Trends in Microbiology

CellPress

Review

Engineering insects from the endosymbiont out

Katherine M. Elston,[1] Sean P. Leonard,[2] Peng Geng,[1] Sarah B. Bialik,[1] Elizabeth Robinson,[1] and Jeffrey E. Barrick [1,*]

Insects are an incredibly diverse group of animals with species that benefit and harm natural ecosystems, agriculture, and human health. Many insects have consequential associations with microbes: bacterial symbionts may be embedded in different insect tissues and cell types, inherited across insect generations, and required for insect survival and reproduction. Genetically engineering insect symbionts is key to understanding and harnessing these associations. We summarize different types of insect–bacteria relationships and review methods used to genetically modify endosymbiont and gut symbiont species. Finally, we discuss recent studies that use this approach to study symbioses, manipulate insect–microbe interactions, and influence insect biology. Further progress in insect symbiont engineering promises to solve societal challenges, ranging from controlling pests to protecting pollinator health.

Highlights

Insects are diverse and integral members of natural ecosystems. Some are pests that harm human health and agriculture, while others have beneficial roles.

Many insects have evolved associations with symbiotic bacteria that are far more intimate and persistent than our interactions with the human microbiome.

Insect symbionts from taxonomically diverse bacterial groups have been cultured and genetically engineered using a variety of techniques.

Genetically engineered symbionts have been used to study symbiosis, to prevent insects from vectoring pathogens, and to control their insect hosts.

Emerging synthetic biology tools will make it possible to use this paratransgenesis approach for new applications in more insect species in the near future.

Introduction

Insects are the most diverse animals on the planet. Over one million species have been described, and the true number is undoubtedly much higher [1]. Many insects have beneficial roles as pollinators or consumers of waste [2], while others are pests that threaten agriculture [3] or vector disease [4]. By studying insects, we can learn about important evolutionary and ecological processes and apply this knowledge to solve societal challenges. Some insects, such as fruit flies, are long-standing model organisms for genetics, and new technologies such as RNA-guided nucleases (e.g., CRISPR/Cas9) have made genetic engineering of new insect species possible [5,6]. However, the sheer diversity of their lifestyles and modes of reproduction has hampered progress in studying and controlling more insects through **transgenesis** (see Glossary).

An alternative to editing the genomes of insects is to engineer the **symbionts** that live within their bodies. Bacterial **endosymbionts** can reside in many different host organs and tissues, even inside insect cells. In some cases, these bacteria are passed directly to an insect's offspring and have evolved to resemble cellular organelles [7]. The guts of many insects harbor distinctive microbial communities that consistently include specific species of bacterial symbionts [8]. Insect–bacteria associations may be ancient and obligate, or they may be transient and show signs of ongoing biological conflict. It has long been recognized that bacterial symbionts represent a potential 'backdoor' for engineering insect biology [9]. Advances in synthetic biology and characterization of more microbial species associated with insects have created new opportunities to apply this **paratransgenesis** paradigm.

In this review, we briefly introduce the variety of insect–bacteria associations. Then, we describe genetic engineering approaches that have been successfully applied to different insect symbionts. Finally, we discuss how engineered symbionts are now being used to study insect biology, thwart pests and disease vectors, and protect pollinator health. We do not cover transplantation of nonengineered *Wolbachia* or other symbionts for control of mosquito-borne diseases, which is the subject of other recent reviews [10,11]. Instead, we focus more broadly and include cases in which symbionts of beneficial insects and vectors of plant diseases have been engineered.

[1]Department of Molecular Biosciences, Center for Systems and Synthetic Biology, The University of Texas at Austin, Austin, TX 78712, USA
[2]Department of Integrative Biology, The University of Texas at Austin, Austin, TX 78712, USA

*Correspondence: jbarrick@cm.utexas.edu (J.E. Barrick).

Insect–bacteria relationships

Insect–bacteria symbioses exhibit tremendous diversity in form and function. These relationships are the subject of several comprehensive reviews [12–16]. Here, we summarize aspects of these symbioses relevant to engineering them: how symbionts have coevolved with insects, where symbionts are found within insect bodies, whether and how hosts transmit symbionts to their progeny and other insects, and to what degree symbionts associate with specific insect species (Figure 1).

Interdependence: obligate or facultative?

The degree to which an insect and a microbe rely on one another for their continued survival is a key consideration when engineering a symbiosis. Some insects have **obligate symbionts** that are found species-wide in every individual, as they are necessary for the continued survival of the insect population. These symbionts, such as *Buchnera aphidicola* in various aphids, including *Acyrthosiphon pisum*, often supply nutrients that the host lacks in its diet [17]. Obligate symbionts usually show hallmarks of long-term coevolution with their insect hosts, such as extreme genome reduction [13]. Consequently, these symbionts generally cannot survive outside of the host and have not been **axenically cultured**. Though insects will die or fail to reproduce when their obligate bacterial symbionts are eliminated with antibiotics, rare replacements of obligate symbionts with other microbes that can fulfill the same roles in supporting insect fitness do sometimes occur and become permanent on evolutionary timescales [13]. For example, some Cerataphidini aphids have lost *Buchnera* and rely on a yeast-like symbiont in its place [18].

Insects may also have **facultative symbionts** that are not essential for their survival. Many, but not all, of these facultative symbionts can survive outside of their insect hosts and have been cultured in the laboratory. Generally, insects can be cured of their facultative symbionts with antibiotics and recolonized with similar symbionts. Facultative symbionts have a range of detrimental, neutral, or beneficial effects on their hosts. For example, *Wolbachia* and *Arsenophonus* can act as parasites, manipulating insect reproduction to improve their transmission to the next generation [19,20]. *Xylella fastidiosa*, which colonizes the leafhopper gut, appears to have no effect on its insect host but is a plant pathogen [21]. Other plant pathogens can benefit their insect hosts, such as the bacterium *Burkholderia gladioli,* which protects *Lagria* beetles from pathogenic microbes [22]. Beneficial facultative symbionts include *Sodalis pierantonius*, which supplies its weevil host with amino acid precursors for cuticle synthesis [23], and *Morganella morganii,* which produces sex pheromones for the grass grub beetle [24]. The relationship between a facultative symbiont and its host may also vary depending on the circumstances. For instance, *Hamiltonella defensa* can benefit aphids by protecting them from parasitoid wasps, but it reduces aphid reproduction and longevity in the absence of parasitism [25].

Location: within insect cells, tissues, or the gut?

Where an engineered symbiont resides within an insect's body can influence its suitability for different applications. Typically, obligate endosymbionts reside intracellularly in specialized insect cells called **bacteriocytes**. This is the case for *Buchnera* in aphids [26] and *Wigglesworthia* in tsetse flies [27]. Some facultative endosymbionts colonize bacteriocytes, even though they may also live extracellularly within the insect, as is illustrated by *Serratia symbiotica* in aphids [28]. Many facultative endosymbionts are found embedded extracellularly in a characteristic set of insect organs and tissues that may include the salivary glands, hemolymph (insect blood), fat bodies, and reproductive organs [12]. The alimentary canals of insects vary widely between species and often include specialized gut sections related to their diets [8]. Extracellular gut symbionts that are acquired orally typically colonize the surfaces of specific portions of this

Glossary

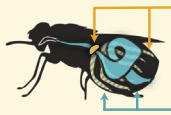
Allelic exchange: recombination between homologous segments in a donor DNA sequence and the chromosome for targeted replacement or insertion of genes.
Axenically cultured: a microbial isolate that has been grown in the complete absence of other organisms (e.g., independently of an insect host or insect cells).
Bacteriocyte: a specialized insect cell that houses intracellular symbionts. Bacteriocytes are organized into an organ called the bacteriome in some insects.
Broad-host-range (BHR) plasmid: a plasmid containing an origin of replication that enables it to replicate in diverse bacterial host species.
Endosymbiont: a symbiont that lives inside of another organism's cells or tissues as opposed to on its surface or in its environment.
Facultative symbiont: a symbiont that is not required for host survival. It may be present in some, but not all, host populations.
Horizontal transmission: any transmission of symbiotic bacteria that does not occur directly between parent and offspring, such as within a colony of social insects or when a symbiont is acquired from an insect's environment during feeding.
Obligate symbiont: a symbiont that is required for host survival. Due to this dependence, obligate symbionts are present in all individuals within a host population.
Paratransgenesis: genetically engineering a microbe to alter or control its host. Originally this term referred specifically to using this approach to eliminate disease-causing pathogens from insect populations.
RNA interference (RNAi): a highly conserved eukaryotic pathway for antiviral defense and gene regulation. Exogenous double-stranded RNA (or other RNA species) can be used to trigger RNAi cleavage of host messenger RNAs with matching sequences.
Shuttle vector: a plasmid containing two origins of replication, one that enables replication in *E. coli* and one that enables replication in the bacterial species of interest.
Suicide plasmid: a plasmid that does not replicate outside of specialized host cells. Suicide plasmids carry DNA cargo

route through an insect's body. For example, gut symbionts associated with honey bees assemble into a community lining the ileum [29,30].

Transmission: vertical, horizontal, or both?

Engineered symbionts will persist and spread within insect populations in different ways depending on how they are inherited or acquired. Obligate intracellular symbionts often undergo **vertical transmission**: they are directly passed from parents to progeny either intracellularly or extracellularly. This transmission is usually maternal, but paternal transmission of symbionts via sperm has been reported in leafhoppers and mosquitoes [31–33]. One of the best-studied examples of intracellular

into other cells for integration into the chromosome.
Symbiont: an organism that has a close association with an organism of a different species. Often symbionts have coevolved with their hosts.
Transgenesis: genetically engineering an organism using a foreign gene.
Vertical transmission: transmission of symbiotic bacteria from parent to offspring.

Insect host (Order)	Endosymbiont species	Dependence (Obligate / Facultative)	Localization (Intracellular / Extracellular)	Transmission (Horizontal / Vertical)	Host specificity (Restricted / Promiscuous)
Darkling beetle (Coleoptera) 	*Burkholderia gladioli*	Pathogen protection	Reproductive accessory glands	Egg smearing Food source	*Lagria* beetles
Mosquito (Diptera) 	*Wolbachia* spp.	Reproductive parasite	Female germ cells, diverse tissues in some hosts	Germline	Many insects
	Asaia spp.	Pathogen protection	Salivary gland, reproductive organs, gut	Egg smearing Food source, Mating	Mosquitoes, Leafhoppers
Tsetse fly (Diptera) 	*Wigglesworthia glossinidia*	Nutrition	Bacteriome Milk glands	Milk gland secretions	Tsetse flies
	Sodalis glossinidius	Unknown function	Fat body cells, milk glands, gut hemolymph	Milk gland secretions Mating	Tsetse flies
Aphid (Hemiptera) 	*Buchnera aphidicola*	Nutrition	Bacteriome	Transovarial	Aphids
	Hamiltonella defensa	Parasitoid defense	Bacteriome Hemolymph	Transovarial	Aphids, Psyllids Whiteflies
Leafhopper (Hemiptera) 	*Sulcia muelleri*	Nutrition	Bacteriome	Transovarial	Leafhoppers
	Pantoea agglomerans	Unknown function	Gut	Food source	Many insects
Honey bee (Hymenoptera) 	*Snodgrassella alvi*	Nutrition, pathogen protection	Hindgut	Fecal-oral	Bees

Trends in Microbiology

Figure 1. Characteristics of insect endosymbionts relevant for engineering. Insect endosymbionts exhibit a wide range of lifestyles and relationships with their hosts. Here we have broken down the characteristics of some important examples and classified them with respect to the level of dependence of the insect on the symbiont, localization of the symbiont in the insect body, the mode of transmission of the symbiont to new insects, and whether the symbiont can colonize one or multiple insect hosts. The image on the left for each insect depicts the tissues and organs colonized by each symbiont.

vertical transmission occurs in aphids undergoing viviparous asexual reproduction: mothers transfer endosymbionts to the bacteriocytes of embryos developing within their bodies [34]. Vertical transmission can also occur when symbionts invade the ovaries or reproductive organs, as happens with *Sodalis glossinidius* in tsetse flies [35] and for *Burkholderia* symbionts of mealybugs and scale insects [36]. Vertical transmission of extracellular symbionts can include a step that takes place outside of the insect body [16]. The insect may inoculate the eggs or the site of oviposition with bacteria, as occurs with mosquitoes [37], or transmit their symbionts to offspring packaged in a special capsule or jelly, as occurs with plataspid stinkbugs [38]. Certain coprophagic insects, including kissing bugs, can efficiently pass symbionts on to their offspring through ingestion of parental feces [39].

Horizontal transmission encompasses all other routes by which symbiotic bacteria can be passed between insects. It usually involves a stage in which the bacteria must replicate or survive outside of the host. As with humans, fecal–oral transmission between insects living in close proximity can spread bacteria. This type of social transmission occurs between honey bees in a hive [29]. Ants and the aphid 'livestock' that they tend have even been reported to exchange symbionts [40]. Many insects also acquire gut symbionts from their food – for example, plants for lepidopteran larvae (caterpillars) [41] and thrips [42] – or from other environmental sources. Gut symbionts that are also plant pathogens often spread to other insects feeding on the same plant [21,22]. Horizontal transmission events can act in conjunction with, or as precursors to, vertical transmission. For example, *Sodalis glossinidius* can be passed horizontally from male to female mosquitoes during mating, then vertically from mother to offspring [43].

Host specificity: restricted or promiscuous?

The range of insects that an engineered symbiont can colonize is another important consideration for implementing paratransgenesis. Generally, obligate symbionts have coevolved with their hosts to such a degree that they cannot colonize other insect species [13]. By contrast, some facultative symbionts have been found in association with different insect families. These cosmopolitan symbionts include bacteria from the genera *Pantoea* [44], *Arsenophonus* [45], *Burkholderia* [46], *Spiroplasma* [47], and *Rickettsia* [48]. Their diverse associations may imply that certain types of bacteria are predisposed to evolve into insect symbionts. Remarkably, host promiscuity can even apply to the same bacterial strain. For example, *Asaia* isolated from mosquitoes colonizes leafhoppers, though it is not vertically transmitted in this new host [49].

Implications for engineering

Given the myriad relationships between bacteria and insects, which are the most promising platforms for paratransgenesis? What types of symbionts are most likely to be engineerable and deployable in a way that is safe and effective? The details of how insect and symbiont biology intersect with a given application will ultimately determine the answers to these questions, but we can offer some general expectations based on the characteristics described in the previous sections (Box 1).

Genetic engineering of insect symbionts

All insect symbionts that have been successfully engineered to date have been cultured outside of insects, and most are facultative symbionts. The first step towards implementing paratransgenesis with a new insect-associated bacterium is to determine which genetic tools and approaches are effective in that species. Typical workflows begin with expressing fluorescent proteins and antibiotic-resistance genes from a plasmid. Usually, these engineered symbionts can stably colonize insects if they are fed to, or microinjected into, aposymbiotic individuals or in conjunction with clearing an individual of any native symbionts via antibiotic treatment. The same basic methodology can then be used to express other transgenes to achieve proof of principle for

Box 1. Which insect symbionts can and should be genetically engineered?

Obligate symbionts, like *Buchnera* of aphids, present an opportunity to engineer bacteria that are tightly integrated with their hosts. Because symbiont transmission is required for continued insect survival and reproduction, engineered versions of these symbionts should be maintained in an insect population for many generations. However, no obligate symbionts have been axenically cultured or genetically manipulated yet due to their coevolved dependence on their hosts. If they could be engineered in the future, their circumscribed lifestyles would be a boon for biocontainment as they are unlikely to survive in the environment or colonize other insects.

Facultative endosymbionts, like *S. symbiotica* of aphids, and gut symbionts, such as *S. alvi* of bees, are typically less reliant on their hosts even though they may also be faithfully transmitted to an insect's offspring or relatives. This independence makes it more likely that it will be possible to culture facultative symbionts. For this reason, all of the current examples of successfully engineered insect symbionts fall into this category. The potential downsides are that these symbionts may not be stably maintained within a target insect population as they can be lost with little to no effect on host fitness, and it may be more likely that they could be acquired by nontarget insect species.

Another overarching consideration is whether a symbiont occupies the right real estate. Molecules produced by intracellular symbionts may be rendered ineffective if they are trapped inside of host cell membranes. Symbionts that colonize the gut or salivary glands may be especially useful for affecting insect feeding and disease transmission or for dosing an insect with an ingestible molecule. Furthermore, endosymbionts embedded in fat bodies or other tissues distributed throughout insect bodies may be able to systemically deliver a molecule for a more powerful and immediate effect on the host. Titer, the number of bacterial cells per insect, is also a key feature of a symbiosis to consider when undertaking an engineering project: more symbiont cells will produce more effector molecules.

Certain symbionts can colonize multiple host species from very different insect families [21,40]. This is particularly the case when transmission occurs outside of the insect and for some generalists like *Arsenophonus* and *Sodalis* species. These endosymbionts may be especially versatile chassis for interchangeably engineering many different insect species, but there is also an increased biocontainment risk since they could potentially colonize off-target hosts in the environment. It will take careful field trials to guide which engineered symbionts can be safely used outside of the laboratory.

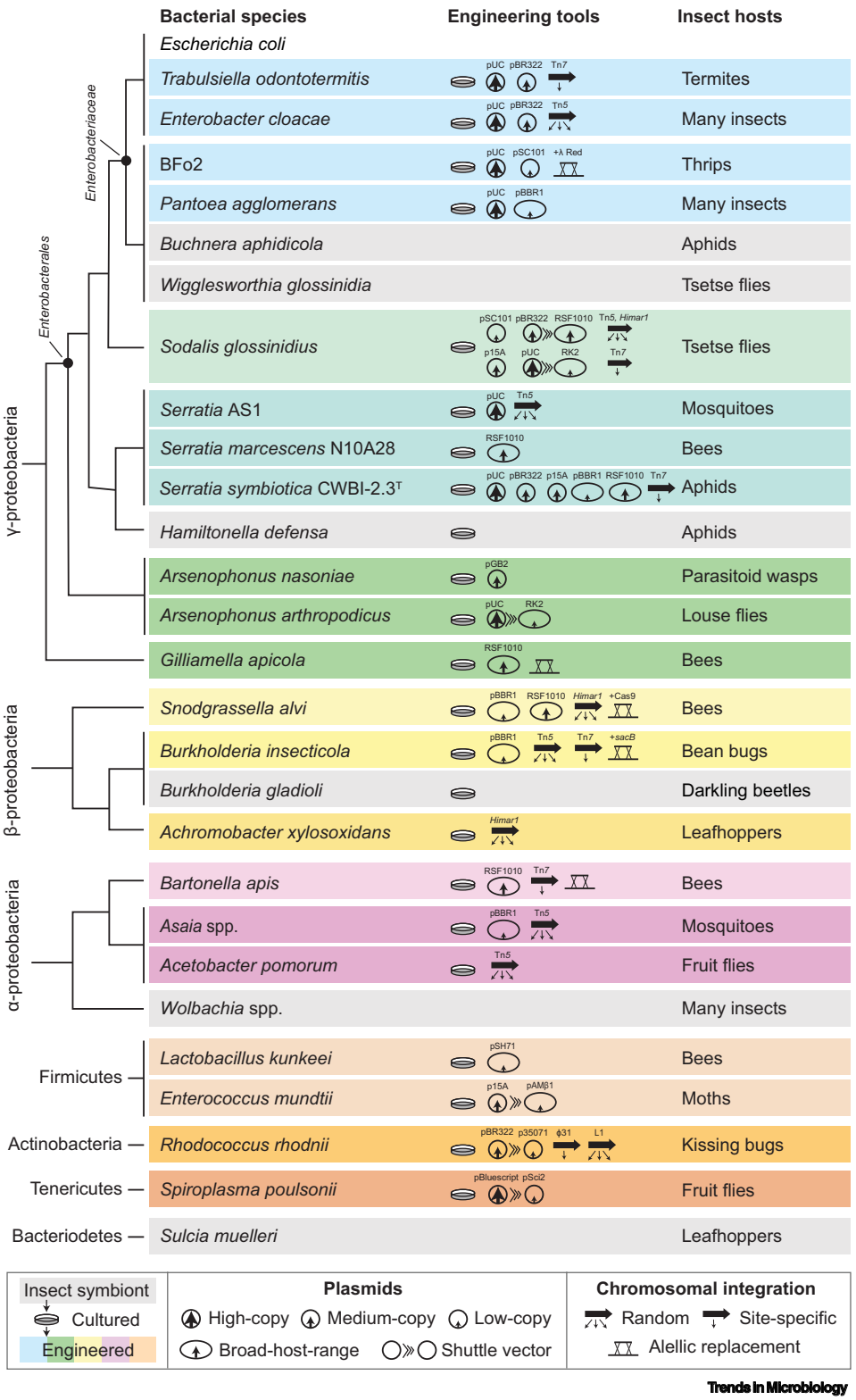
many applications. Further engineering steps often include disrupting symbiont genes to study their functions and inserting transgenes into the symbiont chromosome so that they can be stably maintained without the need for antibiotic selection. Various tools for genetic manipulation have been demonstrated in a variety of insect symbionts (Figure 2).

Engineering the enterobacterium inside

Many cultured insect symbionts are γ-proteobacteria closely related to *Escherichia coli*. It has been possible to transform most of these enterobacteria with *E. coli* plasmids. High-copy pUC plasmids used for recombinant DNA cloning and protein overexpression in *E. coli* function in γ-proteobacterial symbionts of leafhoppers [50,51], mosquitoes [52], thrips [53], aphids [54], and termites [55]. *E coli* plasmids with lower-copy pBR322, p15A, pSC101, and pGB2 origins of replication have also been used to engineer symbionts of thrips [53], parasitoid wasps [20], and tsetse flies [56]. When these tried-and-true plasmids can be added to cells using standard electroporation or chemical transformation protocols, insect paratransgenesis is straightforward. *S. glossinidius*, by contrast, is an example of an enterobacterial insect symbiont that is challenging to work with because it is slow-growing, fastidious, and refractory to these transformation methods. Recent studies show how conjugation with improved counterselection against *E. coli* donor cells and transduction with P1 phagemids can be used to deliver *E. coli* plasmids to this bacterium [57,58]. These methods may prove useful for engineering other bacteria such as *H. defensa*, an enterobacterial endosymbiont of aphids that has only recently been cultured and has not yet been genetically modified [59].

Broad-host-range plasmids and shuttle vectors

Broad-host-range (BHR) plasmids that naturally replicate in diverse bacterial species have been versatile tools for engineering symbionts that are incompatible with *E. coli* plasmids. Many BHR vectors are derived from natural plasmids that are transmissible by conjugation, so they are often



Trends in Microbiology

(See figure legend at the bottom of the next page.)

delivered from a donor *E. coli* strain to a symbiont by this route. BHR plasmids derived from pBBR1 function in *Asaia* species [32,49,60,61] and *Burkholderia insecticola* [62–64]. Both pBBR1 and RSF1010 plasmids were used to engineer the honey bee gut bacterium *Snodgrassella alvi*, and RSF1010 plasmids function in other phylogenetically diverse bacteria from bees [65]. More unusually, a pSH71-based plasmid was used in *Lactobacillus kunkeei* from honey bees [66].

An alternative approach to using a BHR plasmid is to create a **shuttle vector** that has two origins of replication, one specific to *E. coli* and one that functions in the target species. The secondary origin is often derived from a plasmid that is naturally found in the genome of the target bacterium or a close relative. For example, *Rhodococcus rhodnii* from kissing bugs has been engineered with a shuttle plasmid that has a pBR322 origin and an origin derived from its native p35071 plasmid [67]. Similarly, the *Spiroplasma poulsonii* symbiont of fruit flies was engineered using a shuttle vector containing the origin of replication of the pSci2 plasmid native to the closely related bacterium *Spiroplasma citri* and a pBluescript origin that functions in *E. coli* [68].

Combining an *E. coli* origin of replication with a BHR origin is another strategy for constructing shuttle plasmids. Though not always strictly necessary, since many common BHR origins already support replication in *E. coli*, a boost in copy number from an *E. coli*-specific origin when propagating the plasmid in this host simplifies purifying sufficient DNA for cloning and transformation procedures. For example, a vector containing a pUC origin and the BHR IncP origin derived from the RK2 plasmid has been used to engineer *Arsenophonus* from louse flies [69] and *S. glossinidius* from tsetse flies [70]. Another plasmid of this type combines an *E. coli* p15A origin with the pAMβ1 origin, which has a BHR within Gram-positive bacteria, and has been used to engineer *Enterococcus mundtii* from moths [71].

Editing symbiont genomes

Fewer studies have engineered the chromosomes of bacterial symbionts, even though this type of genetic modification is generally more stable and relieves one of needing to administer antibiotics to the insect to prevent plasmid loss by the symbiont. One common approach for chromosomal modification is to use transposons or phage integrases to inactivate a gene of interest or insert a custom cargo. Different elements may integrate randomly or at a specific site in the chromosome. Engineered Tn*5* and *Himar1* mariner transposons have been used to randomly insert reporter genes into the *S. glossinidius* [57], *Serratia* AS1 [52], and *Asaia* genomes [33]; and a reporter gene was inserted at multiple locations in the *R. rhodnii* genome using the L1 mycobacteriophage integrase. Random mutagenesis using the *Himar1* transposon enabled high-throughput characterization of gene function in *S. alvi* [72]. When combined with functional screening or enrichment, researchers have used random Tn*5* transposon mutagenesis to isolate strains with specific gene knock-outs in *B. insecticola* [64], *S. glossinidius* [73], and *Acetobacter pomorum* [74]. Site-specific chromosomal integrations have also been performed in the *R. rhodnii* chromosome using the *Streptomyces* phage φC31 integrase [53,75], and Tn*7* transposon constructs have been used to insert genes at a defined site in the genomes of *S. glossinidius* [43,58], *Bartonella apis* [65], and *B. insecticola* and related *Pandoraea* species [76,77].

Although transposons and integrases can achieve some types of chromosomal modifications, they lack the flexibility of **allelic exchange** methods. Recombination between homology in a

Figure 2. Methods used to engineer bacterial endosymbionts of insects. Whether different species of symbionts have been axenically cultured (Petri dish icon) and/or genetically modified (colored background and additional icons) is indicated according to the legend. Bacteria are organized by their taxonomic classification and relatedness, including by consensus phylogenetic trees constructed for enterobacteria [94,110].

donor plasmid or linear DNA fragment and the chromosome allows for the targeted replacement of native sequences with selectable markers and other transgenes. Successful allelic replacement by λ Red recombination has been achieved in BFo2 [53] and *S. glossinidius* [56]. This phage-derived system allows for recombination facilitated by short homology segments but generally functions only in enterobacteria. Genes have been disrupted via allelic exchange, relying on the more limited native recombination capacity of the bacterial host for integration, with **suicide plasmids** delivered by conjugation in *R. rhodnii* [53], *S. glossinidius* [57], and multiple bee gut bacteria [65]. The efficiency of isolating cells with stable integrations resulting from double-crossover recombination events has been improved in *B. insecticola* by adding a *sacB* counterselectable marker to the donor plasmid [62,63] and in *S. alvi* by targeting Cas9 cleavage to the sequence being replaced [65].

Gene expression not included

After a DNA sequence has been added to a symbiont, another obstacle remains: transgenes need to be expressed. Common gene expression elements used in *E. coli* may be incompatible with distantly related symbiont species. One can sometimes overcome this barrier by using heterologous promoters shown to function in a variety of bacteria, as illustrated by engineering of bee gut symbionts [65]. Mobile elements and BHR plasmids that naturally travel between bacterial hosts are another potential source for versatile gene expression elements. For example, the promoter from the Tn*5 nptII* antibiotic-resistance gene was co-opted to drive transgene expression in *Asaia* [32]. A more unusual challenge was encountered in *S. poulsonii*. Because spiroplasmas use a variant genetic code in which UGA encodes tryptophan rather than a stop codon, a fluorescent protein gene needed to be edited to avoid read-through of its original stop codon for expression in this species [68].

Applications for engineered insect symbionts

Engineered symbionts can be used to study and control insects at several different levels of biological organization (Figure 3, Key figure). The most straightforward applications involve fluorescently tagging the symbiont, so it can be tracked, or altering its genome to disrupt its normal interactions with its host. One can also engineer new microbial interactions, like having a symbiont exclude pathogens from an insect microbiome. Finally, one can introduce transgenes that directly interface with the host insect's biology. Because insects are major agricultural pests and transmit both human and plant diseases, most applications beyond basic science have focused on finding new ways to control insect populations or their vectorial capacity (Table 1).

Studying symbiosis

Simply expressing a fluorescent protein in a symbiont can dramatically improve our understanding of host–bacteria relationships. Many symbionts have been engineered to express GFP to track their infection dynamics and presence in different organs and tissues (Table 1). In contrast to fluorescence *in situ* hybridization (FISH) and other imaging methods that require freezing or fixing specimens, symbionts engineered to be fluorescent or luminescent can be used to follow how an individual, living insect is colonized over time. For example, the dynamics with which native symbionts colonize the guts of bean bugs [78] and aphids [54] have been characterized in this way. This approach also facilitates transmission studies: one can watch infected insects 'light up' versus relying on destructive sampling and colony counting or PCR detection. GFP-tagging has been used to better resolve transmission of *Arsenophonus nasoniae* between parasitoid wasps [20], *S. glossinidius* between tsetse flies [43], and *S. symbiotica* between aphids [79], among many other examples (Table 1).

Key figure

Applications of engineered endosymbionts

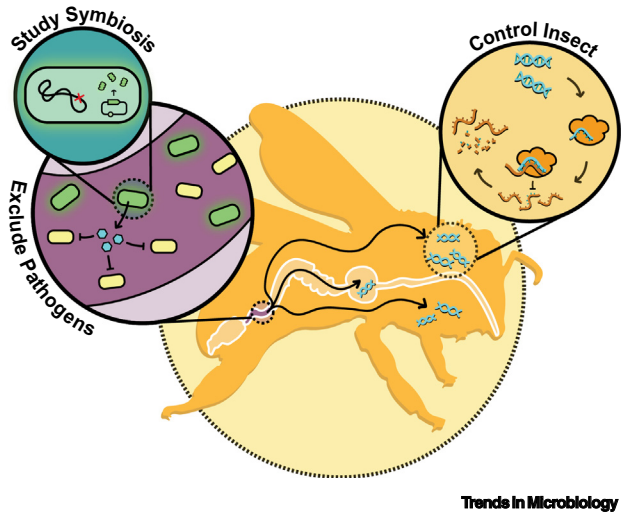


Figure 3. To date, insect symbionts have been engineered primarily to study symbiosis, to prevent insects from vectoring or becoming infected with animal and plant pathogens, and to control the biology of their hosts. The first and third applications have been demonstrated in honey bees, and the second application would also be possible in this insect host.

Genome engineering methods have allowed researchers to identify genes that are important for the establishment of symbioses. In honey bees, transposon insertion sequencing (Tn-seq) of *S. alvi* was carried out to systematically identify genes important for gut colonization [72]. Targeted genome editing methods can be used to validate specific hypotheses concerning symbiont gene function, including those generated by Tn-seq, RNA-seq, and other high-throughput methods. For instance, knockout of the *uppP* cell wall synthesis gene in *Burkholderia* [62] or the *staA* adhesion gene in *S. alvi* [65] resulted in strains that could not effectively colonize their insect hosts.

Excluding pathogens

Historically, paratransgenesis has been used mainly to prevent insect vectors from transmitting pathogens that cause human disease. These applications rely on the fact that insect symbionts often occupy the same niche as the pathogenic microbes vectored by their host. This technique was first demonstrated in the kissing bug, *Rhodnius prolixus*, the vector for the Chagas disease parasite (*Trypanosoma cruzi*). The insect's *R. rhodnii* gut symbiont was engineered to express multiple antitrypanosomal effectors [80,81], including the antimicrobial peptide (AMP) cecropin A [82], which was shown to destroy trypanosome cells in insects colonized with the engineered symbiont.

Several other vector-borne diseases have now been targeted in similar ways (Table 1). Gut symbionts of *Anopheles* mosquitoes, including *Pantoea agglomerans* [83], *Asaia* SF2.1 [60], and *Serratia* AS1 [52], were engineered to express a range of effectors targeting malaria parasites (*Plasmodium* spp.). The most potent of these was the AMP scorpine [52]. The engineered *Serratia* AS1 strain was monitored and shown to be inherited across three generations of mosquitoes, which is promising for being able to stably establish this symbiont in a wild vector population. An indirect method for pathogen exclusion has also been demonstrated in mosquitoes.

Table 1. Examples of insect symbiont engineering

Insect classification	Insect	Bacterial symbiont	Product or modification	Purpose	Refs
Coleoptera; Scarabaeidae	Scarab beetles (*Dermolepida albohirtum*)	Burkholderiales isolate Da-11	Kanamycin resistance	Genetic modification	[111]
Diptera; Culicidae	Mosquitoes (e.g., *Aedes aegypti*, *Anopheles stephensi*, *Ochlerotatus triseriatus*)	*Aerococcus viridans*, *Aeromonas hydrophila*	GFP, YFP	Genetic modification	[112]
		Asaia AE10.8	GFP	Observe colonization	[49]
		Asaia SF2.1	GFP, DsRed	Observe colonization and transmission	[32,33,49]
			Anti-*Plasmodium* effector proteins	Eliminate malaria parasite	[60]
			Wolbachia surface proteins	Reduce transmission of heartworm parasite	[61]
		Cedecea neteri	Gene knockout (*ompA*), mCherry, gentamicin resistance	Study biofilm formation and colonization	[113]
		Enterobacter cloacae	GFP, defensin proteins	Observe colonization dynamics	[114]
			GFP, YFP, RFP	Genetic modification	[112]
		Pantoea agglomerans	GFP	Observe colonization dynamics	[83]
			Anti-*Plasmodium* effector proteins	Eliminate malaria parasite	[83]
		Serratia AS1	GFP, mCherry	Observe colonization and transmission	[52] [115]
			Anti-*Plasmodium* effector proteins	Eliminate malaria parasite	[52]
Diptera; Drosophilidae	Fruit flies (*Drosophila melanogaster*)	*Acetobacter pomorum*	Transposon insertion library	Study symbiotic relationship	[74]
		Spiroplasma poulsonii	tdTomato fluorescent protein	Genetic modification	[68]
Diptera; Glossinidae	Tsetse flies (*Glossina morsitans*)	*Sodalis glossinidius*	Antibiotic resistance	Genetic modification	[116]
			GFP	Observe colonization and transmission	[43,117,118]
			Transposon insertion library	Study symbiont colonization	[73]
			Gene knockouts (*fliM*, *phoP*)	Genetic modification (via recombineering)	[56]
			Anti-trypanosomal nanobodies	Eliminate trypanosome parasite	[70]
			Transposon insertions, gene knockouts	Genetic modification (via conjugation)	[57]
			Reporter genes, gene knockouts	Genetic modification (via transduction)	[58]
Diptera; Hippoboscidae	Louse flies (*Pseudolynchia canariensis*)	*Arsenophonus arthropodicus*	Kanamycin resistance	Genetic modification	[69]
Diptera; Tephritidae	Tephritid flies (*Rhagoletis completa*)	*Klebsiella oxytoca*, *Raoultella* spp.	GFP	Genetic modification, observe colonization dynamics	[119]
		Klebsiella pneumoniae	GFP, ECFP, DsRed	Genetic modification, observe colonization dynamics	[120,121]
		Pantoea agglomerans	GFP	Genetic modification	[120,121]
Hemiptera; Aphididae	Aphids (e.g., *Acyrthosiphon pisum* and *Aphis fabae*)	*Serratia symbiotica* CWBI-2.3^T	GFP	Genetic modification, observe colonization and transmission	[54,79]

Table 1. (continued)

Insect classification	Insect	Bacterial symbiont	Product or modification	Purpose	Refs
Hemiptera; Alydidae	Bean bugs (*Riptortus pedestris*)	*Burkholderia cordobensis*	RFP	Observe colonization dynamics	[77]
		Burkholderia insecticola	GFP, RFP	Observe colonization dynamics	[64,76–78]
			Gene knockouts	Study symbiotic relationship	[62,77,122]
		[a,c]*Burkholderia* spp., [c]*Pandoraea norimbergensis*	GFP	Observe colonization dynamics	[77]
Hemiptera; Cicadellidae	Leafhoppers (*Homalodisca vitripennis* and *Scaphoideus titanus*)	*Achromobacter xylosoxidans*	DsRed	Observe colonization dynamics	[123]
		[b]*Asaia* SF2.1	GFP	Observe colonization dynamics	[49]
		[b]*Pantoea agglomerans* E325	Antimicrobial peptides (melittin and scorpine-like molecule)	Prevent transmission of plant pathogen *Xyllela fastidiosa*)	[50,51]
Hemiptera; Reduviidae	Kissing bugs (*Rhodnius prolixus*)	*Rhodococcus rhodnii*	Cecropin A	Eliminate Chagas disease parasite	[82]
			Murine antibody fragment	Eliminate Chagas disease parasite	[80]
			Arthrobacter β-1,3-glucanase	Eliminate Chagas disease parasite	[81]
			RHBP hairpin RNA, Nitrophorin-1, -2, and Vitellogenin dsRNA, RNaseIII knockout	Symbiont-mediated RNAi, reduce host fitness	[53,87]
			GFP	Observe transmission dynamics	[53]
Hymenoptera; Apidae	Bees (*Apis mellifera* and *Bombus* spp.)	*Bartonella apis*	Gene knockout (*narG*), GFP	Genetic modification	[65]
		Gilliamella apicola	GFP	Genetic modification	[65]
		Lactobacillus kunkeei	NucA reporter protein	Genetic modification	[66]
		Serratia marcescens N10A28	GFP and E2 Crimson	Genetic modification, observe colonization	[65]
		Snodgrassella alvi	Transposon insertion library	Study symbiotic relationship	[72]
			GFP and E2 Crimson	Genetic modification, observe colonization	[65]
			Gene knockout (*staA*)	Study symbiotic relationship	[65]
			Deformed wing virus and *Varroa* mite dsRNA	Protect host insect from virus and parasite	[88]
Hymenoptera; Formicidae	Fire ants (*Solenopsis invicta*)	Enterobacteriaceae isolate 38, *Kluyvera cryocrescens*, *Serratia marcescens*	DsRed	Observe colonization and transmission	[124]
Hymenoptera; Pteromalidae	Parasitoid wasps (*Nasonia vitripennis*)	*Arsenophonus nasoniae*	GFP	Observe colonization and transmission	[20]
Isoptera; Rhinotermitidae	Formosan termites (*Coptotermes formosanus*)	*Enterobacter cloacae*	GFP	Observe colonization and transmission dynamics	[125]
				Genetic modification	[55]
			Insecticidal proteins (TcdA1 and TcdB1)	Biological control of host insect	[85]
		[a]*Trabulsiella odontotermitis* AS-7737	GFP	Observe colonization and transmission dynamics	[55]
				Genetic modification	[126]

(continued on next page)

Table 1. (continued)

Insect classification	Insect	Bacterial symbiont	Product or modification	Purpose	Refs
Lepidoptera; Crambidae and Noctuidae	Moths (*Glyphodes pyloalis* and *Spodoptera littoralis*)	[a]*Enterobacter cloacae*	Ice-nucleation protein (InaA)	Biological control of host insect	[84]
		Enterococcus mundtii	GFP	Genetic modification, observe colonization dynamics	[71]
Thysanoptera; Thripidae	Western flower thrips (*Frankliniella occidentalis*)	BFo2	α-Tubulin dsRNA, RNase III knockout	Symbiont-mediated RNAi, reduce crop damage	[53]

[a]Symbiont originally isolated from a similar type of insect but a different species than the one in which it was studied.
[b]Symbiont originally isolated from a different insect order.
[c]Symbiont originally isolated from an environmental source.

Asaia SF2.1 expressing a *Wolbachia* surface protein that stimulated the host's immune response was able to prevent heartworm parasite (*Dirofilaria immitis*) development in *Aedes aegypti* [61].

Recently, this strategy has been extended to agriculture. Glassy-winged sharpshooters (a type of leafhopper) are vectors for *X. fastidiosa*, which causes Pierce's disease in grapes and is also a pathogen of many other crop plants. *P. agglomerans* E325, which colonizes the sharpshooter gut, was engineered to secrete the AMPs melittin and SLM (scorpine-like molecule) [50,51]. When symbionts expressing each effector were coinoculated into sharpshooters, they destroyed *X. fastidiosa* and reduced the spread of Pierce's disease without harming the insect.

Host control

Engineered symbionts can also be used to alter their host's survival or biology. In several instances, insect symbionts have been converted into biological control agents that express insecticidal effectors. The idea is that these symbionts, or insects infected with them, could be released into an environment infested with a pest. Then, the bacteria would spread within the host population and cause its collapse, as long as symbiont transmission outpaces the effect on insect mortality. Recombinant expression of an ice-nucleation protein from an *Enterobacter cloacae* gut symbiont of moths reduced survival of colonized larvae at cold temperatures, and mortality was greater than when they were treated with plant pathogens that naturally express these proteins [84]. In another study, an *E. cloacae* strain, native to the termite gut, was engineered to express insecticidal proteins [85]. This symbiont was socially transmitted between cohoused termites and resulted in greatly increased insect mortality over the course of several weeks. Thus, this engineered symbiont could potentially be applied as a biocontrol agent that would be brought back to a colony, spread within it, and destroy it.

Most other examples of host control rely on symbiont production of double-stranded RNA (dsRNA) to induce an **RNA interference** (**RNAi**) response that can knock down expression of a matching insect gene. Compared with engineering symbionts to express other types of effector molecules, this method has the benefit of being easily reprogrammable to target different insect genes, and thereby different biological processes. Symbiont-mediated RNAi also has significant advantages over administering dsRNA to insects through injection or feeding. The symbiont provides a continuous dose of dsRNA, and there is no need for complex feeding protocols or traumatic injections once the symbiont has colonized an insect or is stably inherited by an insect lineage [86]. Often, feeding dsRNA-producing *E. coli* cells to an insect is a stepping stone to demonstrating symbiont-mediated RNAi (Box 2).

Box 2. *E. coli* as a testbed for symbiont-mediated RNAi

E. coli is often used in proof-of-principle studies to determine what might be possible by genetically engineering insect symbionts. The natural niche of *E. coli* is the mammalian gut, and strains used in research are often modified in ways that decrease their competitive fitness even in their natural environment [98–100]. Nevertheless, laboratory strains of *E. coli* are capable of robustly colonizing many insects. They can reach high cell densities in the guts of aphids [79,101], *Drosophila* [102], and house flies [103], for instance. Alternatively, *E. coli* cells can be administered to an insect or plant surface with no expectation that they will be able to divide or survive. In this case, the *E. coli* cells are used merely as a convenient container for producing and delivering biomolecules.

To demonstrate potential pest-control applications, researchers have used *E. coli* to induce an RNAi response that causes insects to silence their own essential genes. *E. coli* HT115, a strain that has been modified to have reduced RNase III activity [104], can be used to produce high titers of a double-stranded RNA (dsRNA) sequence targeted to an essential insect gene. Ingesting these *E. coli* induces an RNAi response that is harmful or even lethal to a pest. For example, fitness defects were observed in kissing bugs that ingested *E. coli* producing dsRNAs targeting a heme-binding protein and catalase [87]. Beet armyworm caterpillars, a common crop pest, have been successfully targeted with dsRNA in this manner by several groups [105–107]. It is also effective against caterpillars of the tomato pinworm [108] and the spotted stalk borer [109].

In some of these cases, the dose of dsRNA delivered by *E. coli* may be amplified if the bacterium can replicate within the host insect and continue to produce dsRNA. For this reason, and also to potentially prolong bacterial survival in a relevant environment outside of the laboratory, it has often been suggested that even more effective pest control with RNAi could be achieved by engineering insect or plant symbionts. Before attempting to transition to these more specifically tailored delivery platforms, the laboratory workhorse *E. coli* can serve as a useful 'pseudo-symbiont' for rapidly prototyping the delivery of dsRNA or other bioactive molecules to insects.

Symbiont-mediated RNAi was originally envisioned as a means for achieving pest control. After validating the method using *E. coli*, the native *R. rhodnii* symbiont of kissing bugs was engineered to knock down expression of a gene involved in oviposition [87]. In a separate study, dsRNA production was coupled with knockout of the dsRNA-degrading RNase III enzyme in *R. rhodnii* to improve knockdown of vitellogenin, a protein required for insect fecundity [53]. Similarly, production of dsRNA targeting the essential insect gene α-tubulin by the BFo2 symbiont of western flower thrips was able to cause significant larval mortality and reduce damage to plants [53].

Applying symbiont-mediated RNAi to protect the health of beneficial insects has recently been demonstrated in bees. The platform symbiont was *S. alvi*, a core member of the conserved gut microbiome of honey bees and bumble bees [30]. The efficacy of this approach was initially validated by altering honey bee feeding behavior by targeting an insulin receptor [88]. Then, symbiont-mediated RNAi was used to protect against bee pathogens implicated in colony collapse. *S. alvi* producing dsRNA targeting the deformed wing virus genome primed the bee immune response, reducing viral loads and increasing bee survival after pathogen challenge. Remarkably, *S. alvi* expressing dsRNA matching essential genes of *Varroa* mites (an arthropod parasite of bees) was able to decrease mite survival. Presumably, these mites ingest RNA species that induce a self-targeting RNAi response when feeding on bees colonized with the engineered *S. alvi*. These examples show that symbiont-mediated RNAi is a versatile technique for engineering a symbiont to impact the host insect: it can be used for the biocontrol of pests but also for studying insect gene function and protecting beneficial insects.

Concluding remarks and future perspectives

Paratransgenesis has increased our understanding of insect–bacteria symbioses. The potential for engineering these interactions to protect human health and agriculture has also been demonstrated. However, this work has largely been restricted to a few species and to laboratory settings. We expect that researchers will soon implement paratransgenesis in a wider range of insects and test potential applications of this technology in simulated field conditions. Many questions remain about what approaches will be most effective and safe (see Outstanding questions).

Outstanding questions

Can we use high-throughput methods to discover conditions for axenically culturing additional insect symbionts, even obligate intracellular endosymbionts? How do we engineer symbionts *in situ*, that is, without needing to culture them outside of insects?

What emerging synthetic biology tools will speed up the genetic engineering of new symbiont species? What generic parts for reprogramming insect–symbiont interactions (e.g., dsRNA generators, insect toxins) can we add to genetic toolkits?

How do we ensure that engineered symbionts stably colonize insects, especially without requiring that antibiotics be administered to select for engineered strains?

How will engineered symbionts interact with the native microbiomes of their insect hosts? Do we need to engineer these interactions for symbionts to establish and persist in certain communities (e.g., gut microbiomes)?

Can we engineer symbionts to broaden or restrict their host ranges (which insects they colonize) and to control their routes of transmission in insect populations?

Can we alter aspects of symbiont biology that are important for having them deliver effector molecules to their hosts (e.g., location, titer, export machinery)?

How do characteristics of a particular insect–bacteria relationship (such as symbiont lifestyle, transmission bottlenecks, and insect ecology) influence how rapidly a function engineered into a symbiont will be lost from an insect population?

What types of field trials are needed to adequately understand the risks of using engineered symbionts outside of the laboratory? Are certain symbionts more likely to colonize off-target species or release recombinant DNA? Can we engineer better biocontainment to guard against potential negative effects on natural ecosystems?

What unexplored applications of engineered insect symbionts will have the most beneficial impacts on the environment and society?

Our inability to culture many insect-associated bacteria remains one of the main obstacles to genetically engineering them. In the future, new, high-throughput methods will likely make it possible to culture even more insect symbionts [89]. Even so, many bacteria, including obligate intracellular endosymbionts, may remain difficult or impossible to culture axenically. There is also a concern that culturing and engineering bacterial symbionts risks altering their normal interactions with their insect hosts. For example, it has been possible to axenically culture the aphid symbiont *H. defensa*, but only after passaging for long periods on insect cells, indicating that it may need to mutate in order to grow in an artificial environment [59]. It has also been reported that *S. poulsonii* expressing GFP has a lower titer when colonizing *Drosophila* than the wild-type strain and does not exhibit the normal male-killing phenotype [68].

In situ engineering approaches can potentially be used to genetically modify insect symbionts – including those that have not yet been cultured – without interrupting their host-associated lifestyles. For example, conjugation systems that have successfully delivered BHR plasmids directly from *E. coli* to other bacterial species in the mammalian gut [90] could be adapted for insects. It has also been suggested that BHR bacteriophages, such as P1, which has been used to successfully modify *S. glossinidius* in culture, could also be administered directly to insects by feeding or injection to modify symbiont cells by transduction [58]. These approaches for DNA delivery could be combined with newly discovered CRISPR-Cas transposon systems that have been used to precisely insert cargo genes into the chromosomes of specific bacteria within complex environmental communities [91].

Intracellular endosymbionts pose further challenges, as host membranes may block access to bacterial cells. For these bacteria, a hybrid approach could be applied in which endosymbionts are isolated, transformed, then reinjected back into the host, requiring that they survive for only a short time *ex vivo*. Biolistic (gene gun) and conjugation-based methods used in mitochondrial genetic engineering could also serve as models for how DNA could be delivered to these symbionts [92]. Interestingly, some endosymbionts with reduced genomes, such as *B. aphidicola* and *H. defensa*, have native plasmids that could be developed into vectors for genetic engineering [93,94].

Even successfully engineered symbionts may fail to thrive and persistently function when reintroduced into their hosts. Microbes expressing transgenes often incur a fitness cost for doing so. This burden may result in poorer symbiont colonization and transmission, as has been reported for an engineered *S. glossinidius* strain in tsetse flies [70]. It can also lead to a strong selection pressure favoring the replacement of genetically modified cells with faster-growing mutants that have lost or inactivated an engineered function [95]. Researchers have investigated how to lower the fitness burden on engineered gut bacteria in humans to prolong their stability [96], but the selection pressures and bottlenecks experienced by most insect symbionts as they are vertically or horizontally transmitted remain largely unknown. It is important to address these and other ecological and evolutionary challenges in future studies.

As the obstacles to engineering more and more bacterial symbionts of insects are overcome, there will be the potential to adapt paratransgenesis for new applications. When used for pest and vector control, engineered symbionts may overcome natural resistance mechanisms of insects and cause fewer off-target effects on beneficial insects than chemical pesticides [3,51,53,83]. Another exciting application of engineered symbionts is using them to protect the health of beneficial insects, such as pollinators. Finally, symbiont-mediated RNAi may prove to be a powerful approach for performing functional genomics studies of some insects.

Because symbiont engineering does not rely on germline manipulation of insects, it is expected to be more versatile and less permanent, which may lead to a more rapid development cycle to combat the emergence of new threats and better public acceptance of this technology. Systems appropriate for delivering engineered symbionts to insects in the field have been developed for kissing bugs and leafhoppers [50,97], so these approaches will likely be tested in more authentic conditions in the near future. However, serious questions remain about what types of engineered symbionts can be safely and effectively deployed at scale. For this reason, future translational research efforts must focus on assuring that engineered symbionts and their transgenes do not have off-target effects that harm natural ecosystems.

Acknowledgments

We thank Julie Perreau for helpful discussions. Insect symbiont engineering projects in the Barrick laboratory have been supported by the Defense Advanced Research Projects Agency (HR0011-15-C0095 and HR0011-17-2-0052) and the Army Research Office (W911NF-20-1-0195).

Declaration of interests

J.E.B. and S.P.L. are co-inventors on a patent application (16/029,686) related to the commercial use of engineered bee gut bacteria.

References

1. Stork, N.E. (2018) How many species of insects and other terrestrial arthropods are there on Earth? *Annu. Rev. Entomol.* 63, 31–45
2. Weisser, W.W. and Siemann, E. (2008) *Insects and Ecosystem Function*, Springer
3. Radcliffe, E.B. *et al.* (2008) *Integrated Pest Management*, Cambridge University Press
4. World Health Organization (2014) *A Global Brief on Vector-borne Diseases*, WHO Press
5. Taning, C.N.T. *et al.* (2017) CRISPR/Cas9 in insects: applications, best practices and biosafety concerns. *J. Insect Physiol.* 98, 245–257
6. Chaverra-Rodriguez, D. *et al.* (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nat. Commun.* 9, 3008
7. McCutcheon, J.P. and Moran, N.A. (2012) Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* 10, 13–26
8. Engel, P. and Moran, N.A. (2013) The gut microbiota of insects – diversity in structure and function. *FEMS Microbiol. Rev.* 37, 699–735
9. Beard, C.B. *et al.* (1998) Bacterial symbiosis in arthropods and the control of disease transmission. *Emerg. Infect. Dis.* 4, 581–591
10. Caragata, E.P. *et al.* (2016) Exploiting intimate relationships: controlling mosquito-transmitted disease with *Wolbachia*. *Trends Parasitol.* 32, 207–218
11. Dorigatti, I. *et al.* (2018) Using *Wolbachia* for dengue control: insights from modelling. *Trends Parasitol.* 34, 102–113
12. Perlmutter, J.I. and Bordenstein, S.R. (2020) Microorganisms in the reproductive tissues of arthropods. *Nat. Rev. Microbiol.* 18, 97–111
13. Bennett, G.M. and Moran, N.A. (2015) Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci. U. S. A.* 112, 1–8
14. Douglas, A.E. (2011) Lessons from studying insect symbioses. *Cell Host Microbe* 10, 359–367
15. Kikuchi, Y. (2009) Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes Environ.* 24, 195–204
16. Salem, H. *et al.* (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc. R. Soc. B Biol. Sci.* 282, 20142957
17. Akman Gündüz, E. and Douglas, A.E. (2009) Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proc. R. Soc. B Biol. Sci.* 276, 987–991
18. Fukatsu, T. *et al.* (1994) Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: Implications for host-symbiont coevolution and evolution of sterile soldier castes. *Zool. Sci.* 11, 613–623
19. Hurst, G.D.D. and Frost, C.L. (2015) Reproductive parasitism: maternally inherited symbionts in a biparental world. *Cold Spring Harb. Perspect. Biol.* 7, a017699
20. Nadal-Jimenez, P. *et al.* (2019) Genetic manipulation allows *in vivo* tracking of the life cycle of the son-killer symbiont, *Arsenophonus nasoniae*, and reveals patterns of host invasion, tropism and pathology. *Environ. Microbiol.* 21, 3172–3182
21. Nadarasah, G. and Stavrinides, J. (2011) Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol. Rev.* 35, 555–575
22. Flórez, L.V. *et al.* (2017) Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. *Nat. Commun.* 8, 15172
23. Vigneron, A. *et al.* (2014) Insects recycle endosymbionts when the benefit is over. *Curr. Biol.* 24, 2267–2273
24. Marshall, D.G. *et al.* (2016) *Morganella morganii* bacteria produces phenol as the sex pheromone of the New Zealand grass grub from tyrosine in the colleterial gland. *Sci. Nat.* 103, 59
25. Vorburger, C. *et al.* (2013) Comparing constitutive and induced costs of symbiont-conferred resistance to parasitoids in aphids. *Ecol. Evol.* 3, 706–713
26. Braendle, C. *et al.* (2003) Developmental origin and evolution of bacteriocytes in the aphid–*Buchnera* symbiosis. *PLoS Biol.* 1, 070–076
27. Aksoy, S. (1995) *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *Int. J. Syst. Bacteriol.* 45, 848–851
28. Fukatsu, T. *et al.* (2000) The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* 66, 2748–2758
29. Powell, J.E. *et al.* (2014) Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Appl. Environ. Microbiol.* 80, 7378–7387
30. Kwong, W.K. *et al.* (2014) Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc. Natl. Acad. Sci. U. S. A.* 111, 11509–11514
31. Watanabe, K. *et al.* (2014) Intrasperm vertical symbiont transmission. *Proc. Natl. Acad. Sci. U. S. A.* 111, 7433–7437

32. Favia, G. *et al.* (2007) Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proc. Natl. Acad. Sci. U. S. A.* 104, 9047–9051
33. Damiani, C. *et al.* (2008) Paternal transmission of symbiotic bacteria in malaria vectors. *Curr. Biol.* 18, 1087–1088
34. Koga, R. *et al.* (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proc. Natl. Acad. Sci. U. S. A.* 109, E1230–E1237
35. Dale, C. and Welburn, S. (2001) The endosymbionts of tsetse flies: manipulating host–parasite interactions. *Int. J. Parasitol.* 31, 628–631
36. Michalik, K. *et al.* (2016) Bacteria belonging to the genus *Burkholderia* are obligatory symbionts of the *Eriococcids Acanthococcus aceris* (Signoret, 1875) and *Gossyparia spuria* (Modeer, 1778) (Insecta, Hemiptera, Coccoidea). *Arthropod Struct. Dev.* 45, 265–272
37. Coon, K.L. *et al.* (2014) Mosquitoes rely on their gut microbiota for development. *Mol. Ecol.* 23, 2727–2739
38. Fukatsu, T. and Hosokawa, T. (2002) Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. *Appl. Environ. Microbiol.* 68, 389–396
39. Beard, C. *et al.* (2001) Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. *Int. J. Parasitol.* 31, 621–627
40. Renoz, F. *et al.* (2018) Evidence for gut-associated *Serratia symbiotica* in wild aphids and ants provides new perspectives on the evolution of bacterial mutualism in insects. *Microb. Ecol.* 78, 159–169
41. Mason, C.J. and Raffa, K.F. (2014) Acquisition and structuring of midgut bacterial communities in gypsy moth (Lepidoptera: Erebidae) larvae. *Environ. Entomol.* 43, 595–604
42. De Vries, E.J. *et al.* (2001) Growth and transmission of gut bacteria in the western flower thrips, *Frankliniella occidentalis*. *J. Invertebr. Pathol.* 77, 129–137
43. De Vooght, L. *et al.* (2015) Paternal transmission of a secondary symbiont during mating in the viviparous tsetse fly. *Mol. Biol. Evol.* 32, 1977–1980
44. Walterson, A.M. and Stavrinides, J. (2015) *Pantoea*: insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiol. Rev.* 39, 968–984
45. Wilkes, T.E. *et al.* (2012) The genus *Arsenophonus*. In *Manipulative Tenants: Bacteria Associated with Arthropods* (Zchori-Fein, E. and Bourtzis, K., eds), pp. 225–244, CRC Press
46. Kaltenpoth, M. and Flórez, L.V. (2020) Versatile and dynamic symbioses between insects and *Burkholderia* bacteria. *Annu. Rev. Entomol.* 65, 145–170
47. Bolã, L.M. *et al.* (2015) Arthropod–*Spiroplasma* relationship in the genomic era. *FEMS Microbiol. Ecol.* 91, 1–8
48. Perlman, S.J. *et al.* (2006) The emerging diversity of *Rickettsia*. *Proc. R. Soc. B Biol. Sci.* 273, 2097–2106
49. Crotti, E. *et al.* (2009) *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environ. Microbiol.* 11, 3252–3264
50. Arora, A.K. *et al.* (2015) A delivery system for field application of paratransgenic control. *BMC Biotechnol.* 15, 59
51. Arora, A.K. *et al.* (2018) A paratransgenic strategy to block transmission of *Xylella fastidiosa* from the glassy-winged sharpshooter *Homalodisca vitripennis*. *BMC Biotechnol.* 18, 50
52. Wang, S. *et al.* (2017) Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science* 357, 1399–1402
53. Whitten, M.M.A. *et al.* (2016) Symbiont-mediated RNA interference in insects. *Proc. R. Soc. B Biol. Sci.* 283, 20160042
54. Elston, K.M. *et al.* (2020) Engineering a culturable *Serratia symbiotica* strain for aphid paratransgenesis. *Appl. Environ. Microbiol.* 87, e02245-20
55. Tikhe, C.V. *et al.* (2016) Assessment of genetically engineered *Trabulsiella odontotermitis* as a 'Trojan Horse' for paratransgenesis in termites. *BMC Microbiol.* 16, 202
56. Pontes, M.H. and Dale, C. (2011) Lambda Red-mediated genetic modification of the insect endosymbiont *Sodalis glossinidius*. *Appl. Environ. Microbiol.* 77, 1918–1920
57. Kendra, C.G. *et al.* (2020) Conjugal DNA transfer in *Sodalis glossinidius,* a maternally inherited symbiont of tsetse flies. *mSphere* 5, e00864-20
58. Keller, C.M. *et al.* (2021) Genetic modification of *Sodalis* species by DNA transduction. *mSphere* 6, e01331-20
59. Brandt, J.W. *et al.* (2017) Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. *Proc. R. Soc. B Biol. Sci.* 284, 20171925
60. Bongio, N.J. and Lampe, D.J. (2015) Inhibition of *Plasmodium berghei* development in mosquitoes by effector proteins secreted from *Asaia* sp. bacteria using a novel native secretion signal. *PLoS ONE* 10, e0143541
61. Epis, S. *et al.* (2020) Chimeric symbionts expressing a *Wolbachia* protein stimulate mosquito immunity and inhibit filarial parasite development. *Commun. Biol.* 3, 105
62. Kim, J.K. *et al.* (2013) Bacterial cell wall synthesis gene *uppP* is required for *Burkholderia* colonization of the stinkbug gut. *Appl. Environ. Microbiol.* 79, 4879–4886
63. Kim, J.K. *et al.* (2013) Polyester synthesis genes associated with stress resistance are involved in an insect–bacterium symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 110, E2381–E2389
64. Ohbayashi, T. *et al.* (2015) Insect's intestinal organ for symbiont sorting. *Proc. Natl. Acad. Sci. U. S. A.* 112, E5179–E5188
65. Leonard, S.P. *et al.* (2018) Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. *ACS Synth. Biol.* 7, 1279–1290
66. Rangberg, A. *et al.* (2015) The paratransgenic potential of *Lactobacillus kunkeei* in the honey bee *Apis mellifera*. *Benefic. Microbes* 6, 513–523
67. Beard, C.B. *et al.* (1992) Transformation of an insect symbiont and expression of a foreign gene in the Chagas' disease vector *Rhodnius prolixus*. *Am. J. Trop. Med. Hyg.* 46, 195–200
68. Masson, F. *et al.* (2020) Transformation of the *Drosophila* sex-manipulative endosymbiont *Spiroplasma poulsonii* and persisting hurdles for functional genetic studies. *Appl. Environ. Microbiol.* 86, e00835-20
69. Dale, C. *et al.* (2006) Isolation, pure culture, and characterization of 'Candidatus *Arsenophonus arthropodicus*,' an intracellular secondary endosymbiont from the hippoboscid louse fly *Pseudolynchia canariensis*. *Appl. Environ. Microbiol.* 72, 2997–3004
70. De Vooght, L. *et al.* (2014) Delivery of a functional anti-trypanosome Nanobody in different tsetse fly tissues via a bacterial symbiont, *Sodalis glossinidius*. *Microb. Cell Fact.* 13, 156
71. Teh, B.-S. *et al.* (2016) Colonization of the intestinal tract of the polyphagous pest *Spodoptera littoralis* with the GFP-tagged indigenous gut bacterium *Enterococcus mundtii*. *Front. Microbiol.* 7, 928
72. Powell, J.E. *et al.* (2016) Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. *Proc. Natl. Acad. Sci. U. S. A.* 113, 13887–13892
73. Dale, C. *et al.* (2001) The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U. S. A.* 98, 1883–1888
74. Shin, S.C. *et al.* (2011) *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* 334, 670–674
75. Dotson, E.M. *et al.* (2003) Transformation of *Rhodococcus rhodnii*, a symbiont of the Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage. *Infect. Genet. Evol.* 3, 103–109
76. Kikuchi, Y. and Fukatsu, T. (2014) Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*. *Mol. Ecol.* 23, 1445–1456
77. Itoh, H. *et al.* (2019) Host–symbiont specificity determined by microbe–microbe competition in an insect gut. *Proc. Natl. Acad. Sci. U. S. A.* 116, 22673–22682
78. Kikuchi, Y. *et al.* (2020) *Burkholderia insecticola* triggers midgut closure in the bean bug *Riptortus pedestris* to prevent

secondary bacterial infections of midgut crypts. *ISME J.* 14, 1627–1638
79. Perreau, J. *et al.* (2021) Vertical transmission at the pathogen-symbiont interface: *Serratia symbiotica* and aphids. *mBio* 12, e00359-21
80. Durvasula, R.V. *et al.* (1999) Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. *Med. Vet. Entomol.* 13, 115–119
81. Jose, C. *et al.* (2013) Recombinant *Arthrobacter* β-1, 3-glucanase as a potential effector molecule for paratransgenic control of Chagas disease. *Parasit. Vectors* 6, 65
82. Durvasula, R.V. *et al.* (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 94, 3274–3278
83. Wang, S. *et al.* (2012) Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* 109, 12734–12739
84. Watanabe, K. *et al.* (2001) Biological control of an insect pest by gut-colonizing *Enterobacter cloacae* transformed with ice nucleation gene. *J. Appl. Microbiol.* 88, 90–97
85. Zhao, R. *et al.* (2008) Cloning and heterologous expression of insecticidal-protein-encoding genes from *Photorhabdus luminescens* TT01 in *Enterobacter cloacae* for termite control. *Appl. Environ. Microbiol.* 74, 7219–7226
86. Whitten, M. and Dyson, P. (2017) Gene silencing in non-model insects: overcoming hurdles using symbiotic bacteria for trauma-free sustainable delivery of RNA interference. *BioEssays* 39, 1600247
87. Taracena, M.L. *et al.* (2015) Genetically modifying the insect gut microbiota to control Chagas disease vectors through systemic RNAi. *PLoS Negl. Trop. Dis.* 9, e0003358
88. Leonard, S.P. *et al.* (2020) Engineered symbionts activate honey bee immunity and limit pathogens. *Science* 367, 573–576
89. Lewis, W.H. *et al.* (2021) Innovations to culturing the uncultured microbial majority. *Nat. Rev. Microbiol.* 19, 225–240
90. Ronda, C. *et al.* (2019) Metagenomic engineering of the mammalian gut microbiome *in situ*. *Nat. Methods* 16, 167–170
91. Rubin, B.E. *et al.* (2020) Targeted genome editing of bacteria within microbial communities. *bioRxiv* Published online July 21, 2020. https://doi.org/10.1101/2020.07.17.209189
92. Klucnika, A. and Ma, H. (2020) Mapping and editing animal mitochondrial genomes: Can we overcome the challenges? *Philos. Trans. R. Soc. B Biol. Sci.* 375, 20190187
93. Shigenobu, S. *et al.* (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* 407, 81–86
94. Degnan, P.H. *et al.* (2009) *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proc. Natl. Acad. Sci. U. S. A.* 106, 9063–9068
95. Renda, B.A. *et al.* (2014) Engineering reduced evolutionary potential for synthetic biology. *Mol. BioSyst.* 10, 1668–1678
96. Charbonneau, M.R. *et al.* (2020) Developing a new class of engineered live bacterial therapeutics to treat human diseases. *Nat. Commun.* 11, 1738
97. Durvasula, R.V. *et al.* (1999) Strategy for introduction of foreign genes into field populations of Chagas disease vectors. *Ann. Entomol. Soc. Am.* 92, 937–943
98. Myhal, M.L. *et al.* (1982) Relative colonizing abilities of human fecal and K 12 strains of *Escherichia coli* in the large intestines of streptomycin-treated mice. *Eur. J. Clin. Microbiol.* 1, 186–192
99. Browning, D.F. *et al.* (2013) Laboratory adapted *Escherichia coli* K-12 becomes a pathogen of *Caenorhabditis elegans* upon restoration of O antigen biosynthesis. *Mol. Microbiol.* 87, 939–950
100. Cuenca, M. *et al.* (2016) D-alanine-controlled transient intestinal mono-colonization with non-laboratory-adapted commensal *E. coli* strain HS. *PLoS ONE* 11, e0151872
101. Stavrinides, J. *et al.* (2010) A single genetic locus in the phytopathogen *Pantoea stewartii* enables gut colonization and pathogenicity in an insect host. *Environ. Microbiol.* 12, 147–155
102. Shokal, U. *et al.* (2016) Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. *BMC Microbiol.* 16, 16
103. Petridis, M. *et al.* (2006) Horizontal transfer of Shiga toxin and antibiotic resistance genes among *Escherichia coli* strains in house fly (Diptera: Muscidae) gut. *J. Med. Entomol.* 43, 288–295
104. Timmons, L. *et al.* (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263, 103–112
105. Kim, E. *et al.* (2015) A transformed bacterium expressing double-stranded RNA specific to integrin β1 enhances Bt Toxin efficacy against a polyphagous insect pest, *Spodoptera exigua*. *PLoS ONE* 10, e0132631
106. Tian, H. *et al.* (2009) Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *PLoS ONE* 4, e6225
107. Vatanparast, M. and Kim, Y. (2017) Optimization of recombinant bacteria expressing dsRNA to enhance insecticidal activity against a lepidopteran insect, *Spodoptera exigua*. *PLoS ONE* 12, e0183054
108. Bento, F.M.M. *et al.* (2020) Gene silencing by RNAi via oral delivery of dsRNA by bacteria in the South American tomato pinworm, *Tuta absoluta*. *Pest Manag. Sci.* 76, 287–295
109. Adeyinka, O.S. *et al.* (2019) Identification and validation of potential reference gene for effective dsRNA knockdown analysis in *Chilo partellus*. *Sci. Rep.* 9, 13629
110. Adeolu, M. *et al.* (2016) Genome-based phylogeny and taxonomy of the *'Enterobacteriales'*: proposal for *Enterobacterales* ord. nov. divided into the families *Enterobacteriaceae*, *Erwiniaceae* fam. nov., *Pectobacteriaceae* fam. nov., *Yersiniacea*. *Int. J. Syst. Evol. Microbiol.* 66, 5575–5599
111. Pittman, G.W. *et al.* (2008) Assessment of gut bacteria for a paratransgenic approach to control *Dermolepida albohirtum* larvae. *Appl. Environ. Microbiol.* 74, 4036–4043
112. Muturi, E. *et al.* (2019) Green, yellow, and red fluorescent proteins as markers for bacterial isolates from mosquito midguts. *Insects* 10, 49
113. Hegde, S. *et al.* (2019) CRISPR/Cas9-mediated gene deletion of the *ompA* gene in symbiotic *Cedecea neteri* impairs biofilm formation and reduces gut colonization of *Aedes aegypti* mosquitoes. *PLoS Negl. Trop. Dis.* 13, e0007883
114. Dehghan, H. *et al.* (2017) Dynamics of transgenic *Enterobacter cloacae* expressing green fluorescent protein- defensin (GFP-D) in *Anopheles stephensi* under laboratory condition. *J. Arthropod. Borne Dis.* 11, 515–532
115. Koosha, M. *et al.* (2019) Delivery of a genetically marked *Serratia* AS1 to medically important arthropods for use in RNAi and paratransgenic control strategies. *Microb. Ecol.* 78, 185–194
116. Beard, C.B. *et al.* (1993) Genetic transformation and phylogeny of bacterial symbionts from tsetse flies. *Insect Mol. Biol.* 1, 123–131
117. Cheng, Q. and Aksoy, S. (1999) Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Mol. Biol.* 8, 125–132
118. De Vooght, L. *et al.* (2018) Towards improving tsetse fly paratransgenesis: stable colonization of *Glossina morsitans* with genetically modified *Sodalis*. *BMC Microbiol.* 18, 165
119. Martinez-Sañudo, I. *et al.* (2011) Green fluorescent protein (GFP)-labeling of enterobacteria associated with fruit flies (Diptera: Tephritidae) and persistence in their natural host *Rhagoletis completa* Cresson. *Can. J. Microbiol.* 57, 969–973
120. Peloquin, J.J. *et al.* (2000) Transformation of internal extracellular bacteria isolated from *Rhagoletis completa* Cresson gut with enhanced green fluorescent protein. *Curr. Microbiol.* 40, 367–371
121. Peloquin, J.J. *et al.* (2002) Transformed bacterial symbionts re-introduced to and detected in host gut. *Curr. Microbiol.* 45, 41–45
122. Kim, J.K. *et al.* (2017) The lipopolysaccharide core oligosaccharide of *Burkholderia* plays a critical role in maintaining a proper gut symbiosis with the bean bug *Riptortus pedestris*. *J. Biol. Chem.* 292, 19226–19237

123. Bextine, B. *et al.* (2004) Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr. Microbiol.* 48, 327–331
124. Medina, F. *et al.* (2009) Genetic transformation of midgut bacteria from the red imported fire ant (*Solenopsis invicta*). *Curr. Microbiol.* 58, 478–482
125. Husseneder, C. and Grace, J.K. (2005) Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. *Appl. Microbiol. Biotechnol.* 68, 360–367
126. Tikhe, C.V. *et al.* (2017) Isolation and assessment of gut bacteria from the Formosan subterranean termite, *Coptotermes formosanus* (Isoptera: Rhinotermitidae), for paratransgenesis research and application. *Insect Sci.* 24, 93–102