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**NEW TOOLS AND APPLICATIONS FOR GENETICALLY
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**NEW TOOLS AND APPLICATIONS FOR GENETICALLY
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by

Katherine Marie Elston

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Dedication

This work is dedicated to all things small and seemingly insignificant.

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The Moran lab may not have been my official lab home, but I've been going to happy hours at the Local and stealing reagents from them for years now. I've been lucky enough to make some great friends over the years and I'm excited to see where our paths cross again in the future.

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Abstract

New tools and applications for genetically engineered insect symbionts

Katherine Marie Elston, Ph.D.

The University of Texas at Austin, 2022

Supervisor: Jeffrey Barrick

Insects play a broad range of roles in natural ecosystems. They pollinate plants, recycle resources, and spread diseases. One integral component of the biology of many of these insects is the symbiotic bacteria that live inside of them. The relationships between symbionts and their insect hosts are well-studied in model systems like aphids, but despite this work, the ability to study genetic components of these relationships has been lacking. A major factor in this limitation is the deficit of tools that are available for genetically manipulating both non-model bacteria and insect species. In this work, I examine these limitations, along with the possibilities for the study of insect biology and control of insect pests that may arise if we can overcome them (**CHAPTER 1**). I develop methods to engineer recently isolated symbionts of aphids (**CHAPTER 2**), and fruit flies (**CHAPTER 3**). I then adapt these engineered aphid symbionts to try to alter aphid gene expression through symbiont-mediated RNAi (**CHAPTER 4**). I

conclude with a summary of our results and the implications this work may have for the future of insect symbiont engineering (**CHAPTER 5**).

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Chapter 1: Engineering insects from the endosymbiont out

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All authors contributed to the development of this work. K.M.E., S.P.L., P.G., S.B.B., E.R., and J.E.B. performed independent research and contributed to the writing of this review. K.M.E. and J.E.B. developed figures and performed final edits on the document.

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ABSTRACT

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

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 bacteria that live within them. Bacterial endosymbionts can reside in the insect gut or other organs and tissues, including even inside insect cells. In some cases, these bacteria are inherited by an insect's offspring and have evolved to resemble cellular organelles (7). Other symbiont associations may be transient, conditionally beneficial, or show signs of biological conflict. It has long been recognized that bacterial endosymbionts represent a potential 'backdoor' for engineering insect biology (8). 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 introduce the variety of insect-bacteria associations and genetic engineering approaches that have been applied to insect endosymbionts. Then, we discuss how engineered symbionts have been used to study insect

biology, thwart pests and disease vectors, and protect pollinator health. We do not discuss transplantation of nonengineered *Wolbachia* or other endosymbionts for control of mosquito-borne diseases, which is the subject of other recent reviews (9, 10). Instead, we focus more broadly and include cases in which symbionts of beneficial insects and vectors of plant diseases have been engineered.

INSECT-BACTERIA RELATIONSHIPS

Insect-bacteria symbioses exhibit tremendous diversity in form and function (11–15). Here we summarize aspects of symbioses relevant to engineering them: how symbionts have co-evolved 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 (Fig. 1.1).

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

Figure 1.1: Characteristics of insect endosymbionts relevant for engineering.

Insect endosymbionts exhibit a wide range of lifestyles and relationships with their hosts. This table breaks down the characteristics of some important examples and classifies 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.

Interdependence: obligate versus facultative

Some insects have obligate symbionts that are found species-wide in every individual, as they are necessary for their survival. These symbionts, such as *Buchnera aphidicola* in aphids, often supply nutrients that the host lacks in its diet (16). Obligate symbionts usually show hallmarks of long-term coevolution with their insect hosts, such as extreme genome reduction (12). Typically, they cannot survive outside of their hosts and have not been axenically cultured.

Insects may also have facultative symbionts that are not essential for their survival. These symbionts can have 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 (17, 18). *Xylella fastidiosa*, which colonizes the leafhopper gut, appears to have no effect on its insect host but is a plant pathogen (19). Other plant pathogens can benefit their insect hosts, such as the bacterium *Burkholderia gladioli*, which protects the bean bug from pathogenic microbes (20). Beneficial facultative symbionts include *Sodalis pierantonius*, which supplies its weevil host with chitin precursors (21); and *Morganella morganii*, which produces sex pheromones for the grass grub beetle (22). Unlike their obligate counterparts, many facultative symbionts can survive outside of their insect hosts.

Localization: intracellular versus extracellular

Typically, obligate symbionts reside intracellularly in specialized insect cells called bacteriocytes. This is the case for *Buchnera* in aphids (23) and

Wigglesworthia in tsetse flies (24). Some facultative symbionts can colonize bacteriocytes, even though they may also live extracellularly within the insect, as is illustrated by *Serratia symbiotica* of aphids (25). Extracellular symbionts can colonize a variety of insect organs and tissues such as the salivary glands, gut, hemolymph (insect blood), fat bodies, and reproductive organs (11). Some symbionts are distributed throughout the entire insect body while others are concentrated in one or more of these locations.

Transmission: vertical versus horizontal

Obligate intracellular symbionts often undergo vertical transmission: they are directly passed from parents to progeny either intra- or extracellularly. This transmission is usually maternal, but paternal transmission of symbionts via sperm has been reported in leafhoppers and mosquitoes (26–28). One of the best-studied examples of intracellular vertical transmission occurs in aphids undergoing asexual reproduction. Mothers transfer obligate and facultative symbionts to the bacteriocytes of embryos developing within their bodies (29). Vertical transmission can also occur when symbionts invade the ovaries or reproductive organs, as happens with *Sodalis glossinidius* in tsetse flies (30) and *Burkholderia* symbionts of mealybugs and scale insects (31). Some extracellular symbionts can also be transmitted vertically (15). The insect may simply inoculate the eggs or the site of oviposition with bacteria, as occurs with mosquitoes (32), transmit their symbionts to offspring through a special symbiont capsule or jelly, as occurs with stinkbugs (33). Coprophagic insects, including

kissing bugs, can directly pass symbionts on to their offspring through ingestion of parental feces (34).

Horizontal transmission encompasses every other method by which bacteria can pass from insect to insect. 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 occurs between honey bees in a hive (35) and even between ants and the aphid "livestock" that they tend (36). Many insects acquire symbionts from their food—e.g., plants for lepidopteran larvae (caterpillars) (37) and thrips (38)—or from other environmental sources.

Host specificity: restricted versus promiscuous

Generally, obligate symbionts have co-evolved with their hosts to such a degree that they cannot colonize other insect species (12). In contrast, some facultative symbionts have been found in association with different insect families. These cosmopolitan symbionts include bacteria from the genera *Pantoea* (39), *Arsenophonus* (40), *Burkholderia* (41), *Spiroplasma* (42), and *Rickettsia* (43). 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 mosquitos colonizes leafhoppers, though it is not vertically transmitted in this new host (44).

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.

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 reproduction, engineered versions of these symbionts should be maintained in an insect population for many generations without being lost or displaced by other bacteria. 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, their circumscribed lifestyles would be a boon for biocontainment, as they are unlikely to survive in the environment or colonize other insects.

Facultative symbionts, like *S. symbiotica* of aphids and *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 downside is 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.

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. On the other hand, symbionts 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 endosymbiont cells will produce more effector molecules.

Certain symbionts can colonize multiple host species from very different insect families (19, 36). 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 lab.

GENETIC ENGINEERING OF INSECT ENDOSYMBIONTS

Most efforts to genetically modify insect-associated bacteria begin with expressing a fluorescent protein from a plasmid. The same plasmid can then be used to express a transgene to achieve proof of principle for 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. These genetic manipulations have been demonstrated in a variety of insect endosymbionts (Fig. 1.2).

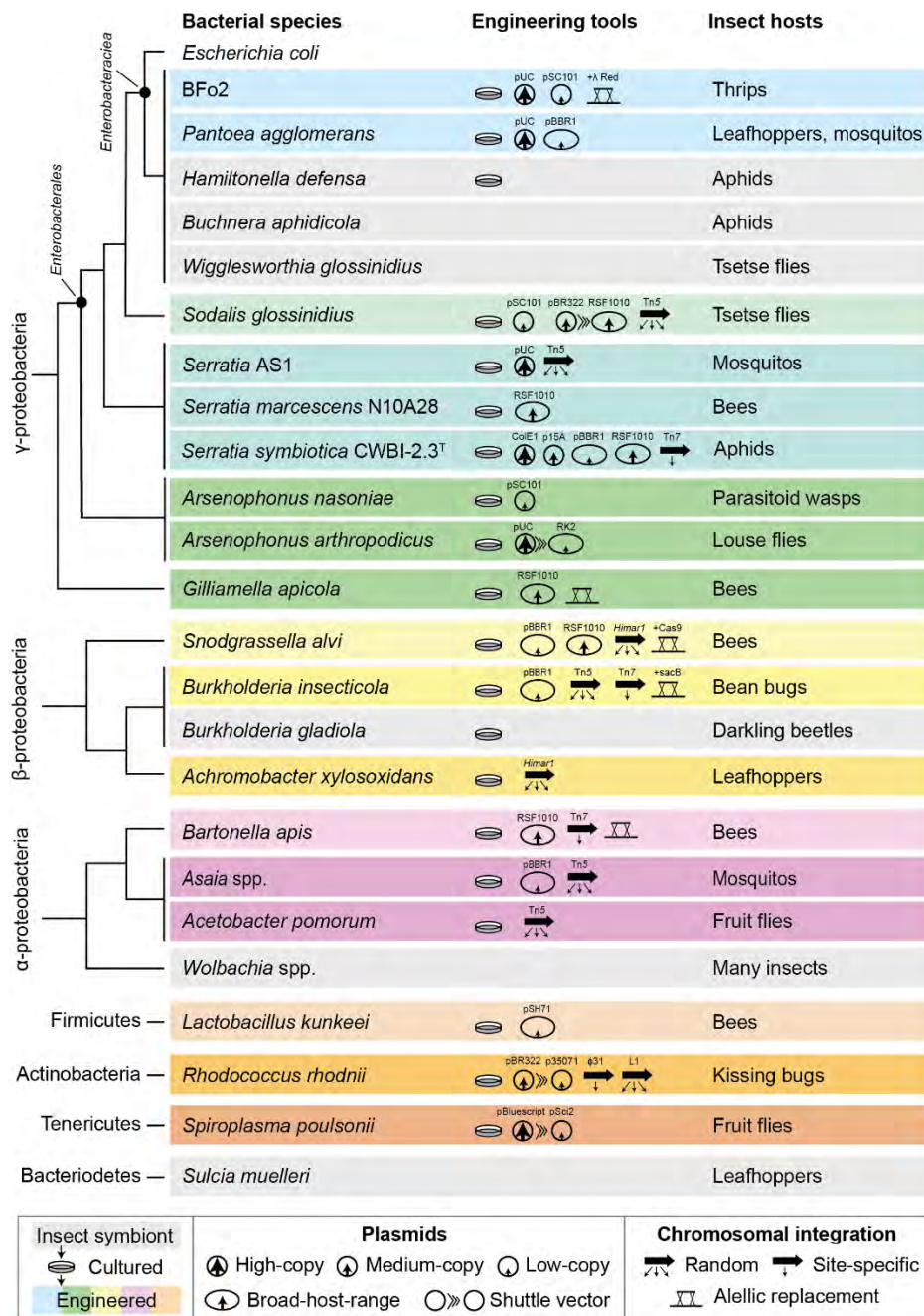


Figure 1.2: Methods used to engineer bacterial endosymbionts of insects.

Whether different species of symbionts have been axenically cultured (petri dish icon) and/or genetically modified (colored background and additional icons) are indicated according to the legend. Bacteria are organized by their taxonomic classification, including a consensus phylogenetic tree for the Enterobacterales (45).

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 (46, 47), mosquitoes (48), thrips (49), and aphids (50). Low-copy plasmids like pSC101 have also been used to engineer symbionts of thrips (49), parasitoid wasps (18) and tsetse flies (51). When these tried-and-true tools work, insect paratransgenesis is especially straightforward.

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. These BHR plasmids are often transferred into symbionts via conjugation from *E. coli*, bypassing the need for developing electroporation or chemical transformation protocols. BHR plasmids derived from pBBR1 function in *Asaia* species (27, 44, 52, 53) and *Burkholderia insecticola* (54–56). Both pBBR1 and RSF1010 BHR plasmids were used to engineer the honey bee gut bacterium *Snodgrassella alvi*, and RSF1010 plasmids function in other phylogenetically diverse bacteria from bees (57). More unusually, a vector containing both a pUC origin and the BHR IncP origin derived from the RK2 plasmid was used to transform *Arsenophonus* from the louse fly (58), and a pSH71-based BHR plasmid was used in *Lactobacillus kunkeei* from honey bees (59).

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. Often, the secondary origin derives from a plasmid that is natively found in the genome of the target bacterium or a close relative. For example, the *Spiroplasma poulsonii* endosymbiont of fruit flies was engineered using a shuttle vector containing the origin of replication of a plasmid native to the closely related bacterium *Spiroplasma citri* and a pBluescript origin that functions in *E. coli* (60).

Editing symbiont genomes

Fewer studies have engineered the chromosomes of bacterial symbionts. One common approach 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 Tn5 transposons were used to randomly insert fluorescent reporter genes into the *Serratia* AS1 (48) and *Asaia* genomes (28), and a reporter gene was inserted at multiple locations in the *Rhodococcus rhodnii* genome using the L1 mycobacteriophage integrase. Random mutagenesis using the *Himar1* mariner transposon enabled high-throughput characterization of gene function in *S. alvi* (61). When combined with functional screening or enrichment, researchers have used random Tn5 transposon mutagenesis to isolate strains with specific gene knock-outs in *B. insecticola* (62), *S. glossinidius* (63), and *Acetobacter pomorum* (64). Site-specific chromosomal integrations have also been performed in the *R. rhodnii* chromosome using the *Streptomyces* phage ϕ C31 integrase (49, 65), and Tn7

transposon constructs have been used to insert genes at a defined site in the genomes of *B. apis* (57), and *B. insecticola* and related *Pandora* species (66, 67).

Although transposons and integrases can achieve some types of chromosomal modifications, they lack the flexibility of allelic exchange methods. Recombination between homology in a donor plasmid or linear DNA fragment and the chromosome allows for the targeted replacement of native sequences with selectable markers and other transgenes. Successful λ Red recombination has been achieved in BFo2 (49) and *S. glossinidius* (51), but this system generally only functions in enterobacteria. Genes have been disrupted via allelic exchange with a suicide plasmid in multiple bee gut bacteria (57) and *R. rhodnii* (49). The efficiency of this technique has been improved in *B. insecticola* by adding a *sacB* counterselectable marker to the donor plasmid (54, 55) and in *S. alvi* by targeting Cas9 cleavage to the sequence being replaced (57).

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 (57). 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 Tn5 *nptII* antibiotic resistance

gene was co-opted to drive transgene expression in *Asaia* (27). 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 (60). A final challenge is that transgenes in a symbiont genome are expected to eventually mutate and cease functioning (68). The selection pressures and bottlenecks experienced by insect symbionts as they are vertically or horizontally transmitted are largely unknown, so it is difficult to predict how evolution will affect the reliability of paratransgenesis.

APPLICATIONS FOR ENGINEERED INSECT SYMBIONTS

Engineered symbionts can be used to study and control insects at several different levels of biological organization (Fig. 1.3). The most straightforward methods 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 those aimed at basic science have focused on finding new ways to control insect populations or their vectorial capacity (Table 1.1).

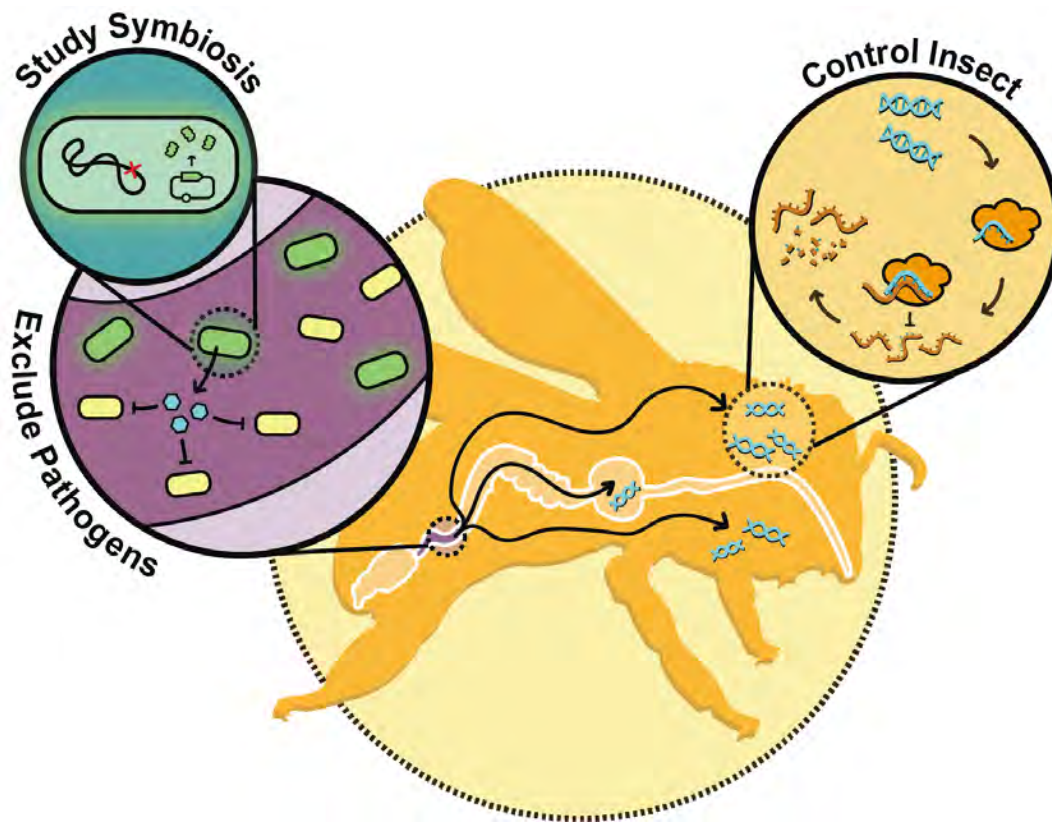


Figure 1.3: Applications of engineered endosymbionts.

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.

Insect	Bacterial Symbiont	Native?	Product or Modification	Purpose	Reference
Aphids (e.g. <i>Acyrtosiphon pisum</i> and <i>Aphis fabae</i>)	<i>Serratia symbiotica</i> CWBI-2.3 ^T	Yes	GFP	Genetically engineer the strain, observe colonization and transmission	(50, 69)
Bean bug (<i>Riptortus pedestris</i>)	<i>Burkholderia cordobensis</i>	Yes	RFP	Observe colonization dynamics	(67)
	<i>Burkholderia insecticola</i>	Yes	GFP, RFP	Observe colonization dynamics	(56, 66, 67, 70)
			Gene knockouts	Study symbiotic relationship	(67, 71)
	<i>Burkholderia spp.</i>	No	GFP	Observe colonization dynamics	(67)
	<i>Pandoraea norimbergensis</i>	No	GFP	Observe colonization dynamics	(67)
Bumble bee (<i>Bombus sp.</i>)	<i>Snodgrassella alvi</i>	Yes	Antibiotic resistance genes	Genetically engineer the strain	(57)
Fruit fly (<i>Drosophila melanogaster</i>)	<i>Acetobacter pomorum</i>	Yes	Transposon insertion library	Study symbiotic relationship	(64)
	<i>Spiroplasma poulsonii</i>	Yes	tdTomato fluorescent protein	Genetically engineer the strain	(60)
Glassy-winged sharpshooter (<i>Homalodisca vitripennis</i>)	<i>Achromobacter xylosoxidans</i>	Yes	dsRed	Observe colonization dynamics	(72)
	<i>Pantoea agglomerans</i> E325	No	Antimicrobial peptides (melittin and scorpine-like molecule)	Prevent transmission of pathogen (<i>Xylella fastidiosa</i>)	(46, 47)

Table 1.1: Examples of insect symbiont engineering

Table 1.1 cont'd

Honey bee (<i>Apis mellifera</i>)	<i>Bartonella apis</i>	Yes	GFP, Gene knockout (<i>gfp</i> , <i>narG</i>)	Genetically engineer the strain	(57)
	<i>Gilliamella apicola</i>	Yes	GFP	Genetically engineer the strain	(57)
	<i>Lactobacillus kunkeei</i>	Yes	NucA reporter protein	Genetically engineer the strain	(59)
	<i>Serratia marcescens</i> N10A28	Yes	GFP and E2-Crimson	Genetically engineer the strain, observe colonization	(57)
	<i>Snodgrassella alvi</i>	Yes	Transposon insertion library	Study symbiotic relationship	(61)
			GFP and E2-Crimson	Genetically engineer the strain, observe colonization	(57)
			Gene knockout (<i>staA</i>)	Study symbiotic relationship	(57)
			Deformed wing virus and <i>Varroa</i> mite dsRNA	Improve host survival under pathogenic and parasitic threat	(73)
Kissing bug (<i>Rhodnius prolixus</i>)	<i>Rhodococcus rhodnii</i>	Yes	Cecropin A	Eliminate sleeping sickness parasite (<i>T. cruzi</i>)	(74)
			Murine antibody fragment	Eliminate sleeping sickness parasite (<i>T. cruzi</i>)	(75)
			Recombinant <i>Arthrobacter</i> β -1, 3-glucanase	Eliminate sleeping sickness parasite (<i>T. cruzi</i>)	(76)
			GFP	Observe transmission dynamics	(49)
			RHBP hairpin RNA, Nitrophorin-1, -2, and Vitellogenin dsRNA, RNaseIII knockout	Test symbiont mediated RNAi, Reduce host fitness	(49, 77)

Table 1.1 cont'd

Leafhopper (<i>Scaphoideus titanus</i>)	<i>Asaia</i> SF2.1	No	GFP	Observe colonization dynamics	(44)
Louse fly (<i>Pseudolynchia canariensis</i>)	<i>Arsenophonus arthropodicus</i>	Yes	Kanamycin resistance	Genetically engineer the strain	(58)
Mosquito (<i>Aedes aegypti</i> and <i>Anopheles stephensi</i>)	<i>Asaia</i> AE10.8	Yes	GFP	Observe colonization of <i>A. aegypti</i>	(44)
	<i>Asaia</i> SF2.1	Yes	GFP, DsRed	Observe colonization and transmission in <i>A. stephensi</i>	(27, 28, 44)
			anti- <i>Plasmodium</i> effector molecules	Eliminate malaria parasites (<i>P. berghei</i>)	(52)
			<i>Wolbachia</i> surface proteins	Reduce parasite transmission (<i>Dirofilaria immitis</i>)	(53)
	<i>Pantoea agglomerans</i>	Yes	GFP	Observe colonization dynamics	(78)
			anti- <i>Plasmodium</i> effector molecules	Eliminate malaria parasites (<i>P. falciparum</i> and <i>P. berghei</i>)	(78)
	<i>Serratia</i> AS1	Yes	mCherry, GFP	Observe colonization and transmission	(48)
			anti- <i>Plasmodium</i> effector molecules	Eliminate malaria parasites (<i>P. falciparum</i> and <i>P. berghei</i>)	(48)
Parasitoid wasp (<i>Nasonia vitripennis</i>)	<i>Arsenophonus nasoniae</i>	Yes	GFP	Observe colonization and transmission	(18)

Table 1.1 cont'd

Tsetse fly (<i>Glossina morsitans</i>)	<i>Sodalis glossinidius</i>	Yes	Antibiotic resistance genes	Genetically engineer the strain	(79)
			GFP	Observe colonization and transmission	(80, 81)
			Transposon insertion library	Study symbiont colonization	(63)
			Chromosomally integrated Rifampicin resistance	Recombineering with lambda red	(51)
Western flower thrips (<i>Frankliniella occidentalis</i>)	BFo2	Yes	alpha-Tubulin dsRNA, RNase III knockout	Test symbiont mediated RNAi, Reduce crop damage	(49)

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 1.1). In contrast to fluorescence *in situ* hybridization (FISH) and other imaging methods that require freezing or fixing specimens, GFP-tagged symbionts can be used to follow how an individual, living insect is colonized over time. The dynamics with which native symbionts colonize the guts of bean bugs (70) and aphids (50, 69) have been characterized in this way. This method also facilitates transmission studies: one can watch infected insects "light up" versus relying on destructive sampling and colony counting or PCR detection. For example, GFP-tagged *Arsenophonus nasoniae*

was used to better resolve its route of transmission between parasitoid wasps (18).

Gene knockouts in symbionts have allowed researchers to identify proteins that are important for the establishment of symbioses. In honeybees, a non-targeted transposon knockout strategy was carried out to broadly understand genes important for gut colonization by *S. alvi* (61). Targeted knockout methods can enhance the precision of these studies and allow researchers to validate the results of RNA-seq or non-targeted knockout approaches. For instance, the *uppP* gene in *Burkholderia* (55) and the *staA* adhesion gene in *S. alvi* (57) were knocked out to demonstrate that these genes are important for microbial colonization of the insect host.

Excluding pathogens

Historically, paratransgenesis has mainly been used 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, the vector for the sleeping sickness parasite (*Trypanosoma cruzi*). A gut symbiont of the kissing bug, *R. rhodnii*, was engineered to express multiple anti-trypanosomal molecules (75, 76), including the antimicrobial peptide (AMP) cecropin A (74), which was shown to destroy trypanosome cells in insects colonized with the engineered symbiont.

Similar work targeted the malaria parasite (*Plasmodium*). Gut symbionts of *Anopheles* mosquitos, including *Pantoea agglomerans* (78), *Asaia* SF2.1 (52),

and *Serratia* AS1 (48), were engineered to express a range of anti-*Plasmodium* effectors. The most potent of these was the AMP, scorpine (48). The engineered *Serratia* AS1 were inherited by three generations of mosquitoes, which is promising for being able to stably establish this solution in a wild vector population. An indirect method for pathogen exclusion has also been shown in mosquitos. *Asaia* SF2.1 designed to express a *Wolbachia* surface protein that stimulated the host's immune response was able to prevent heartworm parasite (*Dirofilaria immitis*) development in *Aedes aegypti* (53).

Recently, this strategy has been extended to agriculture. Glassy-winged sharpshooters (a type of leafhopper) are vectors for *Xylella fastidiosa*, the bacterial pathogen responsible for Pierce's disease in grapes. *Pantoea agglomerans* E325, which colonizes the sharpshooter gut, was engineered to secrete the AMPs melittin and SLM (scorpine-like molecule) (46, 47). When symbionts expressing each effector were co-inoculated into sharpshooters, they destroyed *X. fastidiosa* and reduced the spread of Pierce's disease without harming the insect.

Host control

The most complex applications for engineered symbionts focus on altering their host's biology. Currently, most of these applications in insects 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. While other types of effector molecules could be produced by endosymbionts, this method has the benefit of being easily reprogrammable to

target different genes, and thereby different biological processes. Symbiont-mediated RNAi has 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 (82). Often, feeding dsRNA-producing *E. coli* cells to an insect is a stepping stone to demonstrating symbiont-mediated RNAi.

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 was used to knock down expression of a gene involved in oviposition in kissing bugs (77). 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 (49). Engineering the BFo2 symbiont of western flower thrips to produce dsRNA targeting the essential insect gene alpha-tubulin was also able to cause significant larval mortality and reduce damage to plants (49).

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 (83). Efficacy was initially validated by altering honey bee feeding behavior by targeting an insulin receptor (73). 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) were 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*.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Paratransgenesis has increased our understanding of insect symbionts and their hosts. The potential of this approach for real-world applications in protecting human health and agriculture has also been demonstrated. However, this work has largely been restricted to a few symbionts and laboratory settings. We expect researchers will soon implement paratransgenesis in a wider range of insects and in field-realistic conditions.

In the future, new, high-throughput methods may make it possible to culture, and therefore more easily engineer, additional insect symbionts (84). Even so, it is likely that many bacteria, including obligate intracellular symbionts, will remain impossible to axenically culture. There is also a concern that culturing and engineering host-associated bacteria risks altering their normal interactions with their insect hosts. For example, it has been possible to axenically culture the aphid symbiont *Hamiltonella 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 (85). It has also been reported that *S. poulsonii* expressing GFP colonizes *Drosophila* at lower titers than the wild-type strain and does not exhibit the normal male-killing phenotype (60).

The rapid development of *in situ* engineering approaches may soon enable symbionts to be engineered without interrupting their normal associations

with insects to overcome some of these issues. Synthetic biology continues rapidly advancing. New broad-host-range tools for microbial genetic engineering, such as conjugation systems that can deliver plasmids to bacterial cells within an animal gut (86) and CRISPR-Cas transposase systems that can insert genes into specific members of a microbial community (87), have recently been demonstrated. It remains to be seen, however, exactly how these new approaches can be applied to various insect symbionts.

Engineering endosymbionts is an alternative to using chemical insecticides for pest and vector control that may overcome natural resistance mechanisms (49, 88) and cause fewer off-target effects on beneficial insects (89). 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 sharpshooters (46, 75), so these approaches will likely be tested in more authentic field conditions in the near future. Serious challenges remain as far as proving that engineered symbionts can be safely and effectively deployed in the wild. For this reason, we expect that future engineering of insect symbionts will also need to focus on assuring that engineered symbionts and their transgenes do not have off-target effects that harm natural ecosystems.

Chapter 2: Engineering a culturable *Serratia symbiotica* strain for aphid paratransgenesis

This chapter is reproduced (with minor modifications) from its initial publication:

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Research Contributions

All authors contributed to the development of the research project. K.M.E. and J.P. performed experiments. K.M.E. analyzed data. K.M.E. and J.E.B. wrote the manuscript.

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ABSTRACT

Aphids are global agricultural pests and important models for bacterial symbiosis. To date, none of the native symbionts of aphids have been genetically manipulated, which limits our understanding of how they interact with their hosts. *Serratia symbiotica* CWBI-2.3^T is a culturable, gut-associated bacterium isolated from the black bean aphid. Closely related *Serratia symbiotica* strains are facultative aphid endosymbionts that are vertically transmitted from mother to offspring during embryogenesis. We demonstrate that CWBI-2.3^T can be genetically engineered using a variety of techniques, plasmids, and gene expression parts. Then, we use fluorescent protein expression to track the dynamics with which CWBI-2.3^T colonizes the guts of multiple aphid species, and we measure how this bacterium affects aphid fitness. Finally, we show that we can induce heterologous gene expression from engineered CWBI-2.3^T in living aphids. These results inform the development of CWBI-2.3^T for aphid paratransgenesis, which could be used to study aphid biology and enable future agricultural technologies.

IMPORTANCE

Insects have remarkably diverse and integral roles in global ecosystems. Many harbor symbiotic bacteria, but very few of these bacteria have been genetically engineered. Aphids are major agricultural pests and an important model system for the study of symbiosis. This work describes methods for engineering a culturable aphid symbiont, *Serratia symbiotica* CWBI-2.3^T. These

approaches and genetic tools could be used in the future to implement new paradigms for the biological study and control of aphids.

INTRODUCTION

Many insects have a characteristic bacterial microbiome. These associations can take many different forms, ranging from the conserved gut communities of bees (90) to symbionts of sap-sucking insects that have evolved to resemble organelles (91). These and other bacterial-host relationships have inspired attempts to study and control insects by genetically engineering their resident microbiomes. This approach, known as “paratransgenesis”, has been developed primarily for insects that are vectors of human disease, including kissing bugs, tsetse flies, and mosquitoes (48, 74, 77). Recently, feasibility of paratransgenesis has also been demonstrated in agricultural pests, where it could provide an alternative to chemical pesticides and the development of genetically engineered crops (47, 49, 92, 93).

Aphids are major worldwide agricultural pests and vectors for many plant viruses (94–96). They are also model organisms for understanding insect-endosymbiont coevolution because they have evolved close relationships with multiple species of bacterial symbionts. Typically, aphid symbionts are housed in specialized host cells called bacteriocytes and are reliably vertically transmitted from mother to offspring (91, 97). The obligate symbiont, *Buchnera aphidicola*, plays an essential role producing nutrients lacking in the aphid diet. The relationship between *Buchnera* and its host epitomizes a common dynamic in natural symbioses in which bacteria and host are completely dependent on one another for survival. Aphids can also be associated with facultative symbionts such as *Candidatus* “Hamiltonella defensa”, *Candidatus* “Regiella insecticola”, or *Serratia symbiotica* that provide benefits in stressful environments (98). These

non-essential symbionts have more complex relationships with their aphid hosts (99). *H. defensa* protects aphids from wasp parasitism, but it also reduces host reproduction and longevity (100–102). *Serratia symbiotica* can impose fitness costs on aphids that include increasing their susceptibility to insecticides, but it has also been shown to improve host survival in response to heat stress and wasp parasitism (103–107).

In 2011 a culturable strain of *S. symbiotica*, CWBI-2.3^T, was isolated from the black bean aphid, *Aphis fabae* (108). CWBI-2.3^T is a member of a widespread clade of *S. symbiotica* that is distinct from the bacteriocyte-associated symbiont strains. These gut-associated *S. symbiotica* appear to be at a primitive or transitional stage of symbiosis with aphids (36, 107). More specialized facultative symbiont strains of *S. symbiotica*, such as Tucson and IS, are found primarily in bacteriocytes and the insect hemolymph and are faithfully transmitted to all of a colonized mother's offspring (29). In contrast, *S. symbiotica* CWBI-2.3^T primarily colonizes the digestive tracts of aphids and exhibits only sporadic transmission to progeny (69, 107). Support for CWBI-2.3^T being a transitional stage also comes from its genome, which is intermediate in size between genomes of the strictly symbiotic strains, and genomes of related free-living *Serratia* species (109, 110). CWBI-2.3^T is thought to spread in natural populations of aphids through two horizontal transmission routes. It is excreted in aphid honeydew (liquid feces) onto plant surfaces, which could lead to environmental transmission (36, 107, 111), and it has been reported to spread between aphids feeding on the same plant by colonizing the phloem (112).

We examined the potential of *Serratia symbiotica* CWBI-2.3^T for paratransgenesis in aphids. We found that many plasmids, gene expression

parts, and techniques used in *Escherichia coli* function in CWBI-2.3^T. We used a fluorescently labeled strain to show that *S. symbiotica* CWBI-2.3^T can be reliably established in the guts of multiple species of aphids by feeding. However, colonization with CWBI-2.3^T eventually leads to decreased survival of some species. Finally, we show that we can achieve inducible expression of GFP in engineered CWBI-2.3^T living inside the aphid gut. No other aphid symbionts have been genetically engineered to date. Thus, CWBI-2.3^T represents a promising chassis organism for achieving short-term paratransgenesis, as well as an intriguing organism in which the genetic approaches that we describe could be used to study evolutionary transitions from insect pathogens to symbionts.

RESULTS

Genetic engineering of *S. symbiotica* CWBI-2.3^T

We first tested whether common genetic techniques and DNA parts functioned in *S. symbiotica* CWBI-2.3^T. We began by measuring the growth rate of the wild-type strain. Its doubling time in culture is approximately 4 hours. Accounting for this slower growth rate allowed for the successful transformation of CWBI-2.3^T through conjugation and electroporation procedures used in *E. coli*. By conjugating a nonreplicating donor plasmid encoding a mini-Tn7 construct from *E. coli* into *S. symbiotica* CWBI-2.3^T, we were also able to integrate GFP into a specific location in its chromosome. Integration of engineered constructs in this way enables their functions to be stably maintained without the need for antibiotic selection (113). The resulting GFP-expressing *S. symbiotica* CWBI-2.3^T

strain (CWBI-2.3^T-GFP) can be used to monitor colonization of insects, as we show in the next section.

We also screened CWBI-2.3^T for its ability to maintain plasmids containing different origins of replication and antibiotic resistance cassettes, some of which have been reported to function in *Serratia marcescens* strains (57, 114–116). We transformed plasmids that had either broad-host-range or *E. coli*-specific origins, as well as different copy numbers. Additionally, we tested whether CWBI-2.3^T was compatible with several common antibiotic resistance genes. We achieved successful transformants for every plasmid and antibiotic tested (Table 2.1). CWBI-2.3^T transformed with pBTK570, an RSF1010 plasmid with spectinomycin resistance that expresses E2-Crimson is shown in Figure 2.1A. These results demonstrate great flexibility in how one can genetically modify CWBI-2.3^T using combinations of plasmids and genome integration.

Origin	Copy number	Host range	Plasmid(s)	Reference
RSF1010	Low	Broad	pBTK570, pBTK501, pBTK503, pBTK509, pBTK510	(57)
p15a	Low	Narrow	pBTK409	(57)
pBBR1	Medium	Broad	pAK <i>gfplux1</i>	(117)
ColE1	High	Narrow	pYTK001, mTagBFP2-pBAD	(118, 119)
Antibiotic resistance	Resistance gene	Construct(s)		Reference
Ampicillin/Carbenicillin	<i>bla</i> _{TEM-116}	pAK <i>gfplux1</i> , pBTK409, pBTK501, pBTK503, pBTK509, pBTK510		(57, 117)
Ampicillin/Carbenicillin	<i>bla</i> _{TEM-181}	mTagBFP2-pBAD		(119)
Chloramphenicol	<i>catI</i>	pYTK001		(118)
Gentamicin	<i>aacC1</i>	pBT20		(61, 120)
Kanamycin/Neomycin	<i>aphA-2 (nptII)</i>	miniTn7-GFP-Kan		(57, 121)
Spectinomycin	<i>aadA</i>	pBTK570		(57)

Table 2.1: Plasmid origins and antibiotic resistance genes that function in *S. symbiotica* CWBI-2.3T

To complete our assessment of the capabilities of *S. symbiotica* CWBI-2.3^T we tested the performance of different constitutive and inducible promoters. For applications requiring continuous gene expression, we examined the relative levels of GFP expression from four broad-host-range constitutive promoters: PA1, PA2, PA3, and CP25 (57). Each of these promoters was functional, yielding a fluorescent signal 100-200 times brighter than the background autofluorescence of the wild-type strain (Fig. 2.1B). To allow for controlled gene expression, we also tested the functionality of the pBAD plasmid system, which

uses the *araC* regulator to enable arabinose-inducible expression from the *araBAD* promoter. We found that this system behaves as expected. There was low expression of GFP in the absence of arabinose: the average fluorescence of the uninduced strain containing the pBAD-GFP plasmid was only 1.12 times the background of wild-type CWBI-2.3^T with no plasmid. GFP fluorescence in the strain with the pBAD-GFP plasmid became 39 and 57 times brighter than the wild-type strain when induced with 0.2% or 2% arabinose, respectively (Fig. 2.1C).

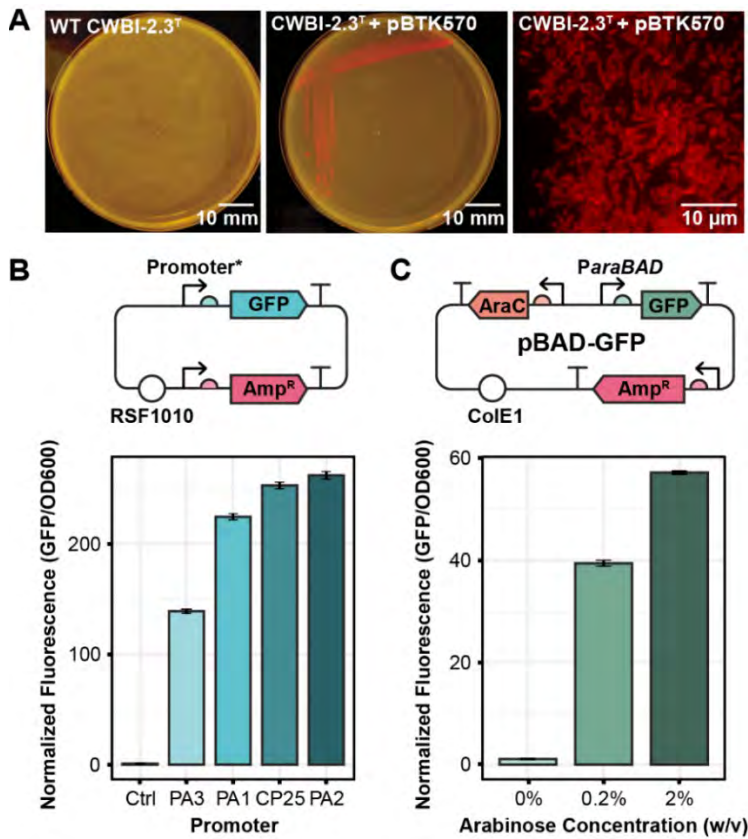


Figure 2.1: Origin and promoter function in *Serratia symbiotica* CWBI-2.3T

(A) Representative images of CWBI-2.3T transformed with an RSF1010 origin plasmid expressing E2-Crimson. The left two images comparing this strain to wild-type CWBI-2.3T were captured at the macro scale using a blue light transducer and edited with linear adjustments. The image on the right was captured at the micro scale using a Nikon Eclipse inverted fluorescence microscope (640 nm excitation, 685 nm emission) and was linearly adjusted and pseudocolored. Plasmid features in (B) and (C) are shown using Synthetic Biology Open Language Visual symbols: promoter (bent arrow), ribosome binding site (semicircle), open reading frame (block arrow), terminator (T bar), and origin of replication (open circle). (B) Normalized expression of GFP from a series of plasmids that are identical except for their GFP promoter sequences. The plasmid map is shown above and expression levels are shown below. Expression for each promoter is normalized to the GFP/OD600 reading for wild-type CWBI-2.3T (Ctrl). (C) Normalized expression of GFP from CWBI-2.3T-pBAD-GFP following induction with arabinose. A schematic of pBAD-GFP is shown above and expression levels are shown below. Fluorescence at each inducer concentration is normalized to the GFP/OD600 reading for wild-type CWBI-2.3T.

Engineered *S. symbiotica* CWBI-2.3^T can colonize multiple species of aphids

To test the versatility of engineered CWBI-2.3T for paratransgenesis we assessed its ability to colonize multiple species of aphids and monitored the colonization dynamics using CWBI-2.3T-GFP. We first established a method for delivering engineered CWBI-2.3T to aphids by adapting the methods of Renoz et al. (111). CWBI-2.3T-GFP was delivered to 3rd instar aphids through feeding on an artificial diet. Three days after feeding, a blue light transilluminator could be used to observe GFP fluorescence from CWBI-2.3T-GFP inside the colonized aphids. Using this feeding technique with *Ac. pisum*, we were able to reliably colonize an average of about 75% of treated aphids.

The fluorescence of the CWBI-2.3T-GFP strain also allowed us to monitor how CWBI-2.3T colonizes living aphids. We observed that over the course of 10 days *S. symbiotica* CWBI-2.3T spreads through the digestive tract of *Ac. pisum* until the entire gut is colonized (Fig. 2.2A, Fig. 2.3). No bacteria were observed outside of the gut in bacteriocytes or embryos (Fig. 2.3), and the gut remained colonized and fluorescent for the duration of experiments (up to 10 days after feeding for *A. pisum*). We also quantified host colonization by tracking the number of colony-forming units (CFUs) per aphid. On 2,3,5 and 10 days post-feeding, colonized *Ac. pisum* were crushed and plated to determine the bacterial load in their bodies. CWBI-2.3T titer increased over time until it reached an average of about 108 CFUs per aphid on day 5. Between day 5 and day 10, no significant change in titer was observed ($p = 0.80$, two-tailed Mann-Whitney U test) (Fig. 2.2B). All of these results are consistent with prior studies that used

fluorescence in situ hybridization and quantitative PCR to monitor *Ac. pisum* colonization (111).

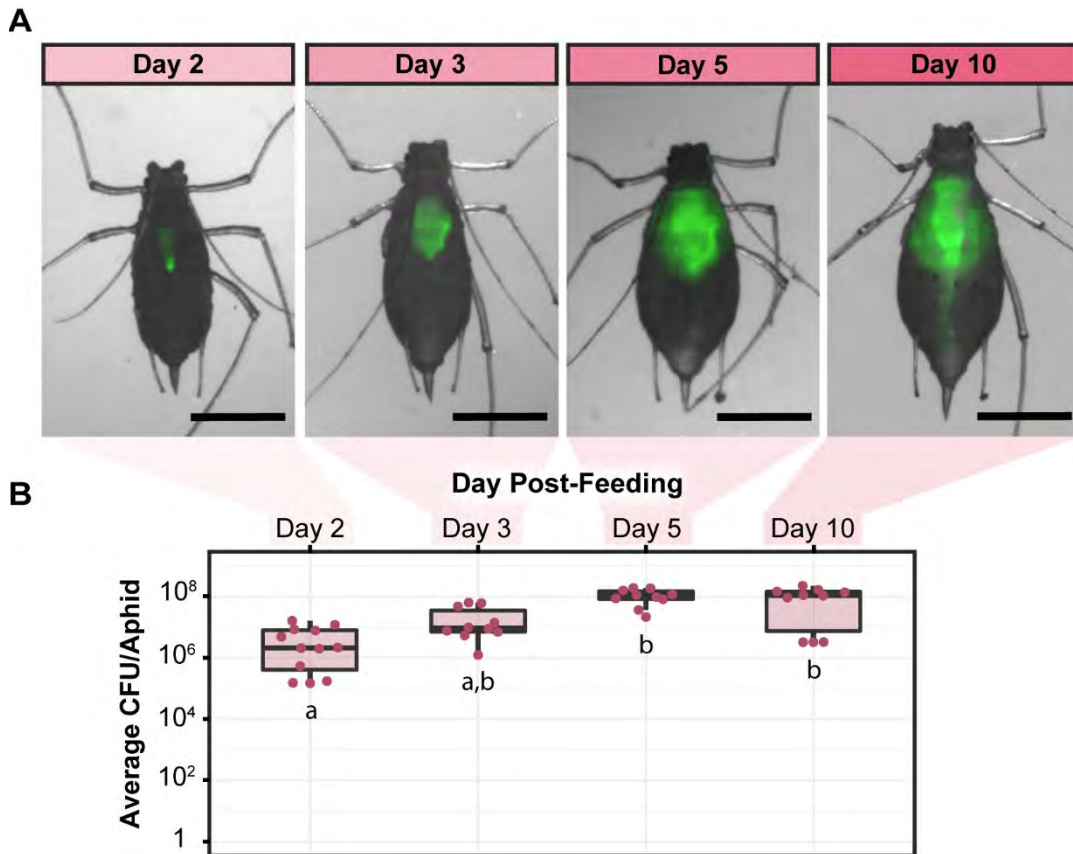


Figure 2.2: Colonization dynamics of CWBI-2.3T in *Ac. pisum*

(A) Composite brightfield and fluorescence images tracking the spread of CWBI-2.3^T-GFP through the gut of a live *Ac. pisum* individual. Dorsal images of the same aphid were captured for each time point. Images were linearly adjusted to enhance the brightness of GFP. Scale bar is 1 mm. (B) CWBI-2.3^T-GFP titer in *Ac. pisum* over time. At least 10 aphids per time point were crushed and plated to perform CFU counts. Lowercase letters (a, b) below the bars designate groups of time points at which significantly different colonization levels were observed (Dunn's test with Bonferroni correction, $p < 0.05$).

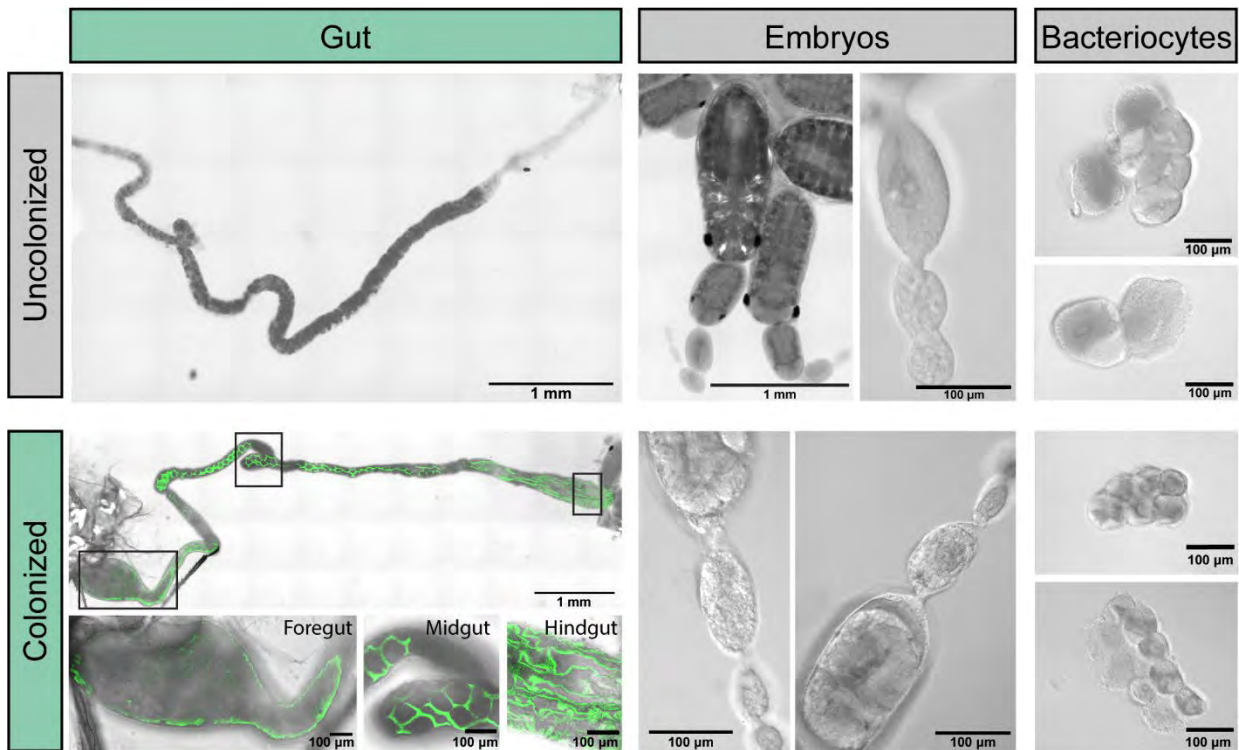


Figure 2.3: CWBI-2.3T-GFP colonizes the aphid gut

A. *pisum* fed on diet alone (uncolonized) and diet containing CWBI-2.3^T-GFP (colonized) were dissected at 10 days following treatment. Laser scanning confocal microscopy was used to capture brightfield and GFP images for each tissue type, and images were linearly adjusted to enhance the brightness of GFP. CWBI-2.3^T-GFP was only observed in the aphid gut and not in embryos, bacteriocytes, or other tissues.

We next tested whether the colonization pattern in *Ac. pisum* was representative of other aphid species by repeating this experiment with *Aphis fabae* (black bean aphid), *Aphis craccivora* (cowpea aphid), and *Lipaphis erysimi* (mustard aphid). We found that CWBI-2.3^T-GFP principally colonizes the gut of these three species as well (Fig. 2.4A). Each of the other aphids tested also showed a similar trend in bacterial titer over time. The CFUs per aphid

consistently reached between 10^7 and 10^8 on the fifth day after treatment for all four species, with averages of 5.3×10^7 , 7.4×10^7 , 2.4×10^7 , and 1.2×10^7 for *Ac. pisum*, *A. fabae*, *A. craccivora*, and *L. erysimi*, respectively (Fig. 2.4B). There were slight, but significant differences in bacterial titers observed in the four species ($p = 0.0018$, Kruskal-Wallis test). Titer was highest in *A. fabae*, which is the natural host from which CWBI-2.3^T was isolated. The trend in the other species may reflect their different body sizes, as *Ac. pisum* is much larger than the other aphids.

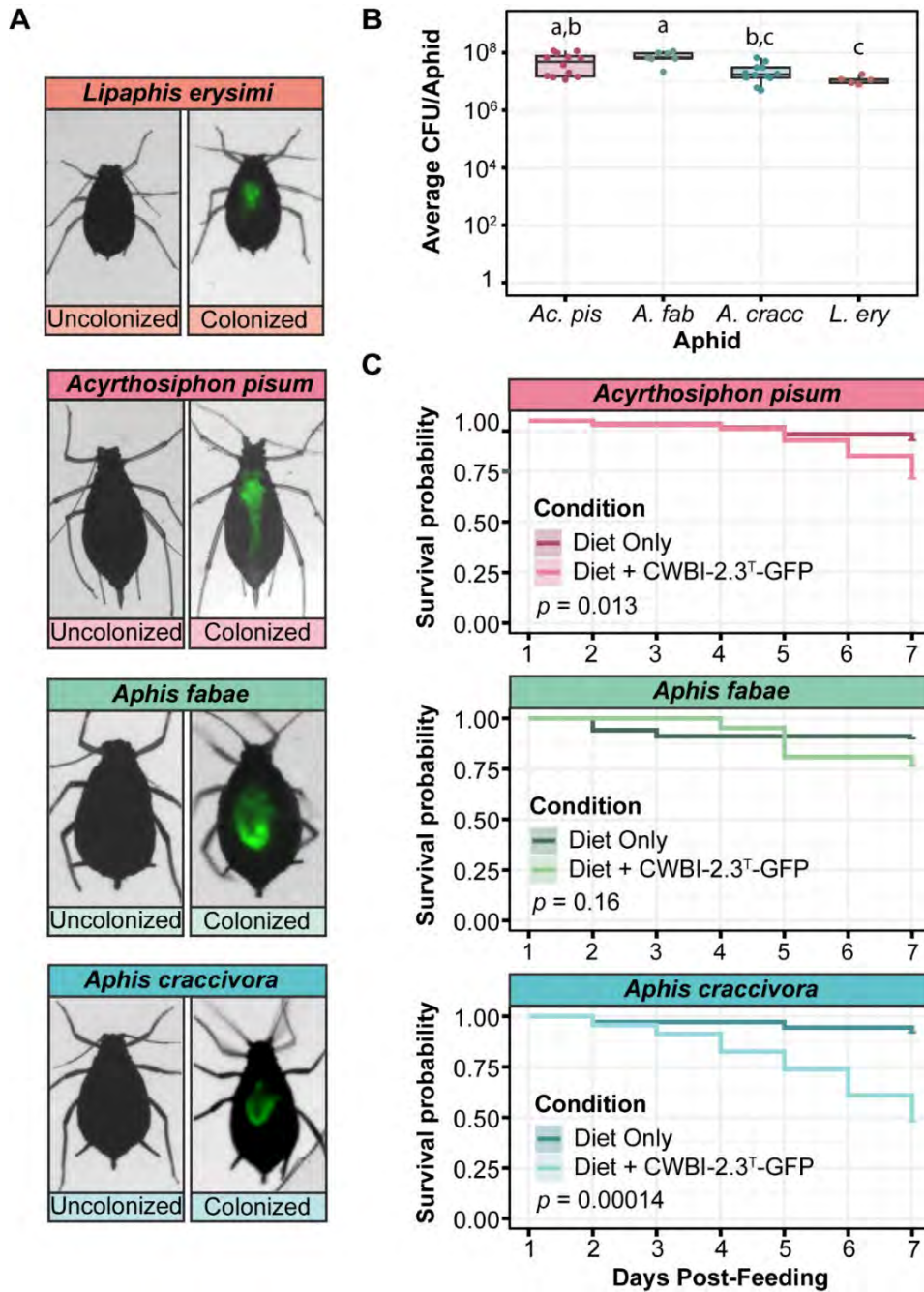


Figure 2.4: CWBI-2.3T-GFP colonization of multiple aphid species

(A) Composite brightfield and fluorescent stereoscope images showing CWBI-2.3^T-GFP colonization of four different species. Photographs for the uncolonized and colonized pairs were captured with the same exposure for comparison.

Figure 2.4 cont'd.

Images were captured from the ventral side of each aphid on the 5th day after feeding. (B) CWBI-2.3^T-GFP titer in each of the 4 species on day 5 post-feeding. At least 5 aphids per species were crushed and plated to perform CFU counts. Lowercase letters (a, b, c) above the bars designate sets of aphid species for which significantly different colonization levels were observed (Dunn's test with Bonferroni correction, $p < 0.05$). (C) Mortality curves showing the survival probability of each aphid colonized with CWBI-2.3^T-GFP compared to uncolonized aphids. Counts began on the first day after feeding to eliminate aphids that died during experimental setup. The starting population was at least 21 aphids for each survival curve. The statistical significance of differences in aphid survival were calculated using a log-rank test (p values shown in each panel).

S. symbiotica CWBI-2.3^T decreases the survival of some aphid hosts

Next, we investigated how CWBI-2.3^T affected a component of aphid fitness. We monitored the survival of aphids for 7 days after colonizing them with CWBI-2.3^T-GFP through feeding (Fig. 2.4C). We observed a similar impact of CWBI-2.3^T-GFP on the survival of *Ac. pisum* and *A. fabae*. After 7 days, the treated *A. fabae* and *Ac. pisum* populations lost 15% and 19% more aphids than their respective control populations. This decrease was not significant for *A. fabae* and only marginally significant for *Ac. pisum* ($p = 0.16$ and $p = 0.013$, log-rank tests, respectively). These results are consistent with previous studies that used wild-type CWBI-2.3^T (107, 111). Colonization with engineered CWBI-2.3^T had a larger effect on *A. craccivora* survival. After 7 days, 44% fewer colonized *A. craccivora* remained alive compared to the uncolonized aphids of this species, and this reduction was highly significant ($p = 0.00014$, log-rank test). The effects of CWBI-2.3^T colonization on *A. craccivora* have not been tested before. Overall,

these results show that CWBI-2.3^T can have a range of fitness effects on different aphid host species.

Induction of *S. symbiotica* CWBI-2.3^T can be performed *in vivo*

To enable the controlled expression of genes inside living aphids, we tested whether we could induce the expression of GFP inside *A. pisum*. We first colonized *Ac. pisum* with wild-type CWBI-2.3^T or CWBI-2.3^T transformed with the plasmid pBAD-GFP (strain CWBI-2.3^T-pBAD-GFP). Three days later we fed those same aphids on leaves embedded in agar containing no arabinose or 2% arabinose. We observed that after one day of feeding, only the aphids that were colonized with CWBI-2.3^T-pBAD-GFP and fed on 2% arabinose showed visible GFP fluorescence (Fig. 2.5A). We quantified the per-aphid fluorescence in each condition and found that arabinose induction significantly increased the GFP signal in the CWBI-2.3^T-pBAD-GFP aphids ($p = 6.9 \times 10^{-8}$, one-tailed Mann-Whitney U test) (Fig. 2.5B). On average, GFP fluorescence was 83.5 times brighter with induction compared to the uninduced condition.

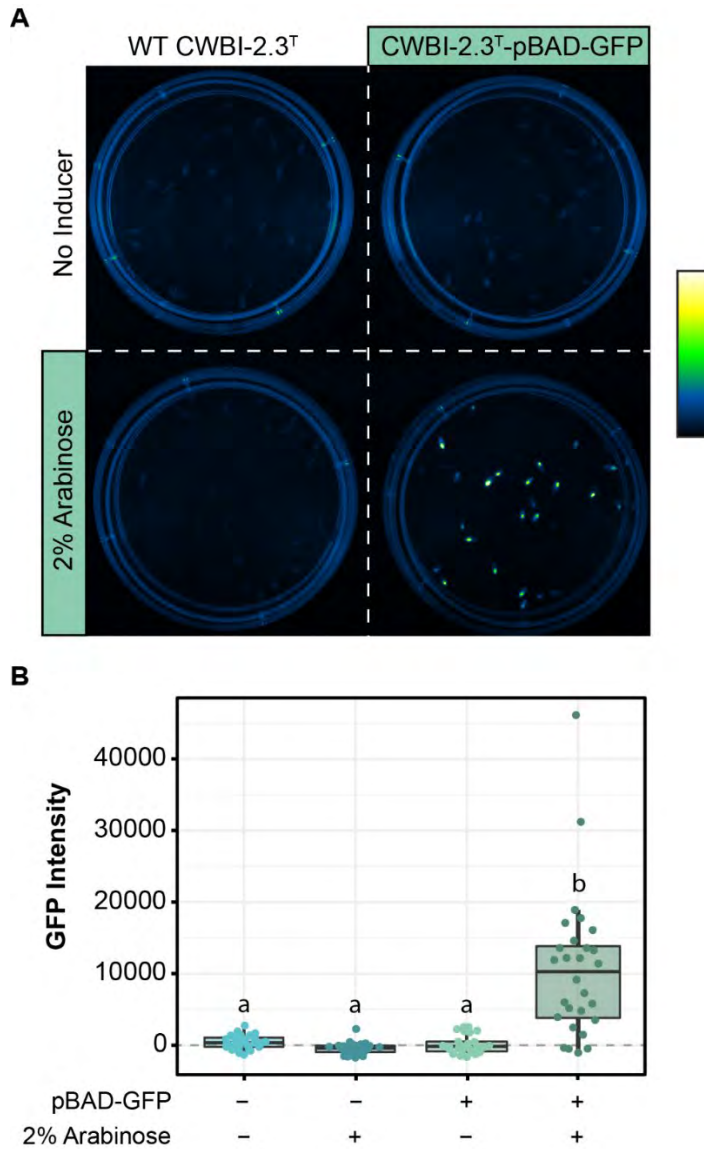


Figure 2.5: GFP induction inside living aphids

(A) Photograph of GFP expression for each of the four conditions tested for induction: CWBI-2.3^T ± Arabinose and CWBI-2.3^T-pBAD-GFP ± Arabinose. The image has been pseudocolored and linearly adjusted for contrast. (B) Boxplots showing the fluorescence distribution of each population of treated aphids. The GFP intensity of at least 24 aphids was measured per condition. Lowercase letters (a, b) above the bars designate treatments for which significantly different colonization levels were observed (Dunn's test with Bonferroni correction, $p < 0.05$).

We confirmed that the lack of fluorescence in the uninduced CWBI-2.3^T-pBAD-GFP condition was not due to poor colonization or loss of plasmid function by crushing the aphids and plating their bacteria on media containing 2% arabinose. We found that 93% and 100% of the aphids in the uninduced and induced CWBI-2.3^T-pBAD-GFP conditions, respectively, were colonized with *S. symbiotica*. In both conditions, the plated bacteria all expressed GFP, indicating that the plasmid had not been lost or mutated. The discrepancy between the percentage of aphids that were colonized (100%) and how many visibly fluoresced (82.8%) in the induced condition and variation in the fluorescence per aphid are likely attributable to differences in when and how much individual aphids fed on the inducer under the experimental conditions.

DISCUSSION

In this study we establish a platform that can be used for aphid paratransgenesis. We found that *Serratia symbiotica* CWBI-2.3^T is compatible with both broad-host-range and *E. coli* plasmids and gene expression parts. We fed engineered CWBI-2.3^T back to multiple aphid species, tracked gut colonization over time, and characterized the effects on insect survival. Lastly, we showed that an engineered function in CWBI-2.3^T could be induced within living aphids. This work provides new tools that can be used to increase our understanding of aphid/symbiont relationships, as well as a foundation for developing methods for the control of aphids pests without the need for insect or plant genetic engineering.

Previous studies of CWBI-2.3^T characterized its relationship with *A. fabae* and *Ac. pisum* (107, 111). These experiments established the basis for the hypothesis that CWBI-2.3^T and related strains may resemble a transitional proto-symbiont of aphids. In particular, *S. symbiotica* CWBI-2.3^T does not colonize aphid bacteriocytes or exhibit vertical transmission to offspring (36, 107), which are important characteristics of obligate and facultative bacterial symbionts that have co-evolved with aphids. Our results with engineered CWBI-2.3^T support the original findings in *Ac. pisum* and *A. fabae*, and we find similar results in two additional aphid species: *A. craccivora* and *L. erysimi*. CWBI-2.3^T colonizes the guts of all these aphids and can have mild to moderate negative effects on aphid fitness. These results support the hypothesis that this strain represents a transitional state of symbiosis.

S. symbiotica provides a unique opportunity to study the evolution of aphid symbioses because the entire spectrum of known host-microbe relationships are represented within a single bacterial species (109, 110). CWBI-2.3^T and its relatives are aphid proto-symbionts and retain their ability to survive outside the host. *S. symbiotica* Tucson, IS, and similar strains are more specialized facultative symbionts of many aphid species. *S. symbiotica* SCt, SCc, and STs have evolved to act as co-obligate symbionts with *B. aphidicola* in Lachninae aphids. As “transitional” symbionts, CWBI-2.3^T and related *S. symbiotica* strains likely have genetic features that distinguish them from the more specialized endosymbiont clades. For example, the genome of the CWBI-2.3^T, while reduced, is about 1 Mb larger than that of the bacteriocyte-associated *S. symbiotica* Tucson strain found in *Ac. pisum* (109). The genomes of the CWBI-2.3^T-like *S. symbiotica* strains encode potential pathogenicity factors that are

missing in the bacteriocyte-associated strains, which could contribute to their more volatile relationships with their hosts (122).

We expect that our work will empower future studies of the proto-symbiont relationship between CWBI-2.3^T and aphids. By engineering CWBI-2.3^T we were able to observe colonization dynamics in living aphids and easily distinguish the colonization status of the aphids due to their fluorescence. This feature simplifies large assays where colonization is required, such as mortality or transmission experiments. Similar work engineering *Arsenophonus nasoniae*, a parasitoid wasp symbiont, to express GFP allowed researchers to better understand how it is vertically transmitted during oviposition (18). In the future, the ability to engineer CWBI-2.3^T and deliver it back to aphids could be combined with engineering tools for gene knockout. This approach was used to study the role of the type III secretion system of *Sodalis glossinidius* in establishing a symbiotic relationship with its tsetse fly host (63). Similar genetic approaches could be used to understand the role of putative symbiotic factors in CWBI-2.3^T that may have enabled its transition from a free-living, plant-associated bacterium to an insect symbiont.

Engineering CWBI-2.3^T to manipulate aphid biology could also be of interest for agricultural applications. It has been proposed that insect paratransgenesis could be used for targeted pest-control strategies that pose less of a risk to the environment than chemical pesticides (93, 123). In aphids, one approach would be to engineer CWBI-2.3^T to reduce the capacity of aphids for vectoring plant diseases. Related techniques have already been carried out by groups performing paratransgenesis in other insects. For instance, *Pantoea agglomerans*, a symbiont of the glassy-winged sharpshooter, was engineered to

produce antimicrobial peptides that selectively kill the phytopathogen *Xylella fastidiosa* that is carried and spread by sharpshooters (47). Another *Serratia* symbiont, *Serratia* AS1, was engineered to limit mosquito transmission of the malaria parasite (48). These approaches took advantage of the fact that both symbiont and pathogen occupied the same gut environment within their host to successfully reduce pathogen transmission. In aphids, CWBI-2.3^T could be used to similarly prevent the spread of bacterial phytopathogens that propagate within aphids such as *Erwinia aphidicola*, *Dickeya dadantii*, and *Pseudomonas syringae* (19).

Our ability to engineer CWBI-2.3^T could also be adapted to enable symbiont-mediated RNA interference (RNAi) to control aphid gene expression and vectorial capacity. This method enables targeted gene silencing by engineering the symbiont to produce double-stranded RNA inside the host to induce its innate RNAi response. Symbiont-mediated RNAi has proven to be effective for genetically manipulating insects, such as kissing bugs and western flower thrips, that are typically less compatible with injecting or feeding double-stranded RNA (49, 77). This approach has also recently been used to protect honeybees from parasitic mites and deformed wing virus (73). In aphids, symbiont-mediated RNAi could be used to directly target plant viruses that circulate and/or propagate in aphids (e.g., Luteoviruses such as potato leaf roll virus and Rhabdoviruses such as lettuce necrotic yellows virus) (124, 125). It might also be used to reduce the expression of the receptors that noncirculative viruses bind to such as the Stylin-01 receptor bound by cauliflower mosaic virus (126).

Overall, the capabilities for engineering *S. symbiotica* CWBI-2.3^T that we have demonstrated could lead to a multitude of new applications in the future. They may help researchers build a better understanding of the evolution of host-microbe symbioses in the well-established aphid model system. These tools also provide a foundation for exploring new synthetic biology approaches for pest management that could lead to safer and more environmentally friendly agricultural practices.

MATERIALS AND METHODS

Growth of *S. symbiotica* CWBI 2.3^T

We obtained *S. symbiotica* CWBI 2.3^T from the German Collection of Microorganisms and Cell Cultures (DSM 23270). Bacteria were cultured at room temperature (~25°C) in BBL Trypticase Soy Broth (TSB) or on Trypticase Soy Agar (TSA) plates (Becton, Dickinson and Company, MD, USA). For liquid cultures, the tubes were incubated with orbital shaking at 180 r.p.m. over a diameter of one inch. Concentrations of antibiotics and other media supplements used in this study are as follows: carbenicillin (100 µg/mL), chloramphenicol (20 µg/mL), gentamicin (40 µg/mL), kanamycin (50 µg/mL), spectinomycin (60 µg/mL), and diaminopimelic acid (DAP)(0.3 mM). To assess growth rate, two-day-old cultures of CWBI-2.3^T were grown up and diluted 1:100 into fresh TSA in a 96 well plate. Ten replicates were included on the plate which was incubated at 25°C with 6 mm amplitude orbital shaking every 15 seconds in an Infinite 200 Pro plate reader (Tecan , Männedorf, Switzerland). OD600 readings were taken

every 10 minutes for 48 hours. Growth curves were fit to a logistic model using Growthcurver (version 0.3.0) (127).

Growth and maintenance of aphid colonies

Acyrtosiphon pisum LSR1 was acquired from long-term stocks maintained in the lab of Nancy Moran (University of Texas at Austin). *Aphis fabae* was obtained from the lab of Thierry Hance (Université catholique de Louvain, Belgium). *Aphis craccivora* and *Lypaphis erysimi* were collected in Austin, TX and identified by COI barcode sequencing using the LepF (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') primer pair (128). *Ac. pisum*, *A. fabae*, and *A. craccivora* were maintained on Broad Windsor *Vicia faba* plants (Mountain Valley Seed Company, UT, USA). *L. erysimi* was reared on *Brassica oleracea* var. Capitata (The Seed Plant, TX, USA). All colonies were maintained in cup cages on their respective plants at 20°C with a long (16L:8D) photoperiod in Percival I-36LLVL incubators (Perry, IA, USA).

Transformation of *S. symbiotica* CWBI 2.3^T

S. symbiotica CWBI 2.3^T was made electrocompetent using a modification of a protocol for *E. coli*. Cultures were first grown for two days until they reached saturation. Fifty microliters of saturated culture were then inoculated into 50 mL of TSB in a 250 mL flask and grown for about 16 h to mid-log phase (OD₆₀₀ = 0.4-0.6). Cells were then centrifuged at 4500 × *g* for 5 min, the supernatant was

discarded, and the cells were resuspended in 40 mL of 10% glycerol. This wash step was repeated 4 additional times. The pellet was then resuspended in 500 μ L of 10% glycerol, divided into 50 μ L aliquots, and frozen at -80°C . For electroporation, 2 μ L plasmid was added to 50 μ L of electrocompetent cells and electroporated at 2.5 V in a 0.1 cm cuvette with the BioRad MicroPulser (Hercules, CA, USA). The cells were resuspended in 950 μ L TSB and allowed to recover overnight (~ 16 h) then plated on TSB agar with selective antibiotic.

For conjugative transformation, cultures of donor *E. coli* strain, MFDpir and the recipient *S. symbiotica* CWBI-2.3^T strain were first grown to saturation. Then, 1 mL of each was centrifuged at $1000 \times g$ for 5 min. The supernatant was discarded, and the pellets were resuspended in 1 mL 145 mM saline and centrifuged again as before. The wash step was repeated, and both pellets were resuspended in 500 μ L of 145 mM saline. A 50 μ L 1:100 ratio of donor:recipient cells was prepared in a separate Eppendorf tube then spot plated on a TSB + DAP plate. After two days of growth on the conjugation plate, a metal loop was used to scrape up a small pellet of bacteria from each spot and place it into 1 mL of saline solution. The pellets were then centrifuged and washed twice with saline as described in the steps for the previous day. Next, 100 μ L of resuspended solution along with 100 μ L of $10\times$ concentrated solution was plated on TSA plates containing selective antibiotics. After two to three days of growth, successfully conjugated CWBI-2.3^T colonies were picked and screened for proper strain identity and presence of the conjugative plasmid.

Mini-Tn7 integration into the CWBI-2.3^T chromosome

Integration of a GFP and kanamycin resistance gene cassette into *S. symbiotica* to create strain CWBI-2.3^T-GFP was carried out using the mini-Tn7 system (121). Integration was performed according to the conjugation steps described above, with modifications for the use of two MFDpir⁺ donor strains. One donor strain expresses the suicide delivery vector containing the genes of interest (GFP/Kan^R), and the other holds the helper plasmid (pTNS2) containing the components of the TnsABCD site-specific transposition pathway. Conjugation of both plasmids should insert the GFP/Kan^R genes into the chromosome of *S. symbiotica* 25 base pairs downstream of the *glmS* gene at the *attTn7* site. Chromosomal integration at the expected site was confirmed through PCR and Sanger sequencing using the GFP-mut3-forward (5'-AGCCGTGACAAACTCAAGAA-3') and *glmS*-reverse (5'-GCCGTTGCAATTGTTGTC-3') primer pair.

Assessing plasmid origin, antibiotic resistance gene, and promoter function in CWBI-2.3^T

The compatibility of different origins of replication and antibiotic resistance genes was tested by transforming CWBI-2.3^T with various plasmids, as shown in Table 1. Transformants were picked, and plasmids were purified from cells and verified by Sanger sequencing to confirm successful transformation.

Gene expression from various synthetic promoters was tested by transforming pBTK501, pBTK503, pBTK509, and pBTK510 from the bee microbiome toolkit into CWBI-2.3^T (57). Each strain was grown in culture for two days, then transferred to a fresh tube and diluted to an OD of 0.05 in triplicate.

Following an additional day of growth, GFP fluorescence was measured in the Tecan Infinite 200 Pro plate reader (485 nm excitation, 535 nm emission). Three technical replicates were measured for each of the three biological replicates.

To construct the pBAD-GFP plasmid, we used Gibson assembly to combine the pBR322 origin, *araC* regulator, *araBAD* promoter, and ampicillin resistance gene from the mTagBFP2-pBAD plasmid (Addgene #54572) with the GFPmut3 gene from pBTK503 (57, 119). Overnight cultures of CWBI-2.3^T transformed with pBAD-GFP (CWBI-2.3^T-pBAD-GFP) were spiked with 0.2 or 2% (w/v) arabinose. Three biological replicates of each condition, including the no arabinose control, were used for this experiment. After 24 additional hours of growth, GFP fluorescence was measured using the Infinite 200 Pro plate reader (485 nm excitation, 525 nm emission).

Feeding *S. symbiotica* CWBI-2.3^T to aphids

Feeding was carried out using age-controlled aphid populations reared on *V. faba*. Third instar aphids were used for all feeding experiments. Cultures of *S. symbiotica* CWBI-2.3^T were first grown to log phase, centrifuged at 1000 × *g* for 5 min, washed twice with 1× PBS (phosphate buffered saline), then resuspended in 1× PBS to an OD of 1. One microliter of this culture, along with 2 µL of yellow food dye to improve aphid feeding rates (Gel Spice Company Inc., NJ, USA), was added per 100 µL of Febvay artificial diet (129). Feeding chambers were assembled using 33 mm petri dishes and two layers of parafilm. A hole was cut into the bottom of one half of the petri dish and covered with a piece of tightly woven mesh for air flow. A single piece of parafilm was stretched thin over the

other half, and 100 μ L of diet was pipetted onto the parafilm. The other piece of parafilm was stretched thin over top of the diet to create a “sandwich”. Up to 30 aphids were added to the other half of the petri dish and both sides were sealed together with parafilm. Aphids were fed for 16-24 h then transferred to *V. faba* plants and observed as needed.

Imaging aphid colonization

Five- to six-day-old aphids were fed on diet plates containing CWBI-2.3^T-GFP as described in the quantification methods above. For the time-course, *Ac. pisum* was checked for colonization on the second day post-feeding using a GBox-F3 Syngene imager, and 4 were selected to be imaged for the time points at 2, 3, 5, and 10 days post-treatment. Between all imaging time points the aphids were maintained on separate *V. faba* plants under normal conditions. For the images of the different aphid species at one time point, aphids were screened for colonization on day 5 and 3 of each were randomly selected to be imaged. A Leica MZ16 Fluorescent Stereoscope was used in conjunction with the Leica Application Suite software to capture all images. Images were captured in virtual stacks using both a brightfield and GFP channel. To qualitatively highlight the localization of the colonizing bacteria, the brightness and contrast of the images were linearly adjusted using ImageJ (version 1.52p) as necessary.

To acquire confocal images of aphids, *A. pisum* was fed on plain diet or diet containing CWBI-2.3^T-GFP and dissected at 10 days after feeding. Guts, bacteriocytes, and embryos from three colonized aphids and one control were mounted in PBS solution on glass slides for imaging. Brightfield and GFP

channel images were captured on a Zeiss LSM 710 laser scanning confocal microscope and the resulting images were processed using ImageJ.

Quantifying colonization of aphids with CWBI-2.3^T

Each species of aphid was first fed on diet containing CWBI-2.3^T-GFP at 5-6 days of age as described above. Aphids were fed in pools of 25-30 aphids per container. After 24 hours, aphids were moved to *V. faba* plants. The day when the aphids were transferred to plants serves as day 0 for the colonization experiments. On days 2, 3, 5, and 10, aphids were collected and processed. First, aphids were surface sterilized by soaking in 200 μ L 10% bleach for 1 minute. Aphids were then rinsed with distilled water, resuspended in 200 μ L saline and squashed with a pestle. The squashed aphid mixture was serially diluted 10-fold to reach final 10⁵ dilution. Five microliters of each dilution mix were spotted onto TSA + kanamycin plates (3 replicates per aphid). The plates were incubated for 3 days and then colonies were counted to determine the CFU per aphid.

Aphid fitness following colonization with CWBI-2.3^T

We assessed the effect of CWBI-2.3^T on the fitness of each aphid species by measuring mortality rates. Aphids were fed on either plain diet or diet containing CWBI-2.3^T-GFP. Following treatment, they were transferred to plants, and the number of living adult aphids was recorded each following day. To determine whether aphids that died prior to day 5 were colonized with CWBI-2.3^T

(before it expressed sufficient GFP to be detected by eye), these dead aphids were collected, washed in bleach, squashed and plated as described in the quantification section above. Pieces of mesh material were wrapped around the base of each plant at the start of the experiment so that dead aphids could be easily collected. On day 5, the remainder of the uncolonized aphids were visually sorted and removed from the experimental pool. Mortality was recorded for a total of 7 days post-treatment.

Inducible control of GFP expression *in vivo*

The assay for the function of *in vivo* induction was carried out in two separate feeding steps: colonization with CWBI-2.3^T and induction with arabinose. To colonize the aphids with the symbiont, aphids were first fed on diet plates containing either wild-type CWBI-2.3^T or CWBI-2.3^T-pBAD-GFP. After feeding on diet for 24 hours the aphids were transferred to fava beans. Induction plates were created using 2 cm diameter petri dishes. Each dish was propped up at an angle, and 1 mL 1.5% agar with or without 2% (w/v) arabinose was added. *V. faba* leaves were added to the agar as it cooled, and plates were stored at 4°C. All induction plates were prepared 2 days before aphids fed on them. The aphids were transferred to the induction plates after resting on the fava bean plants for 3 days. Three plates containing 30 aphids each were set up for each condition: CWBI-2.3^T, CWBI-2.3^T + 2% arabinose, CWBI-2.3^T-pBAD-GFP, and CWBI-2.3^T-pBAD-GFP + 2% arabinose.

After 24 hours of feeding on the induction plates, 30 aphids per condition were randomly selected and pooled, and GFP expression of all the aphids was

photographed using the GBox-F3 Syngene imager using a blue LED excitation light and SW06 emission filter. Plates were photographed together in one image to ensure comparable fluorescence measures. To confirm that the aphids were colonized and that the number of aphids showing fluorescence was equivalent to the number of aphids colonized with CWBI-2.3^T-pBAD-GFP, aphids from the induced and uninduced conditions were squashed and 10-fold dilutions were plated onto TSA + Carb + 2% (w/v) arabinose using the protocol used to quantify colonization of aphids described above.

Images were analyzed using ImageJ (version 1.52p). To measure GFP intensity the background was first subtracted from the image using the rolling ball method. Then, image intensity was linearly adjusted to bring the background value for the plate down to zero. This background subtraction step was performed separately for each plate in the image (i.e., each set of aphids in one condition). Finally, the GFP intensity for each aphid was determined by outlining it and using the measurement tool to calculate the integrated density within this region of interest. To account for aphid autofluorescence, the average integrated density of all aphids in both the wild-type CWBI-2.3^T conditions was subtracted from the measurements made in all four conditions.

Chapter 3: The Pathfinder plasmid toolkit for genetically engineering newly isolated bacteria enables the study of *Drosophila*-colonizing Orbaceae

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Research Contributions

S.P.L. designed and built the Pathfinder plasmid set. E.Y. carried out early Pathfinder experiments. K.M.E. and B.M. carried out Pathfinder conjugation experiments. J.H. leads the group that isolated *Orbus* Bib. T.A. isolated *Orbus* Bib. L.P. isolated *Orbus* IpD01, 02, and 03. K.M.E. and L.P. performed *Drosophila* colonization experiments. N.A.M. and J.E.B. shaped the development of this project. K.M.E., L.P., and S.P.L. contributed to the written manuscript. K.M.E. and J.E.B. edited the manuscript.

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ABSTRACT

Plasmid and genetic part toolkits streamline the process of assembling DNA constructs and using them to engineer microbes. Many of these kits were initially designed with the intention of engineering a few specific, laboratory or industrial microbes. For researchers interested in the study of non-model microbial systems it is often unclear which tools and techniques will function in their newly isolated strains. To address this challenge, we designed the Pathfinder toolkit for the purpose of quickly assaying the compatibility of a bacterium with combinations of different plasmid components. We designed our kit with three different broad-host-range origins of replication, along with multiple antibiotic resistance cassettes and reporters, so that sets of plasmids can be delivered through multiplex conjugation. We demonstrate our kit by using it to engineer *Orbaceae* bacteria isolated from several fly species and utilize our newly engineered strains to characterize how they colonize the *Drosophila melanogaster* gut. This work illustrates a simple framework for the development and application of a toolkit for the study of new microbial systems.

INTRODUCTION

In the pursuit of improving our understanding of the natural world, researchers have isolated and sequenced many new microbes from diverse ecosystems and plant and animal hosts. To characterize these microbes and understand how they have evolved to interact with their native microbiomes and physical environments, one needs genetic tools. However, most newly isolated bacteria have never been genetically manipulated (130–132). This could be due to the unavailability of genetic tools at the time of isolation, a lack of time or interest in the lab that isolated the microbe, or a fundamental incompatibility of a species or strain with standard molecular techniques. However, the primary obstacle in many cases is likely that the requisite resources and know-how for microbial genetic engineering are not easily accessible to many researchers who encounter non-model microbes.

Many toolkits of genetic parts have been developed for bacterial systems. These kits, such as SEVA and MoClo toolkits (133, 134), aim to be flexible and comprehensive, containing promoters with a range of different expression levels and multiple reporter genes and plasmid backbones that can be combined to assemble a genetic construct of interest (57, 133, 135–138). While these systems are ideal for use with well-studied laboratory bacteria, there are still gaps with respect to their applicability to all bacteria. They may only have a few antibiotic resistance cassettes or rely on plasmids that replicate only in specific species (139). While these toolkits make complex genetic engineering tasks easier for molecular microbiology or synthetic biology labs familiar with these

techniques, they are less accessible to researchers from other fields who want to achieve preliminary microbial engineering tasks in a new bacterial species. Often fluorescent protein expression is enough for these researchers to investigate factors that are important for host-microbe interactions, such as bacterial localization, or to track their strain within a microbiome or environmental microbial community during experiments, but even this rudimentary genetic modification can be challenging to achieve in some bacteria (130, 132, 140).

When first isolating a new bacterium, it is unclear which genetic parts and techniques will work. Although toolkits contain a bounty of modular parts, it can be daunting to determine which ones should be prioritized in initial tests as many combinations of parts that work in *Escherichia coli* may be nonfunctional in the new species. A reasonable first step is to select a plasmid with a broad-host-range origin. However, although they have been reported to replicate in a wide range of bacteria, it is often unknown whether they will replicate in any specific bacterial group or species (141). Many toolkits also rely on electroporation or chemical treatments to transform their plasmids into cells. Although these methods work in many strains, it is not clear that they function in all bacteria (142, 143), and for a bacterial species that has been recently isolated there are typically no protocols available for making competent cells or electroporating. Developing these techniques through trial and error is frustrating, particularly when one does not even know if the plasmid being used will be able to successfully replicate if it is transformed into cells. Conjugation is often a more reliable method for delivering DNA to non-model bacteria (86, 131), and it has been incorporated into several kits that focus on engineering a wider range of bacteria, such as BEVA (137), the bee microbiome toolkit (BTK) (57), and the

proteobacterial toolkit developed by Schuster et al. (136). The wide phylogenetic distribution of conjugative and transmissible broad-host-range plasmids, argues that these approaches should be able to reach many bacterial species (144–146).

Although *Drosophila melanogaster* has been a model organism for genetics for decades, its gut microbiome has only recently received attention (147–149). Laboratory studies to this point have primarily focused on *Acetobacter* and *Lactobacillus* species (64, 150, 151), which make up only a fraction of the wild *Drosophila* microbiome (152). A large, mostly unexplored percentage of the *Drosophila* microbiome is composed of Enterobacteria, particularly bacteria from the Orbaceae family. This bacterial family is prevalent in a wide variety of insects (90, 153–155), and was observed in populations of both laboratory and wild flies (153, 156–158). However, only one putative fly-associated Orbaceae strain has been isolated and sequenced (159), and no work has been done to experimentally confirm this connection.

Here we describe the design of the Pathfinder system, a simple and robust toolkit for engineering newly isolated bacteria with plasmids and use it to study Orbaceae that we and others recently isolated from flies. We show how multiplex conjugation with defined subsets of Pathfinder plasmids can be used to determine the compatibility of these bacteria with different genetic parts. We then use our newly engineered bacteria to explore how different Orbaceae colonize the *D. melanogaster* gut. This work provides a useful outline for how researchers can apply these tools and techniques to study newly isolated bacteria of interest.

RESULTS

Pathfinder plasmid toolkit design

We designed our Pathfinder series of plasmids (designated pSL#) to test the function of antibiotic resistance genes and origins in newly isolated or non-engineered bacteria. An overview of the plasmid design and procedure is shown in Figure 3.1A and Table 3.1. Plasmids pSL1, pSL9, and pSL25 have 3 different origins of replication (RSF1010, pBBR1, and RP4) paired with 3 different reporters (Red chromoprotein, E2-Crimson, and Blue chromoprotein), respectively, along with kanamycin resistance. The reporter genes are all expressed from the broad-host-range CP25 promoter. Plasmids pSL1-7 all have an RSF1010 origin and red chromoprotein expression with one of 7 different antibiotic resistances (in order): kanamycin (Kan^R), spectinomycin (Spec^R), gentamicin (Gent^R), chloramphenicol (Cam^R), erythromycin (Ery^R), tetracycline (Tet^R), and ampicillin/carbenicillin (Carb^R). All Pathfinder plasmids are also BsaI dropout vectors (Type 8 parts) compatible with the stage 1 Golden Gate assembly scheme used by the yeast and bee microbiome toolkits (57, 118), so they can be readily reconfigured to hold constructs other than the included reporter genes.

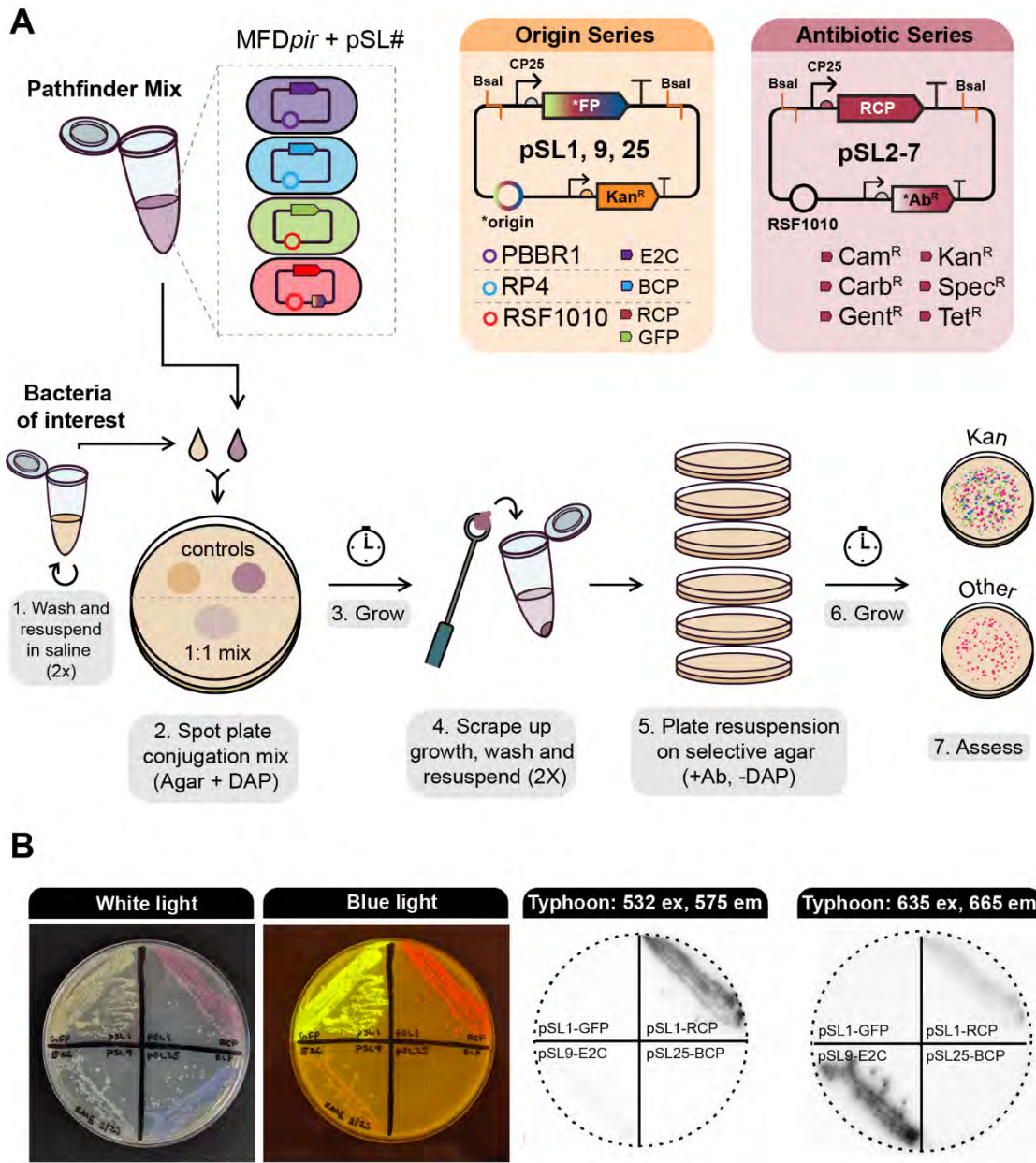


Figure 3.1: The Pathfinder plasmid system.

(A) Plasmid maps and workflow for multiplex conjugation into a bacterium of interest. (B) Visualizing Pathfinder reporters. The same agar plate containing streaks of *E. coli* MFD_{pir} donor strains, each with a plasmid expressing a different fluorescent protein or chromoprotein is shown in all images. The leftmost panel shows the plate under white light, the second shows the plate on a blue light transilluminator, and the last two panels show the plate imaged using a Typhoon 9500 FLA system with two different excitation and emission settings.

Plasmid name	Origin	Origin source	Reporter	Reporter source	Antibiotic resistance gene	Resistance source
pSL1	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Kan (<i>aphA-1</i>)	pBTK402 (57)
pSL1-gfp	RSF1010	pBTK402 (57)	GFP	pBTK520 (57)	Kan (<i>aphA-1</i>)	pBTK402 (57)
pSL2	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Spec (<i>aadA</i>)	pBTK403 (57)
pSL3	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Gent (<i>aacC1</i>)	pKNOCK-Gm (160)
pSL4	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Cam (<i>cat1</i>)	pYTK001 (118)
pSL5	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Ery (<i>ermB</i>)	pMSP3535 (161)
pSL6	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Tet (<i>tetC</i>)	pBMTBX-4 (162)
pSL7	RSF1010	pBTK402 (57)	RCP (E1010)	pBTK569 (57)	Carb (<i>tem116</i>)	pBTK401 (57)
pSL9	pBBR1	pBBR1MC S-2 (163)	E2C	pBTK570 (57)	Kan (<i>aphA-1</i>)	pBTK402 (57)
pSL25	RP4	pTD-C _s YFPTw inStrep (164)	BCP (<i>amilCP</i>)	amilCP chromoprotein (165)	Kan (<i>aphA-1</i>)	pBTK402 (57)

Table 3.1: Parts used for the construction of the pSL Pathfinder plasmids

To confirm that our system was functional we performed an initial test with *E. coli*. All plasmids except pSL5 (RSF1010, RCP, Ery^R) were transformed into MFD_{pir} (166), then combined equally into a mix that could be frozen down and thawed as needed for conjugation. We were able to recover *E. coli* transconjugants using this mix for every plasmid except for pSL3 (RSF1010, RCP, Gent^R) (Fig. 3.2B). The three Pathfinder reporters and GFP are all clearly visible by eye in *E. coli*, but to ensure that they would be visible in strains with lower expression levels, we also demonstrated that fluorescence could be distinguished using a Typhoon FLA 9500 system (Fig. 3.1B).

Pathfinder plasmids can be conjugated into recently isolated insect symbionts

We then applied the Pathfinder plasmid system to a set of bacteria that we isolated from wild flies (newly reported in this study) along with a presumed fly isolate, *Orbus hercynius* CN3 which was collected from boar feces (159). Each of these bacteria are from the Orbaceae family and are related to symbionts of bees and other insects (Fig. 3.2A). The strains are all closely related to one another. LpD01 is the most dissimilar, likely because it was isolated from a more divergent host, a Lauxaniid fly, while the rest were isolated from Drosophilids. Because the isolates are all Gammaproteobacteria, we anticipated no major challenges engineering these strains, yet they are non-model species, so there could have been compatibility issues with standard genetic parts used to engineer *E. coli*.

As expected, we were able to achieve successful conjugation of most plasmids from the Pathfinder system into each of the *Orbus* strains (Fig. 3.2B). In terms of origin of replication compatibility, we only observed conjugation with the pSL1 and pSL1-GFP plasmids from the mix that contain the RSF1010 origin. The pBBR1 and RP4 origin plasmids were absent from our *Orbus* transconjugant plates. For the antibiotic resistance panel, we achieved conjugation with all the plasmids besides pSL6 (TetR) in most strains. Differences mainly arose when strains had high levels of intrinsic resistance to an antibiotic (Table 3.2). For instance, LpD01 was highly resistant to Spec and Cam which prevented us from isolating pSL2 and pSL4 transconjugants, respectively, because untransformed cells grew on these selective plates.

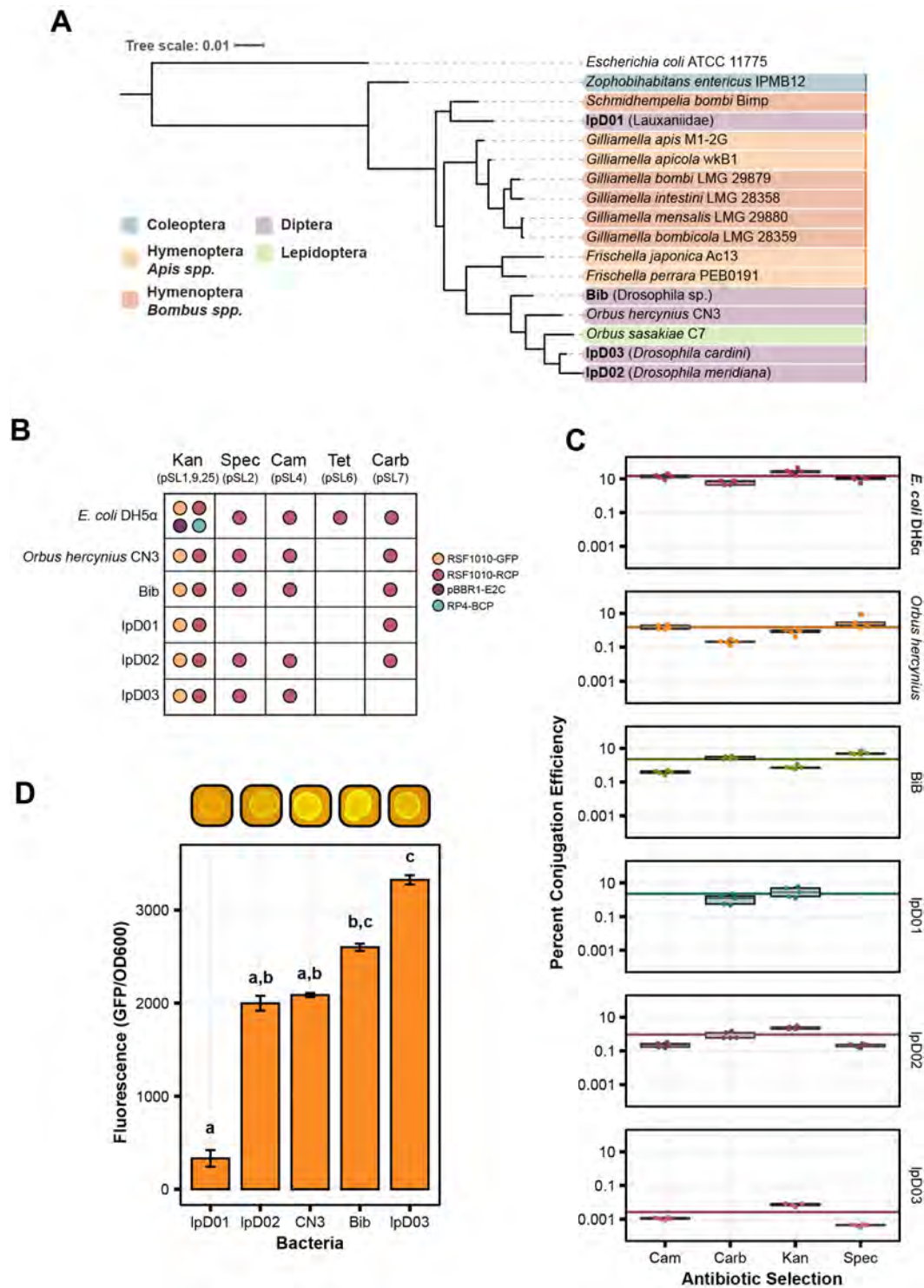


Figure 3.2: Recently isolated *Orbus* strains can be engineered with the Pathfinder plasmid system.

Figure 3.2 cont'd

(A) 16S rRNA phylogeny showing the relationship between the isolated species in this paper (**bolded**) and other insect-associated Orbaceae. Strains are color-coded based on the taxonomic order of their original insect host. For each *Orbus* strain first reported in this study the closest taxonomy identifier that could be established is shown in parentheses. (B) Table depicting the compatibility of the bacteria in this study with each of the Pathfinder plasmids. Compatibility is defined as isolation and verification of at least a single transconjugant colony for each plasmid. Dots are used to represent compatibility and are color-coded according to the origin of replication and reporter gene on the plasmid. (C) The efficiency of conjugation under each of the antibiotic conditions in the study is plotted as a percentage of transconjugants relative to growth on nonselective media. Data are plotted on a log scale. The average conjugation efficiency for each strain is highlighted with a horizontal line across each panel. (D) Bar chart showing the level of GFP expression for each strain engineered with pSL1-GFP normalized to an OD600 reading for that same strain. Images above the chart show the appearance of each strain on a blue light transilluminator. Letters above each bar designate groups that are significantly different at the $p < 0.01$ level calculated using Dunn's test with Bonferroni correction.

		CARB	CAM	KAN	SPEC	TET
Dh5a	MIC	100	20	50	60	10
	Pathfinder	100	20	50	60	10
Bib	MIC	25	6.25	400	100	6.25
	Pathfinder	25	6.25	400	*200	*3
Orbus hercynius CN3	MIC	800	6.25	25	25	6.25
	Pathfinder	800	6.25	25	*100	*3
IpD01	MIC	6.25	400	12.5	400	200
	Pathfinder	*25	400	*50	*800	200
IpD02	MIC	200	6.25	50	100	6.25
	Pathfinder	*400	6.25	*100	100	*3
IpD03	MIC	800	6.25	25	100	6.25
	Pathfinder	*6400	6.25	*100	100	*3

Table 3.2: Antibiotic concentrations used in Chapter 3.

All concentrations are in shown in $\mu\text{g/mL}$. “*” Indicates adjusted concentrations used for the Pathfinder conjugation assay

Rates of conjugation with the Pathfinder plasmids were relatively consistent between the strains and were generally lower than the conjugation efficiency for *E. coli*. The overall average conjugation efficiency for all the *Orbus* strains was $1.4 \pm 0.02\%$. Bib was the easiest strain to conjugate into with an

average conjugation rate of $2.3 \pm 0.1\%$, while IpD03 had the lowest conjugation efficiency at around $0.009 \pm 0.0002\%$ (Fig. 3.2C). Despite the lower conjugation efficiencies observed for some of the *Orbus* strains, we demonstrated that it is possible to engineer each of these newly isolated bacteria with several plasmids from the Pathfinder series.

As a basic test for functionality of the engineered constructs in the uncharacterized *Orbus* strains, we measured GFP fluorescence levels from pSL1-GFP conjugates. We observed significant variation in GFP fluorescence among these strains despite their relatedness ($F_{4,40} = 322.8$, $p < .0001$). In particular, IpD01 had such low expression that it was difficult to determine by eye that it fluoresced on the blue light transilluminator (Fig. 3.2D). Despite the wide variation in expression levels, GFP was still functional enough in each strain that we were able to use it as a signal in downstream applications.

Application of the engineered *Orbus* to study *D. melanogaster* colonization

We next used our engineered strains to assay the colonization patterns for these understudied Orbaceae bacteria in fruit flies. Our first step was to test whether each of these strains could robustly colonize *D. melanogaster* using a method we refer to as arena inoculation (see Methods for additional details). Conventionally reared Canton-S flies were kept in a container along with an agar plate of each of our strains. In this “arena” it was expected that the flies would pick up the bacteria on their bodies and ingest the strains during cleaning or through other means. After inoculation, the flies were transferred to fresh diet every 24 hours to ensure that we were selecting for bacteria that could survive

within the fly and not on the diet. To ensure that the bacteria did not survive on the diet itself, we confirmed lack of outgrowth on the yeast-glucose agar fly diet for each strain. Because we did not perform our inoculation with germ-free flies, we anticipated that there would be other microbes in the fly gut that could complicate our colonization experiment. However, a benefit to performing our assay with engineered fluorescent bacteria that carry an antibiotic resistance marker is that we can easily identify and select for our strain of interest without the necessity of sequencing.

At several time points after arena inoculation, we washed and crushed five flies and plated them on selective media with kanamycin. We showed that each of these strains can colonize the fly better than a bee *Orbaceae* strain, *Gilliamella apicola* M1-2G, which was not retained at any time point (Fig. 3.3). However, there is stochasticity in the colonization results for each of these strains between different time points and flies. In this qualitative experiment we observed that some like IpD01 were able to robustly colonize the fly at every time point, while IpD03 and Bib were lost after day 2 and 4, respectively.

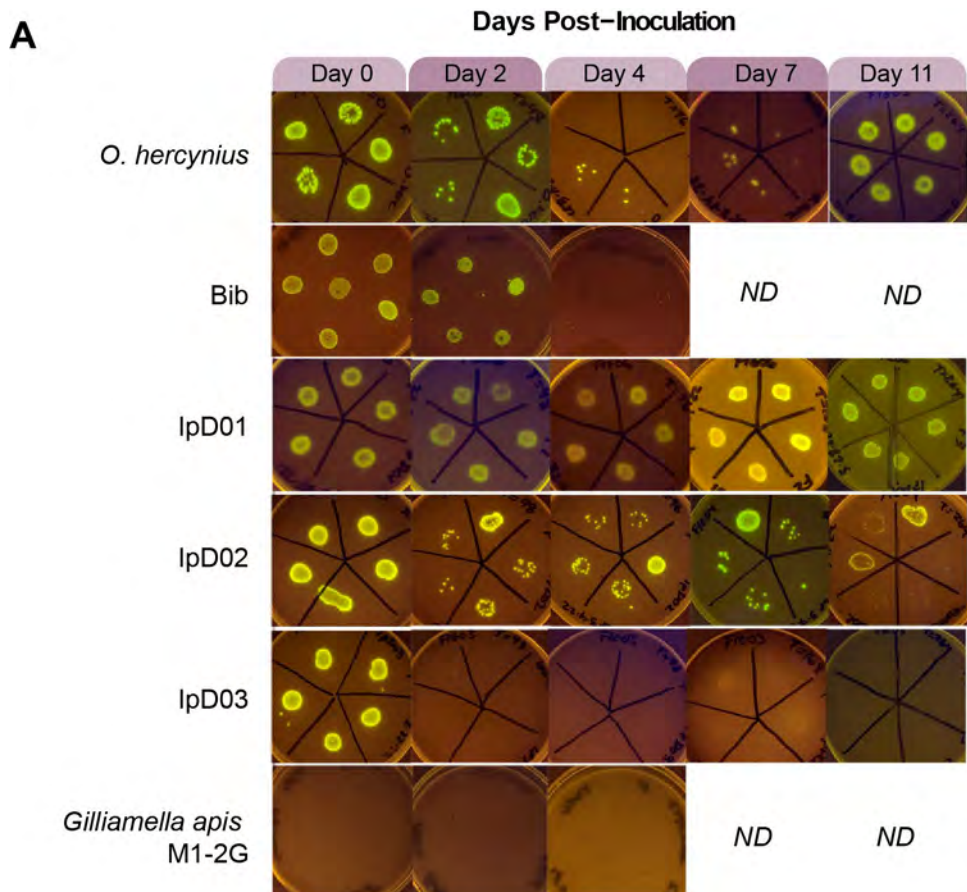


Figure 3.3: Engineered *Orbus* strains can colonize *D. melanogaster*.

(A) Images taken of bacterial growth on selective media containing kanamycin following the growth of bacteria from crushed flies in the days following inoculation. Ability to colonize the host is determined by the presence of GFP-expressing colonies. Days on which we were unable to collect data or stopped the experiment are indicated by “ND”.

Based on these preliminary results, we decided to track bacterial titer over time in flies colonized with IpD02 and IpD01. To account for variation that may occur in different populations of flies, we inoculated three separate arenas of flies per experiment. The overall trend for IpD02 follows the pattern observed in the qualitative experiment. Over time, the number of colony-forming units (CFU) per

fly gradually decreases until most flies are no longer colonized by day 11 (Fig. 3.4A). However, for IpD01 there is a trend in each arena where the average CFU in the population rapidly decreases then increases almost to the initial level seen on day 0 (Fig. 3.4B). Between day 0 and day 4 there is a significant drop in CFU (arena 1, $p = 0.001$, arena 2, $p = 0.0045$, arena 3, $p < 0.0001$, Dunn's test with Bonferroni correction), and in arenas 1 and 3 most of the sampled flies were uncolonized on day 4. The CFU per fly then significantly increased between day 4 and day 11 for arenas 1 and 3 ($p = 0.0022$ and $p = 0.0001$, respectively, Dunn's test with Bonferroni correction). The increase between day 4 and day 11 was not significant for arena 2 ($p = 1$, Dunn's test with Bonferroni correction), but there appears to be a similar trend with a slight temporal delay in the overall dynamics in this arena.

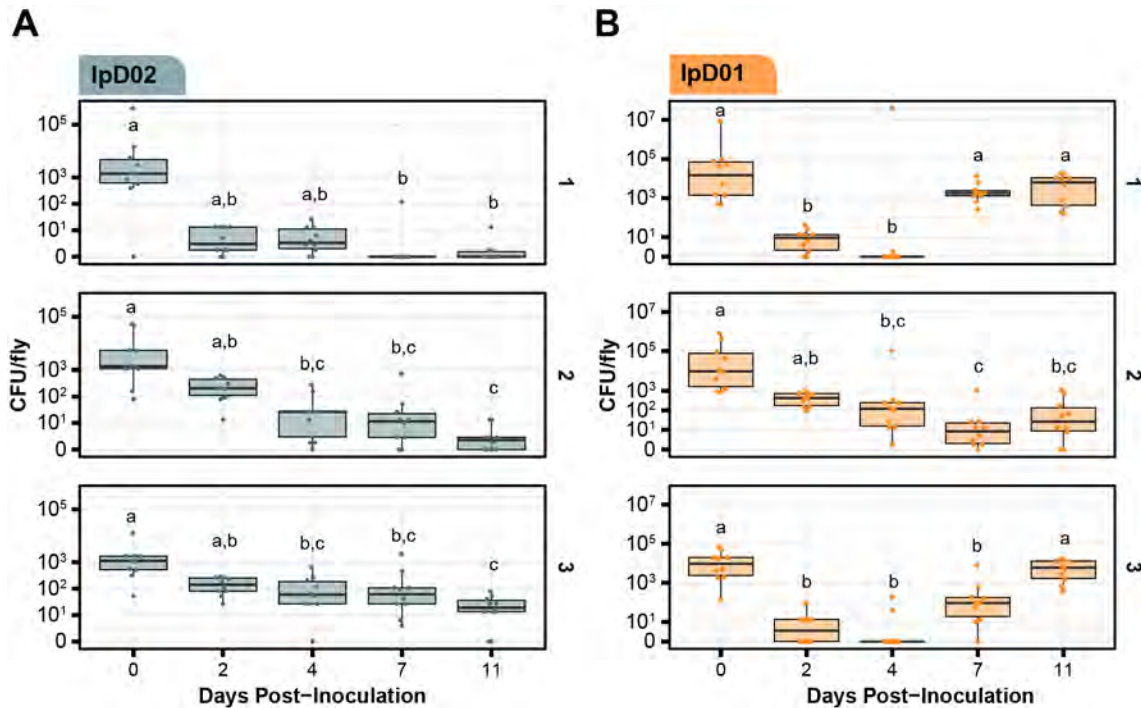


Figure 3.4: Tracking colonization of *D. melanogaster* by two *Orbus* isolates.

CFU/fly were measured following inoculation with either (A) IpD02 or (B) IpD01. CFUs in each of ten flies per arena were measured at each time point. The data are plotted on a pseudo-log scale. Results from three independent arenas are shown in subpanels labeled 1, 2, and 3. Letters above each boxplot represent groupings based on significant differences of $p < 0.05$ (Dunn's test with a Bonferroni correction).

Visualizing IpD01 colonization of *D. melanogaster*.

Based on the results of the bacterial titer assay, we selected IpD01 to visualize *Orbus* colonization of the fly gut. We inoculated 100 flies with the engineered IpD01 + pSL1-GFP strain and reared them for 11 days on fresh diet, replicating the quantification experiment. After this point we dissected the full *Drosophila* gut and used confocal microscopy to assess bacterial localization

(Fig. 3.5). We observed that there was robust colonization of the foregut in the flies we sampled. LpD01 was found in the proventriculus (cardia) of every imaged fly (Fig. 3.5C,D), and for 2 out of 5 flies it could also be found colonizing the crop (Fig. 3.5G,H). In each of these regions, aggregates of bacterial colonies could be found (Fig. 3.5E), indicating that the bacteria may be replicating. Throughout the remainder of the gut, we occasionally observed fluorescent cells (Fig. 3.5J,K), but the strain seems to follow the expected localization patterns for fly gut microbes and does not robustly colonize the midgut or hindgut regions.

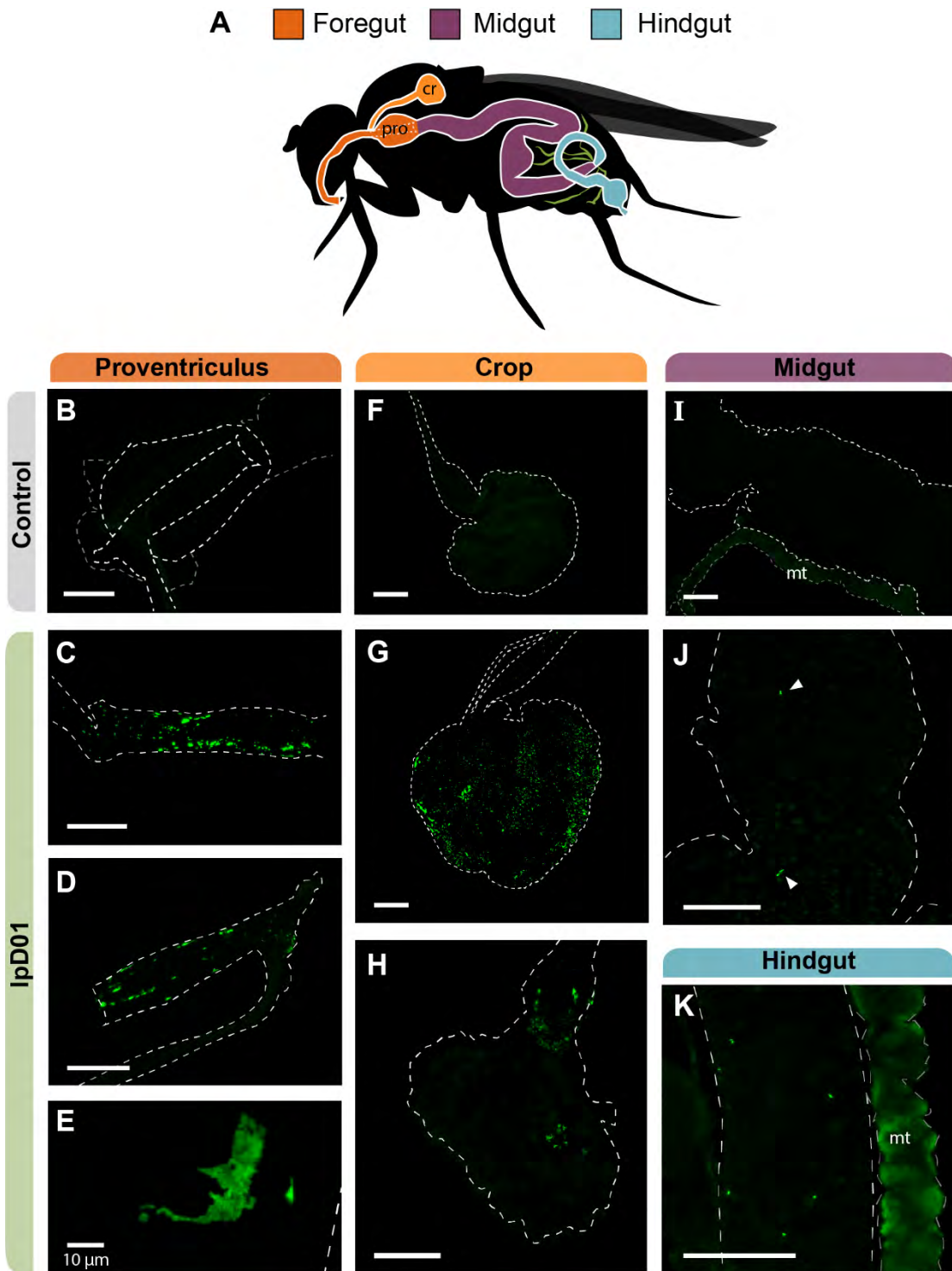


Figure 3.5: Confocal microscopy of the *D. melanogaster* gut colonized with fluorescent IpD01.

Figure 3.5 cont'd

(A) Schematic of the *Drosophila* gut with colors designating three regions, foregut, midgut, and hindgut. The crop (cr) and proventriculus (pro) are labelled due to their relevance for the localization of the strain. Malpighian tubules (mt) are depicted in green. (B-K) Confocal images of the dissected gut of one uncolonized (B, F, I) and three colonized flies. Images (C,G,J and K) are from the same insect, (D,H) are from a second insect, and (E) is a from the third. Images (B-D, F-K) were captured at 10× magnification, and image (E) was captured at 40×. Outlines for each of the relevant gut regions are represented by white dashed lines and arrows were added where necessary to point to individual bacterial cells. The Malpighian tubules (mt) exhibited autofluorescence in all flies. GFP intensities were linearly adjusted in each image to highlight bacterial localization. The scale bar for each image represents 100 µm except where indicated.

DISCUSSION

In this chapter we describe the design of the Pathfinder plasmid system, a simple toolkit with a straightforward methodology for genetically modifying non-model bacteria. With this kit we successfully engineered several candidate *Orbus* species isolated from flies, then utilized their GFP expression to study colonization patterns in *D. melanogaster*. Pathfinder streamlined the process of engineering these strains and enabled us to clearly identify our bacterium during in vivo studies. We found that the *Orbus* isolates differed in how they were able to colonize *D. melanogaster*, with some strains apparently better adapted to survive and/or replicate in the host than others. We observed that one of the strong colonizers mainly colonizes the foregut regions of the crop and proventriculus. This work serves as the first description of the colonization patterns of this understudied member of the fly microbiome.

The Pathfinder toolkit proved effective for generating engineered versions of our newly isolated bacteria. However, our results also demonstrate a common obstacle in this process, that not all plasmid components work well in all bacteria (140). For example, we did not recover *Orbus* spp. transconjugants carrying either the RP4 or pBBR1 origins. This is despite these origins functioning in a wide range of bacteria in other studies (141, 163), and their ability to conjugate into our control *E. coli*. Our results may be skewed by our use of multiplex conjugation. The RP4 and pBBR1 donor strains may be outcompeted by the other donors in the same mix prior to conjugation, or transfer of these plasmids may be less efficient. We cannot rule out that the *Orbus* strains would be successfully transformed with these origins if we tested each donor separately. As expected, we were only successful at transforming our strains with Pathfinder plasmids with certain antibiotic resistance cassettes. The incompatible strains mostly were those with intrinsic resistance to specific antibiotics, but for tetracycline it is unclear what may have been the cause since most of the strains were sensitive to low levels of Tet. It is possible that the Tet resistance gene on the Pathfinder plasmids does not express or function effectively in *Orbus* strains, which would mean that transconjugants would not survive selection. Our results highlight the utility of widely surveying for plasmid component functionality when first working with a new strain. The information from Pathfinder informs how one should select plasmids for future experiments.

There are many other genetic toolkits that have been developed with a similar goal of engineering wild bacteria (86, 137, 167). On its own, the Pathfinder kit is comparatively limited in its components. Because of RSF1010's wide compatibility with different bacteria (168, 169), we prioritized our efforts to

build out the complete antibiotic set with this origin. This origin worked well in the *Orbus* strains, but it may not replicate in other bacteria. There are also many other plasmid origins of replication that function in non-model bacterial species whose host range has not been exhaustively surveyed. In the future it would be ideal if the kit contained additional plasmids in which each of the origins is paired with the entire antibiotic resistance set. The current Pathfinder toolkit also relies on a single promoter to drive all reporter genes. One could expand the combinations to include different promoters in order to rapidly survey their functions in a new bacterium, for example by performing multiplex conjugation and then picking the most highly fluorescent or colored colonies. Combinatorial Golden Gate assembly schemes could be used to create sets of plasmids with new combinations of these components in the future.

Our kit is also limited by its reliance on plasmid-based expression systems. Plasmid transformation is a common first step in engineering a new bacterium, but it is not ideal for ensuring the stability of engineered constructs. Although plenty of systems rely on plasmid expression, these systems can be burdensome to the strain due to their use of resources that the bacterium needs to replicate and compete with other bacteria (68, 170). Because of this, plasmid-based systems are generally more likely to break due to evolving mutations than systems that are engineered into the chromosome and expressed at a lower copy number (171). There is also the complication that plasmids may be rapidly lost from a cell population due to segregation if there is not continued selection for their function. With these concerns in mind, we ensured that our experiments in *Drosophila* were carried out with constant antibiotic selection. This was feasible in fruit flies but may be more challenging in systems that are sensitive to

antibiotics or when sufficient concentrations of antibiotics for selection cannot reach the engineered bacteria during an experiment. Tools designed for chromosomal integration such as transposon systems may be a better option for researchers with this concern (167).

The engineered fluorescent strains that we built enabled us to easily screen for effective colonization of *D. melanogaster*. Through our colonization assays we observed that each strain, although closely related, displayed a range of success. Some, like IpD01 are retained in fly populations over the course of 11 days, while others like IpD03 are lost. We speculate that there could be several reasons for these differences. It could be that the stochasticity of the inoculation process accentuates differences between each of these bacteria that may actually be caused by random environmental factors in our setup (172). This is highlighted by the results for IpD02 in the qualitative and quantitative assays. In the qualitative assay, IpD02 appeared to maintain stable colonization of the host, but this was not the case in the quantitative assay. Repetition of the initial assays that we performed would be needed to confirm this hypothesis, along with quantitative experiments for all our strains.

We also anticipate that variability in the flies themselves affects colonization. We carried out our experiments in non-axenic flies to try to emulate natural conditions for invasion in which other microbes will already be present in the fly gut. The presence of an existing gut microbial community is expected to affect the ability of an introduced bacterium to colonize these flies (172). Repeating these assays with germ-free flies would let us test for host microbiome effects (173). However, experimental flies are still unnaturally housed in a laboratory setting, so their microbiome and diet do not necessarily reflect wild

conditions (174). Finally, we expect that there are genetic differences between the *Orbus* isolates from different flies that affect their ability to colonize *D. melanogaster*. In the future, we plan to further characterize how differences between these bacteria and their hosts impact colonization.

One strain that stood out in our colonization assays was IpD01. Although isolated from a more distantly related Lauxaniid fly, it appeared to outperform the other strains in terms of effectively maintaining itself in the *D. melanogaster* population in the qualitative assay. In the quantitative assay for bacterial titer, we identified a bottleneck pattern where there is a transient decrease in colonization levels after initial inoculation followed by recovery to higher levels. This was similar to the colonization of *Lactobacillus plantarum* described by Obadia et al. (172), although for IpD01 it appears to happen on a shorter time scale. This transient dip in bacterial titer during colonization could be caused by a selective pressures inside of the fly gut that allow only a few bacteria to survive and establish (175).

To further understand IpD01 colonization dynamics, we could collect more images over time to understand what is occurring during our 11-day window. We could also increase the length of our experiment to determine if the CFU past day 11 remains the same, increases, or if it follows a cyclical pattern of decrease/increase. If the CFU were to stay constant or increase after day 11, there is a chance that a beneficial mutant could have swept the bacterial population. This would make sense with IpD01 because it was initially isolated from a Lauxaniid fly and is likely not well-adapted to a Drosophilid host. We could screen for, and sequence, any improved colonizers to determine if there have been any genetic changes that increase their fitness in the fly gut.

However, if bacterial titer were to follow a cyclical pattern, it could imply that there is host pressure that prevents *Orbus* from permanently colonizing the gut. As with some other gut bacteria like *Lactobacillus* and *Acetobacter* species, colonization may be dependent on replenishment from their environment (176). We confirmed that the bacteria do not survive on lab diet, and *Orbus* is not associated with growth on wild diet sources (152), but it may remain in the population after artificial inoculation by spreading between flies through social transmission methods like the fecal-oral route. To keep the bacteria from spreading between flies, they could be housed individually in a replication of the CAFE assay designed by Ja et al. (172, 177). We are at the starting point when it comes to our understanding the dynamics of bacterial colonization in the fly gut. Although we see similar patterns to those observed in other fly gut species, more work is needed to better understand what may be unique to *Orbus*.

Despite its fluctuating colonization, we chose to image IpD01 in the gut to get a sense of where *Orbus* may localize. In the flies we sampled we mainly saw colonization of the crop and proventriculus regions of the foregut. The crop and proventriculus tend to be more hospitable for bacterial colonization than the midgut which has a lower pH and undergoes peristalsis along with continual turnover of the peritrophic membrane (148, 172). This would explain why, in midgut regions, we only observed a few small clusters of cells. Other stable colonizers of *D. melanogaster* like *L. plantarum* and *Acetobacter thailandicus* also principally localize to the crop and proventriculus (172, 174). It is unclear based on our current results whether IpD01 is attaching to and forming biofilms in the gut as was seen for *L. plantarum*.

The Pathfinder plasmid system established a starting point for understanding the patterns of colonization for each of these bacteria, but further genetic studies will be necessary to more fully understand the relevant host-microbe interactions. We will need more tools to identify the genetic factors that promote robust colonization of the fly gut, along with any benefits to the fly resulting from this colonization. We can use the results from our initial Pathfinder assays to select appropriate genetic tools to explore these interactions. Because the Pathfinder plasmids are compatible with established Golden Gate assembly schemes and parts libraries (57, 118), we can build new constructs on these backbones (57). For the genetic study of these strains, we will need to adapt systems for building knockouts (57, 167, 178). Reaching beyond *Drosophila*, the methods demonstrated with these isolates could be adapted to other fly systems where these *Orbus* bacteria are found, such as *Bactrocera dorsalis*, a destructive pest species (157). For those pursuing the population control of these flies, engineering *Orbus* bacteria could lead to new avenues for pest control through paratransgenesis (93, 130).

We developed the Pathfinder toolkit to simplify the engineering process for the study of non-model microbes. We chose to apply this toolkit to a series of fly-associated Orbaceae. Then, we used our engineered bacteria to assay for their colonization patterns in *D. melanogaster*. In the future we will build upon our initial cloning and colonization assays to study the relationship between fruit flies and this understudied member of their microbiome in more depth. For other researchers, we show that the Pathfinder toolkit can be a simple method for engineering newly isolated bacteria that enables future complex engineering tasks. Although similar to other toolkits, we combine our plasmid set with

multiplex conjugation to quickly screen for part function, yielding multiple engineered strains in the process. We expect that, along with other broad-host-range toolkits, Pathfinder will increase the feasibility of genetically engineering non-model microbes so we can more effectively study natural microbiomes.

MATERIALS AND METHODS

Isolation of *Orbus* Bib, IpD01, IpD02, and IpD03 from flies.

Wild flies were collected at the Brackenridge Field Laboratory in Austin, TX (30.284326, -97.778522). Traps were prepared by adding fermented banana, yeast, and twigs to punctured plastic bottles. Traps were hung from trees for 7 days, and flies were collected each day. Flies were placed on ice or at 4°C immediately after collection and processed within 24 h.

Flies were immobilized by placing them on ice, after which they were photographed for morphological identification. Flies were then washed with 10% bleach to remove surface microbes for 1 minute, then washed in sterile water for 1 minute to remove residual bleach. Legs and wings were removed and placed in 100% ethanol for extraction of host DNA. The flies were then placed in 50 µl of Insectagro insect growth medium (IGM) (Corning, VA, USA) and homogenized using a sterile pestle. The homogenate was then diluted down to a factor of 1×10^{-5} and homogenates diluted to 1×10^{-3} through 1×10^{-5} were plated on heart infusion agar (HIA) with 5% sheep's blood. Homogenates were initially placed at 37°C and 5% CO₂ (e.g., in the case of Bib), but 30°C was later used due to superior growth (e.g., in the case of IpD01). Clear or off-white and slower-growing colonies were passaged onto fresh plates multiple times to obtain pure

cultures. To identify the bacteria, we amplified, and Sanger sequenced the 16S rRNA gene using 27F (5'-AGAGTTTGATCMTGGCTCAG) and Orbaceae-specific primer Orb742R (5' -ATCTCAGCGTCAGTATCTGTCCAGAA). Host insects were identified by morphology and by sequencing PCR amplicons of the COI barcoding gene with primers LCO1490F (5'-GGTCAACAAATCATAAAGATATTGG) and HCO2198R (5'-TAAACTTCAGGGTGACCAAAAAATCA). The phylogenetic tree in Figure 3.2A was assembled using the 16S ribosomal RNA sequences for each strain. The sequences were aligned in MUSCLE (EMBL-EBI) (179) then assembled into a phylogeny using IQ-TREE (180). The phylogeny was visualized using iTOL (v5) (181).

Growth and maintenance of bacterial strains.

Escherichia coli DH5α and MFD*pir* were grown in LB broth and on LB agar plates at 37°C, for MFD*pir* growth, media was supplemented with 0.3 mM diaminopimelic acid (DAP). *Orbus hercynius* CN3 was acquired from the DSMZ collection (DSM 22228). Following isolation, Orbaceae strains were determined to be culturable in either IGM, Brain heart infusion (BHI) broth, or on solid BHI or HIA with or without 5% defibrinated sheep blood. For robust growth, IGM and blood agar plates were preferred, but media was selected based on the needs of the assay. All *Orbus* strains were grown at 30°C with 5% CO₂. *G. apis* M1-2G was grown on HIA + 5% sheep's blood at 35°C with 5% CO₂. Antibiotic concentrations used in this study are shown in Table 3.2.

MIC tests to determine antibiotic susceptibility.

We performed MIC assays to determine the appropriate selective antibiotic concentration for each bacterial strain. To do so, we prepared 96-well plates with 2-fold dilutions of each antibiotic, ranging from 400 – 6.25 µg/mL in 100 µL media. One microliter of each strain was inoculated in triplicate for each condition. After allowing the strains to grow for 1-3 days, the plates were visually inspected to determine the minimum inhibitory concentration for each antibiotic. This measure was used to determine selection during the Pathfinder conjugation process (Table 3.2).

Assembly of the Pathfinder plasmids.

All cloning procedures were carried out in *E. coli* strain NEB5alpha (#C2987H, New England Biolabs) cultured overnight aerobically at 37°C in LB broth or solid LB agar. Antibiotics were supplemented when necessary for plasmid selection or maintenance at the following concentrations: Kanamycin (Kan) (50 µg/mL), Spectinomycin (Spec) (60 µg/mL), Gentamicin (Gent) (25 µg/mL), Chloramphenicol (Cam) (20 µg/mL), Erythromycin (Ery) (250 µg/mL), Tetracycline (Tet) (10 µg/mL), and Carbenicillin (Carb) (100 µg/mL).

We designed the Pathfinder plasmid series to have a variety of broad-host-range origins, antibiotic resistance genes, and highly expressed visible reporters suitable for rapid identification and testing in newly isolated bacteria. We started with pBTK402, a broad-host-range plasmid with a RSF1010 origin that we previously engineered to remove any BsaI and BsmBI cut sites and make it suitable for Golden Gate Assembly (57). The pBTK402 plasmid was designed

to function as a Type 8 dropout vector for the BTK Golden Gate Assembly scheme, and all Pathfinder plasmids retain this attribute. We replaced the weakly expressed *rfp* on pBTK402 with a visible Red Chromoprotein (RCP) (Bba_E1010) expressed from the strong CP25 promoter and associated ribosome binding site (RBS) from plasmid pBTK569. This promoter-RBS combination appears to generally lead to robust protein expression in Proteobacteria. This plasmid was re-designated “pSL1” and all subsequent pSL plasmids are derived from pSL1. Plasmid pSL1-GFP replaces the RCP reporter with GFP. Plasmids pSL2 – pSL7 were constructed by replacing the Kanamycin resistance allele (*aphA-1*, Kan^R) present in pSL1 with an alternate antibiotic resistance allele and associated upstream promoter. PSL9 and pSL25 replace the RSF1010/RCP origin and reporter with pBBR1/E2-Crimson (E2C) and RP4/Blue chromoprotein (BCP), respectively (see Table 3.1).

To construct a particular plasmid, we first designed assembly primers using Benchling software (<http://www.benchling.com>) and added Golden Gate-compatible BsmBI cut sites. We ordered DNA primers from IDT (<https://www.idtdna.com/>) and then amplified PCR products using either Q5 Hot-Start Master Mix (#M0494S, New England Biolabs) or KOD XL (#71087-3, Millipore Sigma) according to manufacturer’s instructions. We purified PCR products with a QIAquick PCR Purification Kit (#28104, QIAGEN), assembled them using a NEBridge Golden Gate Assembly Kit (BsmBI-v2) (#E1602S, New England Biolabs), and then electroporated 1 µL of the reaction into electrocompetent NEB5alpha. Cells recovered for 1 h and were then plated on appropriate selective media. Plasmids were initially verified by Sanger

sequencing of the assembly junctions and later verified by whole-plasmid Illumina sequencing.

We next transformed all pSL plasmids into the DAP auxotrophic conjugation donor strain *E. coli* MFD*pir* (166). We could not successfully transform pSL5, however, due to intrinsic Erythromycin resistance in MFD*pir*. These strains of MFD*pir* were used individually or combined for subsequent multiplex conjugation assays.

Conjugation of the Pathfinder plasmids into insect associated bacteria.

We created the Pathfinder conjugation mix by first growing up each of the donor strains separately to saturation. At this point we measured the OD600 of each strain and resuspended it to OD600 = 1. Equal volumes of each strain were combined into a single tube along with 16% glycerol then distributed into PCR tubes and frozen at -80°C. For conjugations, this mix can be thawed and added straight to the first conjugation step.

To perform the conjugation itself, we started with 1 mL of an overnight culture of the target bacteria. The culture was pelleted (1000 × *g* for 6 minutes) and washed once with 145 mM NaCl saline to remove any residual media, and then resuspended in saline to OD600 = 1. Twenty-five µL of the sample was combined with 25 µL of the thawed Pathfinder plasmid mix and spot-plated on media compatible for the growth of both *E. coli* and *Orbus* spp. (BHI for all strains except IpD02, which was grown on BHI + 5% sheep blood) plus DAP. After 1-2 days of growth, we scraped up all the growth from the conjugation spot and suspended it in 1 mL of saline. This was washed and resuspended twice with 1

mL sterile saline to remove residual DAP. The resuspended sample was divided into 5 equal portions and serially diluted 10-fold to a final dilution of 1×10^{-5} . To plate these dilutions, 5 μ L of each replicate and dilution were spotted onto antibiotic plates at each bacterium's MIC, along with a zero-antibiotic control. Plates were left to dry and then placed in an incubator at the optimal temperature for each bacterium. If one of the antibiotic concentrations proved to be too low or too high following conjugation, it was adjusted accordingly, and the procedure was repeated (Table 3.2). To confirm successful conjugation of the construct, colonies were visually confirmed for expression of the fluorescent reporter, and one colony from each antibiotic condition was regrown in liquid media and sequenced with 16SA1F (5'-AGAGTTTGATCMTGGCTCAG) and 16SB1R (5'-TACGGYTACCTTGTTACGACTT) (182). Colonies were counted and the efficiency of conjugation was determined relative to growth on the zero-antibiotic control plate.

Imaging bacterial colonies.

To ensure accurate categorization of each of the fluorescent reporters in strains where the fluorescence was not as bright, we visualized plates on a Typhoon 9500 imager (GE Healthcare Bio-Sciences, Uppsala, Sweden). To visualize RCP fluorescence, we imaged with 532 nm excitation and 575 nm emission. To distinguish E2C fluorescence from RCP, we imaged the plate with 635 nm excitation and 665 nm emission. Images were processed and counted in Fiji (version 1.53q) (183).

Measuring GFP expression in different bacteria.

To measure the level of GFP expression, we assessed three separate colonies picked from the Pathfinder conjugation plate. Each colony was first grown to saturation, then re-grown with a starting OD600 of 0.05 in 5 mL Insectagro in a test tube. After re-growth, 1 mL of each culture was pelleted and resuspended in 600 μ L saline. OD600 and GFP expression (485 nm excitation, 535 nm emission) were measured for 200 μ L of each resuspension in triplicate using a Tecan Infinite 200 Pro plate reader. GFP expression was normalized to the OD600 measurement for each sample.

Growth and maintenance of *Drosophila* stocks.

Canton-S *D. melanogaster* was acquired from the Bloomington Drosophila Stock Center. Fly stocks were reared on Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply Company, Burlington, NC). For experiments, stocks were swapped to a Yeast-Glucose diet containing brewer's yeast, D-glucose, agar, and water (173). Nystatin (10 μ g/mL) and kanamycin (10 μ g/mL) were added to the diet where specified to prevent the growth of fungal contaminants and ensure the maintenance of engineered strains, respectively. Stocks were maintained at 25°C during experiments, with a 12L:12D photoperiod in Percival I-36LLVL incubators (Perry, IA, USA).

Inoculation of *Drosophila* with *Orbaceae* strains.

D. melanogaster was inoculated with the engineered *Orbus* using a method we refer to as arena inoculation. For these experiments, female *D.*

melanogaster adults were first removed from the commercial diet and transferred to YGA diet for 48 hours to clear their gut from the preservatives in the commercial diet. In the meantime, HIA + 5% sheep blood plates + Kan were inoculated with 100 μ L OD600 = 1 *Orbus* isolate + pSL1-GFP and allowed to grow for 1-2 days as needed. The bacterial plate was then taped to the bottom of a lidded 12oz SelecTE plastic container “arena” (Berry Plastics, Evansville, IN) modified with a mesh vent on its lid. YGA flies were transferred to the arena for 24 hours, after which they removed from the arena and transferred to YGA + Nystatin + Kan diet tubes. After this point, flies were transferred to the same fresh diet mix daily.

Quantification of bacterial colonization.

Each bacterial strain was first delivered to newly emerged 10- to 14-day old female adult flies using the methodology described above. For this set of experiments, 3 different arenas were used to inoculate 3 separate populations of female *D. melanogaster*. Equal numbers of flies were placed into each arena, ranging from 100-150 in each arena for each round of this experiment. After inoculation, each separate population of flies was transferred to their own tube of YGA + Nystatin + Kan diet. Flies were transferred to fresh diet daily. At day(s) 0, 2, 4, 7, and 11 post-inoculation, 10 flies from each independent population (30 total) were crushed and plated to determine their quantity of bacterial colonization.

To prepare samples for crushing, we first washed them in bleach to ensure that any bacteria plated would not be from the outside of the fly body.

Flies were immobilized for this procedure by first placing them at -20°C for 1 minute, and then keeping the tubes on ice during the washes. They were soaked in 500 μL 10% bleach for 1 minute, washed with 500 μL 1 $\times$ phosphate buffered saline (PBS), then resuspended in 200ul PBS and homogenized with a micropestle. A set of 1:10 serial dilutions in PBS were then carried out for each sample. Five microliters of each dilution were spotted onto agar plates (HIA + 5% sheep blood, Kan 50 $\mu\text{g}/\text{mL}$, Nystatin 10 $\mu\text{g}/\text{mL}$) in triplicate. The remaining homogenized fly mixture was then centrifuged at 1000 $\times g$ for 6 minutes to pellet the remaining bacteria. The pellet was resuspended in 15 μL saline and plated to ensure the detection of bacteria that were poor colonizers.

Imaging *Drosophila* guts with confocal microscopy.

Fifty female *D. melanogaster* were colonized with IpD01 + pSL1-GFP in two separate arenas and maintained for 11 days along with an uncolonized control population. To ensure that flies were colonized with the bacteria, 3 flies from each population were crushed and plated on day 9 as described in the prior methods section. On day 11, all flies were transferred to a tube without diet for 12-18 hours to empty the gut of any diet-associated particles that may complicate imaging. The entire gut of a selection of the flies was dissected and mounted in PBS on a glass slide. The dissected guts were imaged using a Leica MZ16 Fluorescent Stereoscope in the GFP channel to visualize bacterial colonization. Images were linearly adjusted to highlight the bacterial localization using Fiji software (version 1.53q) (183).

Chapter 4: Addressing the challenges of symbiont-mediated RNAi in aphids

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Research Contributions

K.M.E. carried out experiments for this chapter. G.M. and J.P. helped carry out preliminary experiments and helped shape the chapter. K.M.E. wrote and developed figures for this chapter. K.M.E. and J.E.B. edited the chapter.

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ABSTRACT

Due to the global status that aphids have as agricultural pests, as well as their role as important models for bacterial symbiosis, there is a desire to both study and control their gene function. However, the current methods available for the knockout and knockdown of gene expression are often unreliable and time consuming. In the hopes of addressing these challenges, we attempted to adapt a new method for RNA interference called symbiont mediated RNAi for use in aphids. We engineered laboratory *E. coli*, HT115 and the native aphid symbiont *S. symbiotica* CWBI-2.3^T to produce dsRNA inside of the aphid gut targeting the salivary effector, C002, or ecdysone receptor genes. However, we found that under our conditions symbiont mediated RNAi is not a reliable method for aphid gene knockdown. We conclude with a discussion on the possible avenues through which this technique, and aphid RNAi in general could be improved in the coming years.

INTRODUCTION

Aphids are tiny sap-sucking insects with major agricultural and biological relevance. They are worldwide pests responsible for the spread of devastating plant viruses (94–96), are model species for understanding endosymbiosis (97, 184), and have a plethora of unique plastic traits (185, 186). Despite these interesting phenotypes and the availability of genome sequences for several species, including *Acyrtosiphon pisum* (187), there are limitations to the study of aphid genetics. Aphids have a parthenogenetic reproductive lifestyle in which they give birth to live offspring that are clones of their mothers for a portion of the year. In the laboratory, it is difficult to induce aphid sexual reproduction, which would generate eggs and offspring homozygous for new mutations. Aphids produce low numbers of eggs (1-2 per ovariole) which then take about 100 days to hatch (188). These limits make techniques like chemical mutagenesis and genome editing using RNA-guided nucleases (e.g., CRISPR-Cas9) more challenging to perform (189, 190). Because of this, researchers mainly rely on using RNA interference to knock down expression of aphid genes to study their functions.

RNA interference is not without its issues, however, and few researchers have used this technique to unravel complex gene interactions (191–193). RNAi is typically performed by either injecting or feeding aphids with lab-synthesized double stranded RNA (dsRNA) with a sequence matching the aphid gene target. This dsRNA is processed by the aphid RNAi machinery, which results in knockdown of gene expression from the corresponding host mRNA. However,

injection only delivers a limited dose of dsRNA, and highly active aphid nucleases in the gut limit feeding assays by degrading dsRNA and reducing the knockdown efficiency (194–196). Researchers have overcome these issues by delivering higher, continuous doses of dsRNA through feeding on transgenic plants, and by performing multiple injections (192, 197, 198). These solutions have their own complications, though. Injections are traumatic for these tiny and fragile insects, and generating transgenic plants is time-consuming and untested for many combinations of aphid species and plant hosts.

Bacterial symbionts could serve as an alternative means of delivering dsRNA to insects. Bacteria are typically easier to genetically engineer than plants, and because symbionts are natively associated with insect hosts, they should robustly colonize and then continuously deliver dsRNA throughout the host's life. This methodology, called trans-kingdom RNAi in mammalian systems (199–201), and symbiont-mediated RNAi (smRNAi) in insects, was effective at knocking down gene expression in thrips, kissing bugs, and honey bees (49, 73). Our group recently developed methods for genetically engineering the bacterium *Serratia symbiotica* CWBI-2.3^T and showed that this culturable symbiont robustly colonizes the guts of multiple aphid species (50), making this system a prime candidate for testing smRNAi in aphids.

Here we describe our attempts to knock down *A. pisum* gene expression using symbiont-mediated RNAi. To inform our approach, we first performed an analysis of RNAi studies in aphids to select two targets, the C002 and ecdysone receptor (EcR) genes. We then test the efficacy of smRNAi using two different bacteria, *S. symbiotica* CWBI-2.3^T, which is naturally associated with aphids (36, 69, 108, 111, 112), and *Escherichia coli* HT115 DE3, which is an engineered

laboratory strain that is used for RNAi knockdown in other invertebrates (202, 203). Despite some positive results in certain trials, we did not see reliable effects on aphid phenotypes or knockdown of mRNA levels with the methods tested. However, these experiments may provide insight to others interested in pursuing symbiont-mediated RNAi in aphids and other insects and allow us to better understand the factors necessary for its success.

RESULTS

Analysis of aphid RNAi experiments to select targets for knockdown.

We wanted to select a proven system for testing symbiont-mediated RNAi. To identify genes to target that are expected to exhibit reliable and strong knockdown, we compiled a dataset of aphid RNAi results from 86 peer-reviewed papers. We tabulated how the dsRNA, shRNA, or siRNA was administered, the effectiveness of gene knockdown, and other metadata for a total of 656 experiments described in these publications (Table S1). Looking at the trends in these studies, we can get a sense of what is known about the reproducibility of different RNAi experimental designs in aphids (Fig. 4.1).

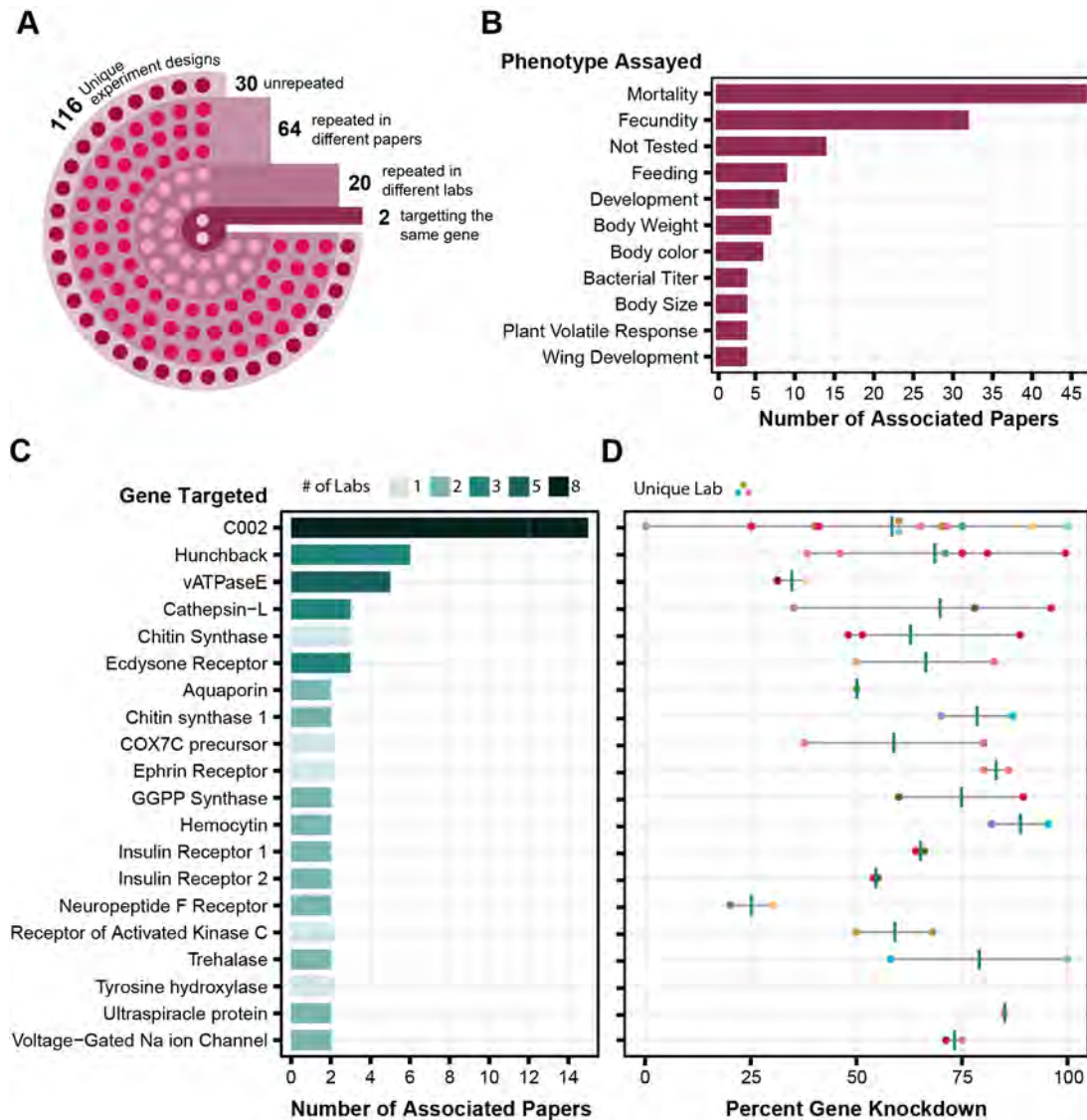


Figure 4.1: Analysis of aphid RNAi experiments.

Each plot is derived from data in Table S1 compiled from various publications. (A) RNAi experimental design. Each circle in the image reflects the number of experimental designs in each category. An experimental design is broadly described based on whether it is an injection, feeding, or topical technique. To further define an experimental design as unique, it is then categorized based on (where applicable) dsRNA concentration, number of targets, number of injections, and feeding source. (B) Phenotypes assayed in aphid RNAi experiments. The top 11 phenotypic outcomes of gene knockdown are plotted against the number of publications in which each was assayed.

Figure 4.1 cont'd

(C) Genes targeted in multiple aphid RNAi publications. Each target is plotted in relation to the number of papers it appears in and the number of labs that have assayed that target. Only targets that have been tested at least twice are shown. Shading is used to indicate the number of different labs that tested each target gene. (D) Corresponding levels of gene expression knockdown achieved in the experiments in C. Each point is colored based on the lab that performed the experiment and shows the maximum effect reported in each publication. Vertical bars are the average of these values.

The main challenge in using this information to judge reliability is that there are few examples of the same gene being targeted using the same RNAi procedure that have been replicated by different labs. Out of the 116 different experimental designs used for dsRNA delivery, 86 have been replicated in different papers, but only 24 of these have been repeated by different labs, and only two of these targeted the same gene (Fig. 4.1A). Most experiments with aphid RNAi are focused on aphid population management. Aphid mortality and reproduction are the phenotypes that are assayed most often (Fig. 4.1B). Replication is also relatively low at the level of the genes targeted: out of 168 different gene targets, only 20 (12%) have appeared in more than one paper (Fig. 4.1C). The reported level of knockdown achieved via RNAi for these genes often varies greatly from study to study (Fig. 4.1D). Nevertheless, there are four targets (C002, cathepsin-L, chitin synthase, and ecdysone receptor) that each have a median reported knockdown of 40% or more of the average across experiments reported in at least three different papers.

The most highly tested target is the C002 gene, one of the first targets that was knocked down by RNAi in aphids (204). This target has been tested by eight different lab groups (194, 196, 198, 205–215). Although it shows high variability

in knockdown effectiveness, we decided that it would be a useful benchmark for testing symbiont-mediated RNAi. Knockdown of the C002 gene prevents aphid feeding and causes aphid mortality (209). In order to choose a second target with a different phenotypic outcome resulting from knockdown, we also selected the ecdysone receptor (EcR) for testing smRNAi. When EcR is knocked down it leads to an increase in the proportion of winged offspring birthed by the affected mother. RNAi results for EcR have been reported by two different labs in three papers using dsRNA delivery via feeding or injection (194, 205, 216), and the effectiveness of EcR knockdown appears to be similar to that observed for C002.

Effects of feeding dsRNA targeting C002.

The effectiveness of RNAi varies in different aphid species and biotypes. We wanted to reproduce prior studies and select an aphid with the largest RNAi response (217), so we assayed whether we could achieve knockdown of C002 in 4 different biotypes of *A. pisum*: LSR1, PA1, Tucson, and 5A. We fed each aphid line on artificial diet supplemented with 100 ng/μL dsRNA for 24 hours, then transferred them to plants and monitored their mortality for 6 days. Overall, mortality rates were low and none of the biotypes showed a significant increase in mortality when treated with C002 dsRNA in our experiment (Fig. 4.2). Out of the four biotypes tested, 5A and LSR1 had the most noticeable effects and lowest p-values when testing whether there was a significant difference associated with dsRNA feeding ($p = 0.16$ and $p = 0.26$, log-rank tests, respectively), so they were selected for the symbiont-mediated RNAi assays to try to achieve a more effective result.

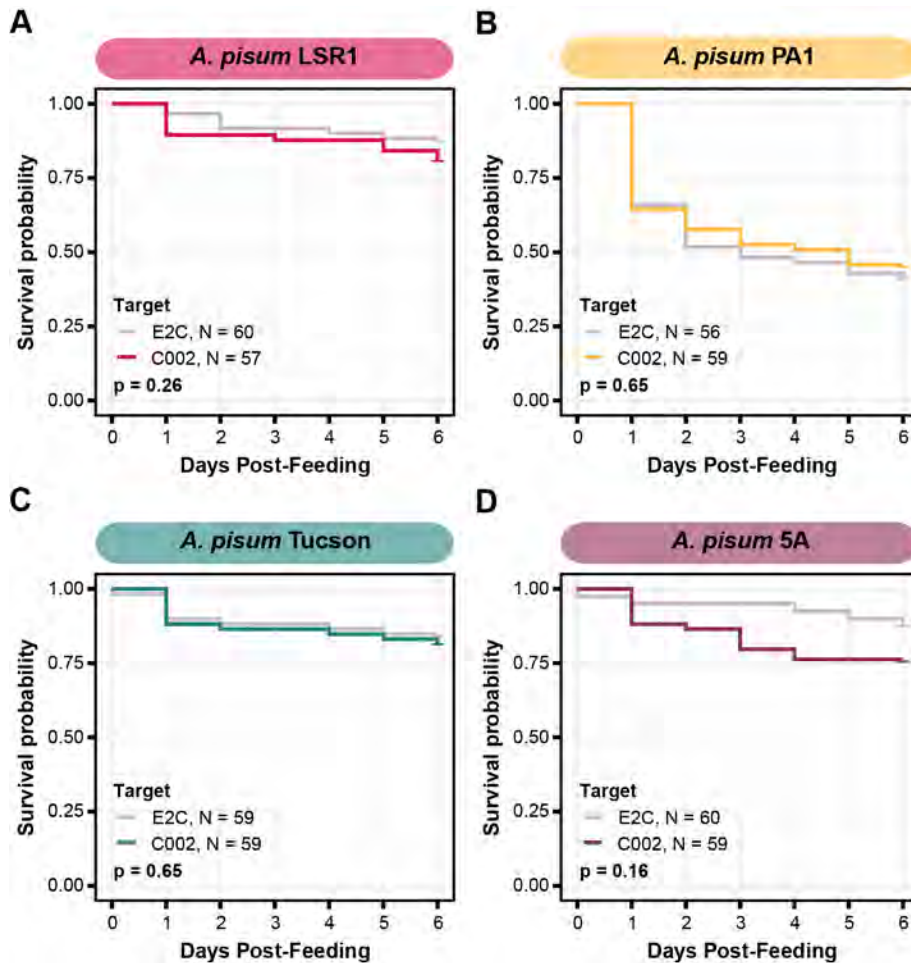


Figure 4.2: Survival of *A. pisum* after feeding dsRNA targeting the C002 gene.

Each plot shows the survival probability of the aphids at each time point following feeding on C002 dsRNA compared to feeding on off-target E2C dsRNA. Each panel shows the results for a different biotype of *A. pisum*, (A) LSR1, (B) PA1, (C) Tucson, and (D) 5A. P-values are calculated based on the log-rank test.

Symbiont-mediated RNAi against C002 does not consistently lead to an increase in aphid mortality.

In order to engineer our strains to produce dsRNA, we built plasmids based on the design used by Leonard et al. (73, 218). These plasmids contain a

sequence matching the targeted gene flanked by two identical inward-facing promoters so that it is expressed as dsRNA. The dsC002-800 construct was built using the same C002 sequence targeted by Mutti et al., 2006 (204), and the off-target dsRNA construct, dsE2C-800, contains a 412 bp fragment of the E2-Crimson (E2C) fluorescent reporter gene. After building these constructs we transformed them into versions of each of our bacteria that express green fluorescent protein (GFP) from a cassette integrated into their chromosomes, HT115-GFP and CWBI-2.3^T-GFP. We can easily identify aphids colonized with our dsRNA-expressing strains because the fluorescent bacteria can be visualized inside the aphid gut on a blue light transilluminator (50).

We decided to first test smRNAi with HT115 *E. coli* because it has an RNase III mutation that increases production of dsRNA. This mutation increases the effectiveness of RNAi in *C. elegans* (202, 203). We fed *A. pisum* on the dsRNA-expressing bacteria and monitored their mortality on fava beans for about one week (Fig. 4.3A). In our first trial with *A. pisum* LSR1, we observed a significant increase in aphid mortality in the C002 knockdown condition compared to the E2C knockdown control ($p = 0.0018$, log-rank test) (Fig. 4.3B). However, when we repeated this trial with a larger number of aphids, we no longer observed a significant change ($p = 0.56$, log-rank test) (Fig. 4.3C). We also did not observe a significant result in an smRNAi experiment in *A. pisum* 5A ($p = 0.18$, log-rank test), though the average effect was in the expected direction (Fig. 4.3D).

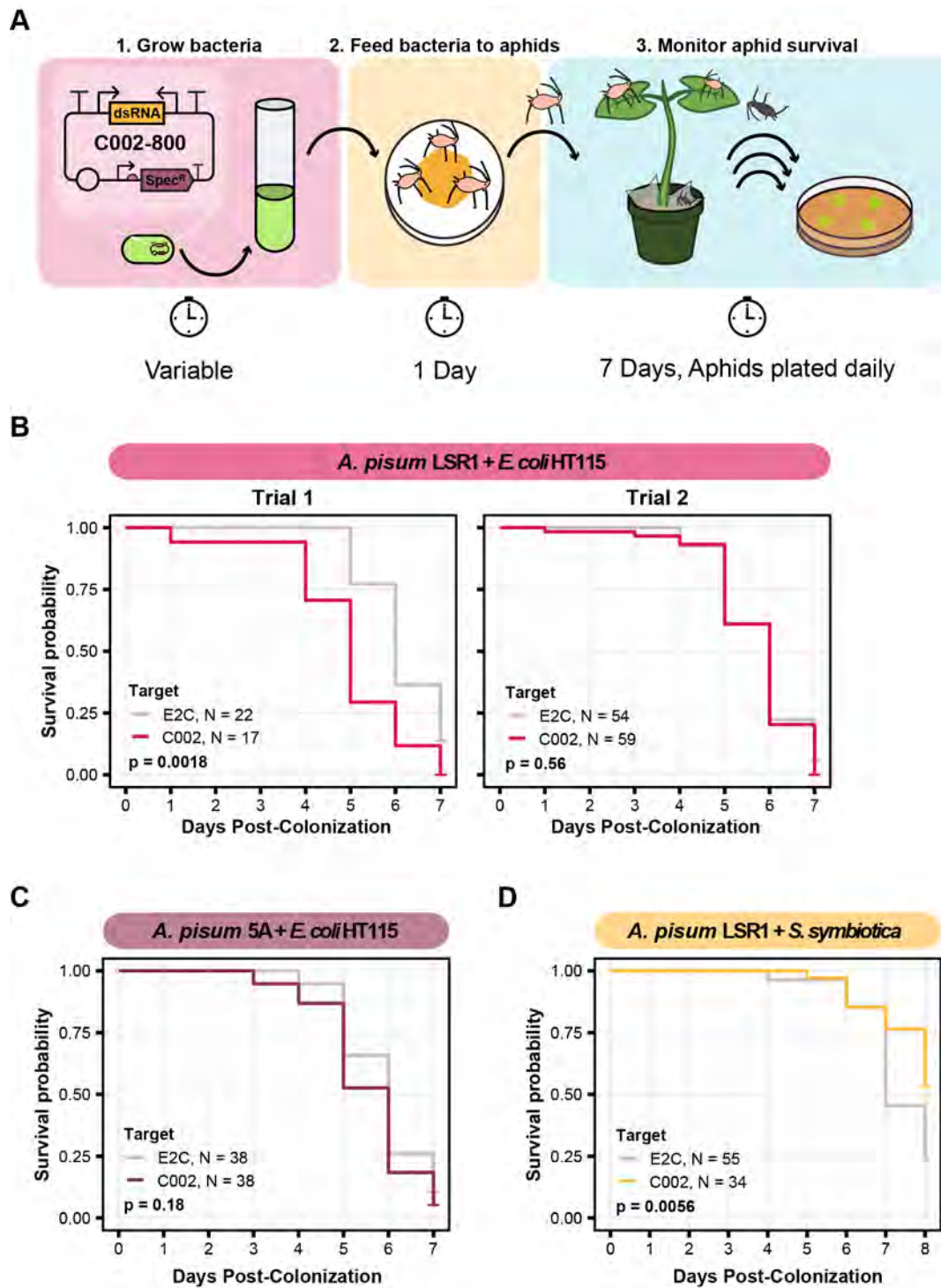


Figure 4.3: Testing knockdown of C002 in aphids using smRNAi.

Figure 4.3 cont'd

(A) Experimental procedure for the C002 assay. This figure shows the plasmid design for the system in the first panel along with the series of steps used to colonize the aphids with the bacteria and assay for a change in mortality. (B-D) Mortality curves for each of the smRNAi assays showing the survival probability of the aphids in the dsC002 condition compared to the dsE2C condition for each day after colonization with the bacteria. Sixty aphids were fed on the bacteria at the start of each experiment except for trial 2 of the *A. pisum* LSR1 + *E. coli* HT115 combination in which 120 aphids were fed. Only aphids that were colonized based on crushing and plating for bacterial growth were counted. P values were calculated with a log-rank test.

Due to the high overall aphid mortality that resulted from colonizing aphids with *E. coli* HT115, we chose to test this knockdown with *S. symbiotica* CWBI-2.3^T as well. Using the same experimental setup, we delivered the dsRNA-expressing bacteria to *A. pisum* LSR1 and monitored their survival. Although the overall level of aphid mortality was lower with CWBI-2.3^T than with HT115 in the off-target controls (Fig. 4.3E) ($p < 0.001$, log-rank test), we found the opposite result of what was expected for dsRNA expression. The off-target control condition with bacteria expressing E2C dsRNA had significantly higher mortality than the C002 condition ($p = 0.0056$, log-rank test). Overall, the relatively small effect size and substantial variation in these results indicates that we are unlikely to achieve a reliable increase in aphid mortality using HT115 or CWBI-2.3^T as hosts for symbiont-mediated RNAi under our conditions.

Additional targeting of aphid Nuclease1 for knockdown does not increase the phenotypic effect of C002 symbiont-mediated RNAi.

To attempt to improve the effectiveness of smRNAi we chose to co-target an aphid nuclease, Nuc1 along with C002. This approach has been shown to increase the potency of RNAi, most likely by decreasing dsRNA degradation in the aphid gut (217, 219). We first tested this technique with *E. coli* HT115. We co-inoculated aphids with two strains, one expressing dsC002-800, and one expressing dsNuc1-800, a construct built to target the same 328 bp of the Nuc1 gene as that were used by Chung et al. (219), and monitored their mortality over one week. In our conditions however, we did not see any improvement in RNAi effectiveness. Mortality in the HT115 double-target C002/Nuc1 condition was not significantly different than the E2C + Nuc1 control ($p = 0.95$, log-rank test) (Fig. 4.4A).

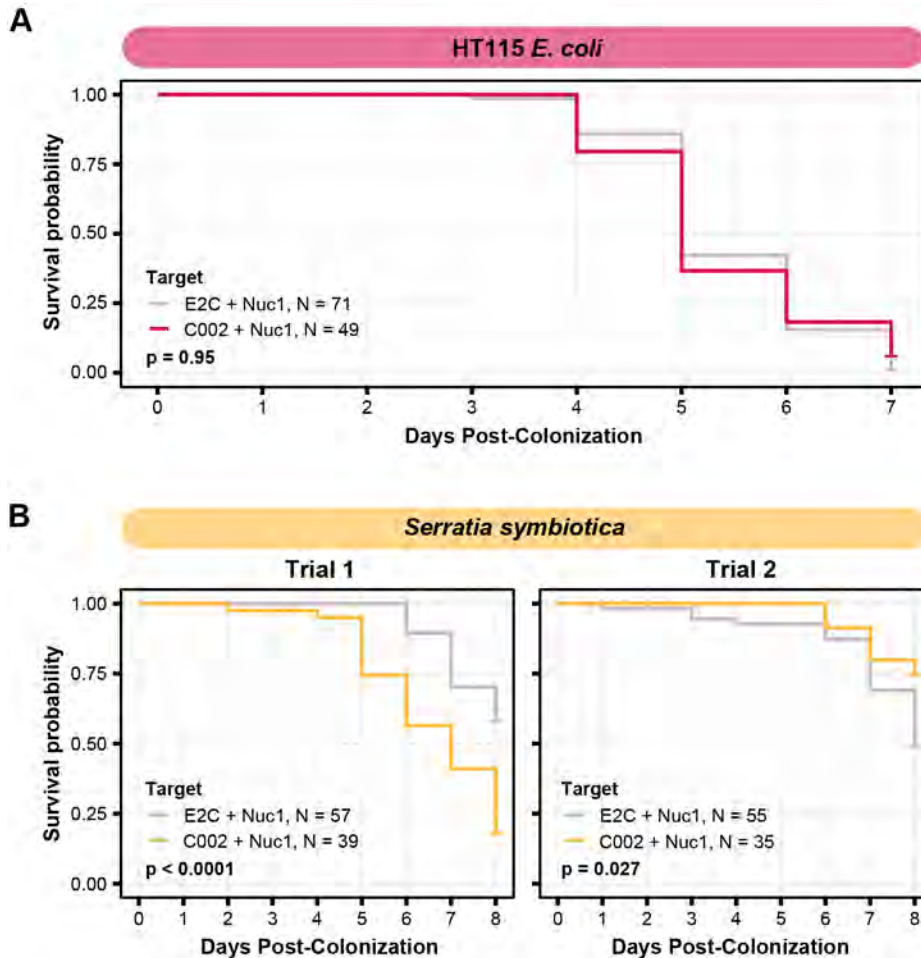


Figure 4.4 Testing co-knockdown of C002 and Nuc1 using smRNAi.

(A) Mortality curve showing the survival of aphids treated with HT115-expressing dsRNA. (B) Mortality of aphid treated with *S. symbiotica* CWBI-2.3^T as the delivery vector. Repeat trials are shown separately. Each panel compares the effect of the on-target C002 combined with Nuc1 knockdown compared to the off-target E2C with Nuc1 knockdown. Sixty aphids were fed on the bacteria at the start of (B) trial one, and 100 were fed for (A) and (B) trial two, but only aphids that were colonized based on crushing and plating for bacterial growth were counted. P values were calculated with a log-rank test.

To see if results would improve with the use of the native symbiont, we performed this same experiment using *S. symbiotica* CWBI-2.3^T. The first attempt at this technique yielded encouraging results: mortality of the aphids in the C002/Nuc1 condition was significantly increased compared to the control condition ($p < 0.0001$, log-rank test) (Fig. 4.4B). However, when we repeated this experiment under the same conditions, we saw the opposite of the expected result: aphids in the control condition died more than in the C002 condition ($p = 0.027$, log-rank test) (Fig. 4.4C). These phenotypic results demonstrate that even with the addition of Nuclease1 dsRNA, this technique is highly inconsistent.

Knocking down EcR with smRNAi does not change numbers of winged offspring.

To test the generality of our results, we performed smRNAi using *S. symbiotica* against a second gene target, EcR. Knockdown of this gene is expected to have a non-lethal phenotype. When the expression of this protein decreases, the proportion of winged (alate) offspring birthed by the treated mother increases. We first performed a control experiment where we injected aphids with EcR dsRNA to assess whether we could recapitulate the results of Vellichirammal et al. (216). As shown in Figure 4.5A, we were able to achieve a significant increase in the number of winged offspring on days 0-2 post-injection ($p = 0.011$, $p = 0.090$, $p = 0.0096$, Fisher's exact test, respectively), though the effect did not persist through day 3 ($p = 0.54$, Fisher's exact test). This mirrors the results of Vellichirammal et al., where significant knockdown was only seen on the first day after injection (216).

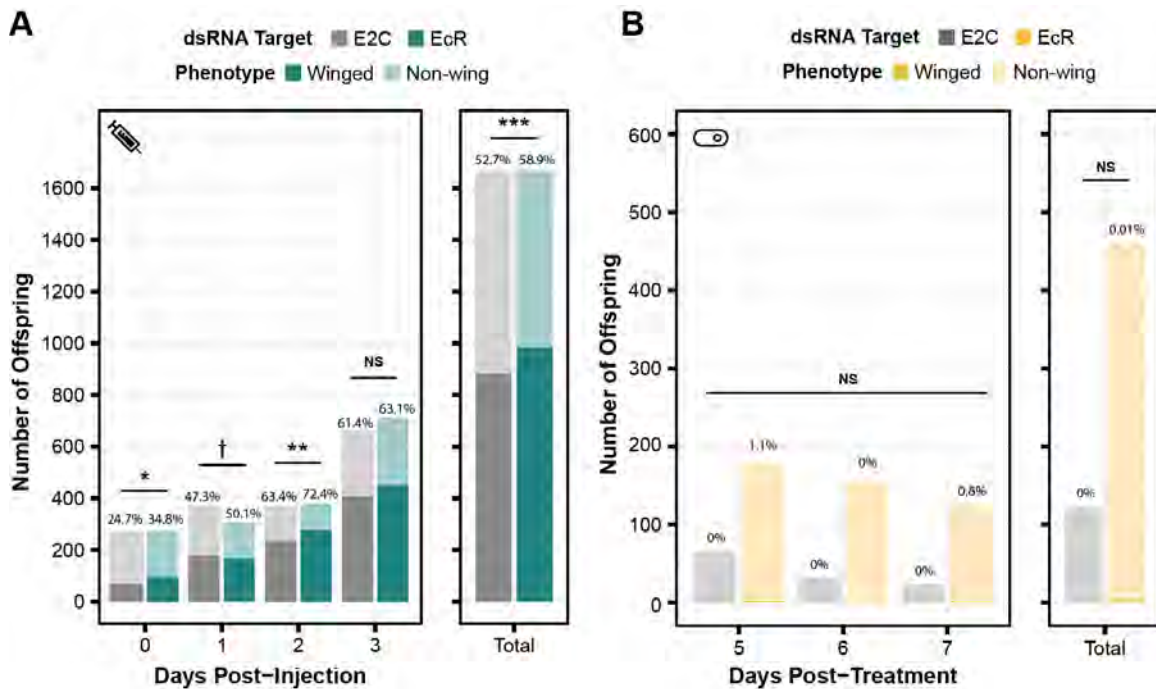


Figure 4.5: Phenotypic results of testing EcR knockdown with two aphid RNAi methods.

(A) The percentage of winged to non-winged offspring birthed by mothers injected with E2C and EcR dsRNA are shown for each day post-injection along with the total for all 4 days. The offspring reflect the totals birthed from 45 mothers split onto 9 separate plants. (B) The percentage of winged to non-winged offspring birthed by aphids fed on bacteria expressing dsE2C or dsEcR. 40 mothers were fed on bacteria per condition and characterized as being colonized based on fluorescence, the colonized mothers were separated onto plants with 4 aphids per plant. P values were calculated based on Fisher's exact test. Significant differences are shown with symbols $\dagger p < 0.1$, $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$.

Because we were able to achieve a significant increase using the injection methodology, we tested knockdown with smRNAi. We designed our knockdown plasmid as before to target the same 466 bp of EcR as Vellichirammal et al., 2017 (216) and transformed it into CWBI-2.3^T-GFP. Aphid stress plays a role in

the production of winged offspring and must have been low because the adults from both the on-target and off-target conditions had considerably fewer winged offspring overall than in the injection iteration of this experiment (Fig. 4.5B). We were thus unable to achieve a significant result using smRNAi ($p = 1$, Fisher's exact test), but the lack of winged offspring complicated determining whether there was any effect from the dsRNA treatment.

QPCR to test for dsRNA expression and RNAi machinery upregulation.

We performed qPCR to attempt to diagnose why the smRNAi system might be ineffective. To do so we set up a repeat of the C002/Nuc1 experiment with five conditions: aphids fed *S. symbiotica* containing an empty vector (pBTK-NR) or plasmids dsE2C, dsC002, dsE2C + dsNuc1, or dsC002 + dsNuc1. After five days we attempted to collect ten colonized aphids from each condition for RNA extraction. However, there were few aphids with GFP+ guts in the dsC002, dsE2C + dsNuc1, and dsC002 + dsNuc1 conditions, so we were only able to obtain 7, 6, and 3 aphids from each, respectively. We did not further analyze the dsC002 + dsNuc1 condition due to its low sample size.

We first sought to confirm production of our dsRNA target sequences by *S. symbiotica* inside of the aphid. We cannot use qPCR to specifically verify production of C002 or Nuc1 dsRNA by engineered symbionts because the exact same sequences are also expressed as RNA by the aphid host, but this test is possible for the E2C off-target control. We used primers targeting E2C to estimate the copy number of dsRNA based on the amount of cDNA generated from 100 ng of total RNA. We were able to see clear expression of the dsRNA

sequence in both the dsE2C and dsE2C + dsNuc1 conditions, although expression was lower in the paired condition (Fig. 4.6A). This difference could be due to unequal colonization by the dsE2C- and dsNuc1-producing bacteria that were co-inoculated in the paired condition.

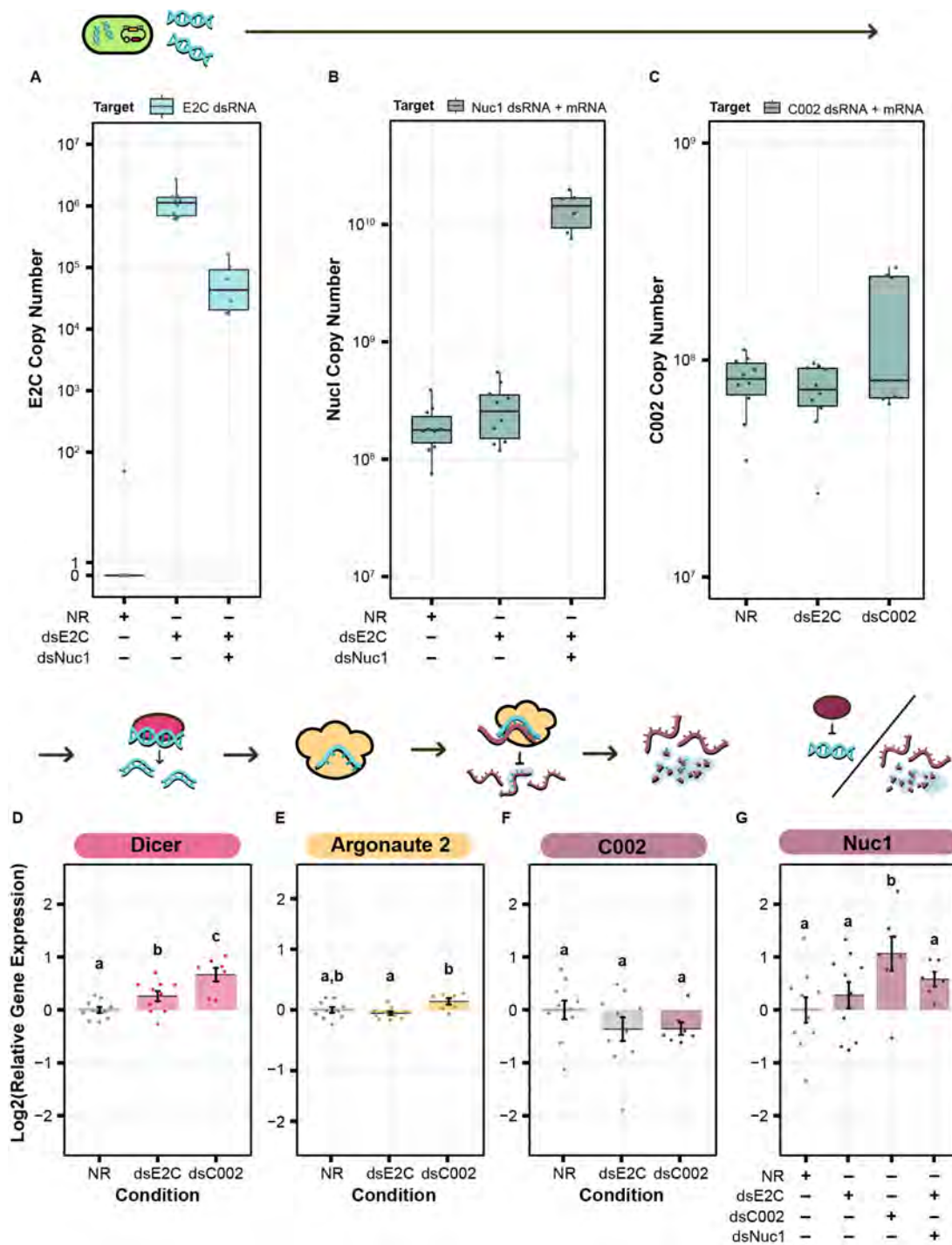


Figure 4.6: qPCR for the level of dsRNA expression and RNAi machinery knockdown after smRNA against C002.

Figure 4.6 cont'd

Above each panel is a simplified depiction of the RNAi process, from expression of dsRNA by the bacterium (green), to processing of dsRNA into siRNA by Dicer (pink), to recognition and knockdown of the matching host mRNA by Argonaute (yellow). (A-C) Copy number of (A) E2-Crimson, (B) Nuc1, and (C) C002 dsRNA sequence after treatment of the aphid with dsRNA expressing bacteria. Copy number is based on the amount of cDNA generated from 100 ng of total RNA. (B,C) For genes that had a corresponding host targets the copy number is shown alongside a the level for a different segment of the gene of interest not found in the dsRNA target. (D-G) Relative level of (D) Dicer, (E) Argonaute 2, (F) C002, and (G) Nuc1 expression calculated with the Pfaffl method. Letters signify groups based on a significant difference of $p < 0.05$. For all qPCR assays, a total of 6-10 aphids were measured for each condition. P values were calculated using the Wilcox test.

For the treatments that included dsNuc1 or dsC002 we assayed the relative expression of the dsRNA construct compared to expression of the corresponding host gene using two separate sets of qPCR primers. One set targets a region found in both the dsRNA sequence and the aphid's mRNA for the gene (dsRNA + mRNA), and the other set targets a region found only in the mRNA (mRNA). The dsNuc1 construct causes an increase in the amount of the dsRNA + mRNA amplicon compared to the other control conditions, consistent with dsRNA expression from the symbiont (Fig. 4.6B). Expression of the dsC002 construct appears to be bimodally distributed (Fig. 4.6C). Three samples show an increased copy number for the dsRNA + mRNA amplicon, reflecting dsRNA production by the symbiont, while the other four samples have similar copy numbers to both control conditions which lack the dsC002 construct.

We next tested to see whether the RNAi pathway is upregulated by screening for changes in Dicer, Argonaute-2 (Ago2). There was significant upregulation of Dicer in both the dsE2C ($p = 0.04326$, two-tailed Mann-Whitney U

test) and dsC002 conditions ($p = 0.001234$, two-tailed Mann-Whitney U test) (Fig. 4.6D). Although Dicer is upregulated for both conditions, we do not see significant upregulation of Ago2 in the dsE2C condition ($p = 0.315$, two-tailed Mann-Whitney U test), and the dsC002 Ago2 is only marginally significantly upregulated compared to the control ($p = 0.055$, two-tailed Mann-Whitney U test) (Fig. 4.6E). These results may indicate that the RNAi pathway has not been fully activated.

Finally, we tested whether smRNAi was effective at reducing C002 expression. For most of the dsC002 samples C002 expression is lower than in the no dsRNA (NR) treatment average. This effect is marginally significant ($p = 0.1331$, two-tailed Mann-Whitney U test) (Fig. 4.6F). Based on our qPCR data, it appears that we were able to produce enough dsRNA to activate Dicer and Ago-2 in the dsC002 condition which may be lowering expression of C002. This effect is still very slight, however, and the effect in the dsE2C condition is even more minimal (0.315 , two-tailed Mann-Whitney U test), despite the level of detected dsRNA being relatively high for all samples. Measurement of our ability to knock down expression of Nuc1 is complicated by the general upregulation of this gene expected in the presence of dsRNA. This upregulation is significant in the dsC002 condition, but not in the dsE2C condition (0.01851 , 0.4813 , two-tailed Mann-Whitney U test, respectively). For the dsE2C + dsNuc1 condition, we see marginally significant upregulation of Nuc1 (0.09341 , two-tailed Mann-Whitney U test), and the upregulation is higher than dsE2C alone. These results indicate that it may be possible to achieve knockdown of at least some aphid genes using smRNAi, but additional experiments are needed to understand where the system needs further optimization.

DISCUSSION

In the configurations that we have tested, symbiont-mediated RNAi is not a reliable tool for gene knockdown in aphids. For both gene targets that we assayed we achieved inconsistent effects on aphid phenotypes. In the C002 trials we saw the expected reduction in aphid survival in one trial but not in others. When testing the ecdysone receptor (EcR) we observed so much variation in our baseline phenotypic outcomes that we could not draw reliable conclusions. Nevertheless, the results of our qPCR experiments show that dsRNA is being expressed within the aphid body and that there is upregulation of genes associated with the host RNAi pathway and some evidence suggesting that mRNA levels of the C002 gene are reduced when it is targeted.

We were careful to attempt to replicate our positive results, including some from preliminary experiments with fewer aphids that are not described here. If we had ended with the first promising result or restricted our reporting to specific gene targets or trials, then we may have judged the success of smRNAi very differently. Some of the limited effectiveness and unreliability that we observed may be specific to smRNAi, such as the choice of symbiont and whether it can stably produce and release enough dsRNA to trigger an effect. Other complications are likely common to many aphid RNAi experiments, such as degradation of dsRNA in the aphid body, judging phenotypes that are highly variable, and other practical difficulties with replicating animal experiments.

The most important factor to consider for implementing smRNAi is the choice of symbiont. In our work we demonstrated a benefit of using a native

symbiont instead of a laboratory *E. coli* strain. HT115 *E. coli* complicated our mortality assay and led to an increase in mortality for all aphids, whereas using *S. symbiotica* CWBI-2.3^T, reduced this complication. It is likely that choosing a well-adapted, native symbiont will generally be a benefit for implementing smRNA. Foreign strains may be under general attack by the host's immune system, which may limit their ability to colonize effectively and produce enough dsRNA to trigger effective gene knockdown. Pathogens that negatively affect host health are likely to have non-specific effects on host gene expression and phenotypes. Although less pathogenic than *E. coli*, *S. symbiotica* CWBI-2.3^T is not a perfect symbiont choice in this respect. It can still reduce aphid longevity and increase aphid mortality (50, 69). If, in the future, a more host-adapted strain of *S. symbiotica* were to be cultured and/or genetically engineered, such as one of the bacteriocyte-associated strains, this would likely be a better symbiont for future aphid smRNAi trials.

Symbiont choice is also expected to affect the production and release of dsRNA. In our experiments we used a strain of *S. symbiotica* that retains expression of RNase III which is likely a limiting factor in the production of dsRNA. Knockout of this gene was unnecessary for successful smRNAi in honeybees (73), but Whitten et al., showed that it was essential to the success of smRNAi in kissing bugs and thrips (49). Deleting RNase III, or mutating it to reduce its expression if a complete knockout is inviable, would be a logical next step to test to increase the effectiveness of our system.

The dsRNA that is produced must also be released by or secreted by the symbiont for delivery to the host. How this occurs during smRNAi is not well understood, even in systems where the technique has been successful. It is

possible that lysis of a portion of the colonizing symbiont population continually releases dsRNA that is taken up by insect cells in the gut in the same way as dsRNA fed in the diet. Small noncoding RNAs can be packaged in outer membrane vesicles (OMVs), which have been proposed to play a role in the export of dsRNA (220, 221) and facilitate interspecies communication (222). OMVs are produced by most Gram-negative bacteria, but their abundance, size, and composition are variable (223). Symbionts may naturally vary in whether they have access to different dsRNA delivery mechanisms. If the relevant mechanisms and genes were known or assays were developed for certain activities, then different symbionts could be computationally or experimentally screened to identify the most promising chassis for smRNAi.

Another general concern with implementing smRNAi in a new system is localization of the symbiont. In most cases, one wants the dsRNA produced by the symbiont, or at least derivatives of the dsRNA that result from processing in host cells, to spread from the symbiont cells and lead to knockdown of the target gene throughout the rest of the host's body. Because *S. symbiotica* CWBI-2.3^T seems to be confined to the aphid gut, this is potentially a concern for our system (50). Based on the results of Zhang et al., dsRNA fed to aphids can enter other tissues (212), and aphids are predicted to have systemic RNAi (224, 225), as exemplified by the success of other feeding studies (Table S1). However, it would be valuable to confirm that systemic knockdown occurs in our system.

To parse whether production or delivery of dsRNA is the primary obstacle for our smRNAi system, studies with different experimental techniques are needed. We were able to observe production of our dsRNA sequences inside the host with qPCR, but this technique does not differentiate amplification products

derived from single- or double-stranded RNA molecules or what proportion of the dsRNA was released into the aphid body by the bacteria. To answer these questions, it may be possible to localize dsRNA in the aphid body with a dsRNA-binding antibody or fluorescence in situ hybridization (FISH) (226, 227). These techniques are widely used to detect RNA-virus associated dsRNAs, and should be adaptable to our system (228). Ideally, dsRNA labelling would allow us to determine that double-stranded dsRNA, not single-stranded RNA, is being expressed and ascertain how it is leaving *S. symbiotica* cells. It could also be used to visualize the spread of dsRNA in the aphid body.

When implementing smRNAi, there may be further complications related to colonizing a host with a genetically engineered microbe. The dsRNA-expression construct may be burdensome to the symbiont, making it possible for mutant cells that inactivate or reduce dsRNA expression to take over during culture in the lab or during insect colonization (68). We observed bimodality in the production of C002 dsRNA between different colonized aphids, which may indicate that this type of evolutionary failure mode is occurring in our experiments. When the aphid phenotype being tested causes mortality, there will be a bias against observing colonization with any symbiont inducing a lethal RNAi response, which would likely further exacerbate this problem. Lastly, an effective insect RNAi response upregulates other immune pathways related to controlling bacterial pathogens which would increase the stress on the strain (229). We encountered challenges achieving reliably high levels of *S. symbiotica* colonization in several C002 trials, which may be related to one or more of these factors. Whether or not the burden is playing a role in our system, it is an important factor to consider when designing a smRNAi experiment as it could

limit overall colonization or favor colonization with mutated symbionts that do not express dsRNA.

Overall, the key to truly improving this technique, along with RNAi in insects at a broader level is to improve the reproducibility of these studies. In the hopes of trying to improve this technique, we, like many others, have introduced a new methodology. However, for the success of this field, there needs to be more emphasis on repeating the methods already tested by other groups and making smaller, more systematic adjustments to optimize them (207, 217, 230, 231). A key to this will be determining which gene targets should be used as positive controls and benchmarks. As of now, there is no established standard in the field to ensure that the technique works in new hands. We attempted to establish a positive control for our experiments based on the most popular gene target. However, prior results reported for the C002 gene still showed wide variation (194, 196), and it's hard to say whether it is a reliable positive control when different methodologies are utilized in each publication. Because of this, perhaps the most popular gene target is not the best candidate for a reliable positive control. A better candidate may be found in survey papers where a variety of targets were tested using consistent methodologies (192, 196, 198, 212). The variability in our baseline results, particularly for the experiment with the ecdysone receptor, suggests that it may also be beneficial to test more genes that are decoupled from aphid viability and stress responses. Genes that affect insect appearance or general behaviors may be more reliable choices (232–236).

In conclusion, although we tried to develop and test a new methodology to improve RNAi knockdown in aphids, we experienced a few pitfalls that have plagued this field. Our results with smRNAi indicate that there may be potential

for this technique using *Serratia symbiotica* CWBI-2.3^T. However, it is likely that this symbiont needs to be further genetically modified, to improve dsRNA production and delivery, for example. If a more aphid-adapted symbiont is cultured and/or engineered in the future, it may be a better platform for this technique. Overall, reproducibility is a major challenge in aphid RNAi studies. Care should be taken to establish standard methodologies and select robust positive controls before it can be reliably used for research and pest control.

MATERIALS AND METHODS

Collection and analysis of aphid RNAi paper data.

To gather the data for the analysis of other aphid RNAi experiments 86 papers on aphid RNAi were read and manually parsed for information. Data was collected related to the aphid tested, the target tested, the method of application of dsRNA, the length and concentration of the RNA, the type of RNA administered (e.g., double-stranded RNA or small hairpin RNA), the level of gene knockdown, and the phenotypic effect of knockdown. To calculate the totals for unique and repeat experimental designs (Fig. 4.1A), an experimental design was broadly described as follows. Each experiment was first filtered by whether it was an injection, feeding, or topical dsRNA application. For injection experiments, a method was defined by the number of injections, the number of targets, and the concentration of dsRNA injected. For feeding experiments, a method was defined by the material fed on, the number of targets, and the concentration of dsRNA in the feeding solution. For topical experiments, an experiment was defined by the

number of targets and the concentration of dsRNA applied to the aphid. All data was analyzed using R (Version 1.2.5042).

Maintaining bacterial and insect stocks.

Serratia symbiotica CWBI-2.3^T strains were grown using Trypticase Soy Broth and agar at room temperature, and HT115-DE3 strains were grown in LB at 30 °C. *Acyrtosiphon pisum* LSR1, PA1, 5A, and Tucson were acquired from long-term stocks maintained in the lab of Nancy Moran (University of Texas at Austin) and were reared on Broad Windsor *Vicia faba* plants (Mountain Valley Seed Company, UT, USA). Aphid cup cages were maintained in Percival I-36LLVL incubators (Perry, IA, USA) at 20°C with a long (16L:8D) photoperiod.

Preparation of dsRNA for injection and feeding experiments.

Ac. pisum LSR1 RNA was extracted from a single aphid using the Zymo Quick RNA Miniprep Kit according to the manufacturer protocols (Zymo Research, CA, USA). The RNA was then reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA). Extended dsRNA regions were amplified from the cDNA template using their respective “Long” primers from Table 4.1. This product served as the template for the second PCR with the “T7” primers to amplify the target dsRNA fragment and add overhangs for T7 transcription. The T7 PCR template was used to prepare dsRNA with the HiScribe T7 Quick High Yield RNA Synthesis Kit according to the manufacturer protocols (New England BioLabs, MA, USA). For the injection experiments, in vitro transcribed (IVT) dsRNA was purified with the Zymo Quick

RNA Miniprep Kit. For the feeding experiments, LiCl precipitation was performed (237). The concentration of the IVT dsRNA was measured using a nanodrop, and the length of the transcripts was confirmed by running samples on a 1% agarose gel (238).

Primer Name	Purpose	Sequence	Amplicon length
c002-long-F	Gene amplification	atctcgtcgtgtatccagt	783 bp
c002-long-R	Gene amplification	gaaagggtaggcgagt	783 bp
Ecr-long-F	Gene amplification	aagaacgccgtgtaccagt	806 bp
Ecr-long-R	Gene amplification	gacaatggcgggtcaacaa	806 bp
Nuc1-long-F	Gene amplification	ggctacgttcgcatagtaaa	672 bp
Nuc1-long-R	Gene amplification	aaaatgccataccgttc	672 bp
c002-T7-F	In vitro dsRNA synthesis	<i>atgtaatacgactcactataggaagttacaaattat</i> <i>acgtagccg</i>	589 bp
c002-T7-R	In vitro dsRNA synthesis	<i>atgtaatacgactcactataggcaaactatttatg</i> <i>gctccc</i>	589 bp
E2C-T7-F	In vitro dsRNA synthesis	<i>atgtaatacgactcactataggaagctgcaagt</i> <i>accaag</i>	412 bp
E2C-T7-R	In vitro dsRNA synthesis	<i>atgtaatacgactcactatagggactgaactcac</i> <i>acaggtag</i>	412 bp
Ecr-T7-F	In vitro dsRNA synthesis	<i>atgtaatacgactcactataggtgtacctaagttc</i> <i>aattgtgcag</i>	466 bp
Ecr-T7-R	In vitro dsRNA synthesis	<i>atgtaatacgactcactataggagcttcacttgagc</i> <i>aagcct</i>	466 bp
GGA-c002-F	Plasmid assembly	<i>gcacgtctcatcgggtctcatatgaagttacaaatta</i> <i>tacgtagccg</i>	589 bp
GGA-c002-R	Plasmid assembly	<i>atgccgtctcagggtctcaggatcaaactatttatgg</i> <i>ctccc</i>	589 bp
GGA-E2C-F	Plasmid assembly	<i>gcacgtctcatcgggtctcatatgaagctgcaagt</i> <i>accaag</i>	412 bp
GGA-E2C-R	Plasmid assembly	<i>atgccgtctcagggtctcaggatgactgaactcac</i> <i>acaggtag</i>	412 bp
GGA-Ecr-F	Plasmid assembly	<i>gcacgtctcatcgggtctcatatgtgtacctgaagtt</i> <i>caatgtgcag</i>	466 bp
GGA-Ecr-R	Plasmid assembly	<i>atgccgtctcagggtctcaggatagcttcacttgagc</i> <i>aagcct</i>	466 bp
GGA-Nuc1-F	Plasmid assembly	<i>gcacgtctcatcgggtctcatatgacctccgaagt</i> <i>ttggtcac</i>	328 bp
GGA-Nuc1-R	Plasmid assembly	<i>atgccgtctcagggtctcaggattgtgccgtacagc</i> <i>tctttg</i>	328 bp

Table 4.1: Primers used for dsRNA amplification and plasmid assembly.

Feeding aphids C002 dsRNA.

For the feeding experiments, four different subspecies of *Ac. pisum*, LSR1, PA1, 5A, and Tucson, were fed IVT dsRNA in an artificial diet mix. Each diet mix contained 100 ng/ μ L dsRNA in a total of 100 μ L Febvay diet + 1ul yellow food dye (Gel Spice Company Inc., NJ, USA) (50, 129). Sixty total 3-5 day old aphids were split into two feeding dishes per condition and fed for 18 hours before being transferred to plants (20 aphids per plant, 3 plants per condition). Aphid mortality was monitored daily for a week and offspring were removed from the plants to prevent overcrowding.

Engineering HT115 and CWBI-2.3^T for the expression of dsRNA.

Construction of CWBI-2.3^T-GFP was described previously (50). HT115-GFP was built using pGRG36::PA1-GFP, a plasmid derived from pGRG-36 (239). For the transformation of this plasmid into HT115, we adapted the method from McKenzie and Craig, 2006 (240). The donor *E. coli* strain, MFDpir, and the recipient HT115 strain were first grown overnight, then washed twice with 145 mM NaCl saline solution, mixed to a 1:2 ratio of donor:recipient, and spot-plated on LB + DAP. After a day of growth, the spot was scraped up and washed with saline as before. The bacteria were plated on LB + Carb and grown at 30°C to select for transformation of the plasmid. Transformants were picked and regrown on LB + Cam at 37°C to enrich for the transposition event. The resulting colonies were picked and regrown at 37°C with Cam or Carb to confirm loss of the plasmid and successful integration.

The design for the dsRNA expression vectors is based on the design from Leonard, et al. (73, 218). In brief, the plasmid has a low to medium copy RSF1010-based origin, spectinomycin resistance, and a dsRNA target sequence flanked by two identical inverted CP25 promoters. Each target for the RNAi experiments was first amplified from *Ac. pisum* LSR1 cDNA with “GGA” primers specified in Table 4.1, then cloned into the dsRNA site on the pBTK800 expression vector (218) with BsaI Golden Gate Assembly. The choice of amplified gene fragment was selected to replicate previous publications by other labs (204, 216, 219). Assembled plasmids were transformed into CWBI-2.3^T-GFP and HT115-GFP for aphid feeding, the exception to this is the dsNuc1-800 plasmid which was transformed into WT CWBI-2.3^T and HT115. Plasmids and strains used in this study are shown in Table 4.2

Plasmid Name	Description	Purpose	Source
pGRG36::PA1-GFP	Plasmid for genome integration of GFP and CamR	Strain construction	Tim Cooper (118)
pYTK095	Golden Gate compatible plasmid, ColE1 origin, AmpR	Plasmid assembly	
pBTK800	Golden Gate compatible plasmid, RSF1010 origin, SpecR	Plasmid assembly	(218)
dsC002-800	Plasmid with an RSF1010 origin, SpecR, expression of dsC002	dsRNA expression	This paper
dsE2C-800	Plasmid with an RSF1010 origin, SpecR, expression of dsE2C	dsRNA expression	This paper
dsNuc1-800	Plasmid with an RSF1010 origin, SpecR, expression of dsNuc1	dsRNA expression	This paper
dsE2C-095	Plasmid with a ColE1 origin, AmpR, expression of dsE2C	qPCR standard	This paper

Table 4.2: Plasmids used in Chapter 4.

Symbiont-mediated RNAi for C002 knockdown.

A. pisum LSR1 was reared to 5 days of age then fed on 98 μ L of Febvay diet, plus 1 μ L of OD=1 dsRNA expressing HT115-GFP or CWBI-2.3^T-GFP, and 1 μ L yellow food dye. Aphids were fed on the diet mix for 24 hours, then transferred to plants. Their mortality was recorded daily for 1 week, and all dead aphids were crushed and plated on selective media as described in Elston et al. to confirm colonization with bacteria expressing dsRNA (50). For the dsNuc1 co-inoculation experiments, each strain was washed and concentrated to OD=1, and 0.5 μ L of each was added to the diet + food dye mixture.

Injection of EcR dsRNA.

To prepare for injection of EcR dsRNA, colonies of *Ac. pisum* LSR1 were reared at low density (5-7 aphids per plant) for at least two generations to decrease colony stress and numbers of alate adults used in the experiments. Next, 45 10-day-old aphids were injected with ~0.1 μ L of 2 μ g/ μ L EcR or off-target E2-Crimson in-vitro transcribed dsRNA. The injected aphids were allowed to recover overnight in petri dishes, then placed onto fava beans with 5 aphids per plant. Every 24 hours the adults were transferred to fresh plants for a total of 3 days. The offspring were then reared to the 4th instar and scored for wing bud development.

Symbiont-mediated RNAi for EcR knockdown.

As described in the injection methods for EcR dsRNA, *Ac. pisum* LSR1 were first reared at a low density for at least two generations. CWBI-2.3T-GFP expressing the dsEcR-800 or dsE2C-800 plasmid was grown to log-phase then prepared for feeding to aphids by washing twice with 1 $\times$ phosphate-buffered saline (PBS) and concentrating to an OD of 1 (50). Forty aphids per condition were fed overnight on a diet mix containing 98 μ L of Febvay diet, 1 μ L blue food dye, and 1 μ L bacteria. After feeding, the aphids were transferred to plants and reared to adulthood (about 5 days post-feeding). At this point the aphids were screened for colonization with fluorescent CWBI-2.3^T bacteria on a blue light transilluminator, and all colonized aphids were transferred to fresh plants (4 aphids per plant). From this point on, the colonized aphids were transferred to new plants every 24 hours for 3 consecutive days and then removed. Their

offspring were reared to the 4th instar and scored for the development of wing buds.

QPCR to assess gene knockdown in aphids.

Bacteria were delivered to *A. pisum* LSR1 in artificial diet as described in the previous methods sections. For these experiments, six conditions were prepared: CWBI-2.3^T-GFP + BTK-NR, CWBI-2.3^T-GFP + dsE2C-800, CWBI-2.3^T-GFP + dsC002-800, CWBI-2.3^T-GFP + dsE2C-800 and CWBI-2.3^T + dsNuc1-800, and CWBI-2.3^T-GFP + dsC002-800 and CWBI-2.3^T + dsNuc1-800. Two sets of 20 aphids were treated to each condition; the aphids were then transferred to plants after 24 hours of feeding. Five days after treatment, 6-10 glowing aphids from each condition were collected and crushed in DNA/RNA shield (Zymo Research, CA, USA). Whole aphid RNA was purified using an RNA Clean & Concentrator kit (Zymo Research, CA, USA) which includes an on-column DNA digestion step. Five hundred nanograms of each RNA sample were then reverse transcribed into cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, MA, USA). Primers for qPCR were designed using IDT's Primerquest tool and are listed in Table 4.3. All qPCR reactions were prepared in triplicate in 384-well plates with Sybergreen reagents (Thermo Fisher, MA, USA), and reactions were carried out using a Viiia-7 machine (Thermo Fisher, MA, USA).

Primer Name	Sequence
Rpl32-qPCR-F	catgtcgatcaagccgaagtatc
Rpl32-qPCR-R	cccttggtttacgccagtt
SDBH-qPCR-F	ccaactcgactcctaagcctaa
SDBH-qPCR-R	ccactatacctaagccagccata
Betatub-qPCR-F	caacttcgtgttcggtcagtc
Betatub-qPCR-R	agtcacagttctcgctctctt
GAPDH-qPCR-F	ctggaatggcttcagagtacc
GAPDH-qPCR-R	tgcggttccttgactttatc
NADH-qPCR-F	tgctcttagacgttacctatct
NADH-qPCR-R	gcgactccatcagctctttc
E2C-qPCR-F	actacctcaagcagtccttcc
E2C-qPCR-R	cttcacgtggtagatgaggg
C002-dsRNA-qPCR-F	gttggcgttcataagagagg
C002-dsRNA-qPCR-R	gcggtcgtgtgcttgatata
C002-host-qPCR-F	taagcgcggtccatacagact
C002-host-qPCR-R	agacgactgtcgaaagggtag
Nuc1-dsRNA-qPCR-F	ccgtacagctcttggacttaatg
Nuc1-dsRNA-qPCR-R	gtttcgacaagtcgaccaagaa
Nuc1-host-qPCR-F	ggtaagggtacagtgggtgttg
Nuc1-host-qPCR-R	cagtcaaccgggtcaaagttaag
Ago2-qPCR-F	tcccgaccaaccaagtatca
Ago2-qPCR-R	gccggatatgaaacagaacgag
Dicer-qPCR-F	ctgacaaggcagcagataaagg
Dicer-qPCR-R	gcggcaaccttagctcttt

Table 4.3: Primers used for qPCR

Control qPCRs were run to determine the efficiency of each primer set and establish the most reliable reference genes for analysis. To determine primer efficiency, PCR products for each qPCR primer pair were synthesized, serially diluted, and run as the template to generate a standard curve. To choose the most reliable reference genes, we tested Beta-tubulin, GAPDH, NADH, Rpl32, and SDBH. We selected SDBH and Rpl32 to serve as references because they

had the lowest standard deviation and variance between conditions. We calculated the gene expression ratio for each aphid target as compared to the geometric mean of both reference genes according to the Pfaffl method (241). The amount of dsRNA production was determined by running the cDNA samples along with a standard curve for the gene of interest. The Cq values returned for the standard curve were then used to calculate the absolute copy number for each condition (242).

Chapter 5: Concluding Remarks

In this dissertation I describe work carried out during my PhD with the express goal of engineering non-model insect symbionts, particularly those of aphids and fruit flies. This chapter will address this work in the context of the questions it brings up for future studies of insect symbioses and applications of insect paratransgenesis.

What counts as a symbiont?

When it comes to paratransgenesis studies, it is important to select a bacterium that is well-adapted to stably co-existing with the host. This choice can prevent complications resulting from strain pathogenicity, improve the reliability of colonization, and can lead to vertical transmission of the engineered bacterium across host generations in some cases. When selecting a “well-adapted” bacterium, the ideal choice would be a “native symbiont”. But this begs the question, what counts as a native symbiont?

In an ideal world, the feasibility of paratransgenesis could initially be tested using *E. coli* because it is so easily engineerable. However, while some insects like flies might be tolerant to colonization by *E. coli* (243, 244), this is not the case for all insect hosts. In our hands, we showed that even a laboratory strain of *E. coli* was toxic to aphids. When assaying colonization of a “native symbiont” that was directly isolated from aphids and is related to vertically inherited symbionts that are widespread in aphid populations, we still encountered pathogenicity, which varied depending on the aphid species that we

colonized (36, 50, 69). Through one of our collaborations, we also learned that the effect of *S. symbiotica* CWBI-2.3^T on mortality is dependent on whether it is injected or fed to aphids, and different isolates are more or less pathogenic (69). In fruit flies we also see that closely related symbionts colonize differently, and others have shown that lab strains are less successful colonizers than wild isolates of the same bacterium (172).

The only way to truly guarantee then, that the symbiont is well-adapted to the host may be to isolate it from your exact host species and/or biotype and prove that it is stable in natural host populations. However, freshly isolating a new symbiont for every species you work with is tedious and impractical. Furthermore, the process of isolation itself can inadvertently lead to “domestication” of a bacterial strain if it gains mutations that improve its growth in culture medium or survival during storage in the lab (85). These mutations may be especially likely to reduce its ability to stably associate with insects, which could potentially negate the benefit of isolating an insect-adapted symbiont. Many symbionts are also unculturable, particularly obligate intracellular symbionts that have co-evolved with the host for the longest (84, 130).

One option to overcome the challenge of working with a true “native” symbiont may be to try to engineer bacterial symbionts while they remain within the host’s body. If successful, this in situ approach may eventually lead to the ability to engineer bacteria that are currently unculturable and avoid the complication of laboratory domestication. This procedure could be carried out through in situ conjugation from a bacterial donor, or through transduction from a broad-host-range phage (245). In situ conjugation of native gut flora has already been carried out in mice (86), and a conjugative element from *Bacillus subtilis*

was used to engineer bacteria in a complex soil environment (131). To expand our study of the fly gut microbiome, we could potentially adapt our arena inoculation method to deliver the Pathfinder donor mix to a fly colonized with its natural microbiome. Ideally, multiplex conjugation would increase the chances of plasmid transfer, and the transconjugants could then be isolated on selective media. This approach eliminates the selective nature of preferentially isolating bacteria of interest before engineering (246). It appears to be feasible in *D. melanogaster*: in a preliminary test we were able to isolate an *Acetobacter* and a *Lactobacillus plantarum* that underwent conjugation inside the host.

Instead of engineering bacterial symbionts inside the host, researchers could alternatively try to adapt a bacterium that has already been isolated/engineered to reduce its pathogenicity or improve its colonization ability. This approach would involve performing mutagenesis on the prospective symbiont, colonizing the host, and selecting for improved colonizers. We could also use a rational genome engineering approach to mutate or remove known pathogenicity factors from the symbiont genome. Finally, we could consider engineering the strain and the host to be reliant on each other. This technique was used to create an unnatural *E. coli* endosymbiont inside a yeast cell (247). Given the genetic tractability of *D. melanogaster* and its symbionts, it may be a promising candidate for engineering this type of co-dependence in a multicellular organism.

Is an aphid the same as a bee? The same as a fly?

From the outset, there are obvious differences in the physiology and lifestyles of aphids, bees, and flies. So why bother comparing them? For researchers in the field of paratransgenesis, where trial and error is mostly the order of the day due to our poor understanding of the critical interactions in these systems, researchers are trying to adapt methods designed for diverse insect/bacterium pairings to new ones and compare their efficacy. Ideally, the field will eventually establish generalizable genetic tools and come to understand at a molecular level why certain approaches work in different systems.

With these goals, we applied the BTK plasmids originally developed for bee symbionts to aphids and flies (57). The basis of these BTK plasmids, is the replicative plasmid origin, RSF1010, which is a common tool for engineering diverse bacteria (86, 136, 169). When we designed the Pathfinder plasmid system, we based it on the RSF1010 origin, while also expanding the chances of successful transformants by including two other common broad-host-range origins, RP4 and pBBR1. In our hands we have yet to encounter an insect symbiont that is incompatible with RSF1010 plasmids, but as these tools are adopted by more groups it will be interesting get a better sense of its range of compatibility. It will be useful to extend the BTK and Pathfinder systems to include other broad-host-range plasmid origins and genomic integration techniques in the future. In particular, CRISPR-associated transposons may be an effective way of integrating genes into symbiont genomes, including for in situ genetic modification (248, 249).

At the level of the insect, differences in physiology play a major role in the success of paratransgenesis. We can highlight these differences in our work with

smRNAi which has only been tested in a handful of species. Although effective in honey bees (73), thrips, and kissing bugs (49), we did not achieve reliable success in aphids. Our results make one wonder if more researchers have encountered smRNAi challenges that have not been reported due to publication biases favoring positive results. In the future, to increase our ability to perform smRNAi in a wider range of insects, comparative studies between these insects may be necessary to uncover the factors for success. Studies that visualize dsRNA within the host would aid in understanding some of these differences, including how the availability, transport, and stability of dsRNA molecules varies for different symbiont-insect pairs. For example, it is known that host RNases released into the gut are a larger barrier to inducing RNAi through dsRNA feeding in certain groups of insects like Lepidoptera (250–252). Differences between symbiont species, like their potential for outer membrane vesicle production, may also affect dsRNA delivery. If there was a robust RNAi system that was tolerant to colonization with different bacteria, this would provide an opportunity to test the effectiveness of different species on a level playing field. There is work using “organ-on-a-chip” systems to perform mammalian RNAi and gut microbiome studies (253, 254). These types of systems could serve as a more carefully controlled environment and testbed for developing smRNA if they were developed for insect systems.

How do we bridge the knowledge gap?

Paratransgenesis is an inherently multidisciplinary technique. You need an understanding of microbiology, synthetic biology, insect physiology, and even environmental biology to achieve a successful outcome. It takes a special

research environment to integrate knowledge across these areas. To expand these capabilities to more researchers, communication between labs with different specialties will be essential to the future of this field. Increasing the accessibility of synthetic biology tools to more labs will expand the boundaries for engineering environmental microbes. Synthetic biology groups often develop toolkits with this intention, but they can only test their application in so many microbes and most of this testing is done outside of hosts and environments that are of interest to microbial ecologists. Ensuring that these genetic tools are inexpensive to obtain and have protocols that are clearly written for researchers without synthetic biology expertise will increase their usage by bridging this gap.

Conclusion

From the outset of my degree, I collaborated with a team of researchers focused on the ambitious goal of using aphids to spread beneficial engineered plant viruses to protect crops against the spread of pathogens. Much of the work in collaboration with these individuals in the Barrick, Moran, and Ellington labs veered away from this initial goal as we encountered challenges in understanding and genetically engineering so many diverse biological systems in new ways. However, knowledge was gained in the realms of engineered plant viruses, endosymbionts, and aphids, including the work in Chapters 2 and 4 of this dissertation (50, 69). Chapter 3 deals with a more standard model system, the fruit fly *Drosophila melanogaster*. In collaboration with Laila Philips, we adapted a toolkit designed and constructed by Sean Leonard to begin to explore some of the native gut residents of flies. These collaborations fostered our ability to take insect symbiont model systems and combine them with synthetic biology tools to

get a better understanding of the symbioses and potential future applications for paratransgenesis. We addressed some of the questions posed in this conclusion: about the definition of a symbiont, how well we can make assumptions about the similarities of host-symbiont systems, and how we can enable the success of multidisciplinary projects. I'm excited to see these collaborations expand to more systems, and I hope that my work contributes to building a solid foundation that will enable many future successes in this field.

References

1. N. E. Stork, How many species of insects and other terrestrial arthropods are there on Earth? *Annu. Rev. Entomol.* **63**, 31–45 (2018).
2. W. W. Weisser, E. Siemann, *Insects and Ecosystem Function* (Springer Berlin Heidelberg, Berlin, Heidelberg, 2008; <http://link.springer.com/book/10.1007/978-3-540-74004-9>), *Ecological Studies*.
3. E. B. Radcliffe, W. D. Hutchison, R. E. Cancelado, *Integrated Pest Management* (Cambridge University Press, Cambridge, 2008; <http://ebooks.cambridge.org/ref/id/CBO9780511626463>).
4. World Health Organization, “A global brief on vector-borne diseases” (WHO/DCO/WHO/2014.1, WHO Press, Geneva, Switzerland, 2014), , doi:WHO/DCO/WHO/2014.1.
5. C. N. T. Taning, B. Van Eynde, N. Yu, S. Ma, G. Smagghe, CRISPR/Cas9 in insects: applications, best practices and biosafety concerns. *J. Insect Physiol.* **98**, 245–257 (2017).
6. D. Chaverra-Rodriguez, V. M. Macias, G. L. Hughes, S. Pujhari, Y. Suzuki, D. R. Peterson, D. Kim, S. McKeand, J. L. Rasgon, Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nat. Commun.* **9**, 3008 (2018).
7. J. P. McCutcheon, N. A. Moran, Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* **10**, 13–26 (2012).
8. C. B. Beard, R. V. Durvasula, F. F. Richards, Bacterial symbiosis in arthropods and the control of disease transmission. *Emerg. Infect. Dis.* **4**, 581–91 (1998).
9. E. P. Caragata, F. O. Rezende, T. C. Simões, L. A. Moreira, Diet-induced nutritional stress and pathogen interference in *Wolbachia*-infected *Aedes aegypti*. *PLoS Negl. Trop. Dis.* **10**, e0005158 (2016).
10. I. Dorigatti, C. McCormack, G. Nedjati-Gilani, N. M. Ferguson, Using *Wolbachia* for dengue control: insights from modelling. *Trends Parasitol.*

34, 102–113 (2018).

11. J. I. Perlmutter, S. R. Bordenstein, Microorganisms in the reproductive tissues of arthropods. *Nat. Rev. Microbiol.* **18**, 97–111 (2020).
12. G. M. Bennett, N. A. Moran, Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci.* **112**, 10169–10176 (2015).
13. A. E. Douglas, Lessons from studying insect symbioses. *Cell Host Microbe.* **10**, 359–367 (2011).
14. Y. Kikuchi, Endosymbiotic bacteria in insects: Their diversity and culturability. *Microbes Environ.* **24**, 195–204 (2009).
15. H. Salem, L. Florez, N. Gerardo, M. Kaltenpoth, An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc. R. Soc. B Biol. Sci.* **282**, 20142957 (2015).
16. E. Akman Gündüz, A. E. Douglas, Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proc. R. Soc. B Biol. Sci.* **276**, 987–991 (2009).
17. G. D. D. Hurst, C. L. Frost, Reproductive parasitism: maternally inherited symbionts in a biparental world. *Cold Spring Harb. Perspect. Biol.* **7**, a017699 (2015).
18. P. Nadal-Jimenez, J. S. Griffin, L. Davies, C. L. Frost, M. Marcello, G. D. D. Hurst, 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 (2019).
19. G. Nadarasah, J. Stavrinides, Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol. Rev.* **35**, 555–575 (2011).
20. L. V. Flórez, K. Scherlach, P. Gaube, C. Ross, E. Sitte, C. Hermes, A. Rodrigues, C. Hertweck, M. Kaltenpoth, Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. *Nat. Commun.* **8**, 15172 (2017).

21. A. Vigneron, F. Masson, A. Vallier, S. Balmand, M. Rey, C. Vincent-Monégat, E. Aksoy, E. Aubailly-Giraud, A. Zaidman-Rémy, A. Heddi, Insects recycle endosymbionts when the benefit is over. *Curr. Biol.* **24**, 2267–2273 (2014).
22. D. G. Marshall, T. A. Jackson, C. R. Unelius, S. L. Wee, S. D. Young, R. J. Townsend, D. M. Suckling, *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 (2016).
23. C. Braendle, T. Miura, R. Bickel, A. W. Shingleton, S. Kambhampati, D. L. Stern, Developmental origin and evolution of bacteriocytes in the aphid–*Buchnera* symbiosis. *PLoS Biol.* **1**, 070–076 (2003).
24. S. Aksoy, *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 (1995).
25. T. Fukatsu, N. Nikoh, R. Kawai, R. Koga, The secondary endosymbiotic bacterium of the pea aphid *Acyrtosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **66**, 2748–2758 (2000).
26. K. Watanabe, F. Yukuhiro, Y. Matsuura, T. Fukatsu, H. Noda, Intrasperm vertical symbiont transmission. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 7433–7437 (2014).
27. G. Favia, I. Ricci, C. Damiani, N. Raddadi, E. Crotti, M. Marzorati, A. Rizzi, R. Urso, L. Brusetti, S. Borin, D. Mora, P. Scuppa, L. Pasqualini, E. Clementi, M. Genchi, S. Corona, I. Negri, G. Grandi, A. Alma, L. Kramer, F. Esposito, C. Bandi, L. Sacchi, D. Daffonchio, Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proc. Natl. Acad. Sci.* **104**, 9047–9051 (2007).
28. C. Damiani, I. Ricci, E. Crotti, P. Rossi, A. Rizzi, P. Scuppa, F. Esposito, C. Bandi, D. Daffonchio, G. Favia, Paternal transmission of symbiotic bacteria in malaria vectors. *Curr. Biol.* **18**, 1087–1088 (2008).
29. R. Koga, X.-Y. X. Y. Meng, T. Tsuchida, T. Fukatsu, Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proc. Natl. Acad. Sci.* **109**, E1230–E1237 (2012).

30. C. Dale, S. . Welburn, The endosymbionts of tsetse flies: manipulating host–parasite interactions. *Int. J. Parasitol.* **31**, 628–631 (2001).
31. K. Michalik, T. Szklarczyk, M. Kalandy-Kołodziejczyk, W. Jankowska, A. Michalik, 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 (2016).
32. K. L. Coon, K. J. Vogel, M. R. Brown, M. R. Strand, Mosquitoes rely on their gut microbiota for development. *Mol. Ecol.* **23**, 2727–2739 (2014).
33. T. Hosokawa, T. Fukatsu, Relevance of microbial symbiosis to insect behavior. *Curr. Opin. Insect Sci.* **39**, 91–100 (2020).
34. C. . Beard, E. . Dotson, P. . Pennington, S. Eichler, C. Cordon-Rosales, R. . Durvasula, Bacterial symbiosis and paratransgenic control of vector-borne Chagas disease. *Int. J. Parasitol.* **31**, 621–627 (2001).
35. J. E. Powell, V. G. Martinson, K. Urban-Mead, N. A. Moran, Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Appl. Environ. Microbiol.* **80**, 7378–7387 (2014).
36. F. Renoz, I. Pons, A. Vanderpoorten, G. Bataille, C. Noël, V. Foray, V. Pierson, T. Hance, 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 (2019).
37. F. E. Acevedo, M. Peiffer, C.-W. Tan, B. A. Stanley, A. Stanley, J. Wang, A. G. Jones, K. Hoover, C. Rosa, D. Luthe, G. Felton, Fall armyworm-associated gut bacteria modulate plant defense responses. *Mol. Plant-Microbe Interact.* **30**, 127–137 (2017).
38. E. J. De Vries, G. Jacobs, J. A. J. Breeuwer, Growth and transmission of gut bacteria in the western flower thrips, *Frankliniella occidentalis*. *J. Invertebr. Pathol.* **77**, 129–137 (2001).
39. A. M. Walterson, J. Stavriniades, *Pantoea*: insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiol. Rev.* **39**, 968–984 (2015).

40. T. E. Wilkes, O. Duron, A. C. Darby, V. Hypša, E. Nováková, G. D. D. Hurst, in *Manipulative Tenants: Bacteria Associated with Arthropods*, E. Zchori-Fein, K. Bourtzis, Eds. (CRC Press, 2012), p. 244.
41. M. Kaltenpoth, L. V. Flórez, Versatile and dynamic symbioses between insects and *Burkholderia* bacteria. *Annu. Rev. Entomol.* **65**, 145–170 (2020).
42. L. M. Bolã, L. E. Servín-Garcidueñas, G. Garcidueñas, E. Martínez-Romero, Arthropod-*Spiroplasma* relationship in the genomic era. *FEMS Microbiol. Ecol.* **91**, 1–8 (2015).
43. S. J. Perlman, M. S. Hunter, E. Zchori-Fein, The emerging diversity of *Rickettsia*. *Proc. R. Soc. B Biol. Sci.* **273**, 2097–2106 (2006).
44. E. Crotti, C. Damiani, M. Pajoro, E. Gonella, A. Rizzi, I. Ricci, I. Negri, P. Scuppa, P. Rossi, P. Ballarini, N. Raddadi, M. Marzorati, L. Sacchi, E. Clementi, M. Genchi, M. Mandrioli, C. Bandi, G. Favia, A. Alma, D. Daffonchio, *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environ. Microbiol.* **11**, 3252–3264 (2009).
45. M. Adeolu, S. Alnajar, S. Naushad, R. S. Gupta, Genome-based phylogeny and taxonomy of the ‘*Enterobacteriales*’: proposal for *Enterobacterales* ord. nov. divided into the families *Enterobacteriaceae*, *Erwiniaceae* fam. nov., *Pectobacteriaceae* fam. nov., *Yersiniaceae*. *Int. J. Syst. Evol. Microbiol.* **66**, 5575–5599 (2016).
46. A. K. Arora, A. Forshaw, T. A. Miller, R. Durvasula, A delivery system for field application of paratransgenic control. *BMC Biotechnol.* **15**, 59 (2015).
47. A. K. Arora, K. N. Pesko, V. Quintero-Hernández, L. D. Possani, T. A. Miller, R. V. Durvasula, A paratransgenic strategy to block transmission of *Xylella fastidiosa* from the glassy-winged sharpshooter *Homalodisca vitripennis*. *BMC Biotechnol.* **18**, 50 (2018).
48. S. Wang, A. L. A. Dos-Santos, W. Huang, K. C. Liu, M. A. Oshaghi, G. Wei, P. Agre, M. Jacobs-Lorena, Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science*. **357**, 1399–1402 (2017).

49. M. M. A. Whitten, P. D. Facey, R. Del Sol, L. T. Fernández-Martínez, M. C. Evans, J. J. Mitchell, O. G. Bodger, P. J. Dyson, Symbiont-mediated RNA interference in insects. *Proc. R. Soc. B Biol. Sci.* **283**, 20160042 (2016).
50. K. M. Elston, J. Perreau, G. P. Maeda, N. A. Moran, J. E. Barrick, Engineering a culturable *Serratia symbiotica* strain for aphid paratransgenesis. *Appl. Environ. Microbiol.* **87** (2021), doi:10.1128/AEM.02245-20.
51. M. H. Pontes, C. Dale, Lambda Red-mediated genetic modification of the insect endosymbiont *Sodalis glossinidius*. *Appl. Environ. Microbiol.* **77**, 1918–1920 (2011).
52. N. J. Bongio, D. J. Lampe, 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 (2015).
53. S. Epis, I. Varotto-Bocazzi, E. Crotti, C. Damiani, L. Giovati, M. Mandrioli, M. Biggiogera, P. Gabrieli, M. Genchi, L. Polonelli, D. Daffonchio, G. Favia, C. Bandi, Chimeric symbionts expressing a *Wolbachia* protein stimulate mosquito immunity and inhibit filarial parasite development. *Commun. Biol.* **3**, 105 (2020).
54. J. K. Kim, Y. J. Won, N. Nikoh, H. Nakayama, S. H. Han, Y. Kikuchi, Y. H. Rhee, H. Y. Park, J. Y. Kwon, K. Kurokawa, N. Dohmae, T. Fukatsu, B. L. Lee, Polyester synthesis genes associated with stress resistance are involved in an insect-bacterium symbiosis. *Proc. Natl. Acad. Sci.* **110**, E2381–E2389 (2013).
55. J. K. Kim, H. J. Lee, Y. Kikuchi, W. Kitagawa, N. Nikoh, T. Fukatsu, B. L. Lee, Bacterial cell wall synthesis gene *uppP* is required for *Burkholderia* colonization of the stinkbug gut. *Appl. Environ. Microbiol.* **79**, 4879–4886 (2013).
56. T. Ohbayashi, K. Takeshita, W. Kitagawa, N. Nikoh, R. Koga, X.-Y. Meng, K. Tago, T. Hori, M. Hayatsu, K. Asano, Y. Kamagata, B. L. Lee, T. Fukatsu, Y. Kikuchi, Insect's intestinal organ for symbiont sorting. *Proc. Natl. Acad. Sci.* **112**, E5179–E5188 (2015).
57. S. P. Leonard, J. Perutka, J. E. Powell, P. Geng, D. D. Richhart, M. Byrom, S. Kar, B. W. Davies, A. D. Ellington, N. A. Moran, J. E. Barrick, Genetic

engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. *ACS Synth. Biol.* **7**, 1279–1290 (2018).

58. C. Dale, M. Beeton, C. Harbison, T. Jones, M. Pontes, 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 (2006).
59. A. Rangberg, G. Mathiesen, G. V. Amdam, D. B. Diep, The paratransgenic potential of *Lactobacillus kunkeei* in the honey bee *Apis mellifera*. *Benef. Microbes.* **6**, 513–523 (2015).
60. F. Masson, F. Schüpfer, C. Jollivet, B. Lemaitre, Transformation of the *Drosophila* sex-manipulative endosymbiont *Spiroplasma poulsonii* and persisting hurdles for functional genetic studies. *Appl. Environ. Microbiol.* **86**, e00835-20 (2020).
61. J. E. Powell, S. P. Leonard, W. K. Kwong, P. Engel, N. A. Moran, Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. *Proc. Natl. Acad. Sci.* **113**, 13887–13892 (2016).
62. T. Ohbayashi, K. Takeshita, W. Kitagawa, N. Nikoh, R. Koga, X.-Y. Meng, K. Tago, T. Hori, M. Hayatsu, K. Asano, Y. Kamagata, B. L. Lee, T. Fukatsu, Y. Kikuchi, Insect’s intestinal organ for symbiont sorting. *Proc. Natl. Acad. Sci.* **112**, E5179–E5188 (2015).
63. C. Dale, S. A. Young, D. T. Haydon, S. C. Welburn, The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 1883–1888 (2001).
64. S. C. Shin, S.-H. Kim, H. You, B. Kim, A. C. Kim, K.-A. Lee, J.-H. Yoon, J.-H. Ryu, W.-J. Lee, *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science.* **334**, 670–674 (2011).
65. E. M. Dotson, B. Plikaytis, T. M. Shinnick, R. V. Durvasula, C. B. Beard, 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 (2003).

66. Y. Kikuchi, T. Fukatsu, Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*. *Mol. Ecol.* **23**, 1445–1456 (2014).
67. H. Itoh, S. Jang, K. Takeshita, T. Ohbayashi, N. Ohnishi, X.-Y. Meng, Y. Mitani, Y. Kikuchi, Host–symbiont specificity determined by microbe–microbe competition in an insect gut. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 22673–22682 (2019).
68. B. A. Renda, M. J. Hammerling, J. E. Barrick, Engineering reduced evolutionary potential for synthetic biology. *Mol. Biosyst.* **10**, 1668–1678 (2014).
69. J. Perreau, D. J. Patel, H. Anderson, G. P. Maeda, K. M. Elston, N. A. Moran, J. E. Barrick, Vertical transmission at the pathogen-symbiont interface: *Serratia symbiotica* and aphids. *MBio.* **12**, e00359-21 (2021).
70. Y. Kikuchi, T. Ohbayashi, S. Jang, P. Mergaert, *Burkholderia insecticola* triggers midgut closure in the bean bug *Riptortus pedestris* to prevent secondary bacterial infections of midgut crypts. *ISME J.* **14**, 1627–1638 (2020).
71. J. K. Kim, H. A. Jang, M. S. Kim, J. H. Cho, J. Lee, F. Di Lorenzo, L. Sturiale, A. Silipo, A. Molinaro, B. L. Lee, 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 (2017).
72. B. Bextine, C. Lauzon, S. Potter, D. Lampe, T. A. Miller, Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr. Microbiol.* **48**, 327–331 (2004).
73. S. P. Leonard, J. E. Powell, J. Perutka, P. Geng, L. C. Heckmann, R. D. Horak, B. W. Davies, A. D. Ellington, J. E. Barrick, N. A. Moran, Engineered symbionts activate honey bee immunity and limit pathogens. *Science.* **367**, 573–576 (2020).
74. R. V. Durvasula, A. Gumbs, A. Panackal, O. Kruglov, S. Aksoy, R. B. Merrifield, F. F. Richards, C. B. Beard, Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proc. Natl. Acad. Sci. U.*

- S. A. **94**, 3274–3278 (1997).
75. R. V. Durvasula, A. Gumbs, A. Panackal, O. Kruglov, J. Taneja, A. S. Kang, C. Cordon-Rosales, F. F. Richards, R. G. Whitham, C. B. Beard, Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. *Med. Vet. Entomol.* **13**, 115–119 (1999).
 76. C. Jose, N. Klein, S. Wyss, A. Fieck, I. Hurwitz, R. Durvasula, Recombinant *Arthrobacter* β -1, 3-glucanase as a potential effector molecule for paratransgenic control of Chagas disease. *Parasit. Vectors.* **6**, 65 (2013).
 77. M. L. Taracena, P. L. Oliveira, O. Almendares, C. Umaña, C. Lowenberger, E. M. Dotson, G. O. Paiva-Silva, P. M. Pennington, Genetically modifying the insect gut microbiota to control Chagas disease vectors through systemic RNAi. *PLoS Negl. Trop. Dis.* **9**, e0003358 (2015).
 78. S. Wang, A. K. Ghosh, N. Bongio, K. A. Stebbings, D. J. Lampe, M. Jacobs-Lorena, Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 12734–9 (2012).
 79. C. B. Beard, S. L. O'Neill, P. Mason, L. Mandelco, C. R. Woese, R. B. Tesh, F. F. Richards, S. Aksoy, Genetic transformation and phylogeny of bacterial symbionts from tsetse flies. *Insect Mol. Biol.* **1**, 123–131 (1993).
 80. Q. Cheng, S. Aksoy, Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Mol. Biol.* **8**, 125–32 (1999).
 81. L. De Vooght, S. Van Keer, J. Van Den Abbeele, Towards improving tsetse fly paratransgenesis: stable colonization of *Glossina morsitans morsitans* with genetically modified *Sodalis*. *BMC Microbiol.* **18**, 165 (2018).
 82. M. Whitten, P. Dyson, Gene silencing in non-model insects: overcoming hurdles using symbiotic bacteria for trauma-free sustainable delivery of RNA interference. *BioEssays.* **39**, 1600247 (2017).
 83. W. K. Kwong, P. Engel, H. Koch, N. A. Moran, Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc. Natl.*

Acad. Sci. U. S. A. **111**, 11509–11514 (2014).

84. W. H. Lewis, G. Tahon, P. Geesink, D. Z. Sousa, T. J. G. Ettema, Innovations to culturing the uncultured microbial majority. *Nat. Rev. Microbiol.* **2020** *194*. **19**, 225–240 (2020).
85. J. W. Brandt, G. Chevignon, K. M. Oliver, M. R. Strand, Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. *Proc. R. Soc. B Biol. Sci.* **284**, 20171925 (2017).
86. C. Ronda, S. P. Chen, V. Cabral, S. J. Yaung, H. H. Wang, Metagenomic engineering of the mammalian gut microbiome in situ. *Nat. Methods.* **16**, 167–170 (2019).
87. B. E. Rubin, S. Diamond, B. F. Cress, A. Crits-christoph, Targeted genome editing of bacteria within microbial communities. *bioRxiv* (2020), doi:10.1101/2020.07.17.209189.
88. M. Vatanparast, Y. Kim, Optimization of recombinant bacteria expressing dsRNA to enhance insecticidal activity against a lepidopteran insect, *Spodoptera exigua*. *PLoS One.* **12**, e0183054 (2017).
89. E. Kim, Y. Park, Y. Kim, E. Ling, A transformed bacterium expressing double-stranded RNA specific to integrin $\beta 1$ enhances Bt Toxin efficacy against a polyphagous insect pest, *Spodoptera exigua*. *PLoS One.* **10**, e0132631 (2015).
90. W. K. Kwong, N. A. Moran, Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family *Neisseriaceae* of the *Betaproteobacteria*, and *Gilliamella apicola*. *Int. J. Syst. Evol. Microbiol.* **63**, 2008–2018 (2013).
91. N. A. Moran, P. H. Degnan, Functional genomics of *Buchnera* and the ecology of aphid hosts. *Mol. Ecol.* **15**, 1251–1261 (2006).
92. A. A. Andongma, C. Greig, P. J. Dyson, N. Flynn, M. M. A. Whitten, Optimization of dietary RNA interference delivery to western flower thrips *Frankliniella occidentalis* and onion thrips *Thrips tabaci*. *Arch. Insect Biochem. Physiol.* **103**, e21645 (2020).

93. A. Berasategui, S. Shukla, H. Salem, M. Kaltenpoth, Potential applications of insect symbionts in biotechnology. *Appl. Microbiol. Biotechnol.* **100**, 1567–1577 (2016).
94. J. C. K. Ng, B. W. Falk, Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu. Rev. Phytopathol.* **44**, 183–212 (2006).
95. S. D. Zapata, R. Dudensing, D. Sekula, G. Esparza-Díaz, R. Villanueva, Economic impact of the sugarcane aphid outbreak in South Texas. *J. Agric. Appl. Econ.* **50**, 104–128 (2018).
96. C.-A. Dedryver, A. Le Ralec, F. Fabre, The conflicting relationships between aphids and men: a review of aphid damage and control strategies. *C. R. Biol.* **333**, 539–553 (2010).
97. K. M. Oliver, P. H. Degnan, G. R. Burke, N. A. Moran, Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol.* **55**, 247–266 (2010).
98. J. A. Russell, A. Latorre, B. Sabater-Munoz, A. Moya, N. A. Moran, Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol.* **12**, 1061–1075 (2003).
99. S. Zytynska, K. Thighiouart, E. Frago, A meta-analysis on the benefits and costs of hosting secondary endosymbionts in sap-sucking insects. *bioRxiv*, 563031 (2019).
100. P. H. Degnan, N. A. Moran, Diverse phage-encoded toxins in a protective insect endosymbiont. *Appl. Environ. Microbiol.* **74**, 6782–6791 (2008).
101. J.-C. Simon, S. Boutin, T. Tsuchida, R. Koga, J.-F. Le Gallic, A. Frantz, Y. Outreman, T. Fukatsu, Facultative symbiont infections affect aphid reproduction. *PLoS One.* **6**, e21831 (2011).
102. C. Vorburger, P. Ganesanandamoorthy, M. Kwiatkowski, Comparing constitutive and induced costs of symbiont-conferred resistance to parasitoids in aphids. *Ecol. Evol.* **3**, 706–713 (2013).
103. R. Koga, T. Tsuchida, M. Sakurai, T. Fukatsu, Selective elimination of

aphid endosymbionts: Effects of antibiotic dose and host genotype, and fitness consequences. *FEMS Microbiol. Ecol.* **60**, 229–239 (2007).

104. M. Skaljic, P. Kirfel, J. Grotmann, A. Vilcinskas, Fitness costs of infection with *Serratia symbiotica* are associated with greater susceptibility to insecticides in the pea aphid *Acyrtosiphon pisum*. *Pest Manag. Sci.* **74**, 1829–1836 (2018).
105. J. A. Russell, N. A. Moran, Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc. Biol. Sci.* **273**, 603–610 (2006).
106. G. Burke, O. Fiehn, N. Moran, Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *ISME J.* **4**, 242–252 (2010).
107. I. Pons, F. Renoz, C. Noël, T. Hance, New insights into the nature of symbiotic associations in aphids: infection process, biological effects, and transmission mode of cultivable *Serratia symbiotica* bacteria. *Appl. Environ. Microbiol.* **85**, e02445-18 (2019).
108. A. Sabri, P. Leroy, E. Haubruge, T. Hance, I. Frère, J. Destain, P. Thonart, Isolation, pure culture and characterization of *Serratia symbiotica* sp. nov., the R-type of secondary endosymbiont of the black bean aphid *Aphis fabae*. *Int. J. Syst. Evol. Microbiol.* **61**, 2081–2088 (2011).
109. A. Manzano-Marín, A. Latorre, Snapshots of a shrinking partner: Genome reduction in *Serratia symbiotica*. *Sci. Rep.* **6**, 32590 (2016).
110. A. Manzano-Marín, A. Latorre, Settling down: the genome of *Serratia symbiotica* from the aphid *Cinara tujafilina* zooms in on the process of accommodation to a cooperative intracellular life. *Genome Biol. Evol.* **6**, 1683–1698 (2014).
111. F. Renoz, C. Noël, A. Errachid, V. Foray, T. Hance, Infection dynamic of symbiotic bacteria in the pea aphid *Acyrtosiphon pisum* gut and host immune response at the early steps in the infection process. *PLoS One.* **10**, e0122099 (2015).
112. I. Pons, F. Renoz, C. Noël, T. Hance, Circulation of the cultivable symbiont *Serratia symbiotica* in aphids is mediated by plants. *Front. Microbiol.* **10**,

764 (2019).

113. K. H. Choi, J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. A. R. Karkhoff-Schweizer, H. P. Schweizer, A Tn7-based broad-range bacterial cloning and expression system. *Nat. Methods*. **2**, 443–448 (2005).
114. J. D. Reid, S. D. Stoufer, D. M. Ogrydziak, Efficient transformation of *Serratia marcescens* with pBR322 plasmid DNA. *Gene*. **17**, 107–112 (1982).
115. N. Sakural, S. Komatsubara, Simple and versatile electrotransformation of *Serratia marcescens* Sr41. *Lett. Appl. Microbiol.* **23**, 23–26 (1996).
116. S. J. Coulhurst, N. R. Williamson, A. K. P. Harris, D. R. Spring, G. P. C. Salmond, Metabolic and regulatory engineering of *Serratia marcescens*: Mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities. *Microbiology*. **152**, 1899–1911 (2006).
117. A. Karsi, M. L. Lawrence, Broad host range fluorescence and bioluminescence expression vectors for Gram-negative bacteria. *Plasmid*. **57**, 286–295 (2007).
118. M. E. Lee, W. C. DeLoache, B. Cervantes, J. E. Dueber, A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synth. Biol.* **4**, 975–986 (2015).
119. O. M. Subach, P. J. Cranfill, M. W. Davidson, V. V. Verkhusha, An enhanced monomeric blue fluorescent protein with the high chemical stability of the chromophore. *PLoS One*. **6**, e28674 (2011).
120. H. D. Kulasekara, I. Ventre, B. R. Kulasekara, A. Lazdunski, A. Filloux, S. Lory, A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial cup genes. *Mol. Microbiol.* **55**, 368–380 (2004).
121. K.-H. H. Choi, J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. R. A. R. Karkhoff-Schweizer, H. P. Schweizer, A Tn7-based broad-range bacterial cloning and expression system. *Nat. Methods*. **2**, 443–448 (2005).
122. F. Renoz, A. Champagne, H. Degand, A. Faber, P. Morsomme, V. Foray,

- T. Hance, Toward a better understanding of the mechanisms of symbiosis: a comprehensive proteome map of a nascent insect symbiont. *PeerJ*. **5**, e3291 (2017).
123. A. K. Arora, A. E. Douglas, Hype or opportunity? Using microbial symbionts in novel strategies for insect pest control. *J. Insect Physiol.* **103**, 10–17 (2017).
 124. A. E. Whitfield, B. W. Falk, D. Rotenberg, Insect vector-mediated transmission of plant viruses. *Virology*. **479–480**, 278–289 (2015).
 125. E. S. Sylvester, Circulative and propagative virus transmission by aphids. *Annu. Rev. Entomol.* **25**, 257–286 (1980).
 126. C. G. Webster, E. Pichon, M. van Munster, B. Monsion, M. Deshoux, D. Gargani, F. Calevro, J. Jimenez, A. Moreno, B. Krenz, J. R. Thompson, K. L. Perry, A. Fereres, S. Blanc, M. Uzzest, Identification of plant virus receptor candidates in the stylets of their aphid vectors. *J. Virol.* **92**, e00432-18 (2018).
 127. K. Sprouffske, A. Wagner, Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics*. **17**, 172 (2016).
 128. M. Hajibabaei, D. H. Janzen, J. M. Burns, W. Hallwachs, P. D. N. Hebert, DNA barcodes distinguish species of tropical Lepidoptera. *Proc. Natl. Acad. Sci.* **103**, 968–971 (2006).
 129. G. Febvay, B. Delobel, Y. Rahbé, Influence of the amino acid balance on the improvement of an artificial diet for a biotype of *Acyrtosiphon pisum* (Homoptera: Aphididae). *Can. J. Zool.* **66**, 2449–2453 (1988).
 130. K. M. Elston, S. P. Leonard, P. Geng, S. B. Bialik, E. Robinson, J. E. Barrick, Engineering insects from the endosymbiont out. *Trends Microbiol.* **30** (2022), pp. 79–96.
 131. J. A. N. Brophy, A. J. Triassi, B. L. Adams, R. L. Renberg, D. N. Stratis-Cullum, A. D. Grossman, C. A. Voigt, Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. *Nat. Microbiol.* **3**, 1043–1053 (2018).

132. M. C. Waller, J. R. Bober, N. U. Nair, C. L. Beisel, Toward a genetic tool development pipeline for host-associated bacteria. *Curr. Opin. Microbiol.* **38**, 156–164 (2017).
133. G. Durante-Rodríguez, V. De Lorenzo, E. Martínez-García, The standard European vector architecture (SEVA) plasmid toolkit. *Methods Mol. Biol.* **1149**, 469–478 (2014).
134. S. V. Iverson, T. L. Haddock, J. Beal, D. M. Densmore, CIDAR MoClo: Improved MoClo assembly standard and new *E. coli* part library enable rapid combinatorial design for synthetic and traditional biology. *ACS Synth. Biol.* **5**, 99–103 (2016).
135. E. Martínez-García, T. Aparicio, A. Goñi-Moreno, S. Fraile, V. De Lorenzo, SEVA 2.0: An update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. *Nucleic Acids Res.* **43**, D1183–D1189 (2015).
136. L. A. Schuster, C. R. Reisch, A plasmid toolbox for controlled gene expression across the Proteobacteria. *Nucleic Acids Res.* **49**, 7189–7202 (2021).
137. B. A. Geddes, M. A. Mendoza-Suárez, P. S. Poole, A Bacterial Expression Vector Archive (BEVA) for flexible modular assembly of golden gate-compatible vectors. *Front. Microbiol.* **10**, 3345 (2019).
138. E. Weber, C. Engler, R. Gruetzner, S. Werner, S. Marillonnet, A modular cloning system for standardized assembly of multigene constructs. *PLoS One.* **6**, e16765 (2011).
139. R. Young, M. Haines, M. Storch, P. S. Freemont, Combinatorial metabolic pathway assembly approaches and toolkits for modular assembly. *Metab. Eng.* **63**, 81–101 (2021).
140. E. Freed, J. Fenster, S. L. Smolinski, J. Walker, C. A. Henard, R. Gill, C. A. Eckert, Building a genome engineering toolbox in nonmodel prokaryotic microbes. *Biotechnol. Bioeng.* **115**, 2120–2138 (2018).
141. A. Jain, P. Srivastava, Broad host range plasmids. *FEMS Microbiol. Lett.* **348**, 87–96 (2013).

142. T. E. V. Aune, F. L. Aachmann, Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. *Appl. Microbiol. Biotechnol.* **85**, 1301–1313 (2010).
143. P. Mazodier, J. Davies, Gene transfer between distantly related bacteria. *Annu. Rev. Genet.* **25**, 147–171 (1991).
144. C. J. H. Von Wintersdorff, J. Penders, J. M. Van Niekerk, N. D. Mills, S. Majumder, L. B. Van Alphen, P. H. M. Savelkoul, P. F. G. Wolffs, Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.* **7**, 173 (2016).
145. T. Akiyama, K. L. Asfahl, M. C. Savin, Broad-host-range plasmids in treated wastewater effluent and receiving streams. *J. Environ. Qual.* **39**, 2211–2215 (2010).
146. L. E. Brooks, M. Kaze, M. Siström, Where the plasmids roam: Large-scale sequence analysis reveals plasmids with large host ranges. *Microb. Genomics.* **5**, e000244 (2019).
147. A. E. Douglas, The *Drosophila* model for microbiome research. *Lab Anim. (NY)*. **47** (2018), pp. 157–164.
148. W. B. Ludington, W. W. Ja, *Drosophila* as a model for the gut microbiome. *PLoS Pathog.* **16**, e1008398 (2020).
149. N. A. Broderick, B. Lemaitre, Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes.* **3** (2012), p. 307.
150. P. D. Newell, J. M. Chaston, Y. Wang, N. J. Winans, D. R. Sannino, A. C. N. Wong, A. J. Dobson, J. Kagle, A. E. Douglas, In vivo function and comparative genomic analyses of the *Drosophila* gut microbiota identify candidate symbiosis factors. *Front. Microbiol.* **5**, 576 (2014).
151. M. E. Martino, P. Joncour, R. Leenay, H. Gervais, M. Shah, S. Hughes, B. Gillet, C. Beisel, F. Leulier, Bacterial adaptation to the host's diet is a key evolutionary force shaping *Drosophila-Lactobacillus* symbiosis. *Cell Host Microbe.* **24**, 109–119.e6 (2018).
152. J. A. Chandler, J. Morgan Lang, S. Bhatnagar, J. A. Eisen, A. Kopp,

Bacterial communities of diverse *Drosophila* species: Ecological context of a host–microbe model system. *PLoS Genet.* **7**, e1002272 (2011).

153. V. G. Martinson, J. Carpinteyro-Ponce, N. A. Moran, T. A. Markow, A distinctive and host-restricted gut microbiota in populations of a cactophilic *Drosophila* species. *Appl. Environ. Microbiol.* **83**, e01551-17 (2017).
154. T. J. Hammer, W. O. McMillan, N. Fierer, Metamorphosis of a butterfly-associated bacterial community. *PLoS One.* **9**, 86995 (2014).
155. J. Y. Kim, J. Lee, N. R. Shin, J. H. Yun, T. W. Whon, M. S. Kim, M. J. Jung, S. W. Roh, D. W. Hyun, J. W. Bae, *Orbus sasakiae* sp. nov., a bacterium isolated from the gut of the butterfly *Sasakia charonda*, and emended description of the genus *Orbus*. *Int. J. Syst. Evol. Microbiol.* **63**, 1766–1770 (2013).
156. V. G. Martinson, A. E. Douglas, J. Jaenike, Community structure of the gut microbiota in sympatric species of wild *Drosophila*. *Ecol. Lett.* **20**, 629–639 (2017).
157. S.-H. Liu, Y. Chen, W. Li, G.-H. Tang, Y. Yang, H.-B. Jiang, W. Dou, J.-J. Wang, Diversity of bacterial communities in the intestinal tracts of two geographically distant populations of *Bactrocera dorsalis* (Diptera: Tephritidae). *J. Econ. Entomol.* **111**, 2861–2868 (2018).
158. A. K. Gupta, D. Nayduch, P. Verma, B. Shah, H. V. Ghate, M. S. Patole, Y. S. Shouche, Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). *FEMS Microbiol. Ecol.* **79**, 581–593 (2012).
159. M. Volkmann, E. Skiebe, T. Kerrinnes, F. Faber, D. Lepka, Y. Pfeifer, G. Holland, N. Bannert, G. Wilharm, *Orbus hercynius* gen. nov., sp. nov., isolated from faeces of wild boar, is most closely related to members of the orders “*Enterobacteriales*” and *Pasteurellales*. *Int. J. Syst. Evol. Microbiol.* **60**, 2601–2605 (2010).
160. M. F. Alexeyev, The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. *Biotechniques.* **26**, 824–6, 828 (1999).

161. E. M. Bryan, T. Bae, M. Kleerebezem, G. M. Dunny, Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid*. **44**, 183–190 (2000).
162. J. E. Prior, M. D. Lynch, R. T. Gill, Broad-host-range vectors for protein expression across gram negative hosts. *Biotechnol. Bioeng.* **106**, 326–332 (2010).
163. M. E. Kovach, P. H. Elzer, D. Steven Hill, G. T. Robertson, M. A. Farris, R. M. Roop, K. M. Peterson, Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*. **166**, 175–176 (1995).
164. T. Dammeyer, K. N. Timmis, P. Tinnefeld, Broad host range vectors for expression of proteins with (Twin-) Strep-tag, His-tag and engineered, export optimized yellow fluorescent protein. *Microb. Cell Fact.* **12** (2013), doi:10.1186/1475-2859-12-49.
165. J. Liljeruhm, S. K. Funk, S. Tietscher, A. D. Edlund, S. Jamal, P. Wistrand-Yuen, K. Dyrhage, A. Gynnå, K. Ivermark, J. Lövgren, V. Törnblom, A. Virtanen, E. R. Lundin, E. Wistrand-Yuen, A. C. Forster, Engineering a palette of eukaryotic chromoproteins for bacterial synthetic biology. *J. Biol. Eng.* **12**, 8 (2018).
166. L. Ferrières, G. Hémerly, T. Nham, A. M. Guérout, D. Mazel, C. Beloin, J. M. Ghigo, Silent mischief: Bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *J. Bacteriol.* **192**, 6418–6427 (2010).
167. T. J. Wiles, E. S. Wall, B. H. Schlomann, E. A. Hay, R. Parthasarathy, K. Guillemin, Modernized tools for streamlined genetic manipulation and comparative study of wild and diverse proteobacterial lineages. *MBio*. **9**, e01877-18 (2018).
168. R. Meyer, Replication and conjugative mobilization of broad host-range IncQ plasmids. *Plasmid*. **62**, 57–70 (2009).
169. B. Bishé, A. Taton, J. W. Golden, Modification of RSF1010-based broad-host-range plasmids for improved conjugation and cyanobacterial bioprospecting. *iScience*. **20**, 216–228 (2019).

170. F. Ceroni, R. Algar, G.-B. B. Stan, T. Ellis, Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nat. Methods.* **12**, 415–418 (2015).
171. J. A. Englaender, J. A. Jones, B. F. Cress, T. E. Kuhlman, R. J. Linhardt, M. A. G. Koffas, Effect of genomic integration location on heterologous protein expression and metabolic engineering in *E. coli*. *ACS Synth. Biol.* **6**, 710–720 (2017).
172. B. Obadia, Z. T. Güvener, V. Zhang, J. A. Ceja-Navarro, E. L. Brodie, W. W. Ja, W. B. Ludington, Probabilistic invasion underlies natural gut microbiome stability. *Curr. Biol.* **27**, 1999-2006.e8 (2017).
173. M. L. Koyle, M. Veloz, A. M. Judd, A. C. N. Wong, P. D. Newell, A. E. Douglas, J. M. Chaston, Rearing the fruit fly *Drosophila melanogaster* under axenic and gnotobiotic conditions. *J. Vis. Exp.*, 54219 (2016).
174. I. S. Pais, R. S. Valente, M. Sporniak, L. Teixeira, *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS Biol.* **16**, e2005710 (2018).
175. D. N. Lesperance, N. A. Broderick, Microbiomes as modulators of *Drosophila melanogaster* homeostasis and disease. *Curr. Opin. Insect Sci.* **39**, 84–90 (2020).
176. J. E. Blum, C. N. Fischer, J. Miles, J. Handelsman, Frequent replenishment sustains the beneficial microbiome of *Drosophila melanogaster*. *MBio.* **4** (2013), doi:10.1128/mBio.00860-13.
177. W. W. Ja, G. B. Carvalho, E. M. Mak, N. N. De La Rosa, A. Y. Fang, J. C. Liong, T. Brummel, S. Benzer, Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 8253–8256 (2007).
178. A. T. Tucker, E. M. Nowicki, J. M. Boll, G. A. Knauf, N. C. Burdis, M. S. Trent, B. W. Davies, Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *MBio.* **5**, e01313-14 (2014).
179. F. Madeira, M. Pearce, A. R. N. Tivey, P. Basutkar, J. Lee, O. Edbali, N. Madhusoodanan, A. Kolesnikov, R. Lopez, Search and sequence analysis

tools services from EMBL-EBI in 2022. *Nucleic Acids Res.*, gkac240–gkac240 (2022).

180. B. Q. Minh, H. A. Schmidt, O. Chernomor, D. Schrempf, M. D. Woodhams, A. Von Haeseler, R. Lanfear, E. Teeling, IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Mol. Biol. Evol.* **37**, 1530–1534 (2020).
181. I. Letunic, P. Bork, Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* **49**, W293–W296 (2021).
182. T. Fukatsu, N. Nikoh, Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **64**, 3599–3606 (1998).
183. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: An open-source platform for biological-image analysis. *Nat. Methods.* **9** (2012), pp. 676–682.
184. P. Baumann, N. A. Moran, L. Baumann, in *The Prokaryotes* (2006; <http://link.springer.com/10.1007/0-387-30747-8>), pp. 403–438.
185. B. J. Parker, J. A. Brisson, A laterally transferred viral gene modifies aphid wing plasticity. *Curr. Biol.* **29**, 2098-2103.e5 (2019).
186. M. E. Grantham, J. A. Brisson, Extensive differential splicing underlies phenotypically plastic aphid morphs. *Mol. Biol. Evol.* **35**, 1934–1946 (2018).
187. Y. Li, H. Park, T. E. Smith, N. A. Moran, N. Singh, Gene family evolution in the pea aphid based on chromosome-level genome assembly. *Mol. Biol. Evol.* **36**, 2143–2156 (2019).
188. G. Trionnaire, J. Hardie, S. Jaubert-Possamai, J.-C. Simon, D. Tagu, Shifting from clonal to sexual reproduction in aphids: physiological and developmental aspects. *Biol. Cell.* **100**, 441–451 (2008).

189. D. Tagu, G. Le Trionnaire, S. Tanguy, J.-P. Gauthier, J.-R. Huynh, EMS mutagenesis in the pea aphid *Acyrtosiphon pisum*. *G3 Genes|Genomes|Genetics*. **4**, 657–667 (2014).
190. G. Le Trionnaire, S. Tanguy, S. Hudaverdian, F. Gleonnec, G. Richard, B. Cayrol, B. Monsion, E. Pichon, M. Deshoux, C. Webster, M. Uzest, A. Herpin, D. Tagu, An integrated protocol for targeted mutagenesis with CRISPR-Cas9 system in the pea aphid. *Insect Biochem. Mol. Biol.* **110**, 34–44 (2019).
191. G. Wang, Y. Gou, S. Guo, J. J. Zhou, C. Liu, RNA interference of trehalose-6-phosphate synthase and trehalase genes regulates chitin metabolism in two color morphs of *Acyrtosiphon pisum* Harris. *Sci. Rep.* **11**, 1–12 (2021).
192. C. X. Sun, Z. X. Li, Biosynthesis of aphid alarm pheromone is modulated in response to starvation stress under regulation by the insulin, glycolysis and isoprenoid pathways. *J. Insect Physiol.* **128**, 104174 (2021).
193. C. Ye, X. S. Hu, Z. W. Wang, D. Wei, G. Smagghe, O. Christiaens, J. Niu, J. J. Wang, Involvement of clathrin-dependent endocytosis in cellular dsRNA uptake in aphids. *Insect Biochem. Mol. Biol.* **132**, 103557 (2021).
194. O. Christiaens, L. Swevers, G. Smagghe, DsRNA degradation in the pea aphid (*Acyrtosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides*. **53**, 307–314 (2014).
195. M. Cao, J. Gatehouse, E. Fitches, A systematic study of RNAi effects and dsRNA stability in *Tribolium castaneum* and *Acyrtosiphon pisum*, following injection and ingestion of analogous dsRNAs. *Int. J. Mol. Sci.* **19**, 1079 (2018).
196. A. B. Ghodke, R. T. Good, J. F. Golz, D. A. Russell, O. Edwards, C. Robin, Extracellular endonucleases in the midgut of *Myzus persicae* may limit the efficacy of orally delivered RNAi. *Sci. Rep.* **9**, 11898 (2019).
197. J. Mao, C. Liu, F. Zeng, Hunchback is required for abdominal identity suppression and germband growth in the parthenogenetic embryogenesis of the pea aphid, *Acyrtosiphon pisum*. *Arch. Insect Biochem. Physiol.* **84**, 209–221 (2013).

198. C. Ye, X. An, Y.-D. Jiang, B.-Y. Ding, F. Shang, O. Christiaens, C. N. T. Taning, G. Smagghe, J. Niu, J.-J. Wang, Induction of RNAi core machinery's gene expression by exogenous dsRNA and the effects of pre-exposure to dsRNA on the gene silencing efficiency in the pea aphid (*Acyrtosiphon pisum*). *Front. Physiol.* **9**, 1906 (2019).
199. T. A. Nguyen, J. H. Fruehauf, Transkingdom RNA interference (tkRNAi): a novel method to induce therapeutic gene silencing. *Methods Mol. Biol.* **514**, 27–34 (2009).
200. S. Xiang, J. Fruehauf, C. J. Li, Short hairpin RNA-expressing bacteria elicit RNA interference in mammals. *Nat. Biotechnol.* **24**, 697–702 (2006).
201. L. Zhang, L. Gao, L. Zhao, B. Guo, K. Ji, Y. Tian, J. Wang, H. Yu, J. Hu, D. V. Kalvakolanu, D. J. Kopecko, X. Zhao, D. Q. Xu, Intratumoral delivery and suppression of prostate tumor growth by attenuated *Salmonella enterica* serovar typhimurium carrying plasmid-based small interfering RNAs. *Cancer Res.* **67**, 5859–5864 (2007).
202. L. Timmons, D. L. Court, A. Fire, Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene.* **263**, 103–112 (2001).
203. S. Dasgupta, L. Fernandez, L. Kameyama, T. Inada, Y. Nakamura, A. Pappas, D. L. Court, Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III — the effect of dsRNA binding on gene expression. *Mol. Microbiol.* **28**, 629–640 (1998).
204. N. S. Mutti, Y. Park, J. C. Reese, G. R. Reeck, RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrtosiphon pisum*. *J. Insect Sci.* **6**, 1–7 (2006).
205. T. Yan, H. Chen, Y. Sun, X. Yu, L. Xia, RNA interference of the ecdysone receptor genes EcR and USP in grain aphid (*Sitobion avenae* F.) affects its survival and fecundity upon feeding on wheat plants. *Int. J. Mol. Sci.* **17**, 2098 (2016).
206. Y. Sun, C. Sparks, H. Jones, M. Riley, F. Francis, W. Du, L. Xia, Silencing an essential gene involved in infestation and digestion in grain aphid through plant-mediated RNA interference generates aphid-resistant wheat

plants. *Plant Biotechnol. J.* **17** (2019), pp. 852–854.

207. J. Niu, W. Yang, Y. Tian, J. Fan, C. Ye, F. Shang, B. Ding, J. Zhang, X. An, L. Yang, T. Chang, O. Christiaens, G. Smagghe, J. Wang, Topical dsRNA delivery induces gene silencing and mortality in the pea aphid. *Pest Manag. Sci.* **75**, 2873–2881 (2019).
208. S. Jacques, J. Reidy-Crofts, J. Sperschneider, L. G. Kamphuis, L. L. Gao, O. R. Edwards, K. B. Singh, An RNAi supplemented diet as a reverse genetics tool to control bluegreen aphid, a major pest of legumes. *Sci. Rep.* **10**, 1604 (2020).
209. N. S. Mutti, J. Louis, L. K. Pappan, K. Pappan, K. Begum, M.-S. Chen, Y. Park, N. Dittmer, J. Marshall, J. C. Reese, G. R. Reeck, A protein from the salivary glands of the pea aphid, *Acyrtosiphon pisum*, is essential in feeding on a host plant. *Proc. Natl. Acad. Sci.* **105**, 9965–9969 (2008).
210. M. Pitino, A. D. Coleman, M. E. Maffei, C. J. Ridout, S. A. Hogenhout, Silencing of aphid genes by dsRNA feeding from plants. *PLoS One.* **6**, e25709 (2011).
211. M. Pitino, S. A. Hogenhout, Aphid protein effectors promote aphid colonization in a plant species-specific manner. *Mol. Plant-Microbe Interact.* **26**, 130–139 (2013).
212. M. Zhang, Y. Zhou, H. Wang, H. Jones, Q. Gao, D. Wang, Y. Ma, L. Xia, Identifying potential RNAi targets in grain aphid (*Sitobion avenae* F.) based on transcriptome profiling of its alimentary canal after feeding on wheat plants. *BMC Genomics.* **14**, 560 (2013).
213. A. D. Coleman, R. H. M. M. Wouters, S. T. Mugford, S. A. Hogenhout, Persistence and transgenerational effect of plant-mediated RNAi in aphids. *J. Exp. Bot.* **66**, 541–548 (2015).
214. D. Wang, Q. Liu, X. Li, Y. Sun, H. Wang, L. Xia, Double-stranded RNA in the biological control of grain aphid (*Sitobion avenae* F.). *Funct. Integr. Genomics.* **15**, 211–223 (2015).
215. Y. Zhang, J. Fan, J. rui Sun, J. lian Chen, Cloning and RNA interference analysis of the salivary protein C002 gene in *Schizaphis graminum*. *J.*

Integr. Agric. **14**, 698–705 (2015).

216. N. N. Vellichirammal, P. Gupta, T. A. Hall, J. A. Brisson, Ecdysone signaling underlies the pea aphid transgenerational wing polyphenism. *Proc. Natl. Acad. Sci.* **114**, 1419–1423 (2017).
217. J.-S. Yoon, H. Tian, J. G. McMullen, S. H. Chung, A. E. Douglas, Candidate genetic determinants of intraspecific variation in pea aphid susceptibility to RNA interference. *Insect Biochem. Mol. Biol.* **123**, 103408 (2020).
218. P. J. Lariviere, S. P. Leonard, R. D. Horak, J. E. Powell, J. E. Barrick, *bioRxiv*, in press, doi:10.1101/2022.04.22.489157.
219. S. H. Chung, X. Jing, Y. Luo, A. E. Douglas, Targeting symbiosis-related insect genes by RNAi in the pea aphid-*Buchnera* symbiosis. *Insect Biochem. Mol. Biol.* **95**, 55–63 (2018).
220. S. Goodfellow, D. Zhang, M.-B. Wang, R. Zhang, Bacterium-mediated RNA interference: potential application in plant protection. *Plants*. **8**, 572 (2019).
221. A. Ghosal, B. B. Upadhyaya, J. V. Fritz, A. Heintz-Buschart, M. S. Desai, D. Yusuf, D. Huang, A. Baumuratov, K. Wang, D. Galas, P. Wilmes, The extracellular RNA complement of *Escherichia coli*. *Microbiologyopen*. **4**, 252–266 (2015).
222. H. J. Lee, Microbe-host communication by small RNAs in extracellular vesicles: Vehicles for transkingdom RNA transportation. *Int. J. Mol. Sci.* **20**, 1487 (2019).
223. R. D. Pathirana, M. Kaparakis-Liaskos, Bacterial membrane vesicles: Biogenesis, immune regulation and pathogenesis. *Cell. Microbiol.* **18**, 1518–1524 (2016).
224. W. Xu, Z. Han, Cloning and phylogenetic analysis of Sid-1-like genes from aphids. *J. Insect Sci.* **8** (2008), doi:10.1673/031.008.3001.
225. R. Bansal, A. P. Michel, Core RNAi machinery and Sid1, a component for systemic RNAi, in the hemipteran insect, *Aphis glycines*. *Int. J. Mol. Sci.* **14**, 3786–3801 (2013).

226. B. D. Stollar, V. Stollar, Immunofluorescent demonstration of double-stranded RNA in the cytoplasm of sindbis virus-infected cells. *Virology*. **42**, 276–280 (1970).
227. P. Targett-Adams, S. Boulant, J. McLauchlan, Visualization of double-stranded RNA in cells supporting hepatitis C virus RNA replication. *J. Virol.* **82**, 2182–2195 (2008).
228. F. Weber, V. Wagner, S. B. Rasmussen, R. Hartmann, S. R. Paludan, Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J. Virol.* **80**, 5059–5064 (2006).
229. L. M. Brutscher, K. F. Daughenbaugh, M. L. Flenniken, Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Sci. Rep.* **7**, 6448 (2017).
230. C. Ye, Y. Di Jiang, X. An, L. Yang, F. Shang, J. Niu, J. J. Wang, Effects of RNAi-based silencing of chitin synthase gene on moulting and fecundity in pea aphids (*Acyrtosiphon pisum*). *Sci. Rep.* **9**, 3694 (2019).
231. J. Qiao, Y. Fan, T. Bai, B. Wu, X. Pei, D. Wang, T. Liu, Lipophorin receptor regulates the cuticular hydrocarbon accumulation and adult fecundity of the pea aphid *Acyrtosiphon pisum*. *Insect Sci.* **28**, 1018–1032 (2021).
232. X. X. Wang, Z. J. Feng, Z. S. Chen, Z. F. Zhang, Y. Zhang, T. X. Liu, Use of tyrosine hydroxylase RNAi to study *Megoura viciae* (Hemiptera: Aphididae) sequestration of its host's L-DOPA for body melanism. *J. Insect Physiol.* **114**, 136–144 (2019).
233. Y. Zhang, X. X. Wang, H. G. Tian, Z. F. Zhang, Z. J. Feng, Z. S. Chen, T. X. Liu, The L-DOPA/Dopamine pathway transgenerationally regulates cuticular melanization in the pea aphid *Acyrtosiphon pisum*. *Front. Cell Dev. Biol.* **8**, 311 (2020).
234. B.-Y. Ding, J. Niu, F. Shang, L. Yang, T.-Y. Chang, J.-J. Wang, Characterization of the geranylgeranyl diphosphate synthase gene in *Acyrtosiphon pisum* (Hemiptera: Aphididae) and its association with carotenoid biosynthesis. *Front. Physiol.* **10**, 1398 (2019).

235. V. Tzin, X. Yang, X. Jing, K. Zhang, G. Jander, A. E. Douglas, RNA interference against gut osmoregulatory genes in phloem-feeding insects. *J. Insect Physiol.* **79**, 105–112 (2015).
236. J. Fan, Y. Zhang, F. Francis, D. Cheng, J. Sun, J. Chen, Orco mediates olfactory behaviors and winged morph differentiation induced by alarm pheromone in the grain aphid, *Sitobion avenae*. *Insect Biochem. Mol. Biol.* **64**, 16–24 (2015).
237. New England Biolabs, Purification of Synthesized RNA, (available at <https://www.neb.com/protocols/2013/04/02/purification-of-synthesized-rna-e2050>).
238. T. Nolan, R. E. Hands, S. A. Bustin, Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* **13**, 1, 1559–1582 (2006).
239. K. N. Phillips, T. F. Cooper, The cost of evolved constitutive *lac* gene expression is usually, but not always, maintained during evolution of generalist populations. *Ecol. Evol.* **11**, 12497–12507 (2021).
240. G. J. McKenzie, N. L. Craig, Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn7 without need for selection of the insertion event. *BMC Microbiol.* **6**, 39 (2006).
241. M. W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45 (2001).
242. S. A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**, 169–193 (2000).
243. U. Shokal, S. Yadav, J. Atri, J. Accetta, E. Kenney, K. Banks, A. Katakam, J. Jaenike, I. Eleftherianos, Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. *BMC Microbiol.* **16**, 16 (2016).
244. M. Petridis, M. Bagdasarian, M. K. Waldor, E. Walker, 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

(2006).

245. R. U. Sheth, V. Cabral, S. P. Chen, H. H. Wang, Manipulating bacterial communities by in situ microbiome engineering. *Trends Genet.* **32**, 189–200 (2016).
246. P. O. Cuív, W. J. Smith, S. Pottenger, S. Burman, E. R. Shanahan, M. Morrison, Isolation of genetically tractable most-wanted bacteria by metaparental mating. *Sci. Rep.* **5**, 13282 (2015).
247. A. P. Mehta, L. Supekova, J.-H. Chen, K. Pestonjamas, P. Webster, Y. Ko, S. C. Henderson, G. McDermott, F. Supek, P. G. Schultz, Engineering yeast endosymbionts as a step toward the evolution of mitochondria. *Proc. Natl. Acad. Sci.*, 201813143 (2018).
248. B. E. Rubin, S. Diamond, B. F. Cress, A. Crits-Christoph, Y. C. Lou, A. L. Borges, H. Shivram, C. He, M. Xu, Z. Zhou, S. J. Smith, R. Rovinsky, D. C. J. Smock, K. Tang, T. K. Owens, N. Krishnappa, R. Sachdeva, R. Barrangou, A. M. Deutschbauer, J. F. Banfield, J. A. Doudna, Species- and site-specific genome editing in complex bacterial communities. *Nat. Microbiol.* **2021 71. 7**, 34–47 (2021).
249. J. R. Rybarski, K. Hu, A. M. Hill, C. O. Wilke, I. J. Finkelstein, Metagenomic discovery of CRISPR-associated transposons. *Proc. Natl. Acad. Sci. U. S. A.* **118** (2021), doi:10.1073/pnas.2112279118.
250. J. Liu, L. Swevers, K. Iatrou, H. Huvenne, G. Smagghe, *Bombyx mori* DNA/RNA non-specific nuclease: Expression of isoforms in insect culture cells, subcellular localization and functional assays. *J. Insect Physiol.* **58**, 1166–1176 (2012).
251. R. B. Guan, H. C. Li, Y. J. Fan, S. R. Hu, O. Christiaens, G. Smagghe, X. X. Miao, A nuclease specific to lepidopteran insects suppresses RNAi. *J. Biol. Chem.* **293**, 6011 (2018).
252. O. Christiaens, M. G. Tardajos, Z. L. M. Reyna, M. Dash, P. Dubruel, G. Smagghe, Increased RNAi efficacy in *Spodoptera exigua* via the formulation of dsRNA with guanylated polymers. *Front. Physiol.* **9**, 316 (2018).

253. S. Lee, S. Kim, D. J. Koo, J. Yu, H. Cho, H. Lee, J. M. Song, S. Y. Kim, D. H. Min, N. L. Jeon, 3D microfluidic platform and tumor vascular mapping for evaluating anti-angiogenic RNAi-based nanomedicine. *ACS Nano*. **15**, 338–350 (2021).
254. S. Jalili-Firoozinezhad, F. S. Gazzaniga, E. L. Calamari, D. M. Camacho, C. W. Fadel, A. Bein, B. Swenor, B. Nestor, M. J. Cronic, A. Tovaglieri, O. Levy, K. E. Gregory, D. T. Breault, J. M. S. Cabral, D. L. Kasper, R. Novak, D. E. Ingber, A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. *Nat. Biomed. Eng.* 2019 37. **3**, 520–531 (2019).