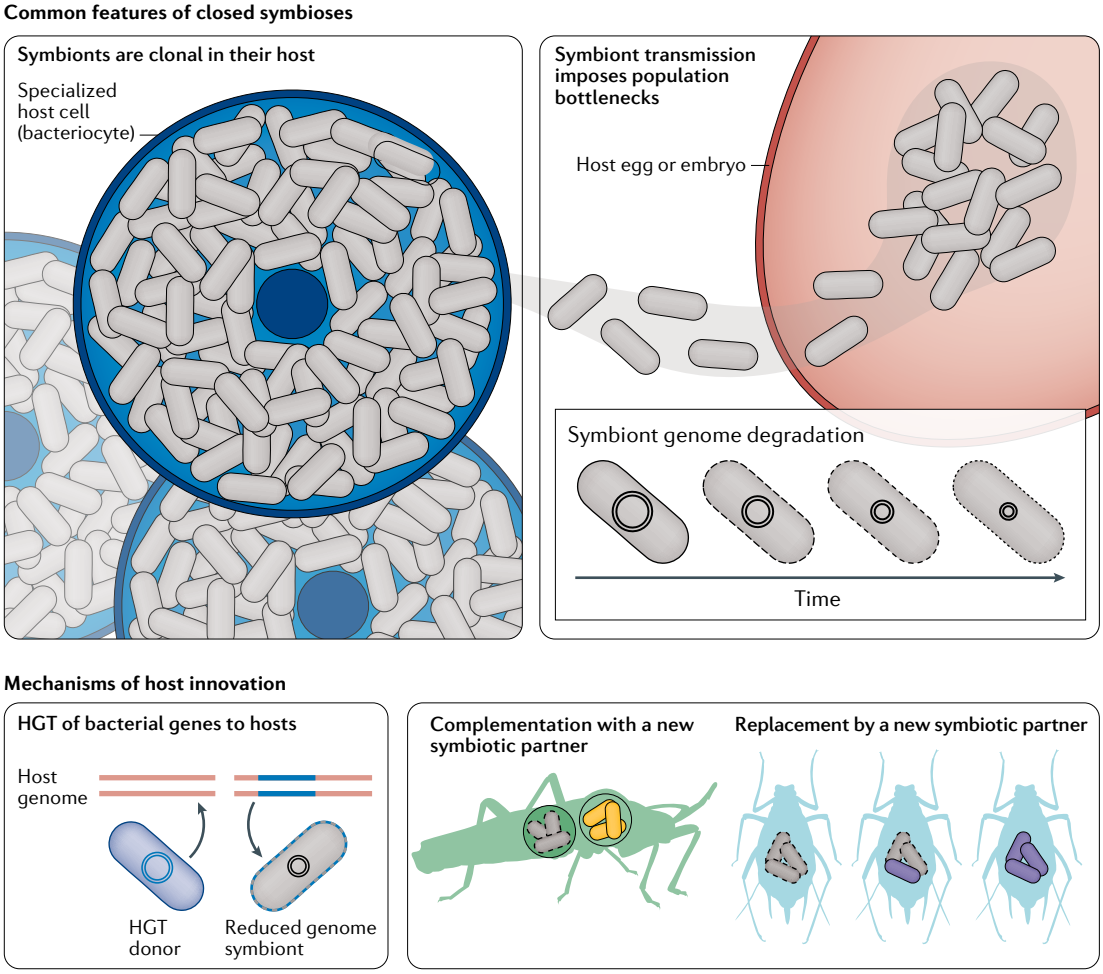

Fig. 5 | **Common features and mechanisms of innovation in closed symbiotic communities.** Closed symbiotic communities are clonal and face population bottlenecks when transmitted to offspring. As a consequence, bacteria in closed communities accumulate deleterious mutations that they are unable to purge and their genomes degrade with time (top). To maintain degrading symbionts, hosts innovate by acquiring bacterial genes from other bacteria, by acquiring additional symbionts or by replacing degraded symbionts altogether (bottom). HGT, horizontal gene transfer.

repair genes and the consequent increase in mutation rates, which are typically elevated to varying degrees in heritable symbionts[40,102].

One limit to genome reduction in obligate, heritable symbionts is the number of genes required to serve host needs. For example, symbionts of *Paracatenula* flatworms contribute relatively complex functions involving energy production and storage and retain relatively large genomes (1.34 Mb) despite the estimated age of 500 My for this symbiosis[8]. By contrast, *Sulcia muelleri* symbionts in sap-feeding insects are also ancient (~280 My) but have genomes of 0.15–0.28 Mb; the larger genome supplies eight essential amino acids and the smaller only three[27,103]. Sometimes a single symbiont retains genes underlying a variety of functions beneficial to hosts; thus, *Profftella* symbionts of the psyllid *Diaphorina citri* synthesize vitamins and carotenoids as well as polyketide toxins that function in host defence[104,105]. In another example, the reduced genome (~500 kb) symbionts of reed beetles (Donaciinae) alternate between provisioning amino acids during larval stages and secreting digestive pectinases during adult stages and transcriptome analyses show that the underlying genes are expressed at corresponding beetle life stages[106].

Innovations to ensure transmission. When host progeny require symbionts for survival, hosts show adaptations to ensure transmission as revealed by a variety of microscopy methods often using fluorescent in situ hybridization to resolve symbiont cells[17]. In gut-inhabiting obligate symbionts, mothers sometimes deposit an inoculum on or near eggs to be ingested by newly hatched progeny. This transmission route can involve striking adaptations: in the extracellular midgut symbionts of stinkbugs (family Urostylididae), large ovaries produce a voluminous jelly-like substance that contains symbiont inocula as well as nutrition upon ingestion by hatchlings[33]. Plataspid stinkbug females produce massive amounts of a specialized protein that is deposited with the reduced genome symbiont, *Ishikawaella*, during transmission within maternally produced capsules; RNAi knockdown of this host protein results in transmission failure[34]. Many other heritable symbionts colonize eggs or progeny within the mother's body using a variety of routes. In whiteflies,

remarkably, an entire maternal bacteriocyte, containing a nuclear genome as well as resident *Portiera* symbionts, is transferred into the egg; sequencing of germ-line and bacteriocyte genomes show that the transferred bacteriocyte persists throughout development and forms a genetic lineage divergent from the main germ-line lineage[107]. More often, symbionts are transmitted to eggs or embryos as microbial inocula. This transfer can be largely host controlled as in aphids, in which the passive *Buchnera* symbionts lack flagella and mobility[30]. Alternatively, transmission can require symbiont participation as in tsetse flies, in which transcriptomic and immunohistochemistry analyses show that *Wigglesworthia* symbionts activate flagellar motility machinery to colonize developing larvae via maternal milk glands[108]. In some hosts, symbionts must colonize different tissues during host development. For example, *Sodalis pierantonus* moves between larval and adult bacteriomes during the development of cereal weevil hosts. A combination of bacteriocyte imaging and RNA sequencing revealed that this movement is achieved through a coordinated sequence of changes in host and symbiont gene expression and cellular features, including migration of the larval bacteriocytes to a new location and activation of the symbiont type III secretion system machinery upon colonization of the adult bacteriocytes[109].

Innovations to compensate for genomic degradation. The one-way ratchet towards ever more genomic degradation and loss of function can lead to extreme outcomes as has been elucidated recently by large-scale genome sequencing of symbionts in many closed symbioses. In *Buchnera* symbionts of aphids, sequencing of genomes from across the host phylogeny reveals an unrelenting ratchet of gene loss in each lineage, with this loss more pronounced for some loci and some lineages[42]. Likewise, in cicadas, genomes of the symbiont *Hodgkinia* often incur deletions of essential genes, requiring hosts to maintain multiple *Hodgkinia* genomes with complementary gene sets[110,111].

How do closed symbioses persist, despite ongoing losses of genes and functions? Some endosymbiont genomes encode nearly complete biosynthetic pathways, with only a single enzyme not encoded, suggesting that another gene has expanded function to complete the missing step[4]. While functional studies of non-cultivable symbionts are challenging, one approach to study gene function is to use heterologous expression in a laboratory model. Using heterologous expression in *E. coli* missing the same gene, a *Buchnera* enzyme from the branched-chain amino acid pathway was shown to have expanded its substrate affinity so as to complete a missing step in pantothenate biosynthesis[112]. Thus, promiscuous enzyme activities may sometimes enable a reduced genome to retain capabilities.

Even for genes that are retained, ongoing mutation accumulation in closed systems results in the thermal instability of proteins, such that symbionts are highly heat sensitive, which can, in turn, limit the thermal range of the host[113,114]. A conspicuous feature, observed repeatedly for symbionts in closed systems, is the constitutive overexpression of molecular chaperones, including GroEL[115], which has been shown to compensate for the effects of destabilizing mutations[116]. Proteomic analyses of *Buchnera* cells using mass spectrometry show that GroEL constitutes up to 10% of protein and other chaperones are also abundant[117].

Functional novelty in closed symbioses. In closed symbioses, adaptations to preserve the symbiosis largely fall to the host and recent discoveries show two surprising routes (FIG. 5). First, genome and transcriptome sequencing has revealed that hosts themselves acquire horizontally transferred bacterial genes that are expressed exclusively or primarily in bacteriocytes (Supplementary Table 1). For example, mealybugs harbouring *Tremblaya* and *Moranella* and aphids harbouring *Buchnera* have acquired genes underlying the biosynthesis or recycling of peptidoglycan components[118–120]. Many leafhoppers possess two bacterial symbionts, each housed in a distinct bacteriocyte type characterized by distinct gene expression profiles, which are predicted to complement the capabilities of the resident symbiont type. These bacteriocyte-specific genes include numerous genes acquired through horizontal transfer from bacteria as well as ancestral host genes that seem to acquire novel functions in bacteriocytes[121].

A second evolutionary route to innovation by hosts in closed symbioses is the gain of new microbial partners that retain intact pathways for supporting themselves and their hosts (Supplementary Table 2). A combination of genome sequencing, transcriptome analyses and phylogenetic reconstruction shows that these new symbionts may supplement or supplant ancient symbionts. For example, in cixiid planthoppers, two ancient symbionts (*Sulcia* and *Vidania*) with tiny genomes (157 kb and 136 kb, respectively) are joined by a novel symbiont, *Purcelliella* (Enterobacterales)[103]. *Purcelliella* is closely related to plant pathogens and retains a somewhat larger genome (480 kb) that encodes pathways for the biosynthesis of B vitamins and of cysteine, the latter of which may complement the metabolites needed for methionine synthesis by *Vidania*. Likewise, multiple cicada lineages have replaced their *Hodgkinia* symbionts, which have fragmented and deteriorated genomes, with symbiotic fungi[122]. In lachnine aphids, *Buchnera* is co-resident with *Serratia symbiotica* strains that have taken over amino acid biosynthesis functions and the acquisition of this novel symbiont has enabled further erosion of the *Buchnera* genomes[123]. Blood-feeding ticks rely on bacterial endosymbionts for B vitamin biosynthesis and some tick species have replaced the more ancient *Coxiella* symbiont with a *Franciscella* partner experimentally demonstrated to serve this function[124]. Potentially, replacing an ancient, degraded symbiont with a more robust one can trigger loss of host support mechanisms. Thus, sharpshooters (Cicadellinae) have replaced the ancient *Nasuia* symbiont with a newer arrival (*Baumannia*) and transcriptome studies of the distinct bacteriocyte types show that those housing *Baumannia* express fewer host genes predicted to assist symbionts with cell envelope generation and central information processing[125].

In some cases, host genes have undergone adaptation to control and support symbionts with highly

reduced capabilities. For example, *Buchnera* receives non-essential amino acid substrates abundant in the aphid diet and returns the essential amino acids required by hosts. However, *Buchnera* genomes have lost genes for membrane-bound transporters; instead, immunolocalization studies show that host-encoded transport proteins are localized to both the bacteriocyte membrane and the host-derived 'symbiosomal' membrane enclosing each *Buchnera* cell[126]. Furthermore, the expression of these transport proteins in frog oocytes revealed their capacity to transport multiple amino acids between the insect body cavity, bacteriocyte cytoplasm and the symbiosomal space surrounding each symbiont cell, in some cases using feedback regulation to adjust the movement based on host needs[4,126,127].

Innovations to evade immune responses. A challenge for all animal–bacterial symbioses is that of establishing stable, regulated populations despite innate immune pathways, which are universal in animals and are triggered by widespread components of bacterial cell envelopes. Obligate heritable symbioses often have solved this challenge through unusual modifications in hosts. One apparent solution, found in aphids, is the elimination or reduction of innate immune capabilities as revealed by the absence of many immune-related genes from the sequenced pea aphid genome[128] as well as by a lack of the usual insect immune responses following experimental challenge[129]. Additionally, the aphid enzymes AmiD and LdcA, acquired by HGT from bacteria and expressed in bacteriocytes, are predicted to degrade peptidoglycan components and the acquisition and expression of these genes have been hypothesized as a host adaptation to suppress remaining immune responses. However, RNA interference to knockdown the expression of these genes reduced *Buchnera* numbers, suggesting that these HGT products support *Buchnera* growth[130].

A constitutive reduction of innate immunity is likely only possible for organisms such as aphids, which use largely sterile diets (phloem sap) and have short lifespans that minimize pathogen impacts. In contrast to aphids, cereal weevils maintain a complete set of innate immune pathways but express a bacteriocyte-specific isoform of peptidoglycan recognition protein (PGRP); the bacteriocyte PGRP isoform was shown experimentally to cleave tracheal cytotoxin (TCT), a symbiont-derived peptidoglycan component that otherwise causes a systemic immune response[131]. Furthermore, experiments using RNA interference to knockdown PGRP resulted in TCT escape from the bacteriome and a deleterious systemic immune response.

Evasion of immune responses may also be accomplished in part by adaptive gene losses in symbionts: obligate symbionts commonly lose genes involved in the synthesis of cell envelope components, including peptidoglycan components and outer membrane proteins that would otherwise trigger host immune responses[27]. In several cases in which a more ancient and more recent symbiont reside within a host, microscopy studies combined with genome sequencing have revealed that the more recent symbionts, which retain normal Gram-negative cell walls, sequester themselves within the cytoplasm of the more ancient symbiont that lacks cell wall components[132–134]. These rare instances of a bacterium living within another bacterium may represent mechanisms by which a new symbiont can avoid host immune receptors.

Innovations in mixed symbioses

Similar to symbionts in closed systems, symbionts in mixed systems are predominantly vertically transmitted and clonal within their hosts. However, they are also occasionally transferred to other host lineages of the same or different species. Upon arrival in a new host, symbiont success depends on genetic innovations that allow them to evade the host immune response, replicate without excessive virulence, achieve vertical transmission and alter hosts in order to increase the frequency of infected matrilines (FIG. 6). The best-studied and most widespread of such groups is *Wolbachia*. Phylogenomic analyses show that the success of *Wolbachia* has depended on its capacity for horizontal transfer between arthropod species and frequent HGT enabling the acquisition of symbiont-beneficial genes[135,136]. Other examples include lineages within *Hamiltonella*, *Riesia*, *Arsenophonus*, *Sodalis*, *Spiroplasma*, *Serratia* and *Rickettsia*. Based on surveys to date, mixed symbioses are concentrated in terrestrial arthropods, including diverse insects and ticks as well as many species important as disease vectors, agricultural pests, or beneficial biocontrol agents.

Innovations for establishment and spread. Mechanisms for achieving vertical transmission vary among symbiont groups. Experiments with mutant *Drosophila melanogaster* lacking a functional yolk protein receptor revealed that *Spiroplasma* symbionts invade eggs via a conserved pathway for endocytosis of yolk protein, a route that may also be used by other symbionts[137]. Sometimes, symbionts co-opt the transmission routes of more ancient obligate symbioses as in facultative symbionts of aphids that enter progeny via the route used by the obligate symbiont, *Buchnera*[30,138]. Other bacterial lineages have repeatedly managed to enter new hosts: a prime example is *Sodalis*, a clade that has formed independent, maternally transmitted symbioses in diverse insects, including tsetse flies[108], grain weevils[131], spittlebugs[139] and mealybugs[133]. This repeated success at symbiotic life reflects a pre-adaptation: when an isolate of the proto-symbiont *Sodalis praecaptivus* is experimentally introduced to tsetse flies, it uses quorum sensing to attenuate virulence, enabling host survival and transmission to progeny[140].

Highly successful symbionts in mixed systems possess a variety of genetic innovations that enable them to increase the proportion of infected matrilines within host populations (FIG. 6). Many, including *Wolbachia*, *Rickettsia* and *Spiroplasma* in arthropods, act as reproductive manipulators. They shift progeny sex ratios towards females, kill sons or cause infected males to sterilize uninfected females[141,142]. The underlying mechanisms are diverse. For example, within *Drosophila* hosts, both *Spiroplasma* and *Wolbachia* target the X chromosome dosage compensation mechanisms to selectively kill male progeny but employ different mechanisms; *Spiroplasma* use an ankyrin-associated peptide

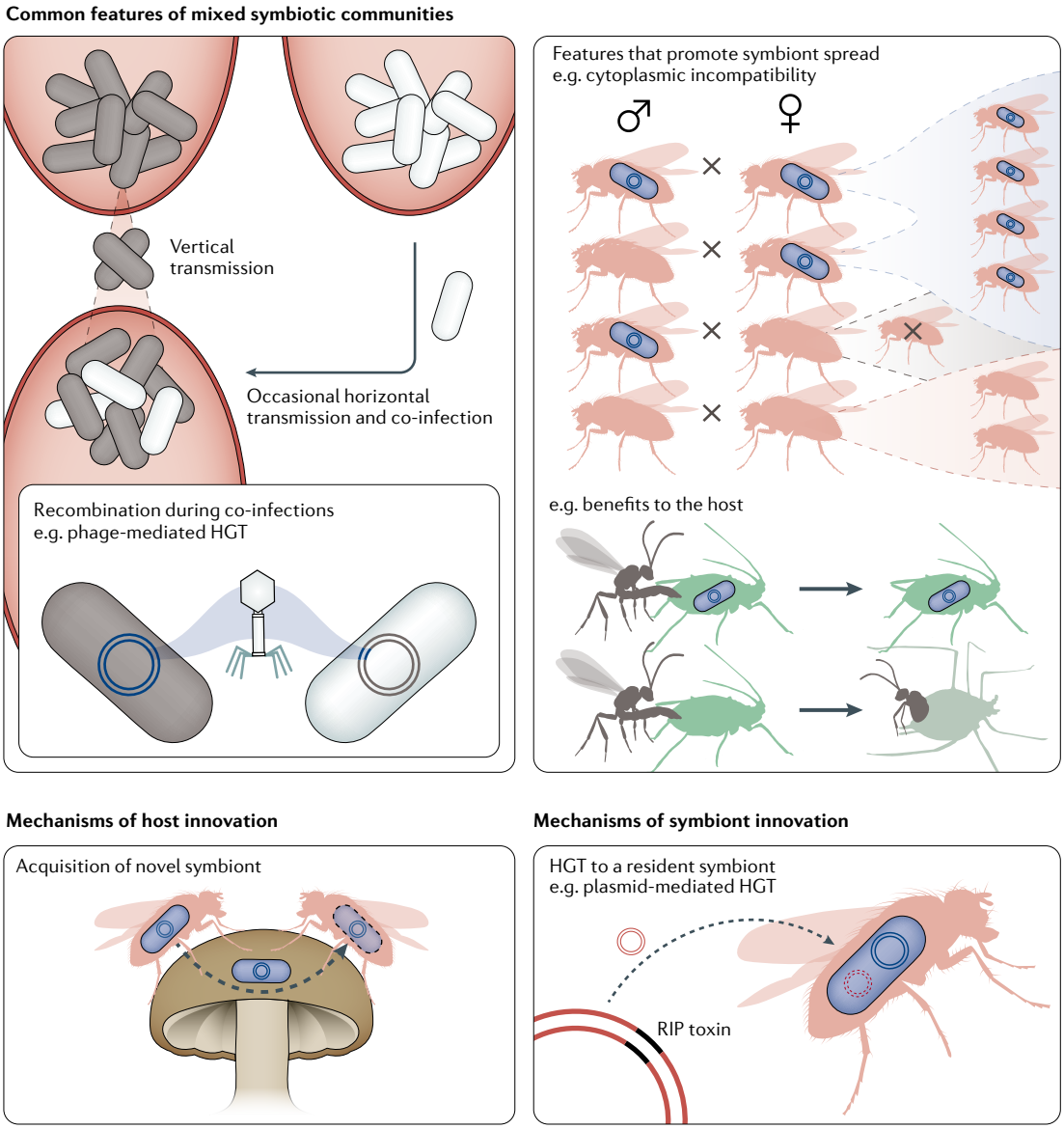

Fig. 6 | **Common features and mechanisms of innovation in mixed symbiotic communities.** Mixed symbiotic communities are mainly clonal because of ongoing vertical transmission but symbionts are also occasionally acquired from other hosts or the environment. Symbionts that co-infect a host can recombine and exchange genes through horizontal gene transfer (HGT), which is often mediated by phages (top left). Successful symbionts in mixed systems possess innovations that have helped them to infect new hosts and spread in host populations (top right). These innovations include the ability to manipulate host reproduction in a way that favours symbiont-bearing hosts (for example, cytoplasmic incompatibility, whereby infected males induce sterility of non-infected females) or to provide a benefit that increases host survival or reproduction (for example, by providing defence against parasitic wasps). Hosts can innovate by acquiring novel symbionts (bottom left) and symbionts are known to innovate through horizontal gene transfer (bottom right). These mechanisms of innovation are illustrated by the symbiosis between *Drosophila* flies and their defensive *Spiroplasma* symbionts[148]. RIP, ribosome-inactivating protein.

toxin[142,143] but the mechanisms are still unclear for *Wolbachia*, even though the responsible genes have been experimentally identified and shown to have varying potencies[144,145]. Recent comparative genomic analyses, discussed in published[146] and preprint[147] articles, show that these genes evolve rapidly and undergo frequent phage-mediated HGT.

Another symbiont strategy for expanding the proportion of infected matrilines is to provide direct fitness advantages to female hosts; this common effect is often combined with reproductive manipulation. These fitness advantages fall into two main categories: defence against parasites and nutritional support. In contrast to most closed systems, symbionts in mixed systems often defend hosts against natural enemies, swapping out novel mechanisms to meet the dynamic 'arms-race' nature of host–parasite co-evolution. One method for demonstrating these effects is pathogen challenges that compare the susceptibility of uninfected hosts with that of genetically similar hosts experimentally infected

with a symbiont. This approach has shown that heritable symbiont-based defence against parasites or pathogens is widespread in insect symbioses. Examples include protection by *Wolbachia* against insect viruses[45,46], by *Spiroplasma* against parasitic nematodes[148] and by *H. defensa* against aphid parasitoids[149–151]. These protective mechanisms are diverse but are usually based on genes acquired through HGT. Thus, the *Spiroplasma* symbionts of some *Drosophila* spp. have acquired varying repertoires of ribosome-inactivating proteins that protect hosts against both parasitic nematodes and wasps[148].

Mobile gene pools in mixed symbioses. A ubiquitous feature of symbionts in mixed systems is their ability to pick up new capabilities and quickly adapt — whether to benefit hosts by adopting new defences against natural enemies or to harm hosts by overcoming host resistance to reproductive manipulation. Comparative genome analyses point to a mobile gene pool shared among distant symbiont lineages, which have the opportunity to exchange genes within co-infected hosts. For example, the complete genomes of *H. defensa* and *Arsenophonus nasoniae* share numerous HGT cassettes that are also present in other insect symbionts[41,152].

In the case of *H. defensa*, defence of aphid hosts against parasitoid wasps depends on phages that jump among symbiont strains. Recent comparative genomic studies reveal that the phages themselves undergo extensive exchange of gene cassettes that encode toxins active against eukaryotic parasites, including homologues of cytolethal distending toxin (CdtB)[43,153]. Remarkably, the gene encoding CdtB is sometimes transferred to the host nuclear genome as observed in some aphids and some *Drosophila* spp., suggesting that the defensive machinery is deployed directly by the host[154]. Likewise, genome sequencing surveys of the bacteriophage WO, which is central to *Wolbachia*'s adaptations for reproductive parasitism, show that WO is responsible for transferring the genes underlying both reproductive incompatibility[146] and male killing[155].

Symbionts in mixed systems sometimes supply nutrients to hosts, while also exerting selfish effects. In *Hamiltonella* and *Arsenophonus* strains living in whitefly species, the nutritional provisioning itself reduces proportions of sons as demonstrated by experiments that manipulate symbiont titre and nutritional status. Thus, the same process confers dual symbiont fitness advantages, increasing overall host fecundity while also biasing towards daughters[156,157]. In general, the genes underlying nutrient provisioning are stable within symbiont genomes and represent widespread bacterial biosynthetic pathways retained from non-symbiont ancestors. However, even genes underlying nutritional functions can jump between symbiont species as genomic analyses have revealed for vitamin-biosynthetic genes in *Erwinia*, *Sodalis* and *Hamiltonella* symbionts in insects[158].

Conclusions and future perspectives

The success of symbiotic relationships, including their ability to overcome changing environmental conditions, depends on genetic innovations accrued by either partner. Symbiont innovations include those that allow them to more successfully invade or compete in hosts or to influence host biology in ways that favour their own spread. Host innovations may allow for better transmission of beneficial symbionts to offspring or for better maintenance, support and control of symbionts.

Genomic sequencing has shown that symbiotic relationships evolve under the constraints of the underlying symbiont population structure and that the symbiont transmission route has major consequences for the kinds of genetic innovations available. In open systems, hosts freely sample diverse bacterial strains and genes from the environment and innovate by gain or loss of trait-bearing symbionts. Likewise, symbionts, as members of diverse pools, innovate by recombination including HGT, often mediated by phages. By contrast, symbionts in closed systems are strictly clonal and evolve largely through gene loss and genomic decay, leaving hosts with no other choice but to provide support to or to replace their symbionts. Additionally, in mixed systems, symbionts are mostly clonal but occasional horizontal transmission allows hosts to gain new symbionts and allows host-associated symbionts to acquire genes through HGT, often from one another.

The study of animal–microorganism symbioses has been complicated by the intractability of most hosts and symbionts. Bacterial culture has long been a prerequisite for common genetic manipulation tools such as mini-Tn7, recombineering and CRISPR–Cas9. However, many symbionts, especially those that reside intracellularly, have complex nutritional or environmental requirements that make them resistant to cultivation[9]. Several common approaches have been adopted to overcome these limitations (FIG. 7). In some cases, genomic data and empirical approaches have elucidated symbiont metabolism and thereby informed the development of axenic culture media[9,159,160]. In other studies, insect cell lines have been successfully used to culture symbionts, facilitating the sequencing of symbionts that reside at low densities in their host[43,161] and providing validation of genes underlying symbiotic functions. Analyses of *H. defensa* cultured in insect cell lines have confirmed phage toxins as the active killers of parasites of insect hosts, for example[150]. Other productive approaches include heterologous expression of symbiont gene products and experimental evolution studies, in which genetic changes in symbiont populations can be directly observed[86,112].

Genetic manipulation of bacteria is commonly accomplished by conjugation of plasmids from a donor to recipient. Conjugal or transduction-based strategies have succeeded for some culturable symbionts, including *Sodalis glossinidius*[162,163], and have enabled validation of genes involved in establishing symbiotic interactions[140]. Conjugation has also been used to produce *Asaia* and *Arsenophonus* strains with integrated fluorescent proteins for in vivo tracking of infections in insect hosts[164,165]. While culturability has long been a prerequisite for reliable conjugation, recent approaches with a single delivery vector now allow for in situ microbial genetics targeting specific DNA sequences or community members as shown in one published study and one preprint[166,167]. We are also witnessing an extension of genetic tools

Axenic culture
The culture of a single microbial strain, in the absence of additional strains or hosts, in laboratory culture media.

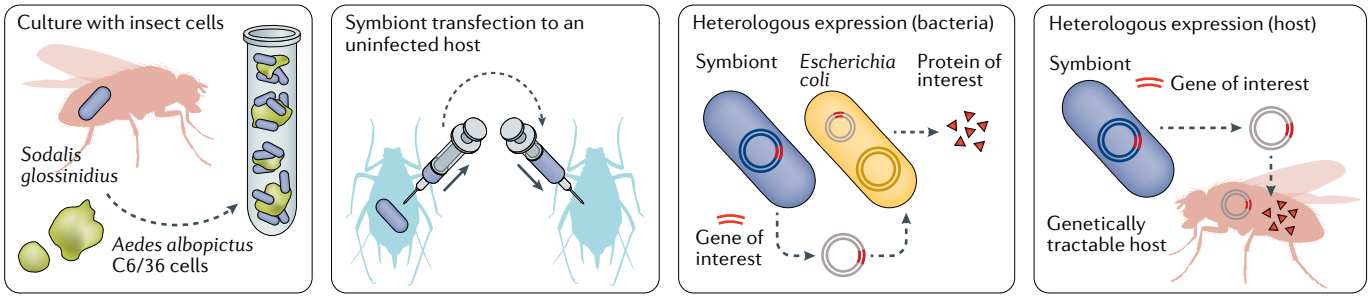

Fig. 7 | **Commonly used tools for the study of symbiont genetics.** Symbionts that live intracellularly often possess reduced genomes and are difficult to culture or genetically engineer, limiting the study of symbiont genetics. Some common strategies have been applied to overcome these limitations. Certain symbionts can be cultured in eukaryotic cell lines and others can be transferred from infected to uninfected hosts. Where sequencing has uncovered variation in gene content across symbiont strains, symbiont culture or symbiont transfer has been used to validate the role of certain host-beneficial genes. Lastly, symbiont gene function can be studied by heterologous expression, that is, expression of symbiont genes in genetically tractable bacteria or hosts.

to non-model, host-associated bacteria, producing resources that promise to facilitate studies of symbiont innovations in alternative host communities[10,168–170].

Advances in genomic sequencing have complemented experimental approaches to provide a better basic understanding of the genetic innovations underlying symbioses. Symbiont effects on host phenotypes have been identified by the transfection of uninfected hosts as for aphid symbionts, in which microinjection into hosts results in stably infected matrilines with altered resistance to parasites or to heat stress[171,172]. Transfection has also enabled the development of symbiont-based biotechnology for practical purposes such as fighting animal and plant diseases. *Wolbachia* strains that act as reproductive manipulators and suppress viral load have been exploited to reduce the capacity of crop pest insects to vector plant viruses[173] and the capacity of mosquitoes to vector human dengue virus[174] as well as to suppress vector population numbers via male sterility[175]. In other cases, the ability of symbiotic bacteria to colonize and persist within hosts makes them attractive chassis organisms for the delivery of synthetic pathways, thereby acting as living therapeutics. Recently, commensal *E. coli* of the human gut have been engineered to detect inflammation and for mitigating inflammatory bowel disease and phenylketonuria[176–178]. In mosquitoes, bacterial symbionts have been genetically manipulated to express anti-*Plasmodium* compounds that reduce vectoring capacity[179]. Furthermore, in honeybees, a specialized gut bacterium engineered to express double-stranded RNA was able to prime the RNA interference pathway of bees to protect them against viruses and mites, which are major causes of bee decline[180].

Although large-scale metagenomic sequencing is a major source of our knowledge of animal symbioses, the usual short-read metagenomic data cannot readily resolve genetic changes in individual symbiont lineages. Some new developments, including long-read sequencing, experimental evolution approaches and genetic engineering of non-culturable organisms, are just beginning to be applied to the study of symbioses and will enable finer scale elucidation of these changes. Likewise, studies of host innovations for symbiosis are relatively few, as non-model animals are often a challenge for genetic studies. This gap is starting to be filled. For example, a genome-enabled study of gene expression in the two symbiotic organs in the bobtail squid revealed distinct genetic underpinnings[181] and other studies have begun to elucidate the genetics and development of host organs that house symbionts[182]. Far better genome assemblies for hosts are now feasible, enabled by proximity ligation and long-read sequencing (for example, REF.[183]). These approaches, combined with experimental work, will help to illuminate the host's role in maintaining symbiotic partnerships. Thus, we can look forward to an ever-clearer picture of the innovations and constraints that govern the evolution of symbioses.

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