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

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Toolkits of plasmids and genetic parts streamline the process of assembling DNA constructs and engineering microbes. Many of these kits were designed with specific industrial or laboratory microbes in mind. For researchers interested in non-model microbial systems, it is often unclear which tools and techniques will function in newly isolated strains. To address this challenge, we designed the Pathfinder toolkit for quickly determining the compatibility of a bacterium with different plasmid components. Pathfinder plasmids combine three different broad-host-range origins of replication with multiple antibiotic resistance cassettes and reporters, so that sets of parts can be rapidly screened through multiplex conjugation. We first tested these plasmids in *Escherichia coli*, a strain of *Sodalis praecaptivus* that colonizes insects, and a *Rosenbergiella* isolate from leafhoppers. Then, we used the Pathfinder plasmids to engineer previously unstudied bacteria from the family *Orbaceae* that were isolated from several fly species. Engineered *Orbaceae* strains were able to colonize *Drosophila melanogaster* and could be visualized in fly guts. *Orbaceae* are common and abundant in the guts of wild-caught flies but have not been included in laboratory studies of how the *Drosophila* microbiome affects fly health. Thus, this work provides foundational genetic tools for studying microbial ecology and host-associated microbes, including bacteria that are a key constituent of the gut microbiome of a model insect species.

INTRODUCTION

Researchers have isolated and sequenced many new microbes from different ecosystems and from diverse plant and animal hosts. To characterize these microbes and study how they interact with their physical environments and with other organisms, one needs genetic tools. However, most described bacterial species have never been genetically manipulated [1–3]. The primary obstacle in many cases is likely that the requisite resources and know-how for microbial genetic engineering are not easily accessible to researchers who encounter non-model microbes.

Toolkits of genetic parts have been developed for molecular microbiology and synthetic biology. These kits, such as the modular cloning (MoClo) and Standard European Vector Architecture (SEVA) toolkits [4, 5], aim to be flexible and comprehensive. Their collections of interchangeable parts include 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 [4, 6–11]. While these systems facilitate complex genetic engineering tasks in well-studied laboratory and industrial bacteria, such as *Escherichia coli* and *Pseudomonas putida*, there are still gaps with respect to their applicability to all bacteria. For example, they may only have a few antibiotic resistance cassettes or rely on plasmids that replicate only in specific species. Researchers may also

find assembling new plasmids according to the schemes in these kits daunting and overly cumbersome if all they want to achieve are basic tasks like expressing a single protein in a new bacterial species.

Fluorescent protein expression alone is often enough to investigate aspects of host-microbe interactions, such as bacterial localization, or to track a strain of interest within a microbiome or environmental community. However, even this rudimentary genetic modification can be challenging [1, 3, 12]. A reasonable first step is to start with a broad-host-range plasmid that has been reported to replicate in diverse bacteria, but one must still empirically test whether one of these plasmids is compatible with each new species [13]. Additionally, electroporation or chemical treatments to transform plasmids into cells do not work in all bacteria [14, 15]. Developing these techniques through trial and error can be frustrating and time-consuming, particularly when one does not know if the plasmid being used will successfully replicate after transformation. Conjugation is often a more reliable method for delivering DNA to non-model bacteria [2, 16], and it has been incorporated into several kits that focus on engineering a wider range of bacteria, such as the Bacterial Expression Vector Archive (BEVA) [9], the bee microbiome toolkit (BTK) [6], and the Proteobacteria toolbox [8]. The wide phylogenetic distribution of natural conjugative and transmissible broad-host-range

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plasmids suggests that this approach should work for many bacterial species [17–19].

Although *Drosophila melanogaster* has been a model organism for genetics for over a century, research focused on its gut microbiome is a relatively new field [20–22]. Laboratory studies have focused primarily on *Acetobacter* and *Lactobacillus* species [23–25], which make up only a fraction of the microbiome that is normally present in wild *Drosophila* [26]. A large percentage of the natural *Drosophila* microbiome is composed of bacteria in the recently-described *Orbaceae* family [27, 28]. *Orbaceae* are prevalent in a wide variety of insects [28–31] and are observed in 16S rRNA gene surveys of populations of laboratory-reared and wild flies of different species, including *D. melanogaster* [26, 29, 32–34]. How *Orbaceae* colonize and interact with their hosts is relatively unexplored despite how prevalent they are in insect microbiomes.

Here we describe the Pathfinder plasmid system, a simple and robust toolkit for engineering newly cultured bacteria. First, we show how multiplex conjugation with defined subsets of Pathfinder plasmids can be used to quickly determine the compatibility of bacteria with different genetic parts. Then, we then use the Pathfinder plasmids to engineer recently cultured *Orbaceae* isolates from flies and characterize how they colonize the *D. melanogaster* gut.

RESULTS
Pathfinder plasmid toolkit design
An overview of the Pathfinder plasmid design and procedure is shown in Fig. 1A and Table 1. Plasmids pSL1, pSL9, and pSL25 have three different origins of replication (RSF1010, pBBR1, and RP4) paired with three different reporters (red chromoprotein, RCP; E2-Crimson, E2C; and blue chromoprotein, BCP), respectively, along with kanamycin resistance. The reporter genes are all expressed from the broad-host-range CP25 promoter. Plasmids pSL1-7 all have an
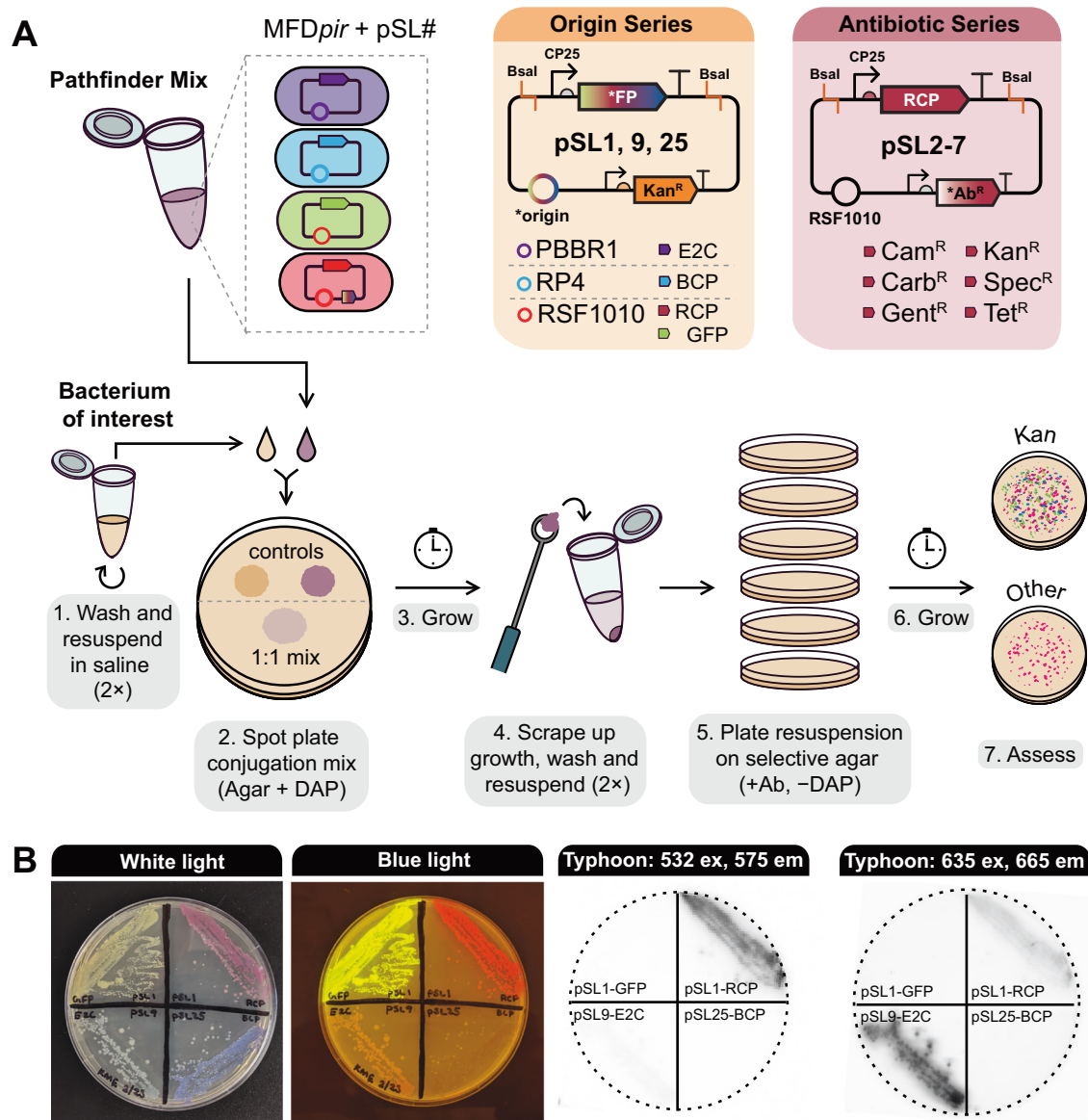

Fig. 1 The Pathfinder plasmid system. A Plasmid maps and workflow for multiplex conjugation into a bacterium of interest. **B** Visualizing Pathfinder reporters. The same agar plate containing streaks of *E. coli* MFD*pir* donor strains, each with a plasmid expressing a different fluorescent protein 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.

Table 1. Pathfinder plasmids.

Plasmid	Addgene accession	Origin	Origin source	Reporter	Reporter source	Antibiotic resistance gene	Resistance source
pSL1	180422	RSF1010	pBTK402 [6]	RCP (mRFP1)	BBa_E1010 [65]	Kan (aphA-1)	pBTK402 [6]
pSL1-GFP[a]	180420	RSF1010	pBTK402 [6]	GFP	pBTK520 [6]	Kan (aphA-1)	pBTK402 [6]
pSL2[b]	190998	RSF1010	pBTK402 [6]	RCP (mRFP1)	Bba_E1010 [65]	Spec (aadA)	pBTK403 [6]
pSL3	190999	RSF1010	pBTK402 [6]	RCP (mRFP1)	Bba_E1010 [65]	Gent (aacC1)	pKNOCK-Gm [66]
pSL4[b]	191000	RSF1010	pBTK402 [6]	RCP (mRFP1)	Bba_E1010 [65]	Cam (cat1)	pYTK001 [35]
pSL5[b]	191001	RSF1010	pBTK402 [6]	RCP (mRFP1)	Bba_E1010 [65]	Ery (ermB)	pMSP3535 [67]
pSL6	191002	RSF1010	pBTK402 [6]	RCP (mRFP1)	Bba_E1010 [65]	Tet (tetC)	pBMTBX-4 [68]
pSL7	191003	RSF1010	pBTK402 [6]	RCP (mRFP1)	Bba_E1010 [65]	Carb (tem116)	pBTK401 [6]
pSL9	191004	pBBR1	pBBR1MCS-2 [43]	E2C	pBTK570 [6]	Kan (aphA-1)	pBTK402 [6]
pSL25	191005	RP4	pTD-C_sYFPTwinStrep [69]	BCP (amilCP)	amilCP chromoprotein [70]	Kan (aphA-1)	pBTK402 [6]

[a]Plasmid is not compatible with Golden Gate assembly.
[b]Plasmid contains a duplication of one BsaI site and the adjacent CP25 promoter and ribosome binding site for RCP. It is still compatible with Golden Gate assembly.

RSF1010 origin and red chromoprotein expression with one of seven different antibiotic resistance cassettes (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]). Each antibiotic resistance gene is expressed from its native promoter. All Pathfinder plasmids are also BsaI dropout vectors (Type 8 parts) compatible with stage 1 of the Golden Gate assembly scheme used by the yeast and bee microbiome toolkits [6, 35]. Thus, the Pathfinder plasmids can be readily reconfigured to convey and express DNA sequences other than the included reporter genes. For example, we created pSL1-GFP to express GFP instead of RCP from the same backbone as pSL1 in this way.

To confirm the functionality of the Pathfinder plasmids, we performed an initial test with *E. coli*. All plasmids except pSL5 (RSF1010, RCP, Ery[R]) were transformed into the *E. coli* donor strain MFD*pir*, which is resistant to erythromycin [36]. Transformants were combined equally into a Pathfinder 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]). Colonies of *E. coli* cells containing Pathfinder plasmids expressing each of the three reporters or GFP can be identified by eye under normal white light illumination or on a blue light transilluminator (Fig. 1B). Fluorescence can also be used to distinguish colonies with different plasmids from one another (Fig. 1B), which might be useful if the markers are expressed at lower levels in other species. To demonstrate functionality in a more distantly-related bacterium, we tested conjugation of the Pathfinder mix into *Sodalis praecaptivus* HS[T], a human wound isolate previously shown to colonize weevils and tsetse flies [37–39]. As with *E. coli*, we were able to recover transconjugants for every plasmid except for pSL3. This negative result may be due to inherent gentamicin resistance of the MFD*pir* strain interfering with conjugation. We also established that Pathfinder plasmids function in a new *Rosenbergiella* bmE01 strain we isolated from *Empoasca* leafhoppers. For bmE01, we tested only pSL1-GFP and pSL7 and successfully isolated transconjugants for both.

Pathfinder plasmids function in recently isolated fly symbionts
We then applied the Pathfinder plasmid system to a set of bacteria that we isolated from wild flies (members of order Diptera), along with an isolate, *Orbus hercynius* CN3, collected by Volkmann et al. [27], which likely originates from a non-*Drosophila* dipteran species breeding in boar feces. Based on phylogenies constructed using 16S rRNA genes, all of these isolates are closely related and belong to the *Orbaceae* family within the *Gammaproteobacteria*, which includes symbionts of bees and other insects (Fig. 2A).

We were able to successfully conjugate Pathfinder plasmids into each of the *Orbaceae* (Fig. 2B). In terms of origin of replication compatibility, we only observed conjugation with the pSL1 and pSL1-GFP plasmids that contain the RSF1010 origin. The pBBR1 and RP4 origin plasmids were absent from our *Orbaceae* transconjugant plates. For the antibiotic resistance panel, we achieved conjugation with all plasmids other than pSL6 (TetR) in most strains. Differences arose when strains had elevated levels of intrinsic resistance to an antibiotic (Table 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. Rates of conjugation with the Pathfinder plasmids were similar across strains and comparable to the rates observed for *E. coli* for many combinations of plasmids and strains (Fig. 2C). The overall average conjugation efficiency for all the *Orbaceae* strains was $1.4 \pm 0.02\%$. BiB had the highest average conjugation efficiency ($2.3 \pm 0.1\%$), while lpD03 had the lowest ($0.0026 \pm 0.0002\%$). Despite the lower conjugation efficiencies observed for some *Orbaceae* strains, we demonstrated that each of these newly isolated bacteria can be engineered with several plasmids from the Pathfinder series.

As a basic test for functionality of the engineered constructs in the uncharacterized *Orbaceae* strains, we measured fluorescence levels from pSL1-GFP conjugants. We observed significant variation in GFP fluorescence among these strains ($F_{4,40} = 322.8$, $p < 0.0001$). In particular, the engineered lpD01 strain had such a low GFP signal that it was difficult to detect by eye that it fluoresced using a blue light transilluminator (Fig. 2D), but even this weak GFP signal was sufficient for further studies of this strain (see below).

D. melanogaster colonization by engineered *Orbaceae*
We next used our engineered strains to determine if fly-associated *Orbaceae* can colonize *D. melanogaster*. We colonized conventionally reared Canton-S flies using a method we refer to as arena
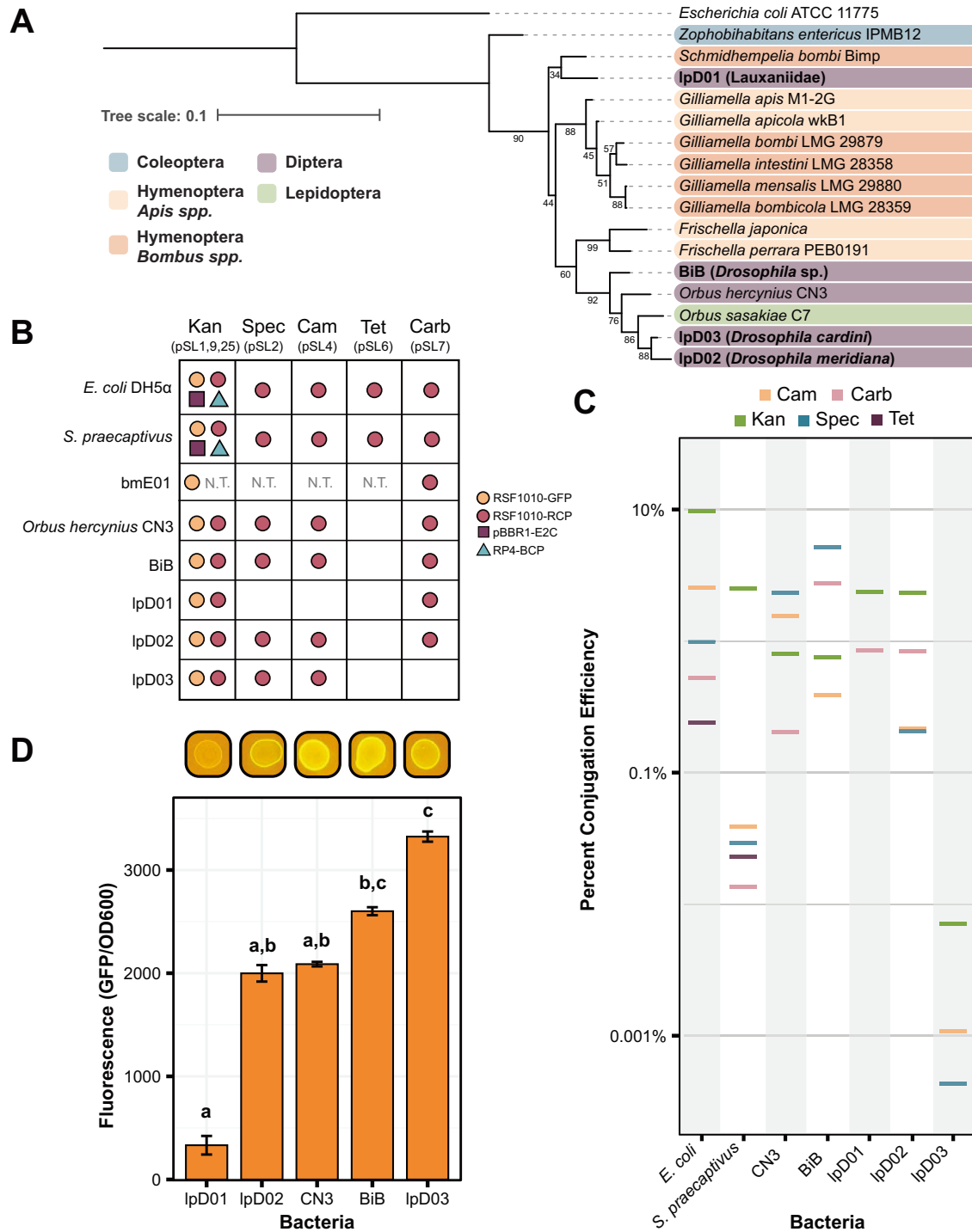
Fig. 2 Recently isolated *Orbaceae* strains can be engineered with the Pathfinder plasmid system. A 16S rRNA gene sequence phylogeny showing the relationship between the isolated species in this paper (in bold) and other members of the family *Orbaceae*. Strains are color-coded based on the taxonomic order of their original insect host. For each *Orbaceae* strain first reported in this study the closest taxonomic identifier that could be established for the host is shown in parentheses. Bootstrap values are depicted next to their respective branches. **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. Except in the Kan condition, where multiple plasmids may be present as shown in (**B**), all plasmids are RSF1010-RCP. **D** Bar chart showing the level of fluorescence for each strain engineered with pSL1-GFP (RSF1010-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. Without the pSL1-GFP plasmid, all strains exhibited negligible autofluorescence (average fluorescence/OD600 levels ≤8.0).

Table 2. Antibiotic concentrations used in this study[a].

		CARB	CAM	KAN	SPEC	TET
DH5α	MIC	100	20	50	60	10
	Pathfinder	100	20	50	60	10
Sodalis praecaptivus	MIC	200	6.25	6.25	50	12.5
	Pathfinder	200	6.25	6.25	50	12.5
bmE01	MIC	400	25	200	400	6.25
	Pathfinder	*800	*50	*400	*800	*12.5
Orbus hercynius CN3	MIC	800	6.25	25	25	6.25
	Pathfinder	800	6.25	25	*100	*3
BiB	MIC	25	6.25	400	100	6.25
	Pathfinder	25	6.25	400	*200	*3
lpD01	MIC	6.25	400	12.5	400	200
	Pathfinder	*25	400	*50	*800	200
lpD02	MIC	200	6.25	50	100	6.25
	Pathfinder	*400	6.25	*100	100	*3
lpD03	MIC	800	6.25	25	100	6.25
	Pathfinder	*6400	6.25	*100	100	*3

Starred (*) and bolded values indicate when concentrations different from the MIC were used for Pathfinder conjugation assays.
[a]All concentrations are provided in µg/ml.

inoculation, in which flies were kept in a container along with an agar plate grown with a lawn of one of our strains (see "Methods" for additional details). We expected that flies would ingest the bacteria while feeding. After 24 h of inoculation, we transferred flies to fresh diet every 24 h to eliminate bacteria that survived on the diet rather than within the flies. To ascertain whether bacteria could persist by replicating on the diet itself, we confirmed lack of growth on the yeast-glucose agar fly diet. No growth was observed on the fly diet for strains lpD01, lpD02, and lpD03. BiB and *O. hercynius* CN3 had light growth after 4–5 days, which could potentially be a source of fly recolonization after the initial inoculation arena. Because we did not perform our inoculation with germ-free flies, we anticipated that other microbes in the fly gut might 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 within a microbiome containing other microbes.

At several time points after inoculation, we washed and crushed 5–6 flies and plated them on selective media with kanamycin. We found that each of these strains can colonize flies to some extent, in contrast to the bee-associated *Orbaceae*, *Gilliamella apicola* M1-2G, which was not retained at any time point (Fig. 3). Colonization of the fly-derived strains varied between time points and between individual flies in these initial tests. The most consistent findings were that lpD01 was able to robustly colonize flies at every time point, while lpD03 and BiB were lost after day 2 and 4, respectively.

Based on these preliminary results, we decided to track bacterial titer over time in flies colonized with lpD02 and lpD01. To account for variation among experimental populations of flies, we inoculated three separate arenas of flies per trial. The overall trend for lpD02 colonization follows the pattern observed in the qualitative experiment. Over time, the number of colony-forming units (CFU) per fly gradually decreases until day 11, when most flies are no longer colonized (Fig. 4A), as judged from observing zero CFUs after plating whole-fly homogenates. However, for lpD01, the average CFU in each arena decreases then increases almost to the initial level seen on day 0 (Fig. 4B). Between day 0 and day 4, average CFU drops (arena 1, $p = 0.001$, arena 2, $p = 0.0045$, arena 3, $p < 0.0001$, Dunn's test with Bonferroni correction), and most sampled flies were uncolonized on day 4
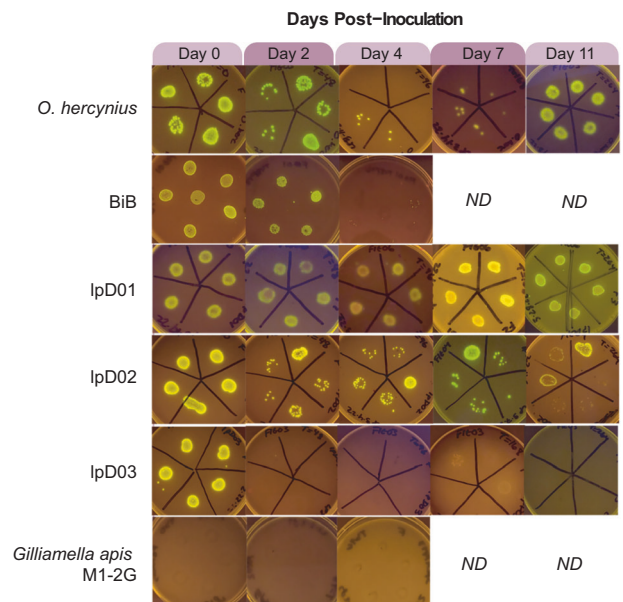

Fig. 3 Engineered *Orbaceae* strains can colonize *D. melanogaster*. Images were taken of bacterial growth on selective media containing kanamycin following the growth of bacteria from crushed flies in the days following inoculation. Ability to colonize the host is determined by the presence of many GFP-expressing colonies. Days on which we were unable to collect data or stopped the experiment are indicated by "ND".

in arenas 1 and 3. The CFU per fly then increases 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 appears to show a similar trend with a slight temporal delay.

Visualizing lpD01 in the gut of *D. melanogaster*
Based on the results of the bacterial titer assay, we selected lpD01 to visualize colonization of the fly gut by *Orbaceae*. We inoculated
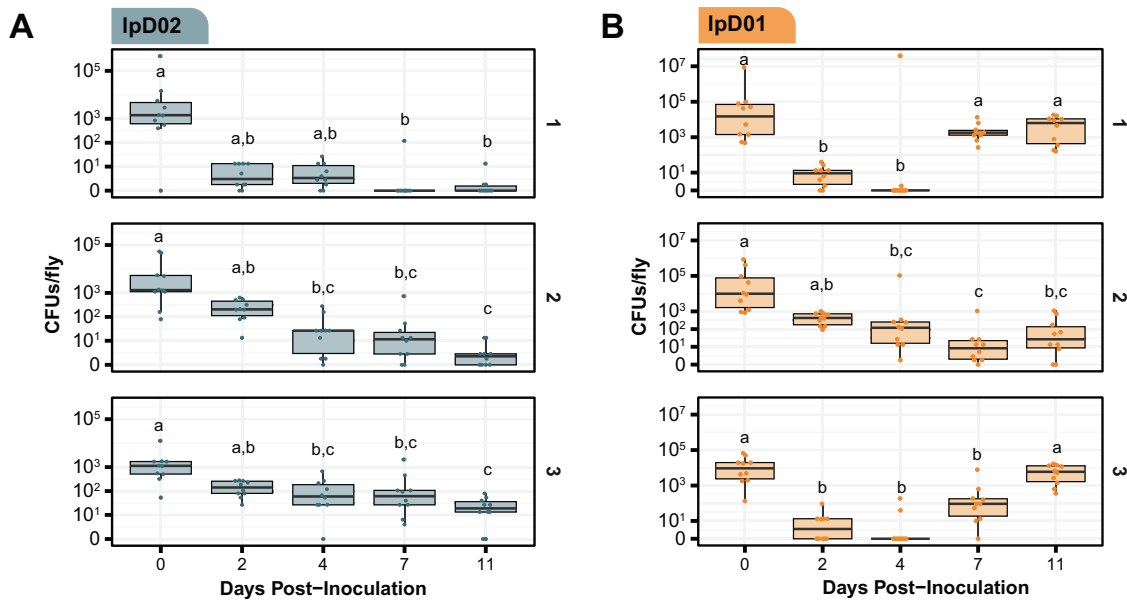
Fig. 4 Dynamics of *D. melanogaster* colonization by two newly isolated *Orbaceae* strains. CFU/fly was measured following inoculation with either (**A**) lpD02 or (**B**) lpD01. CFUs in each of ten flies per arena were measured at each time point. Data are plotted on a pseudo-log scale so the full range of colonization levels can be shown. Results from three independent arenas are shown in subpanels labeled 1, 2, and 3. Letters above each boxplot represent groups that are significantly different from one another ($p < 0.05$, Dunn's test with Bonferroni correction).

100 flies with the engineered lpD01 + pSL1-GFP strain and reared them for 11 days on fresh diet, replicating the quantification experiment. After this point we dissected the *D. melanogaster* gut and used confocal fluorescence microscopy to assess bacterial localization (Fig. 5). We observed the presence of fluorescent lpD01 in the proventriculus (cardia) of every imaged fly (Fig. 5C, D), and for 2 out of 5 flies lpD01 could also be found colonizing the crop (Fig. 5G, H). These locations are consistent with where gut-associated *Acetobacteraceae* and *Lactobacillus* strains have been observed in *D. melanogaster* [40–42]. Bacterial aggregates were present in both the crop and cardia (Fig. 5E), suggesting active replication. Throughout the remainder of the gut, we occasionally observed fluorescent cells (Fig. 5J, K), but lpD01 did not robustly colonize the midgut or hindgut regions.

DISCUSSION

The Pathfinder plasmid system provides a simple toolkit with a straightforward methodology for genetically modifying non-model bacteria. With this kit we successfully engineered several *Orbaceae* strains isolated from wild flies and utilized their GFP expression and antibiotic resistance cassettes to facilitate colonization experiments in *D. melanogaster*. To our knowledge, our study is the first to demonstrate that *D. melanogaster* can be experimentally colonized with natural fly symbionts from the *Orbaceae* family. These *Orbaceae* join wild *Lactiplantibacillus plantarum* and *Acetobacter indonesiensis* isolates as the only symbionts that have been shown to stably colonize the *D. melanogaster* gut to date [40–42]. All of these bacteria, including the *Orbaceae*, predominantly colonize the crop and proventriculus.

Our results illustrate a common obstacle in synthetic biology: not all plasmid components work well in all bacteria [12]. For example, we did not recover *Orbaceae* transconjugants carrying either the RP4 or pBBR1 origins, despite reports that these origins function in a wide range of bacteria in other studies [13, 43]. It is possible that our screening method was not sensitive enough to detect low rates of transformation with these origins during multiplex conjugation, and low levels of reporter expression could lead to challenges in their identification. Screening for transconjugants with each origin one-by-one could confirm this result. In

terms of antibiotic resistance cassette compatibility, our results also matched our expectation that each resistance cassette would not function in every strain. The incompatible strains were mostly those with intrinsic resistance to specific antibiotics. However, the basis of the incompatibility of the Tet resistance cassette with all *Orbaceae* tested is unclear since most were sensitive to low levels of Tet. Possibly, the Tet resistance gene on the Pathfinder plasmids does not express or function effectively in *Orbaceae*. Our varied results highlight the utility of widely surveying for plasmid component functionality when first working with a new strain. The information from Pathfinder informs the selection of plasmids for future experiments.

Many other genetic toolkits have been developed with a similar goal of engineering wild bacteria [9, 16, 44], and Pathfinder has fewer components than other kits. We prioritized building out the complete antibiotic set with RSF1010 because of this origin of replication's wide compatibility with different bacteria [45, 46]. RSF1010 worked well in the *Orbaceae* strains, but it may not replicate in other bacteria. The host ranges of many plasmid origins of replication that function in non-model bacterial species have not yet been exhaustively surveyed. In the future, the kit might be improved by including additional plasmid origins and pairing each of these with the entire antibiotic resistance set. The current Pathfinder toolkit also relies on a single promoter to drive all reporter genes and various native promoters to express antibiotic resistance genes. One could expand the combinations to include different promoters to rapidly survey their functions in a new bacterium, for example by performing multiplex conjugation and then picking the most highly fluorescent or vividly colored colonies. Building out additional origin and promoter components could also address potential issues with low reporter levels or detecting transconjugants, as these outcomes could result from low plasmid copy number and/or poor expression of reporter or antibiotic resistance genes. Combinatorial Golden Gate assembly schemes could be used to create sets of plasmids with new combinations of these components.

The Pathfinder kit is limited to plasmid-based expression systems. Plasmid transformation is a common first step in engineering a new bacterium, but it is not ideal for ensuring the long-term stability of engineered constructs. Multicopy plasmids can be especially burdensome when their gene products divert resources from host
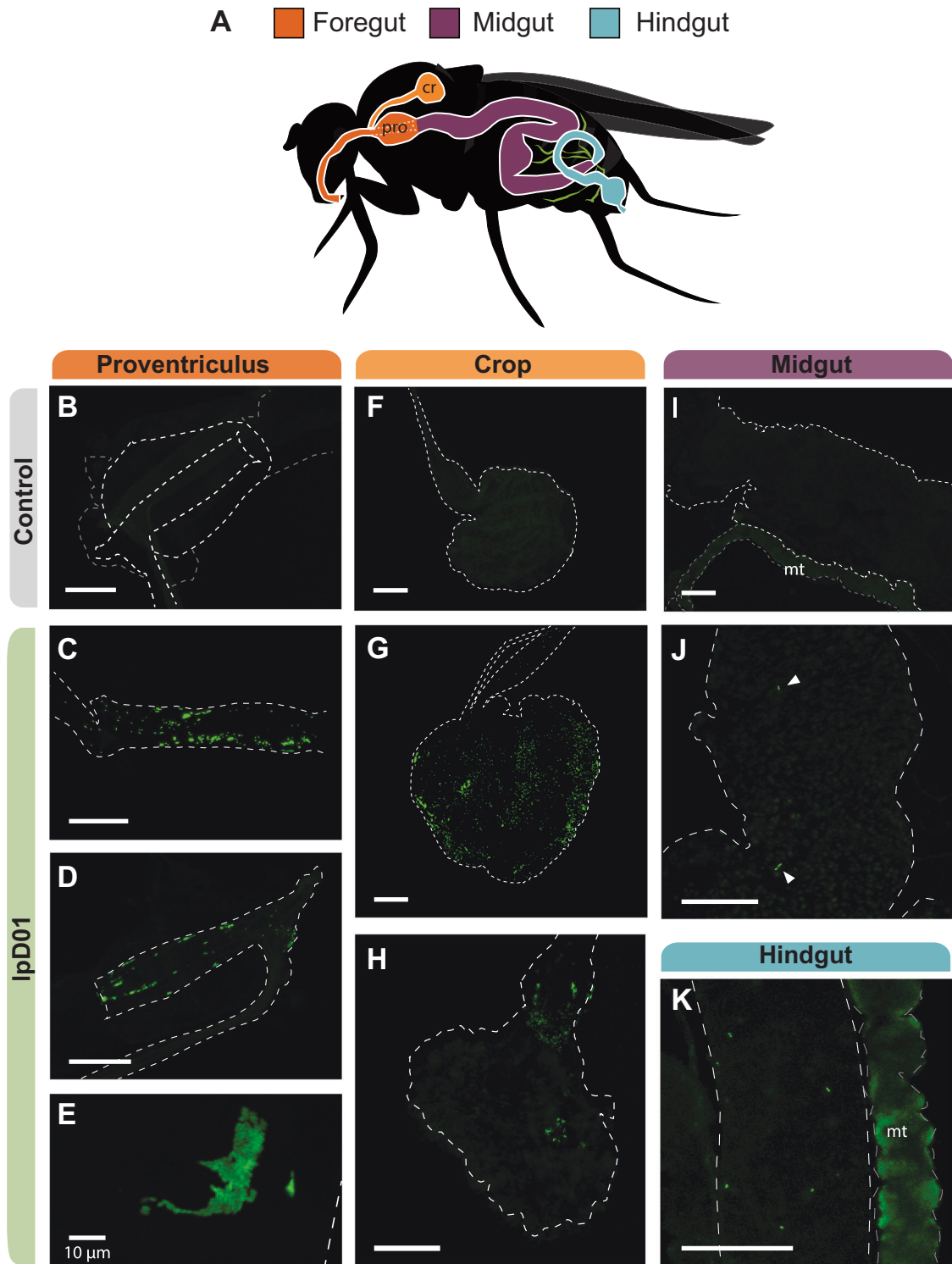
Fig. 5 **Confocal microscopy of the *D. melanogaster* gut colonized with fluorescent lpD01. A** Schematic of the *Drosophila* gut with colors designating three regions, foregut, midgut, and hindgut. The crop (cr) and proventriculus (pro) are labeled due to their relevance for the localization of the 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**, **K**) are from the same fly, (**D**, **H**) are from a second fly, and (**E**) is a from a 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 to (**J**) to point out 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.

cell replication [47, 48]. Because of this, plasmid-based systems tend to be more likely to rapidly lose function due to the takeover of cells with mutated plasmids that alleviate this burden by inactivating engineered functions, as compared to systems engineered into the chromosome [49]. Another potential complication is rapid plasmid loss from a cell population due to segregation in the absence of antibiotic selection. Our colonization experiments in *D. melanogaster* used constant antibiotic selection to prevent plasmid loss. However, administering sufficient levels of antibiotics for selection may be challenging in other environments. Tools designed for chromosomal integration such as transposon or recombineering systems may be a better option for researchers with these concerns [44, 50]. Conjugation has been used to deliver DNA to engineer bacteria in situ in gut and soil communities [2, 16, 51]. The Pathfinder plasmids are compatible with this approach and could potentially be used to engineer bacteria that are currently unculturable outside of their hosts [1, 52].

The fluorescent *Orbaceae* strains that we built enabled us to easily screen for effective colonization of *D. melanogaster*. We observed differences among isolates in their abilities to colonize flies, as well as variable colonization levels among individual flies inoculated with the same isolate and between the preliminary trials and follow-up experiments with the lpD01 and lpD02 isolates. We colonized non-axenic flies to emulate invasion conditions in which other microbes are already present in the fly gut [40]. Differences in the established gut communities of the cohorts of flies that we used could explain some of the variation in our results [41]. Using germ-free flies would eliminate these effects [53]. Since our flies were housed in a laboratory setting, their diets and microbiomes do not necessarily reflect wild conditions [40], which could also impact the success of *Orbaceae* relative to other gut-associated species of bacteria.

We observed fluorescent *Orbaceae* cells in the crop and proventriculus regions of the foregut in a majority of the flies we colonized with lpD01. The *Drosophila* foregut tends 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 [21, 41]. Accordingly, we observed very few lpD01 cells in the midgut, and other stable colonizers of the *D. melanogaster* gut like *Lactobacillus plantarum* and *Acetobacter thailandicus* also principally localize to the crop and proventriculus [40, 41]. Our current results do not reveal whether lpD01 is attaching to and forming biofilms in the gut as has been observed for *L. plantarum* [41].

Orbaceae are widespread insect symbionts, but their roles in host biology, reasons for their host specificity, and interactions with other microbes are largely unexplored. *D. melanogaster* offers sophisticated genetic resources for understanding the host side of these microbiome interactions. Our results show that it is also possible to genetically modify *Orbaceae* to begin to dissect these relationships. Because the Pathfinder plasmids are compatible with established Golden Gate assembly schemes and parts libraries [6, 35], they can be used to build new constructs, including systems for knocking out or inserting genes into the bacterial chromosome [6, 44, 54]. Such tools would facilitate future studies of insect-*Orbaceae* interactions and how *Orbaceae* fit into the microbial ecology of insect gut communities. It may also be possible to use these genetic tools to control agricultural pests [1, 55], by isolating and engineering *Orbaceae* native to the tephritid fruit fly *Bactrocera dorsalis* [33], for example.

MATERIALS AND METHODS
Isolation of bmE01 from leafhoppers
Empoasca sp. leafhoppers were collected by sweep netting *Salvia* sp. plants on the University of Texas campus in Austin, TX (30.289160, −97.738927). Individual leafhoppers were washed by soaking in 70% ethanol for 1 min, followed by another minute in 10% bleach. Next, each leafhopper was rinsed

three times with sterile water and then crushed in 100 μl of sterile saline. Then, 50 μl of five different leafhopper samples were plated onto separate Brain Heart Infusion (BHI) agar plates containing cycloheximide at 100 μg/ml. Colonies were picked and identified based on PCR and Sanger sequencing of the 16S rRNA gene with primers 16SA1F (5′-AGAGTTTGATCMTGGCTCAG) and 16SB1R (5′-TACGGYTACCTTGTTACGACTT) [56]. The bme01 isolate studied here was predicted with high confidence to be a *Rosenbergiella* species by both the Ribosomal Database Project classifier tool [57], and BLAST searches of the NCBI 16S ribosomal RNA sequence database [58].

Isolation of BiB, lpD01, lpD02, and lpD03 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 on ice and photographed for morphological identification. They were then washed with 10% bleach for 1 min to remove surface microbes followed by rinsing in sterile water for 1 min to remove residual bleach. Legs and wings were removed and placed in 95% ethanol to preserve host DNA. Next, each fly was placed in 50 μl of Insectagro DS2 insect growth medium (IGM) (Corning, VA, USA) and homogenized using a sterile plastic pestle. Dilutions of homogenate were plated on heart infusion agar (HIA) with 5% sheep's blood. Agar plates were initially incubated at 37 °C and 5% CO_2 (e.g., in the case of BiB), but 30 °C was later used due to superior growth (e.g., in the case of lpD01). Clear or off-white and slower-growing colonies were passaged onto fresh plates multiple times to obtain pure cultures.

To identify the *Orbaceae*, we amplified and Sanger sequenced the 16S rRNA gene using 27F (5′-AGAGTTTGATCMTGGCTCAG) and *Orbaceae*-specific primer Orb742R (5′-ATCTCAGCGTCAGTATCTGTCCAGAA). Host insects were identified both by morphology and by sequencing PCR amplicons of the barcode region of the COI gene with primers LCO1490F (5′-GGTCAACAAATC ATAAAGATATTGG) and HCO2198R (5′-TAAACTTCAGGGTGACCAAAAAATCA) [59]. The phylogenetic tree was assembled from 16S ribosomal RNA gene sequences. 16S rRNA gene sequences were aligned in Geneious using MUSCLE [60], and all sites containing ≥50% gaps were stripped. This masked alignment was used to infer a phylogenetic tree using IQ-TREE with default options and nonparametric bootstrapping [61]. The tree was visualized using iTOL (v5) [62].

Growth and maintenance of bacterial strains
Escherichia coli DH5α, *E. coli* MFD*pir*, and *Sodalis praecaptivus* HS^T were grown in LB broth and on LB agar at 37 °C. Media was supplemented with 0.3 mM diaminopimelic acid (DAP) for MFD*pir* growth. Following isolation, *Rosenbergiella* was grown at 30 °C on BHI broth or agar. *Orbus hercynius* CN3 was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM 22228). Following isolation, *Orbaceae* strains were determined to be culturable in either IGM or BHI broth, and on BHI agar or HIA with or without 5% defibrinated sheep's blood. For robust growth, BHI and HIA + 5% sheep's blood were preferred, but media were selected based on the needs of the assay. All fly *Orbaceae* were grown at 30 °C with 5% CO_2. *Gilliamella apis* M1-2G was grown on HIA + 5% sheep's blood at 35 °C with 5% CO_2. Antibiotic concentrations used in this study are shown in Table 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 two-fold dilutions of each antibiotic, ranging from 400–6.25 μg/ml, in 100 μl of medium per well. 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 inspected visually to determine the minimum inhibitory concentration for each antibiotic. These MIC values were used to guide how much antibiotic was used for selection during the Pathfinder conjugation process (Table 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 on 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 BsaI and BsmBI cut sites to make it suitable for Golden Gate Assembly [6]. The pBTK402 plasmid was designed to function as a Type 8 dropout vector for the BTK Golden Gate assembly scheme, and all of the main 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 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 1).

To construct plasmids, we first designed assembly primers using Benchling (http://www.benchling.com) and added Golden Gate-compatible BsmBI cut sites. We ordered DNA primers from Integrated DNA Technologies 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 by whole-plasmid Illumina sequencing on an iSeq 100. Three plasmids (pSL2, pSL4, and pSL5) contain a duplication of a BsaI restriction site and the adjacent CP25 promoter and ribosome binding site for RCP. Whether this duplication affects RCP expression is unknown. These plasmids still function as Type 8 dropout vectors for Golden Gate assembly.

We next transformed all pSL plasmids into the DAP auxotrophic conjugation donor strain *E. coli* MFDpir [36]. We could not successfully transform pSL5, however, due to intrinsic erythromycin resistance in MFDpir. These strains of MFDpir were used individually or combined for subsequent multiplex conjugation assays. A preliminary study examined various aspects of how the RSF1010-based Pathfinder plasmids function in *E. coli*, including how different antibiotic markers and concentrations affect plasmid copy number and reporter output and how stably they are maintained over many serial transfers in laboratory cultures with or without antibiotic selection [63].

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 optical density at 600 nm (OD600) of each strain and resuspended it at an OD600 of 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 bacterial strain. The culture was pelleted (1000 × *g* for 6 min) 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 with the growth of both *E. coli* and *Orbaceae* spp. (BHI for all strains except lpD02, 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 five equal portions and serially diluted 10-fold to a final dilution of 1×10^{-5}. To plate these dilutions, 5 µl 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 2). To confirm successful conjugation, colonies were first visually examined for expression of fluorescent reporter genes. Then, one colony from each antibiotic condition was regrown in a liquid medium, and a dilution of this culture was used as template for whole-cell PCR and 16S rRNA gene sequencing with primers 16SA1F and 16SB1R, as described above. 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) [64].

Measuring GFP expression in different bacteria
To measure the level of GFP expression, we assessed three separate colonies picked from the Pathfinder conjugation plate compared to their wild-type counterparts. Each colony was first grown to saturation, then re-grown with a starting OD600 of 0.05 in 5 ml Insectagro DS2 in a test tube. After re-growth, 1 ml of each culture was pelleted and resuspended in 600 µl saline. OD600 and GFP expression (485 nm excitation, 535 nm emission) were measured for 200 µl 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
D. melanogaster Canton-S were acquired from the Bloomington Drosophila Stock Center (Bloomington, IN). 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 agar (YGA) diet containing brewer's yeast, D-glucose, agar, and water [53]. Nystatin (10 µg/ml) and kanamycin (10 µg/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 *Orbaceae* using a method we refer to as arena inoculation. For these experiments, adult female *D. melanogaster* were first removed from the commercial diet and transferred to YGA diet for 48 h to clear their gut from the preservatives in the commercial diet. In the meantime, HIA + 5% sheep blood + Kan plates were inoculated with 100 µl of OD600 = 1 *Orbaceae* + pSL1-GFP and allowed to grow for 1–2 days as needed. The bacterial plate was then taped to the bottom of a lidded 12 oz SelecTE plastic container "arena" (Berry Plastics, Evansville, IN) modified with a mesh vent on its lid and a small port for administering CO2. YGA flies were added to an arena and allowed to feed for 24 h. After this inoculation step, flies were transferred daily to fresh YGA + nystatin + Kan diet tubes.

Quantification of bacterial colonization
Each bacterial strain was first delivered to female adult flies 3–5 days post-eclosion using the methodology described above. For this set of experiments, three different arenas were used to inoculate three 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, ten flies from each independent population (30 total) were crushed and plated to determine their quantity of bacterial colonization.

To prepare flies for crushing, we first washed them in 10% bleach to eliminate any bacteria located on the outsides of their bodies. Flies were immobilized for this procedure by first placing them at −20 °C for 1 min, and then keeping the tubes on ice during the washes. They were soaked in 500 µl 10% bleach for 1 min, washed with 500 µl 1× phosphate buffered saline (PBS), then resuspended in 200 ul PBS and homogenized with a sterile plastic

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 µg/ml, nystatin 10 µg/ml) in triplicate. The remaining homogenized fly mixture was then centrifuged at $1000 \times g$ for 6 min to pellet the remaining bacteria. The pellet was resuspended in 15 µl saline and plated to ensure the detection of bacteria that were present in low abundance.

Imaging *Drosophila* guts with confocal microscopy

Fifty female *D. melanogaster* were colonized with lpD01 + pSL1-GFP in two separate arenas and maintained for 11 days along with an uncolonized control population. To ensure that flies were colonized with these 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 h 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) [64].

DATA AVAILABILITY

The data that support the findings of this study and newly isolated bacteria reported here are available from the authors upon reasonable request. The Pathfinder plasmids have been deposited in Addgene (Table 1).

REFERENCES

1. Elston KM, Leonard SP, Geng P, Bialik SB, Robinson E, Barrick JE. Engineering insects from the endosymbiont out. Trends Microbiol. 2022;30:79–96.
2. Brophy JAN, Triassi AJ, Adams BL, Renberg RL, Stratis-Cullum DN, Grossman AD, et al. Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. Nat Microbiol. 2018;3:1043–53.
3. Waller MC, Bober JR, Nair NU, Beisel CL. Toward a genetic tool development pipeline for host-associated bacteria. Curr Opin Microbiol. 2017;38:156–64.
4. Durante-Rodríguez G, De Lorenzo V, Martínez-García E. The standard European vector architecture (SEVA) plasmid toolkit. Methods Mol Biol. 2014;1149:469–78.
5. Iverson SV, Haddock TL, Beal J, Densmore DM. CIDAR MoClo: improved MoClo assembly standard and new *E. coli* part library enable rapid combinatorial design for synthetic and traditional biology. ACS Synth Biol. 2016;5:99–103.
6. Leonard SP, Perutka J, Powell JE, Geng P, Richhart DD, Byrom M, et al. Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. ACS Synth Biol. 2018;7:1279–90.
7. Martínez-García E, Aparicio T, Goñi-Moreno A, Fraile S, De Lorenzo V. SEVA 2.0: an update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. Nucleic Acids Res. 2015;43:D1183–9.
8. Schuster LA, Reisch CR. A plasmid toolbox for controlled gene expression across the Proteobacteria. Nucleic Acids Res. 2021;49:7189–202.
9. Geddes BA, Mendoza-Suárez MA, Poole PS. A Bacterial Expression Vector Archive (BEVA) for flexible modular assembly of golden gate-compatible vectors. Front Microbiol. 2019;10:3345.
10. Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S. A modular cloning system for standardized assembly of multigene constructs. PLoS ONE. 2011;6:e16765.
11. Young R, Haines M, Storch M, Freemont PS. Combinatorial metabolic pathway assembly approaches and toolkits for modular assembly. Metab Eng. 2021;63:81–101.
12. Freed E, Fenster J, Smolinski SL, Walker J, Henard CA, Gill R, et al. Building a genome engineering toolbox in nonmodel prokaryotic microbes. Biotechnol Bioeng. 2018;115:2120–38.
13. Jain A, Srivastava P. Broad host range plasmids. FEMS Microbiol Lett. 2013;348:87–96.
14. Aune TEV, Aachmann FL. Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. Appl Microbiol Biotechnol. 2010;85:1301–13.
15. Mazodier P, Davies J. Gene transfer between distantly related bacteria. Annu Rev Genet. 1991;25:147–71.
16. Ronda C, Chen SP, Cabral V, Yaung SJ, Wang HH. Metagenomic engineering of the mammalian gut microbiome in situ. Nat Methods. 2019;16:167–70.
17. Von Wintersdorff CJH, Penders J, Van Niekerk JM, Mills ND, Majumder S, Van Alphen LB, et al. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front Microbiol. 2016;7:173.
18. Akiyama T, Asfahl KL, Savin MC. Broad-host-range plasmids in treated wastewater effluent and receiving streams. J Environ Qual. 2010;39:2211–5.
19. Brooks LE, Kaze M, Sistrom M. Where the plasmids roam: Large-scale sequence analysis reveals plasmids with large host ranges. Microb Genomics. 2019;5:e000244.
20. Douglas AE. The *Drosophila* model for microbiome research. Lab Anim. 2018;47:157–64.
21. Ludington WB, Ja WW. *Drosophila* as a model for the gut microbiome. PLoS Pathog. 2020;16:e1008398.
22. Broderick NA, Lemaitre B. Gut-associated microbes of *Drosophila melanogaster*. Gut Microbes. 2012;3:307–21.
23. Newell PD, Chaston JM, Wang Y, Winans NJ, Sannino DR, Wong ACN, et al. In vivo function and comparative genomic analyses of the *Drosophila* gut microbiota identify candidate symbiosis factors. Front Microbiol. 2014;5:576.
24. Shin SC, Kim S-H, You H, Kim B, Kim AC, Lee K-A, et al. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science. 2011;334:670–4.
25. Martino ME, Joncour P, Leenay R, Gervais H, Shah M, Hughes S, et al. Bacterial adaptation to the host's diet is a key evolutionary force shaping *Drosophila-Lactobacillus* symbiosis. Cell Host Microbe. 2018;24:109–19.e6.
26. Chandler JA, Morgan Lang J, Bhatnagar S, Eisen JA, Kopp A. Bacterial communities of diverse *Drosophila* species: ecological context of a host–microbe model system. PLoS Genet. 2011;7:e1002272.
27. Volkmann M, Skiebe E, Kerrinnes T, Faber F, Lepka D, Pfeifer Y, et al. *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. 2010;60:2601–5.
28. Kwong WK, Moran NA. 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. 2013;63:2008–18.
29. Martinson VG, Carpinteyro-Ponce J, Moran NA, Markow TA. A distinctive and host-restricted gut microbiota in populations of a cactophilic *Drosophila* species. Appl Environ Microbiol. 2017;83:e01551–17.
30. Hammer TJ, McMillan WO, Fierer N. Metamorphosis of a butterfly-associated bacterial community. PLoS ONE. 2014;9:86995.
31. Kim JY, Lee J, Shin NR, Yun JH, Whon TW, Kim MS, et al. *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. 2013;63:1766–70.
32. Martinson VG, Douglas AE, Jaenike J. Community structure of the gut microbiota in sympatric species of wild *Drosophila*. Ecol Lett. 2017;20:629–39.
33. Liu S-H, Chen Y, Li W, Tang G-H, Yang Y, Jiang H-B, et al. Diversity of bacterial communities in the intestinal tracts of two geographically distant populations of *Bactrocera dorsalis* (Diptera: Tephritidae). J Econ Entomol. 2018;111:2861–8.
34. Gupta AK, Nayduch D, Verma P, Shah B, Ghate HV, Patole MS, et al. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). FEMS Microbiol Ecol. 2012;79:581–93.
35. Lee ME, DeLoache WC, Cervantes B, Dueber JE. A highly characterized yeast toolkit for modular, multipart assembly. ACS Synth Biol. 2015;4:975–86.
36. Ferrières L, Hémery G, Nham T, Guérout AM, Mazel D, Beloin C, et al. 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. 2010;192:6418–27.
37. Chari A, Oakeson KF, Enomoto S, Grant Jackson D, Fisher MA, Dale C. Phenotypic characterization of *Sodalis praecaptivus* sp. nov., a close non-insect-associated member of the *Sodalis*-allied lineage of insect endosymbionts. Int J Syst Evol Microbiol. 2015;65:1400–5.
38. Medina Munoz M, Spencer N, Enomoto S, Dale C, Rio RVM. Quorum sensing sets the stage for the establishment and vertical transmission of *Sodalis praecaptivus* in tsetse flies. PLoS Genet. 2020;16:e1008992.
39. Enomoto S, Chari A, Clayton AL, Dale C. Quorum sensing attenuates virulence in *Sodalis praecaptivus*. Cell Host Microbe. 2017;21:629–36.
40. Pais IS, Valente RS, Sporniak M, Teixeira L. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. PLoS Biol. 2018;16:e2005710.
41. Obadia B, Güvener ZT, Zhang V, Ceja-Navarro JA, Brodie EL, Ja WW, et al. Probabilistic invasion underlies natural gut microbiome stability. Curr Biol. 2017;27:1999–2006.e8.
42. Dodge R, Jones EW, Zhu H, Obadia B, Martinez DJ, Wang C, et al. A symbiotic physical niche in *Drosophila melanogaster* regulates stable association of a multi-species gut microbiota. Nat Commun. 2023;14:1557.
43. Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop RM, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 1995;166:175–6.
44. Wiles TJ, Wall ES, Schlomann BH, Hay EA, Parthasarathy R, Guillemin K. Modernized tools for streamlined genetic manipulation and comparative study of wild and diverse proteobacterial lineages. MBio. 2018;9:e01877–18.
45. Meyer R. Replication and conjugative mobilization of broad host-range IncQ plasmids. Plasmid. 2009;62:57–70.

46. Bishé B, Taton A, Golden JW. Modification of RSF1010-based broad-host-range plasmids for improved conjugation and cyanobacterial bioprospecting. iScience. 2019;20:216–28.

47. Ceroni F, Algar R, Stan G-BB, Ellis T. Quantifying cellular capacity identifies gene expression designs with reduced burden. Nat Methods. 2015;12:415–8.

48. Renda BA, Hammerling MJ, Barrick JE. Engineering reduced evolutionary potential for synthetic biology. Mol Biosyst. 2014;10:1668–78.

49. Englaender JA, Jones JA, Cress BF, Kuhlman TE, Linhardt RJ, Koffas MAG. Effect of genomic integration location on heterologous protein expression and metabolic engineering in *E. coli*. ACS Synth Biol. 2017;6:710–20.

50. Elmore JR, Dexter GN, Baldino H, Huenemann JD, Francis R, Peabody GL 5th, et al. High-throughput genetic engineering of nonmodel and undomesticated bacteria via iterative site-specific genome integration. Sci Adv. 2023;9:eade1285.

51. Rubin BE, Diamond S, Cress BF, Crits-Christoph A, Lou YC, Borges AL, et al. Species- and site-specific genome editing in complex bacterial communities. Nat Microbiol. 2021;7:34–47.

52. Lewis WH, Tahon G, Geesink P, Sousa DZ, Ettema TJG. Innovations to culturing the uncultured microbial majority. Nat Rev Microbiol. 2020;194 19:225–40.

53. Koyle ML, Veloz M, Judd AM, Wong ACN, Newell PD, Douglas AE, et al. Rearing the fruit fly *Drosophila melanogaster* under axenic and gnotobiotic conditions. J Vis Exp. 2016:113:54219.

54. Tucker AT, Nowicki EM, Boll JM, Knauf GA, Burdis NC, Trent MS, et al. Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. MBio. 2014;5:e01313–14.

55. Berasategui A, Shukla S, Salem H, Kaltenpoth M. Potential applications of insect symbionts in biotechnology. Appl Microbiol Biotechnol. 2016;100:1567–77.

56. Fukatsu T, Nikoh N. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta: Homoptera). Appl Environ Microbiol. 1998;64:3599–606.

57. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261–7.

58. Sayers EW, Bolton EE, Brister JR, Canese K, Chan J, Comeau DC, et al. Database resources of the national center for biotechnology information. Nucleic Acids Res. 2022;50:D20–6.

59. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.

60. Madeira F, Pearce M, Tivey ARN, Basutkar P, Lee J, Edbali O, et al. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res. 2022;50:W276–9.

61. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A, et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol. 2020;37:1530–4.

62. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 2021;49:W293–6.

63. Young E. Antibiotic markers: an overlooked design choice in synthetic biology? Honors thesis. Austin, TX: The University of Texas at Austin; 2021. https://doi.org/10.26153/tsw/43814.

64. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: An open-source platform for biological-image analysis. Nat Methods. 2012;9:676–82.

65. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, et al. A monomeric red fluorescent protein. Proc Natl Acad Sci USA. 2002;99:7877–82.

66. Alexeyev MF. 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. 1999;26:824–6.

67. Bryan EM, Bae T, Kleerebezem M, Dunny GM. Improved vectors for nisin-controlled expression in gram-positive bacteria. Plasmid. 2000;44:183–90.

68. Prior JE, Lynch MD, Gill RT. Broad-host-range vectors for protein expression across gram negative hosts. Biotechnol Bioeng. 2010;106:326–32.

69. Dammeyer T, Timmis KN, Tinnefeld P. Broad host range vectors for expression of proteins with (Twin-) Strep-tag, His-tag and engineered, export optimized yellow fluorescent protein. Microb Cell Fact. 2013;12:49.

70. Liljeruhm J, Funk SK, Tietscher S, Edlund AD, Jamal S, Wistrand-Yuen P, et al. Engineering a palette of eukaryotic chromoproteins for bacterial synthetic biology. J Biol Eng. 2018;12:8.

AUTHOR CONTRIBUTIONS
KME and LEP contributed equally to this work. Their author order was determined on the basis of seniority. KME, LEP, SPL, JCH, NAM, and JEB conceptualized the project and designed experiments. KME, SPL, EY, and BM constructed and tested the Pathfinder plasmids. LEP, JCH, TA, and BM isolated insect symbionts. KME and LEP performed *Drosophila* colonization experiments. KME, LEP, and JEB wrote the first draft of the manuscript and analyzed data. KME, LEP, JCH, SPL, NAM, and JEB revised the manuscript. NAM and JEB provided supervision and obtained funding.

COMPETING INTERESTS
NAM, JEB, and SPL are co-inventors on US Patent 11,382,989 which details methods for genetically engineering bacteria native to insect microbiomes.

ADDITIONAL INFORMATION
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