

1 **Modernized Tools for Streamlined Genetic Manipulation of Wild and Diverse Symbiotic**
2 **Bacteria**

3 Travis J. Wiles^{1,*}, Elena S. Wall¹, Brandon H. Schlomann^{1,2}, Edouard A. Hay², Raghuveer
4 Parthasarathy^{1,2}, and Karen Guillemin^{1,3}

5 ¹Institute of Molecular Biology, University of Oregon, Eugene, Oregon, United States of
6 America

7 ²Department of Physics and Materials Science Institute, University of Oregon, Eugene,
8 Oregon, United States of America

9 ³Humans and the Microbiome Program, Canadian Institute for Advanced Research, Toronto,
10 Ontario M5G 1Z8, Canada

11 *Correspondence: twiles@uoregon.edu

12 **ABSTRACT**

13 The capacity to associate symbiotic bacteria with vital aspects of plant and animal biology is
14 outpacing our understanding of the mechanisms shaping these interactions. A major barrier to
15 mechanistic studies is the paucity of tools for genetically manipulating wild and diverse
16 bacterial isolates. Solving this problem is crucial to elucidating the cellular and molecular rules
17 that govern symbiotic relationships and ultimately harnessing them for agricultural and
18 biomedical applications. Therefore, we constructed a series of vectors that expedite genetic
19 knock-in and knock-out procedures across a range of bacterial lineages. This was
20 accomplished by developing strategies for domestication-free bacterial conjugation, designing
21 plasmids with customizable features, and streamlining allelic exchange using visual markers of
22 homologous recombination. These tools enabled a comparative study based on live imaging of
23 diverse bacterial symbionts native to the zebrafish intestine, with which we discovered
24 heterogeneous colonization patterns and a striking correlation between bacterial population
25 biogeography and cellular behavior.

26 INTRODUCTION

27 High-throughput metagenomic sequencing has exposed the previously unseen diversity of
28 symbiotic bacteria that live in close contact with plants and animals throughout the
29 biosphere^{1,2}. Associations are being made at breakneck speed between the membership and
30 activity of resident bacteria and the health, development, and evolution of their hosts³⁻⁷.
31 However, the cataloging of symbiotic relationships—whether mutualistic, commensal, or
32 pathogenic—is vastly outpacing their cellular and molecular interrogation^{8,9}. Elucidating the
33 mechanisms by which symbiotic bacteria live and interact with each other and their hosts will
34 inform how they can be harnessed for agricultural and biomedical applications^{2,10-12}.

35 Characterizing the biology of symbiotic bacteria requires methods for precisely
36 manipulating their genomes. For example, stable chromosomal insertion of genes encoding
37 fluorescent proteins allows cellular behaviors and interactions to be directly observed within
38 native host-associated environments^{13,14}. Additionally, gene deletion and complementation
39 studies are essential for rigorously dissecting the genetic pathways that control specific
40 phenotypes¹⁵. Such knock-in and knock-out technologies have long been mainstays in
41 microbiology labs working with entrenched model organisms like *E. coli*, but established
42 genetic approaches are often inadequate for manipulating wild and novel species or strains¹⁶.
43 This is largely because legacy protocols can involve cumbersome and outdated procedures
44 that are difficult to use across lineages. Consequently, the in-depth study of most symbiotic
45 bacteria remains out of reach.

46 A major bottleneck within the field of symbiosis research is that locating appropriate
47 genetic tools and methods or developing them de novo are arduous and time-consuming
48 tasks. This problem is especially burdensome for investigators aiming to manipulate multiple

49 bacterial lineages derived from complex communities. To overcome these barriers, we have
50 employed the zebrafish intestinal microbiota as a source of wild and diverse symbiotic
51 bacteria¹⁷—which includes representatives of the *Vibrio*, *Aeromonas*, *Pseudomonas*,
52 *Acinetobacter*, *Enterobacter*, and *Plesiomonas* genera—to develop and test streamlined tools
53 and methods for bacterial genetic manipulation. We identified three main deficiencies inherent
54 to current genetic approaches that if resolved, will immediately improve the genetic tractability
55 of many bacteria. First, although conjugation is a robust and reliable method for delivering
56 DNA into bacteria, strategies for selecting individual cells carrying the transferred DNA are not
57 broadly compatible between different lineages and sometimes rely on deleterious
58 domestication steps. Second, most vectors used for making genetic manipulations are not
59 readily customized, which restricts their versatility and prevents further innovation. And third,
60 techniques for generating chromosomal modifications via allelic exchange often depend on
61 specific selection conditions that can vary between bacterial lineages and are difficult to
62 troubleshoot when they fail. To address these shortcomings, we rationally designed a
63 centralized set of genetic engineering vectors with new and updated functionalities. For DNA
64 delivery, we developed alternative schemes for post-conjugation counterselection that avoid
65 initial domestication of engineered bacteria, thereby preserving their natural physiology and
66 behavior. For customization, we designed gene expression scaffolds with interchangeable
67 sequence elements that can be tailored to different bacterial genomes, and with these
68 produced a variety of ready-made vectors for fluorescently tagging bacteria. Moreover, an
69 extensive collection of marked zebrafish intestinal symbionts was generated during this work
70 that will accelerate research in the growing zebrafish–microbiota community. Lastly, we

71 devised a means of visually following homologous recombination events during allelic
72 exchange protocols for more tractable generation of markerless chromosomal alterations.

73 To demonstrate the potential of these modernized tools to uncover new aspects of
74 host–microbe interactions, we examined the colonization patterns of several bacterial
75 symbionts native to the larval zebrafish intestine by light sheet fluorescence microscopy¹⁸. The
76 intestinal microbiota is an especially important target for exploration because of its impact on
77 host health and disease; however, its phylogenetic diversity and concealed location make it
78 difficult to investigate in situ by conventional techniques. Unexpectedly, live imaging of
79 bacterial symbiont behavior within larval zebrafish revealed that genome sequences and in
80 vitro-based phenotypes were poor predictors of whether a given bacterium exhibits free-
81 swimming motility in vivo. Most strikingly, we also discovered a general relationship between
82 the growth mode of individual bacteria and their overall biogeography; namely, the average
83 location of a population along the intestinal tract is strongly correlated with the fraction of
84 planktonic cells it contains. In addition to revealing the existence of previously undocumented
85 interactions within a vertebrate intestine, this exploratory experiment underscores how tools for
86 genetically manipulating diverse bacterial symbionts facilitates comparative studies involving
87 multiple species.

88 In total, the tools and step by step protocols described here will empower a wide range
89 of researchers studying different host–microbe systems as well as free-living bacteria to
90 explore deeper into the inner workings of wild and novel bacterial isolates. Our solutions
91 equally enhance the genetic manipulation of both established and newly emerging model
92 bacterial lineages and will speed the research pipeline from metagenomics to mechanistic
93 microbiology.

94

95 **RESULTS**

96 ***The lack of compatible post-conjugation counterselection strategies and the deleterious***
97 ***nature of bacterial domestication***

98 The process of genetically manipulating a bacterium typically begins with the delivery of
99 recombinant DNA into its cell. Several methods can be used for this purpose, but conjugation
100 or bacterial mating—which is the transfer of DNA from a donor cell to a target cell—is highly
101 efficient and versatile, working with a wide range of bacterial lineages. During this procedure,
102 successfully modified target cells are made drug resistant to facilitate their selective recovery.
103 However, a constraint of conjugation is that it also depends on a strategy for simultaneously
104 counterselecting against the donor cells that harbor the same drug resistance. This can be
105 problematic when dealing with novel and uncharacterized bacteria because counterselection
106 schemes often rely on known aspects of a target strain’s physiology. For example, many donor
107 strains (e.g., *E. coli* SM10) are auxotrophic for amino acids and vitamins and thus, can be
108 counterselected on defined growth media lacking specific metabolites. But for this approach to
109 work the target strain cannot itself be auxotrophic and the right mixture of nutrients and ions
110 must be formulated, which is not always straightforward due to the complex metabolic needs of
111 different bacteria. We found that this drawback represents an immediate hurdle when working
112 with several zebrafish-derived bacterial symbionts. None of the isolates we aimed to
113 genetically manipulate could grow on a standardized defined growth medium (i.e., M9 minimal
114 media) that is used for *E. coli* SM10 counterselection. This ultimately meant that customized
115 counterselection media would need to be painstakingly developed for each new target strain.

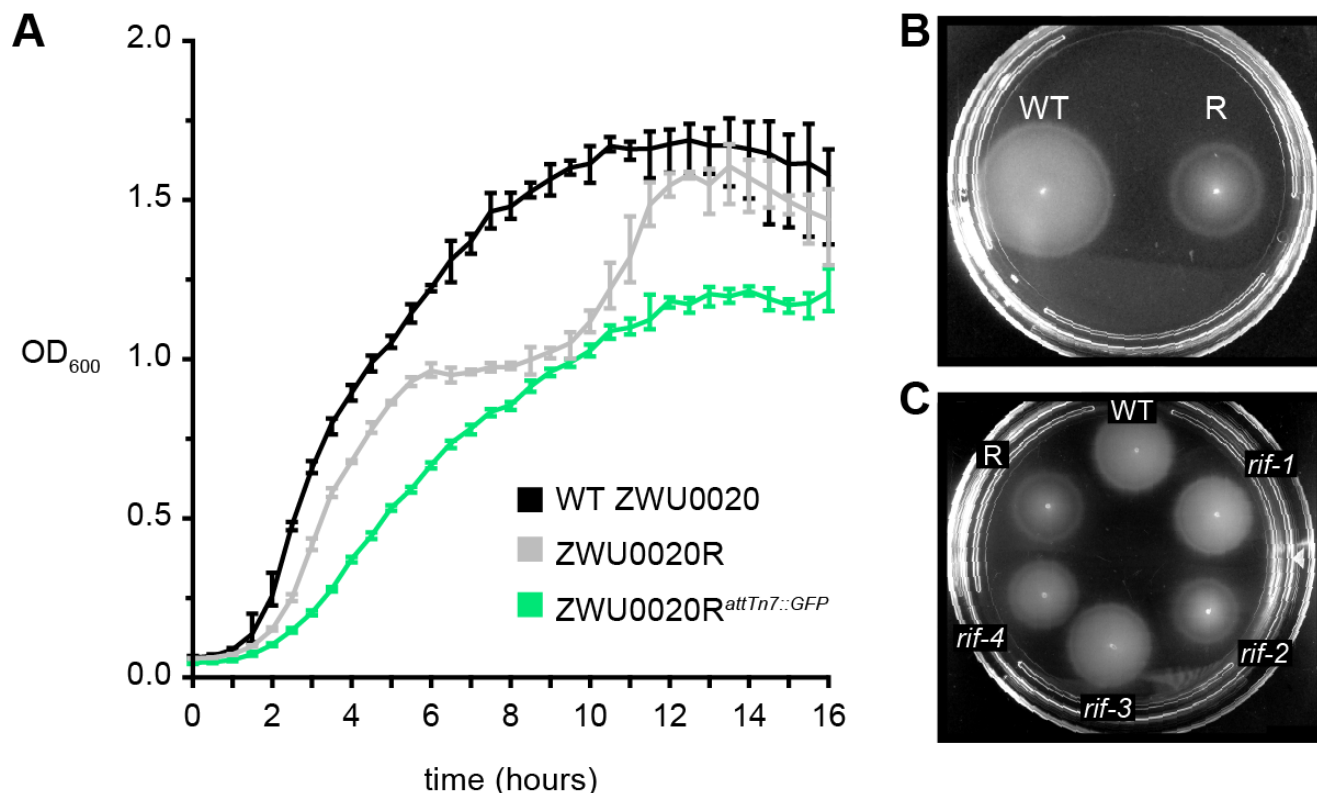
116 Faced with this scenario, we turned to domestication, which is the process of modifying
117 target strains with a selective trait that is not expressed by the donor. This is commonly done

118 by isolating target strain variants with spontaneous antibiotic resistance. However, despite the
119 wide use of this technique, antibiotic resistances associated with domestication (e.g., to
120 rifampicin or streptomycin) typically arise because of mutations within critical cellular
121 machinery, such as RNA polymerase or components of the ribosome, which can severely
122 impair the natural physiology of bacteria and render them unsuitable for study^{19–25}. Consistent
123 with the deleterious consequences of domestication, we found that a rifampicin-resistant
124 variant of the zebrafish intestinal symbiont *Vibrio cholerae* ZWU0020, denoted ZWU0020R,
125 exhibits highly perturbed growth kinetics in vitro (Figure 1A). In addition, although
126 domestication of *Vibrio* ZWU0020 did allow us to successfully insert a gene encoding green
127 fluorescent protein (GFP) within its genome, this modification further aggravated its poor
128 growth phenotype (Figure 1A). As another assessment of ZWU0020R's altered physiology, we
129 characterized its motility phenotype and observed that it displays attenuated swimming in soft
130 agar compared to wild-type (Figure 1B). To determine if altered swimming is a phenotype of all
131 rifampicin-resistant *Vibrio* ZWU0020 variants, we inspected the motility phenotype of four
132 independently derived clones. Interestingly, two of the clones are attenuated like the original
133 ZWU0020R strain, whereas the other two perform similar to wild-type, suggesting that they
134 may carry alternative and/or compensatory mutations (Figure 1C). Altogether, our experience
135 attempting to genetically manipulate a variety of novel zebrafish bacterial symbionts using
136 conventional approaches highlights the limitations of current counterselection strategies and
137 the deleterious nature of domestication.

138

139

Figure 1



140

141 **Figure 1.** Domestication results in physiological defects. (A) Plotted is the average optical
142 density at 600nm (OD₆₀₀) vs. time (hours) of wild-type (WT) *Vibrio* ZWU0020, and its
143 rifampicin-domesticated derivatives ZWU0020R and ZWU0020R^{attTn7::GFP}, during shaking
144 growth in LB broth at 30°C. Range bars are based on four technical replicates. (B) Swim
145 motility of WT and ZWU0020R (R) in 0.2% tryptic soy agar at 30°C. (C) Swim motility of four
146 rifampicin-resistant (*rif*) *Vibrio* ZWU0020 variants compared to WT and ZWU0020R performed
147 as in B.

148

149

150 **Temperature and kill switch-based systems for domestication-free counterselection of**
151 **donor cells.**

152 To address the lack of adequate post-conjugation counterselection methods, we set out to
153 develop strategies that are technically straightforward and not reliant on inherent or
154 domesticated traits of target strains. We devised two plasmid-based counterselection systems

155 that control donor cell growth by a mechanism similar to that of common suicide vectors. The
156 first system is temperature-based and works through a temperature-sensitive origin of
157 replication that restricts donor cell growth in the presence of antibiotic selection at or above
158 37°C. Temperature-based control of plasmid replication is well established, but has not been
159 widely implemented as a method of post-conjugation counterselection despite its amenability
160 and previous indications that it can be used in this way²⁶. The second system restricts donor
161 cell growth through a genetic kill switch that, when induced, leads to the expression of three
162 toxic peptides. These two approaches differ in their mode of action and offer slightly different
163 procedural advantages. Notably, we chose to develop plasmid-based counterselection
164 systems because their portability allows the use of alternative donor strains. To initially test the
165 utility of each counterselection system, we incorporated them into existing vectors that are
166 commonly used for making targeted Tn7 transposon-based chromosomal insertions²⁷.

167 Temperature-based counterselection was achieved by replacing the R6K origin of
168 replication of the Tn7-tagging vector pUC18R6KT-mini-Tn7T-GM (pTW56) with the
169 temperature-sensitive origin of replication *ori*₁₀₁/*repA*101^{ts}²⁸ (Figure 2A). The resulting vector,
170 pTn7xTS (**T**emperature-**S**ensitive), mediates temperature-dependent growth of *E. coli* SM10
171 (Figure 2-Figure Supplement 1). At the permissive temperature of 30°C, SM10/pTn7xTS grows
172 normally on rich media in the presence of antibiotic selection. At the restrictive temperature of
173 37°C, the vector is unable to be maintained, leading to loss of antibiotic resistance and a drop
174 in viability by up to three orders of magnitude (Figure 2-Figure Supplement 1). In the context of
175 an example Tn7-tagging protocol, conjugation is performed at 30°C without antibiotic selection
176 between two SM10 donor strains and a *Vibrio* target strain (Figure 2B, left). The SM10 donors
177 carry either pTn7xTS (donor^{Tn}) or the transposase-encoding helper plasmid pTNS2

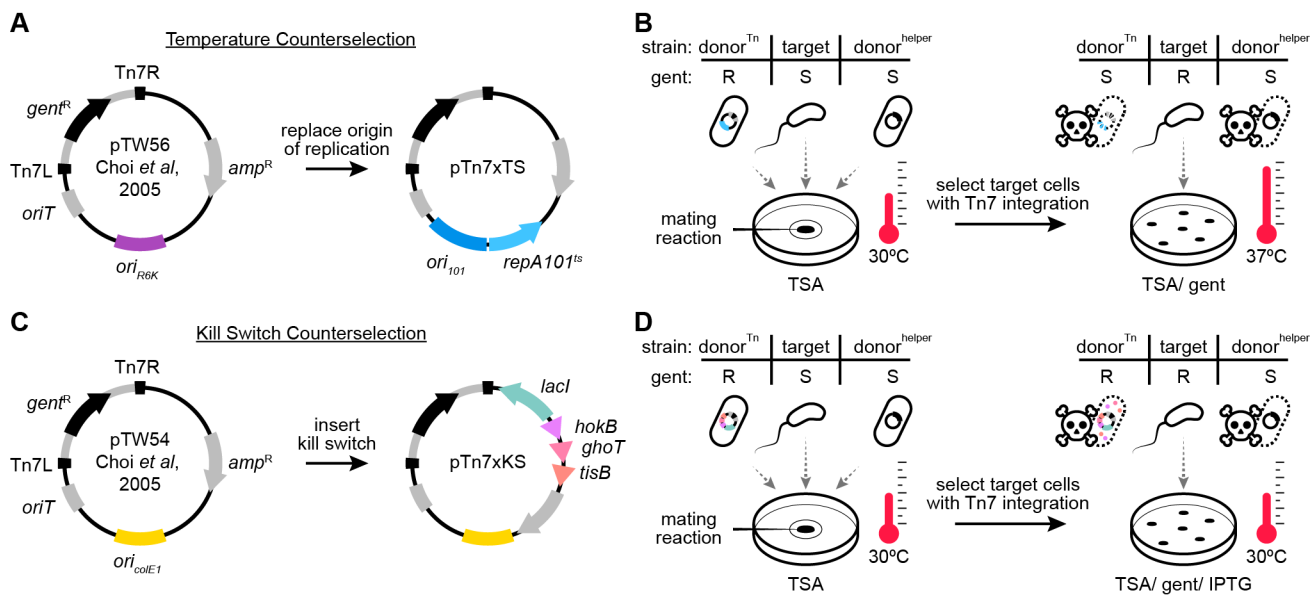
178 (donor^{helper}). At this point in the procedure, only the donor^{Tn} strain is resistant to the selective
179 antibiotic being used, which in this scenario is gentamicin. Successfully modified *Vibrio* cells
180 harboring a chromosomal copy of the Tn7 transposon, along with the gentamicin resistance
181 gene it encodes, are then selected for by plating the mating mixture in the presence of
182 gentamicin at 37°C (Figure 2B, right). The donor^{Tn} strain is counterselected because it is
183 unable to maintain plasmid-based resistance at 37°C, whereas the donor^{helper} strain remains
184 sensitive to gentamicin throughout the procedure.

185 A strength of temperature-based counterselection is that it is technically simple,
186 requiring only a shift in growth temperature, but it is limited to target strains that can grow at
187 37°C. This constraint is problematic for several bacterial lineages native to zebrafish as well as
188 other ectotherms, such as stickleback or fruit flies, which cannot survive at temperatures above
189 the growth temperature of their host (in these cases, $\leq 30^\circ\text{C}$). Therefore, we developed a
190 second strategy based on an inducible kill switch that functions independently of growth
191 temperature.

192 The kill switch we designed consists of two elements: a constitutively expressed *lacI*
193 gene, which encodes the lac repressor, and a synthetic operon containing three *E. coli*-derived
194 genes encoding toxic peptides—HokB, GhoT, and TisB—placed under the control of the LacI-
195 repressible promoter P_{tac} (Figure 2-Figure Supplement 2A)^{29–33}. Upon induction by the
196 allolactose analogue isopropyl- β -D-thiogalactoside (IPTG) these toxic peptides act to disrupt
197 the proton-motive force within donor cells, leading to impaired ATP synthesis and death. We
198 built this kill switch counterselection system into the backbone of the Tn7-tagging vector
199 pUC18T-mini-Tn7T-GM (pTW54), producing pTn7xKS (**K**ill **S**witch) (Figure 2C). In the
200 presence of antibiotic selection and IPTG, pTn7xKS is capable of inhibiting SM10 growth by up

201 to four orders of magnitude (Figure 2-Figure Supplement 2B). Of note, initial kill switch
 202 prototypes carrying only a single toxin gene were less potent, which may be an important
 203 consideration in future kill switch designs (Figure 2-Figure Supplement 2B). In the context of a
 204 Tn7-tagging scenario, kill switch-based counterselection is carried out in much the same way
 205 as temperature-based counterselection, except that selection of modified target cells is done
 206 on media containing IPTG at a growth temperature suitable for the target strain being used
 207 (Figure 2D).

Figure 2



210

211 **Figure 2.** Construction and application of domestication-free counterselection systems. **(A)**
 212 Temperature-based counterselection was achieved by replacing the R6K origin of replication
 213 (*ori_{R6K}*) of pUC18R6KT-mini-Tn7T-GM (pTW56) with the temperature-sensitive origin of
 214 replication *ori₁₀₁/repA101^{ts}*. Tn7L and Tn7R inverted repeats flank the Tn7 transposon (gray
 215 stroke). *gent^R*, gentamicin resistance gene; *amp^R*, ampicillin resistance gene; *oriT*, origin of
 216 transfer. **(B)** Left: triparental conjugation between SM10 donor strains carrying either a
 217 temperature-sensitive Tn7-tagging vector (donor^{Tn}) or transposase helper vector (donor^{helper})
 218 and a *Vibrio* target strain. Gentamicin (gent) phenotype of each strain is indicated as resistant
 219 (R) or sensitive (S). Mating reactions are incubated at 30°C upon a filter disc on a tryptic soy

220 agar (TSA) plate. Right: post-conjugation counterselection of donor cells is done on TSA/ gent
221 plates at 37°C. **(C)** Kill switch-based counterselection was achieved by inserting a LacI-
222 regulated toxin array, comprised of the genes *hokB*, *ghoT*, and *tisB*, into the backbone of
223 pUC18T-mini-Tn7T-GM (pTW54). *ori_{ColE1}*, high copy number origin of replication. **(D)** Left:
224 triparental conjugation as in **B**, except donor^{Tn} carries a kill switch Tn7-tagging vector. Right:
225 post-conjugation counterselection of donor cells is done on TSA/ gent/ IPTG plates at 30°C.

227

228 ***Chromosomal insertion of rationally designed gene expression scaffolds into wild and***
229 ***uncharacterized bacterial lineages using domestication-free counterselection systems.***

230 To test the effectiveness of our domestication-free counterselection systems, we employed
231 them to integrate genetically encoded fluorescent proteins into the chromosome of various
232 uncharacterized zebrafish bacterial symbionts. However, while exploring available gene
233 expression constructs, we found that many are inflexible and inadequately designed.
234 Specifically, vectors often contain extraneous DNA sequences left over from previous
235 imprecise subcloning procedures and have little to no options for customizing important
236 sequence motifs. The ability to customize expression constructs is critical when working with
237 lineages that differ in, for instance, optimal promoter sequences or ribosome binding sites.
238 Therefore, we first addressed the need for standardized expression constructs by rationally
239 designing a modular gene expression scaffold.

240 An expression scaffold containing four interchangeable elements—a promoter, 5' and 3'
241 untranslated regions (UTR), and an open reading frame (ORF)—was built into the multiple
242 cloning site (mcs) of pGEN-mcs³⁴, producing pXS (e**X**pression **S**caffold) (Figure 3). pGEN-mcs
243 was chosen to house the expression scaffold because it enables fast and easy prototyping of
244 scaffold parts in *E. coli*, which like many zebrafish bacterial symbionts, is a member of the
245 Gammaproteobacteria and shares basic genetic control elements. Restriction sites underlie

246 the modular architecture of the scaffold and allow each part to be customized (Figure 3-Figure
247 Supplement 1A). Sequence motifs can be replaced individually or the entire scaffold can be
248 subcloned. As initially built, a minimal P_{tac} promoter without the lac operator sequence, which
249 avoids interference from an endogenously encoded lac repressor if present, is used to achieve
250 constitutive transcription. A synthetic 5' UTR containing both an epsilon enhancer sequence
251 and ribosome binding site controls translation^{35,36}. The 3' UTR, which was originally present
252 within pGEN-mcs, contains a *trpL* attenuator sequence for transcriptional termination³⁷. Lastly,
253 three different ORFs encoding the fluorescent proteins sfGFP³⁸, dTomato³⁹, and mPlum⁴⁰ were
254 each used to produce three separate expression scaffold variants, pXS-sfGFP, pXS-dTomato,
255 and pXS-mPlum (Figure 3-Figure Supplement 1A). After assembly, each expression scaffold
256 was subcloned into Tn7-tagging vectors with either temperature (i.e., pTn7xTS) or kill switch
257 (i.e., pTn7xKS) counterselection systems (Figure 3-Figure Supplement 1B and C).

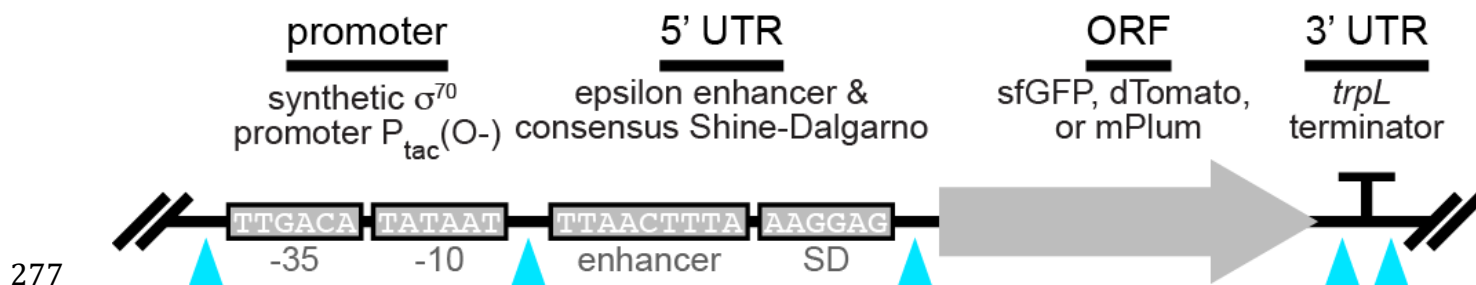
258 Tn7-tagging vectors equipped with rationally designed expression scaffolds were next
259 used to carry out chromosomal tagging of *Vibrio* ZWU0020 as outlined in Figure 2B and 2D.
260 Unlike previous attempts (Figure 1A), domestication-free manipulation of *Vibrio* ZWU0020—
261 using either temperature or kill switch counterselection systems—both worked and preserved
262 this strain's normal physiology (Figure 3-Figure Supplement 2). To demonstrate the
263 compatibility of these tools across different zebrafish symbiont lineages, we tagged 10 strains
264 representing 6 different genera—including *Vibrio*, *Aeromonas*, *Pseudomonas*, *Acinetobacter*,
265 *Enterobacter*, and *Plesiomonas* (Supplementary File 1). Multiple variants that express either
266 *sfGFP*, *dTomato*, or *mPlum* were generated for many of the lineages (Supplementary File 1).
267 This collection of marked zebrafish symbionts serves as a resource of ready-made strains for
268 the zebrafish microbiota community. Notably, several of the lineages manipulated are novel

269 and have yet to be assigned species designations, which is typical of symbionts associated
270 with complex host-associated communities. We also verified that domestication-free
271 counterselection systems can be used to manipulate other species not associated with the
272 zebrafish intestinal microbiota, including a human-derived strain of *E. coli* (HS)⁴¹, the leech
273 symbiont *Aeromonas veronii* HM21⁴², and an environmental isolate of *Shewanella oneidensis*
274 (MR-1)⁴³ (Supplementary File 1).

275

276

Figure 3



278 **Figure 3.** Gene expression scaffold design features. Each interchangeable element is flanked
279 by restriction sites (cyan arrowheads). Promoter: constitutively active P_{tac} promoter without lac
280 operator sequence (O-) drives transcription. 5' untranslated region (UTR): epsilon enhancer
281 sequence and consensus ribosome binding site (i.e., Shine-Dalgarno sequence) promote
282 strong translation. Open reading frame (ORF): encodes a single fluorescent protein. 3' UTR:
283 *trpL* attenuator sequence terminates transcription.

284

285

286 ***Streamlining allelic exchange by visualizing homologous recombination events using a*** 287 ***fluorescent tracker***

288 Allelic exchange is a robust and versatile homologous recombination technique for making
289 targeted genetic knock-ins and knock-outs in bacteria⁴⁴⁻⁴⁶. Therefore, to extend the utility of
290 our domestication-free counterselection systems, we incorporated them into currently available

291 vectors that are used for mediating allelic exchange. As expected, these updates facilitated the
292 domestication-free engineering of gene deletions in several uncharacterized symbiotic
293 bacteria. However, not all bacteria tested could be successfully manipulated using current
294 allelic exchange protocols, highlighting another breakdown in the compatibility.

295 Allelic exchange involves two successive homologous recombination events between
296 an allelic exchange vector and the bacterial chromosome. The crux of allelic exchange is
297 isolating rare unmarked mutant cells from large populations of heterozygous intermediates
298 known as merodiploids that arise after the vector integrates into the chromosome during the
299 first recombination step. A longstanding strategy for recovering variants that have undergone
300 the second recombination, which results in vector loss, works by restricting merodiploid
301 growth. This is typically done by expressing a gene called *sacB* located within the allelic
302 exchange vector backbone that confers growth inhibition in the presence of sucrose⁴⁷.
303 Although widely used, *sacB* counterselection of merodiploids does not always work and can be
304 difficult to troubleshoot when it fails. We experienced these shortcomings while attempting to
305 delete a gene associated with chemotactic behavior in a zebrafish symbiont, *Vibrio furnissii*
306 ZOR0035, using the common *sacB*-based allelic exchange vector pDMS197⁴⁸. *Vibrio*
307 ZOR0035 merodiploids are refractory to *sacB* counterselection, which made it impossible to
308 isolate cells with the desired mutation. We surmise that the counterselection fails in some
309 bacterial lineages because the expression or activity of the levansucrase enzyme encoded by
310 *sacB*, which synthesizes high-molecular-weight fructose polymers, is inadequate. To overcome
311 lineage-specific limitations of *sacB* counterselection, we developed a more tractable strategy
312 based on visual markers.

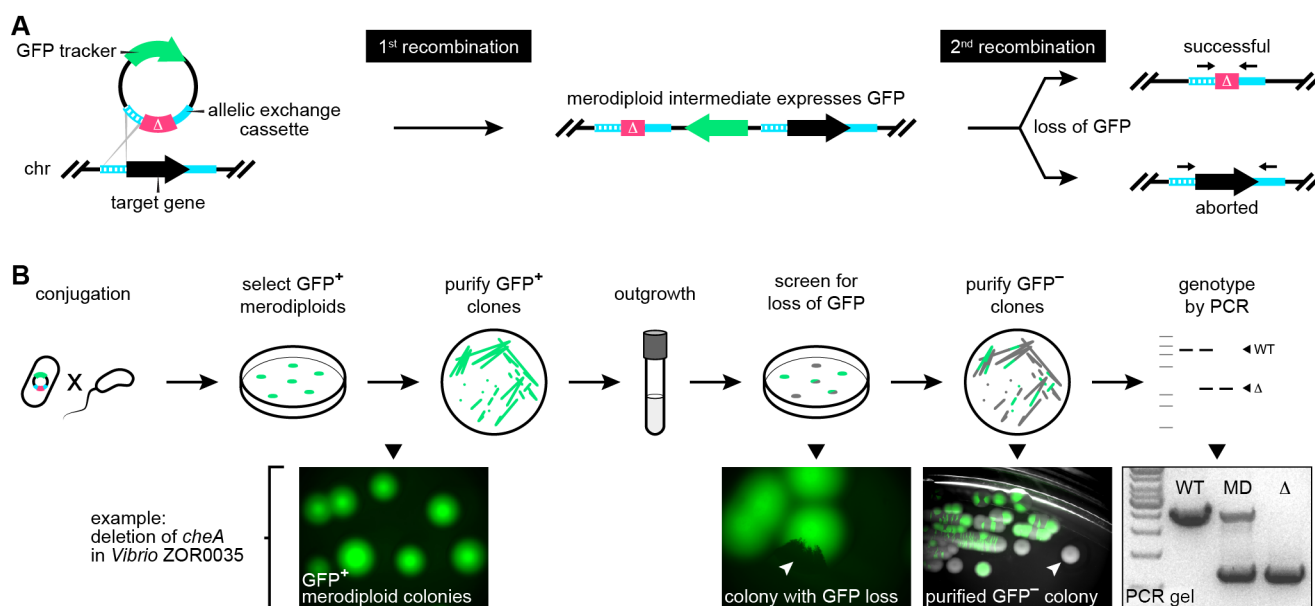
313 Our solution uses GFP to track the merodiploid status of target cells (Figure 4A). In this
314 way, the initial recombination step generates GFP-positive merodiploid populations that can be
315 readily screened for cells where the second recombination step has occurred, producing GFP-
316 negative mutants (i.e., instances of “successful” allelic exchange), which typically occur with
317 equal frequency as wild-type revertants (i.e., instances of “aborted” allelic exchange) (Figure
318 4A). To test the feasibility of this approach, we revisited the engineering of a gene deletion in
319 *Vibrio* ZOR0035. A constitutively expressed GFP gene was inserted into the backbone of a
320 prototype pDMS197 vector containing a kill switch counterselection system and an allelic
321 exchange cassette targeting the chemotaxis gene *cheA*. At the time of this work, *cheA* was the
322 focus of an unrelated project, and it is used here merely to demonstrate proof of concept. As
323 outlined in Figure 4B, the GFP marked allelic exchange vector was delivered into *Vibrio*
324 ZOR0035 via conjugation. GFP-positive merodiploids, harboring an integrated copy of the
325 allelic exchange vector, were readily isolated and purified. Of note, over the course of this work
326 we empirically determined that the kill switch toxins do not interfere with merodiploid growth in
327 several different bacteria, indicating that either they have restricted activity and are only lethal
328 to *E. coli* donor cells or they fail to reach toxic levels when expressed from a single
329 chromosomal locus. Next, populations of merodiploids were expanded in liquid culture and
330 plated on nonselective media at a density that allowed discrete colonies to form. Colonies
331 exhibiting sectorized regions of GFP loss were then purified to obtain isogenic clones, and
332 putative mutants were genotyped by polymerase chain reaction (PCR). Genotyping was done
333 using PCR primers that flank the *cheA* locus, yielding a single large amplification product if the
334 *cheA* gene is present and a smaller sized product if the mutant allele is present. Because they
335 are heterozygous, merodiploids produce both products. Ultimately, our visual merodiploid

336 tracking strategy proved extremely efficient and straightforward to perform, allowing us to
337 successfully engineer a targeted gene deletion in a bacterial strain that was otherwise
338 genetically intractable using previous methodologies.

339

340

Figure 4



341

342 **Figure 4.** Performing allelic exchange with a fluorescent merodiploid tracker. **(A)** Outline of
343 recombination events during allelic exchange with a fluorescent tracker. Depicted is an allelic
344 exchange vector that expresses GFP and carries a cassette comprised of a mutant allele (Δ ,
345 magenta) flanked by regions (hashed and solid cyan strokes) homologous to regions flanking a
346 target gene located on the bacterial chromosome (chr). The first recombination event—which
347 randomly occurs between either homology region—integrates the vector into the chromosome,
348 producing a GFP-expressing merodiploid. The second recombination event results in GFP
349 loss. If it occurs between the unused homology region (i.e., the “solid” region in this scenario),
350 then allelic exchange is successful. If it occurs between the same region (i.e., the “hashed”
351 region), the original wild-type locus is restored. Black arrows above final allelic exchange
352 products denote primer annealing sites for PCR-based genotyping depicted in **B**. **(B)** Top row
353 illustrates the procedural steps of allelic exchange using a fluorescent merodiploid tracker.
354 Bottom row shows example images acquired during the engineering of a gene deletion in
355 *Vibrio ZOR0035*. White arrowheads indicate colonies with partial or complete loss of GFP
356 expression. WT, wild-type *Vibrio ZOR0035*; MD, merodiploid; Δ , Δ *cheA* mutant.

357

358 ***Gene deletion and complementation with modernized engineering vectors***

359 To complete the genetic toolkit for manipulating wild and diverse bacterial isolates, we
360 combined the tools and approaches described thus far to construct a set of adaptable allelic
361 exchange vectors that further improve the tractability of making markerless genetic alterations.
362 These modernized vectors incorporate fluorescent merodiploid tracking and domestication-free
363 counterselection systems within a highly customizable plasmid architecture (Figure 5, “vector
364 design”). Molecular scaffolds for holding antibiotic selection markers, fluorescent trackers, and
365 a counterselection kill switch were built into a pUC-derived vector backbone that has a
366 temperature-sensitive origin of replication and a single blunt restriction site for straightforward
367 insertion of allelic exchange cassettes. This modular design allows virtually every functional
368 element to be customized for different bacterial lineages (Figure 5-Figure Supplement 1).

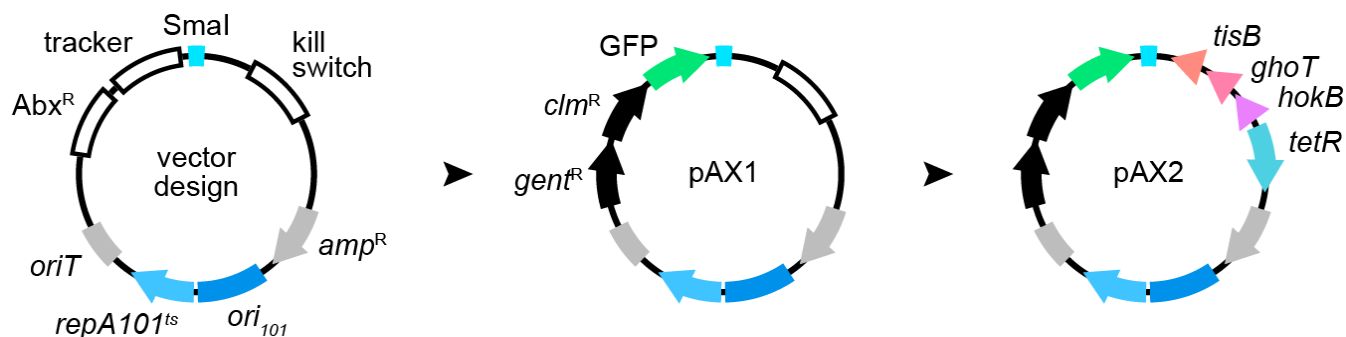
369 In total, two allelic exchange vectors were generated, pAX1 and pAX2 (**A**llelic
370 **e**Xchange), which differ in their domestication-free counterselection systems (Figure 5). Both
371 vectors mediate temperature-based counterselection of SM10 donor cells, but pAX2 also
372 contains a TetR-regulated kill switch that can be induced by anhydrotetracycline (Figure 5-
373 Figure Supplement 2). Notably, the dual temperature/kill switch counterselection activity of
374 pAX2 is quite potent, reducing SM10 viability by over five orders of magnitude (Figure 5-Figure
375 Supplement 2B). Two resistance markers for gentamicin and chloramphenicol were included to
376 give pAX1 and pAX2 greater “off the shelf” compatibility with different target strains.

377

378

Figure 5

379



380 **Figure 5.** Rational design of customizable allelic exchange vectors. “vector design” illustrates
381 vector architecture. Features include customizable molecular scaffolds for holding antibiotic
382 selection markers (*Abx^R*), a merodiploid tracker, a single *SmaI* restriction site for insertion of
383 allelic exchange cassettes, and an option for kill switch-based counterselection of donor cells.
384 pAX1 was initially constructed, and carries two antibiotic selection markers encoding
385 resistance to gentamicin (*gent^R*) and chloramphenicol (*clm^R*) along with a GFP tracker. pAX2
386 was derived via the insertion of a tet-inducible kill switch. *oriT*, origin of transfer;
387 *ori₁₀₁/repA101^{ts}*, temperature-sensitive origin of replication; *amp^R*, ampicillin resistance gene.

388

389

390 These new allelic exchange vectors were next used to engineer markerless gene
391 deletions. For this proof of concept, we designed an allelic exchange cassette to delete two
392 neighboring genes in *Vibrio* ZWU0020, *pomA* and *pomB*, which encode components of the
393 polar flagellar motor. After inserting the cassette into an early, but functionally equivalent,
394 version of pAX1 (see Materials and Methods), GFP-positive merodiploids were generated and
395 isolated as before and screened for loss of GFP expression and thus, the integrated vector
396 (Figure 6-Figure Supplement 1). Mutants harboring the desired mutation, which fused the start
397 codon of *pomA* with the stop codon of *pomB*, were confirmed by PCR (Figure 6A and 6B). As
398 anticipated, ZWU0020 $\Delta pomAB$ exhibited complete loss of swimming motility in soft agar
399 (Figure 6C, bottom left) without overt growth defects in liquid media (Figure 6-Figure

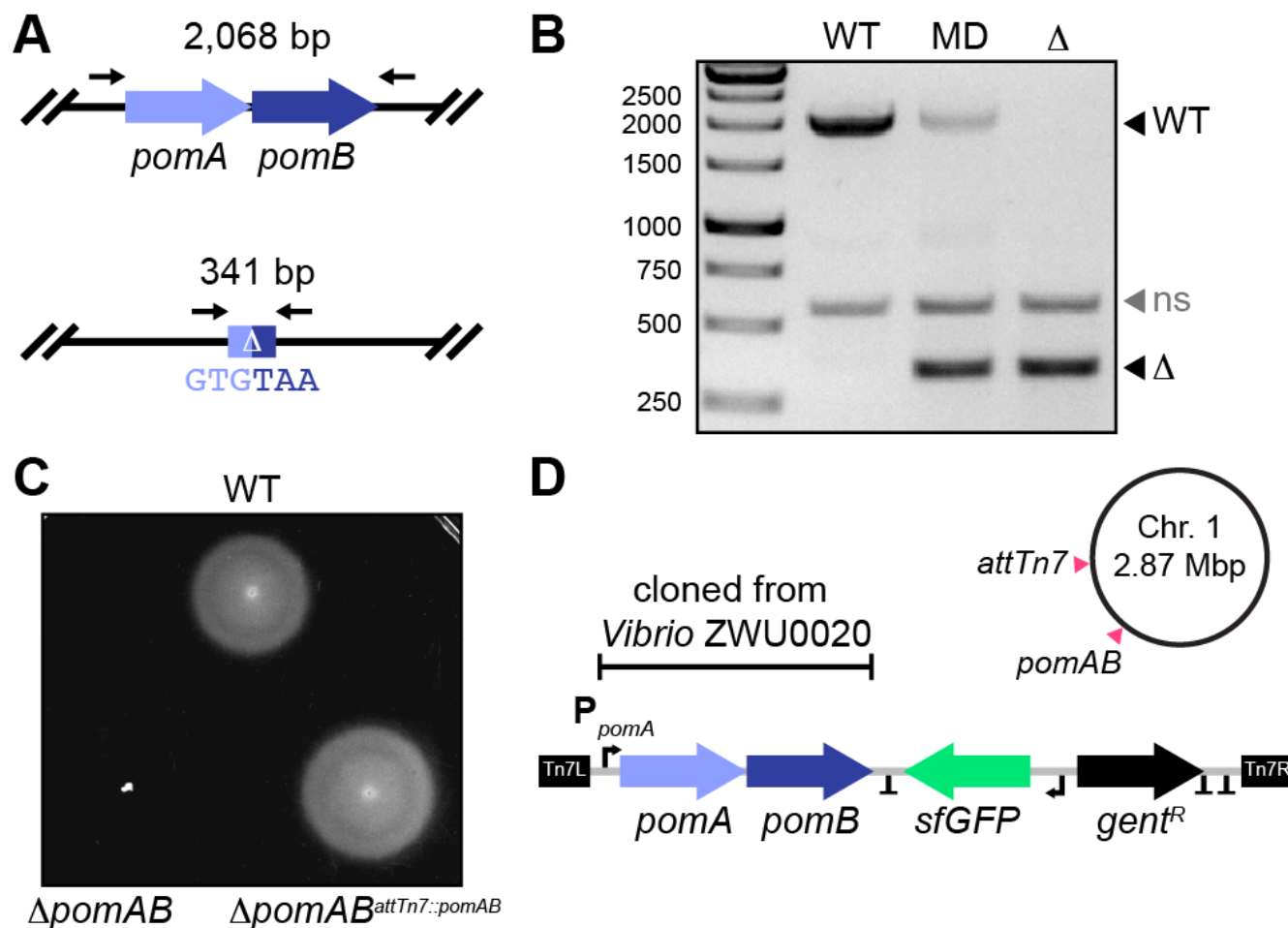
400 Supplement 1). To demonstrate the cross-lineage compatibility of these tools, we successfully
401 employed pAX2 to create a similar markerless deletion of a homologous *pomAB* locus in
402 another zebrafish symbiont, *Aeromonas veronii* ZOR0001 (Figure 6-Figure Supplement 2).
403 Notably, while screening *Vibrio* ZWU0020 and *Aeromonas* ZOR0001 merodiploid colonies for
404 putative mutants, we observed that many different patterns of GFP loss can arise (Figure 6-
405 Figure Supplement 3). Remarkably, even in situations where mutant cells reside within
406 miniscule GFP-negative patches, a single colony purification step can often be used to isolate
407 them. The ability to readily identify and recover mutant cells with such sensitivity highlights the
408 robustness of our visual screening approach.

409 Constructing deletion mutants is just the first step in dissecting the genetic pathways
410 that underlie a given activity or behavior. Complementation must also be performed to
411 rigorously confirm that a mutant phenotype is the result of a specific genetic disruption and not
412 polar effects or other unintended consequences of chromosomal manipulation¹⁵. Therefore, we
413 wanted to demonstrate how our domestication-free Tn7-tagging vectors could be employed to
414 complement the ZWU0020 $\Delta pomAB$ mutant. The *pomAB* locus of ZWU0020, including the
415 native *pomA* promoter, was PCR-amplified and inserted within the Tn7 transposon of the
416 tagging vector pTn7xTS-sfGFP, which also contains a constitutively expressed *sfGFP* (Figure
417 6D). Chromosomal insertion of this construct at the *attTn7* site of ZWU0020 $\Delta pomAB$ fully
418 restores wild-type motility, thus confirming that sole disruption of these genes caused the loss
419 of motility in the mutant (Figure 6C, bottom right).

420

421

Figure 6



422

423 **Figure 6.** Gene deletion and complementation with modernized engineering vectors. (A) Top:
 424 wild-type *pomAB* locus in *Vibrio ZWU0020*. Bottom: result of markerless *pomAB* deletion via
 425 allelic exchange. Black arrows mark approximate primer annealing sites for genotyping and the
 426 size of each amplification product is indicated. (B) Agarose gel showing PCR-based
 427 genotyping of wild-type (WT), merodiploid (MD), and a $\Delta pomAB$ (Δ) mutant. Migration
 428 distances of WT and mutant alleles are indicated. ns, nonspecific amplification product. (C)
 429 Swim motility of WT, $\Delta pomAB$, and the complemented $\Delta pomAB^{attTn7::pomAB}$ variant in 0.2%
 430 tryptic soy agar at 30°C. (D) Shown is a schematic of the Tn7 transposon from pTn7xTS-
 431 sfGFP used for complementation, which was modified to carry the native *pomAB* locus of
 432 *Vibrio ZWU0020*. Also depicted are the relative positions of where the *pomAB* genes were
 433 deleted and reintroduced at the Tn7 insertion site (*attTn7*) on chromosome 1 of *Vibrio*
 434 *ZWU0020*. “T” denotes transcriptional terminators; Tn7L and Tn7R, Tn7 inverted repeats,
 435 P_{*pomA*}, native *pomA* promoter; *gent^R*, gentamicin resistance gene; sfGFP, fluorescent tag.

436

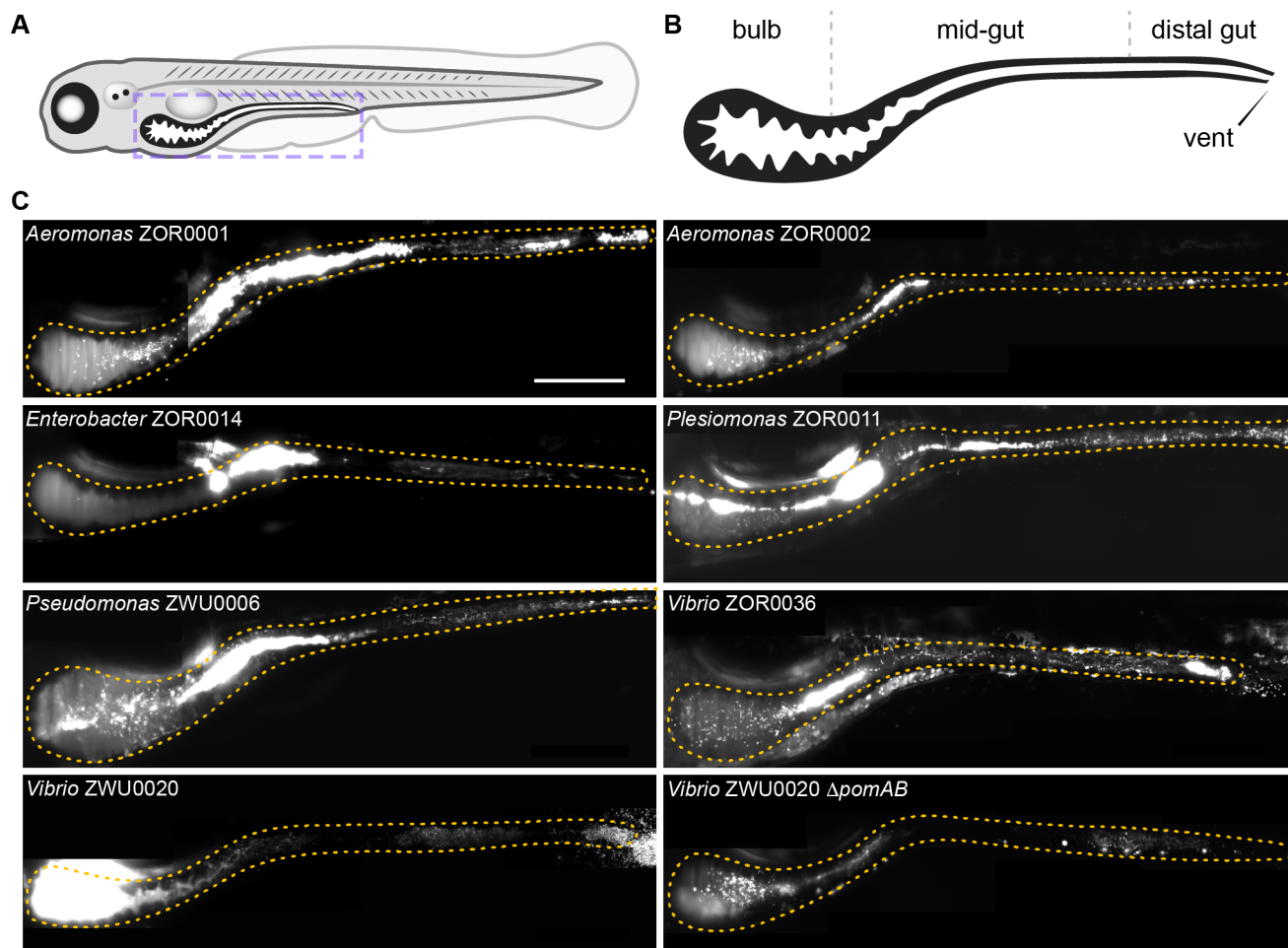
437 ***Live imaging of diverse bacterial symbionts yields insights into host–microbe***
438 ***interactions within the vertebrate intestine.***

439 The power to genetically manipulate a diverse range of bacterial symbionts provides an
440 opportunity for comparative studies focused on identifying unique or broadly conserved
441 features of host–microbe interactions. To illustrate how the tools developed in this work
442 facilitate such investigations, we examined the intestinal colonization patterns and cellular
443 behaviors of seven fluorescently tagged zebrafish symbionts by light sheet fluorescence
444 microscopy^{13,49}. The strains chosen included: *Aeromonas* ZOR0001, *Aeromonas* ZOR0002,
445 *Enterobacter* ZOR0014, *Plesiomonas* ZOR0011, *Pseudomonas* ZWU0006, *Vibrio* ZOR0036,
446 and *Vibrio* ZWU0020. A tagged version of the ZWU0020 $\Delta pomAB$ motility mutant was also
447 analyzed. Prior to imaging, bacteria were associated individually with 4-day old, germ-free
448 larval zebrafish for 24 hours. Mono-association provides unrestricted access to the intestinal
449 environment free of competition with resident microbiota, and therefore allows interactions
450 between a single symbiont and its host to be studied in isolation. For each strain, real-time
451 two-dimensional movies and three-dimensional images spanning the entire volume of the
452 larval intestine were acquired from three separate hosts (Figure 7, Figure 7-Figure Supplement
453 1, and Supplementary Movies 1–8). From these data, distinct population structures are readily
454 identified, and can be summarized by three properties: cell motility, growth mode (i.e.,
455 planktonic vs aggregated), and biogeography. Additional features of each strain, including
456 estimated abundance, are provided in Figure 7-Figure Supplement 2.

457

458

Figure 7



459

460 **Figure 7.** Intestinal colonization patterns and growth modes of zebrafish symbionts. (A)
461 Cartoon diagram of a 5-day old larval zebrafish. Purple dashed box outlines region imaged in
462 C. (B) Diagram shows the boundaries of the bulb, mid-gut, and distal gut within the larval
463 intestine. The estimated bulb to mid-gut boundary is located where the bulb begins to become
464 patently narrow. The mid-gut to distal gut boundary is approximately located where intestinal
465 epithelial cells begin transitioning to a more colonic epithelial cell type⁵⁰. (C) Maximum intensity
466 projections of 3D image stacks acquired by light sheet fluorescence microscopy for indicated
467 bacterial strains. Orange dotted outline marks the intestine in each image. Scale bar: 200μm.

468

469

470 Four of the seven wild strains examined display active motility within the intestine.

471 Surprisingly, we found several discrepancies between the motility phenotype of strains in vivo

472 and their predicted capacity for motility based on phylogenetic relatedness, genome sequence,
473 and performance during in vitro assays (Figure 7-Figure Supplement 3 and Supplementary
474 Movies 1–7). Each strain carries genes for flagellum biogenesis and displays free-swimming
475 motility in liquid media. In addition, all strains were found to swim in soft agar, with the
476 exception of *Vibrio* ZOR0036 (Figure 7-Figure Supplement 4). Yet despite these attributes,
477 motile individuals were not observed within intestinal populations of *Enterobacter* ZOR0014,
478 *Aeromonas* ZOR0001, and *Aeromonas* ZOR0002 (Supplementary Movies 2–4). We note that
479 in a previous live imaging study involving *Aeromonas* ZOR0001 motile cells were detected, but
480 that their occurrence was incredibly rare¹³. In addition, although *Vibrio* ZOR0036 exhibits little
481 motility in soft agar, it gives rise to a considerable number of highly motile cells in vivo
482 (Supplementary Movie 5); however, its overall motility phenotype is muted compared to closely
483 related *Vibrio* ZWU0020 (Figure 7-Figure Supplement 3 and 4, and Supplementary Movie 1).

484 The growth mode of cells is categorized as either planktonic, which includes both motile
485 and non-motile individuals, or aggregated. Both modes are typically represented across
486 different populations, but the ratio of cells in each mode tends to be strain-specific. For
487 example, at one extreme, populations of *Vibrio* ZWU0020 are almost entirely comprised of
488 highly motile planktonic cells; this is more apparent in movies than three-dimensional image
489 scans because of this strain's high population density and fast movement (Supplementary
490 Movie 1). At the opposite extreme, *Enterobacter* ZOR0014 forms large multicellular aggregates
491 without motile individuals (Figure 7C and Supplementary Movie 2). The remaining strains
492 produce populations with intermediate mixtures of planktonic and aggregated cells (Figure 7C
493 and Figure 7-Figure Supplement 1).

494 Regarding biogeography, we observed a range of strain-specific spatial distributions
495 along the length of the intestine. We can coarsely classify the location of bacterial populations
496 as primarily residing in one of two regions, the proximal gut (referred to as the “bulb”) or the
497 mid-gut, which we approximate in Figure 7B based on previous studies of larval zebrafish
498 intestinal development^{50,51}. For most strains, the bulk of their population is distributed
499 throughout the mid-gut (e.g., *Aeromonas* ZOR0001, *Aeromonas* ZOR0002, *Enterobacter*
500 ZOR0014, *Plesiomonas* ZOR0011, *Pseudomonas* ZWU0006, and *Vibrio* ZOR0036) (Figure
501 7C). By contrast, populations of *Vibrio* ZWU0020 are located within the proximal portion of the
502 bulb, consistent with previous findings (Figure 7C)¹³. Notably, compared to wild-type, the
503 ZWU0020 $\Delta pomAB$ motility mutant exhibits a reduction in overall population size and a slight
504 shift in distribution to an area between the bulb and mid-gut (Figure 7C), indicating that for this
505 strain, motility controls abundance and biogeography.

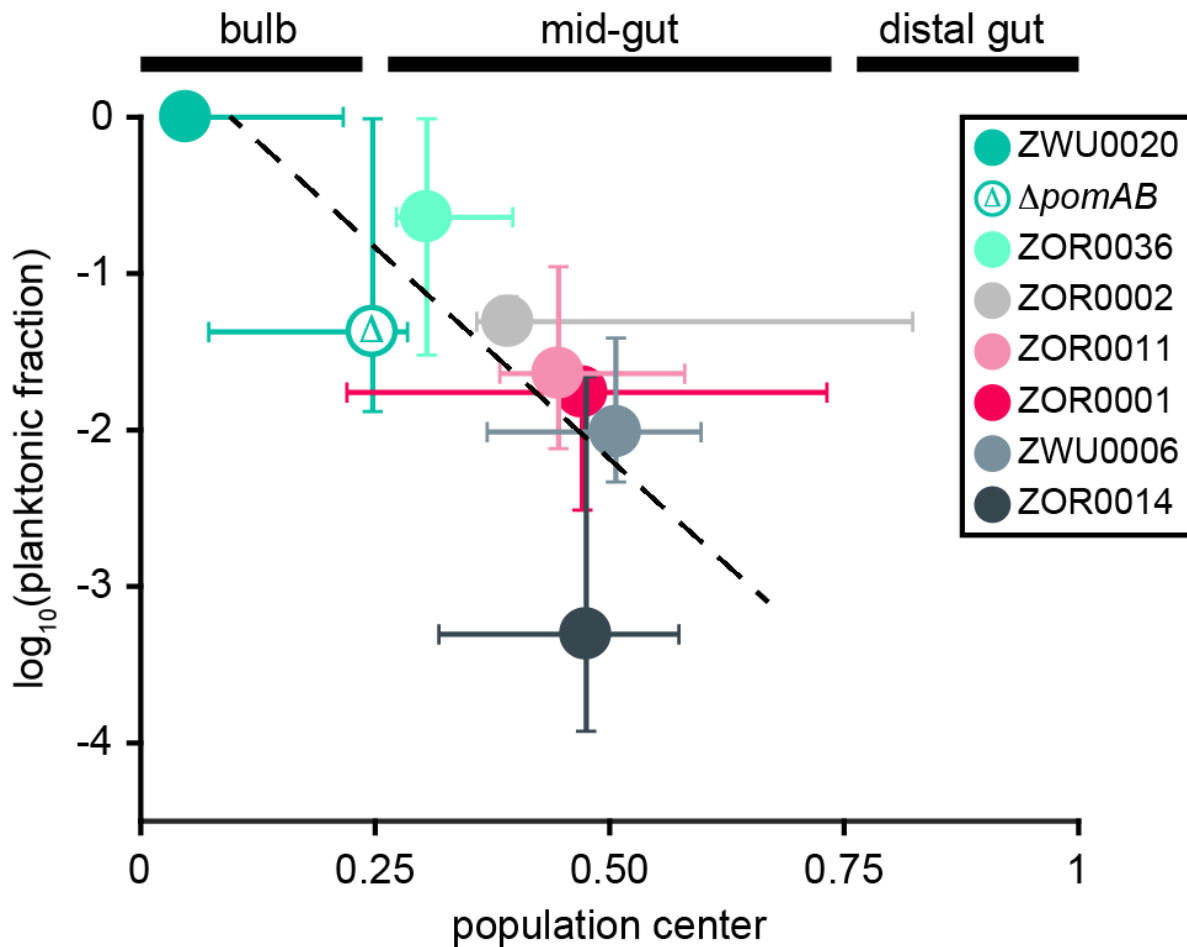
506 To distill and quantify our observations, we devised scalar metrics of biogeography and
507 growth mode using computational image analysis (Materials and Methods). For each
508 population, we computed the center of mass along the anterior–posterior axis of the intestine
509 to represent biogeography and enumerated the fraction of cells that exist as planktonic
510 individuals to represent growth mode. Plotting these data against each other shows a striking
511 and unanticipated relationship: the fraction of planktonic cells within a strain’s population is
512 strongly correlated with its biogeography (Figure 8). We quantified this relationship by linear
513 regression of log-transformed planktonic fraction and population center medians, excluding the
514 ZWU0020 $\Delta pomAB$ motility mutant (Figure 8, dashed trend line; $r^2 = 0.6762$). Remarkably, the
515 ZWU0020 $\Delta pomAB$ motility mutant conforms to this trend (Figure 8). As expected, populations
516 of ZWU0020 $\Delta pomAB$ contain no motile individuals, but unexpectedly they adopt a more

517 aggregated state compared to wild-type ZWU0020 (Supplementary Movie 8). This change in
518 growth mode and the concomitant change in biogeography moves the ZWU0020 motility
519 mutant along the trend line.

520

521

Figure 8



522

523 **Figure 8.** Relationship between growth mode and population biogeography. Plotted is the
524 median log-transformed fraction of planktonic cells and median population 1D center of mass
525 for each bacterial strain. Bars represent ranges. Data were derived from three animals (N = 3)
526 per strain and are based on segmented 3D images from Figure 7 and Figure 7-Figure
527 Supplement 1. Dashed trend line was generated by linear regression of median values (N = 7
528 data points, $r^2 = 0.6762$). Corresponding boundaries for the bulb, mid-gut, and distal gut are
529 indicated above the plot by black bars.

530
531

532 **DISCUSSION**

533 **Impact on Bacterial Symbioses Research**

534 Over the last decade, the landscape of bacterial symbiosis research has shifted due to an
535 explosion in omics technologies and large-scale initiatives like the Human and Earth
536 Microbiome Projects^{52,53}. The traditionally static “one host, one microbe” view has given way to
537 one that is more dynamic and complex, taking into account the highly contextual nature of
538 symbiotic relationships and involvement of diverse multi-member microbial communities. This
539 paradigm shift has generated several new challenges; chiefly, the demand for more efficient
540 genetic manipulation of wild and novel symbiotic bacterial lineages. Addressing this problem is
541 critical to experimentally unravelling the cellular and molecular determinants of host–microbe
542 systems.

543 The impetus behind the genetic tools and approaches described in this work emerged
544 from setbacks encountered while attempting to manipulate members of the zebrafish intestinal
545 microbiota. The diversity of species and strains exposed several key inadequacies and
546 weaknesses in conventional techniques, including the lack of domestication-free strategies for
547 donor cell counterselection, poor modularity of available tools, and intractability of allelic
548 exchange. Therefore, we designed tools and methods to circumvent these points of failure and
549 quickly adapt to unforeseen idiosyncrasies of species or strains. In this way, an individual
550 researcher or laboratory can focus on a single operating procedure using a centralized set of
551 tools while being empowered to innovate when needed.

552

553

554

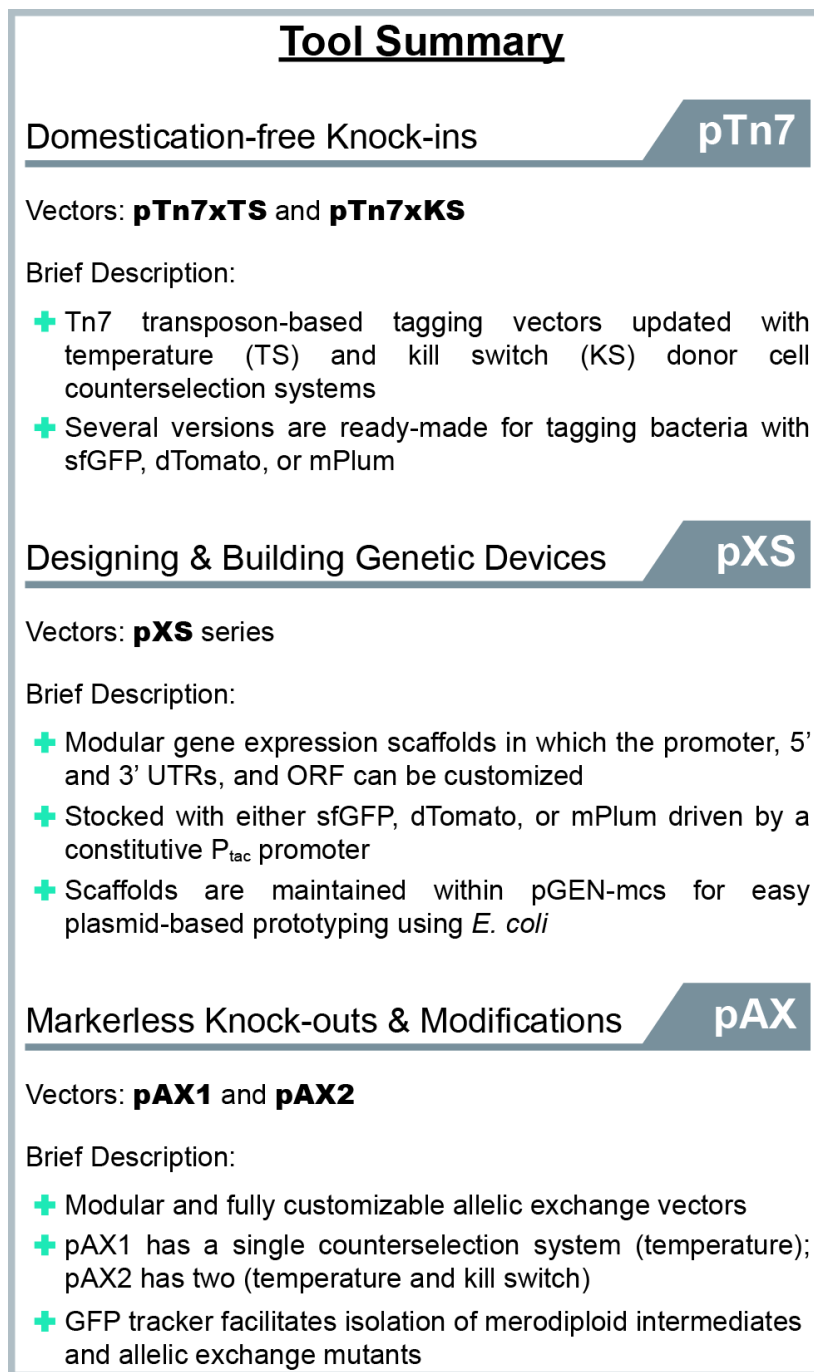
555 **Utility of the Tools Produced by this Work**

556 An extensive collection of molecular tools is available for genetically manipulating bacteria;
557 however, many can only be used with a small number of species or strains. The siloed nature
558 of genetic tools puts a significant burden on researchers looking to manipulate diverse
559 bacterial lineages because it forces them to sift through and become familiar with numerous
560 different vectors and protocols. Furthermore, for those working with novel and uncharacterized
561 bacteria or new to performing genetic manipulations altogether, developing a molecular starter
562 kit is overwhelming. We addressed these problems by constructing a set of standardized
563 engineering vectors that streamline the process of making genetic knock-ins and knock-outs
564 across different lineages. These tools are briefly summarized in Figure 9 and their features are
565 discussed below.

566

567

Figure 9



568

569 **Figure 9.** Summary of the genetic tools described in this work. The functions and features of
570 each engineering vector is briefly summarized. Guide is organized based on technique or
571 intended use.

572

573 *Minimizing laboratory-based domestication of wild bacterial isolates*

574 The deleterious nature of domestication is well-documented, yet it is often overlooked and
575 domestication steps are unfortunately routinely performed because of their convenience^{23,24}.
576 Domestication is commonly used to improve genetic tractability or to help discern specific
577 bacterial strains from other lineages within complex environments (e.g., the vertebrate
578 intestine, water, or soil). Compensatory mutations can rescue or mask physiological defects
579 associated with domestication in vitro²⁵; however, there is no guarantee that critical aspects of
580 symbiont biology, such as those involved in host engagement, are left unperturbed. Therefore,
581 the accurate modeling of symbiotic interactions requires careful attention to preserving natural
582 symbiont behaviors. The incorporation of temperature and kill switch-based domestication-free
583 counterselection systems into a previously described Tn7 tagging vector²⁷, which produced
584 pTn7xTS and pTn7xKS (Figure 2), and the novel allelic exchange vectors pAX1 and pAX2
585 developed in this work (Figure 5), offers ready-made tools for manipulating various symbiotic
586 bacterial lineages in a way that preserves their natural physiology.

587

588 *Achieving broad utility through modularity*

589 The incredible genetic and phenotypic diversity of bacteria challenges cross-lineage
590 compatibility of genetic tools. A major contributing factor to this problem is that many available
591 tools are irreversibly constructed, which impedes the customization of important sequence
592 motifs for different bacteria. We addressed this by building tools with highly modular
593 architectures so that they can be easily reconfigured and are thus molecularly nimble. This
594 feature makes it possible to continuously innovate and build off original designs. For example,
595 expanding on the expression scaffolds within the pXS series of vectors (Figure 3 and Figure 3-

596 Figure Supplement 1), we have engineered more elaborate genetic devices, including
597 reporters of gene expression and genetic switches. Additionally, the modularity of these
598 vectors can be advantageous in situations where rational design is not possible due to the
599 unavailability of suitable sequence elements; libraries in which constructs containing a single
600 variable motif (e.g., a promoter or ribosome binding site) can be readily assembled and
601 screened for optimal activity. While the flexibility of our expression scaffolds is conducive to
602 further engineering, as built they have immediate utility for stably tagging bacteria and thus,
603 facilitating the direct observation of symbionts within their natural host-associated
604 environments. Illustrating this point, the mere fluorescent tagging of the zebrafish symbionts
605 *Vibrio* ZWU0020 and *Aeromonas* ZOR0001 using these tools revealed that each bacterium
606 interacts with the physically dynamic confines of the intestine in distinct ways and that this
607 differential interplay unexpectedly shapes their apparent competition¹³. We also incorporated
608 modularity into the design of the allelic exchange vectors pAX1 and pAX2 (Figure 5 and Figure
609 5-Figure Supplement 1). Several elements within these vectors can be customized, including
610 the antibiotic resistance genes, the fluorescent merodiploid tracker, and the kill switch. With
611 this modularity, we are exploring alternative utilities of these vectors. For example, swapping
612 the GFP merodiploid tracker for one that encodes a red fluorescent protein has allowed us to
613 engineer GFP fusions at endogenous chromosomal loci. In total, tailorable tools such as those
614 described here offer a way of tuning or customizing functionality to increase the experimental
615 potential of bacterial lineages.

616

617

618

619 *Streamlining genetic manipulations with visual screening*

620 To improve the tractability of allelic exchange we used GFP to visually track recombination
621 events (Figure 4). This simple update proved extremely powerful. It allowed merodiploids to be
622 confidently identified and isolated while final mutant derivatives could be screened for with
623 incredible sensitivity, sometimes being found as small subpopulations within merodiploid
624 colonies growing on an agar plate (Figure 4 and Figure 6-Figure Supplements 1–3). The
625 successful manipulation of a previously intractable bacterium (i.e., *Vibrio* ZOR0035) highlights
626 the utility of this approach. Although conventional selection schemes (e.g., based on *sacB*) are
627 adept at recovering mutants that arise at low frequencies due to rarely occurring recombination
628 events, their use is contingent on specific conditions that can be difficult to translate between
629 species or strains. By contrast, our visual screening approach operates freely across lineages
630 and differs from selections in that it allows for the progression of recombination events to be
631 more precisely monitored. As a result, the engineering and isolation of bacterial mutants is
632 more efficient and attainable.

633

634 **Demonstrating New Avenues for Research: Comparative Study of Bacterial** 635 **Biogeography in the Zebrafish Gut**

636 The technical flexibility of the tools and methods developed in this work makes it easier to
637 genetically manipulate many different bacterial lineages in parallel. This functionality greatly
638 facilitates comparative studies aiming to disentangle unique and widely conserved aspects of
639 host–microbe systems. Such investigations are important because many properties of complex
640 host-associated microbial communities, like those comprising human microbiota^{54,55}, are
641 extremely variable and remain unexplained. Illustrating the potential for comparative

642 approaches, we exposed several uncharacterized phenomena by probing the colonization
643 patterns and behaviors of multiple bacterial lineages native to the zebrafish intestine.

644 Upon initial examination, we found discordance between predicted and observed
645 motility phenotypes in vivo. All strains examined carry flagellar genes and tend to be highly
646 motile in vitro, yet several of them (e.g., *Enterobacter* ZOR0014, *Aeromonas* ZOR0001, and
647 *Aeromonas* ZOR0002) display no obvious motility during intestinal colonization
648 (Supplementary Movies 2–4). By contrast, some strains robustly sustain motility in vivo, most
649 notably *Vibrio* ZWU0020, which appears to produce populations almost entirely made up of
650 swimming cells (Supplementary Movie 1). There are several possible explanations for these
651 behaviors that are not necessarily mutually exclusive. For example, they may be the result of
652 bacteria dynamically responding to the intestinal environment and executing strain or lineage-
653 specific colonization strategies. On the other hand, bacteria could be differentially susceptible
654 to some form of host-mediated motility interference, which has recently been documented in
655 mouse models^{56,57}. Work focused on distinguishing these interactions is ongoing. Ultimately,
656 this observation highlights the disconnect that can occur among in silico, in vitro, and in vivo
657 approaches for studying bacterial symbioses. Considering how to best capture and interpret
658 mechanistic insights across model systems will be critical to progress.

659 Our data additionally indicate that some strains display a relatively large amount of
660 variation in growth mode and biogeography between hosts (e.g., *Aeromonas* ZOR0001,
661 *Aeromonas* ZOR0002, and *Enterobacter* ZOR0014), while others do not (e.g., *Plesiomonas*
662 ZOR0011, *Pseudomonas* ZWU0006, and *Vibrio* ZWU0020) (Figure 8). This variance is
663 consistent with earlier reports showing that the structure of bacterial populations within the
664 larval zebrafish gut can be highly dynamic, which is attributable in part to the physical forces of

665 intestinal peristalsis^{13,49}. Yet some bacteria—for example, *Vibrio* ZWU0020—remain stable in
666 the face of this perturbation through still undefined mechanisms¹³. Our observations with the
667 *Vibrio* ZWU0020 $\Delta pomAB$ mutant suggest cell motility is involved.

668 Most strikingly, we discovered a strong correlation between the dominant growth mode
669 of bacterial populations and their biogeography (Figure 8). While cell behavior is recognized to
670 influence local population structure^{58,59}, linking cell aggregation with a global pattern of spatial
671 organization throughout the intestine is unanticipated and profound. A possible explanation for
672 this pattern is that physical properties of the intestinal environment (e.g., its shape and/or
673 peristaltic movement) act to spatially segregate planktonic and aggregated cells. Alternatively,
674 bacteria may toggle between different growth modes in response to spatial cues generated by
675 physiologically distinct regions along the length of the intestine. Going forward, a major
676 objective will be to dissect the potential mechanisms of this relationship and importantly,
677 understand how it generalizes both within and across host–microbe systems.

678

679 **Outlook**

680 Elucidating the rules that govern the assembly and function of bacterial symbioses requires
681 studying a wide range of bacterial symbiont lineages^{1,2}. Whether abundant, rare, divergent, or
682 closely related, all potentially hold clues to how host–microbe systems work and how they can
683 be exploited for biotechnology applications—from boosting food production to treating human
684 disease. As more symbiotic relationships are uncovered and new model systems come online,
685 the continued design and modernization of genetic approaches for streamlined manipulation of
686 diverse bacterial lineages will be paramount. Although symbiotic bacteria were the primary
687 subject for tool development in this work, the approaches we have described are equally

688 applicable to the study of free-living environmental bacteria. Importantly, the growing
689 appreciation for the ubiquity of bacterial symbioses and their far-reaching influence on the lives
690 of plants and animals is inspiring a highly cross-disciplinary generation of microbiologists with
691 mixed and varied backgrounds. Therefore, it will be beneficial to work toward standardized
692 tools and methods that foster the rigorous and accurate investigation of symbiont biology.

693 **MATERIALS AND METHODS**

694 **Animal care**

695 All experiments with zebrafish were done in accordance with protocols approved by the
696 University of Oregon Institutional Animal Care and Use Committee and following standard
697 protocols⁶⁰.

698

699 **Gnotobiology**

700 Wild-type (AB x TU strain) zebrafish were derived germ-free (GF) and colonized with bacterial
701 strains as previously described with slight modification⁶¹. Briefly, fertilized eggs from adult
702 mating pairs were harvested and incubated in sterile embryo media (EM) containing ampicillin
703 (100 μ g/ml), gentamicin (10 μ g/ml), amphotericin B (250ng/ml), tetracycline (1 μ g/ml), and
704 chloramphenicol (1 μ g/ml) for ~6h. Embryos were then washed in EM containing 0.1%
705 polyvinylpyrrolidone–iodine followed by EM containing 0.003% sodium hypochlorite. Sterilized
706 embryos were distributed into T25 tissue culture flasks containing 15ml sterile EM at a density
707 of one embryo per ml and incubated at 28–30°C prior to bacterial colonization. Embryos were
708 sustained on yolk-derived nutrients and not fed during experiments. For bacterial mono-
709 association, bacterial strains were grown overnight in LB liquid media with shaking at 30°C,
710 and prepared for inoculation by pelleting 1ml of culture for 2 min at 7,000 x g, and washing
711 once in sterile EM. Bacterial strains were individually added to the water column of single
712 flasks containing 4-day old larval zebrafish at a final density of ~10⁶ bacteria/ml. Bacterial
713 colonization patterns were assessed 24h later by live imaging of three separate 5-day old
714 zebrafish hosts per bacterial strain. Three animals were determined to be adequate for

715 capturing general colonization features of each bacterial strain based on at least two previous
716 independent qualitative assessments of colonization patterns.

717

718 **Bacterial strains and culture**

719 All wild and recombinant bacterial strains used or created in this study are listed in
720 Supplementary File 1. Archived stocks of bacteria are maintained in 25% glycerol at -80°C.
721 Prior to manipulations or experiments, bacteria were directly inoculated into 5ml Luria-Bertani
722 (LB) media (10g/L NaCl, 5g/L yeast extract, 12g/L tryptone, 1g/L glucose) and grown for ~16h
723 (overnight) shaking at 30°C, except for *E. coli* HS, which was grown at 37°C. For growth on
724 solid media, tryptic soy agar was used. 10µg/ml gentamicin was used to select recombinant
725 strains tagged with the Tn7 transposon, which was modified to carry a gentamicin resistance
726 gene. When selecting merodiploid intermediates made using pAX1 or pAX2, which carry
727 resistance to both gentamicin and chloramphenicol, either 10µg/ml gentamicin or 5µg/ml
728 chloramphenicol was used. Selection of rifampicin-domesticated variants was done using
729 100µg/ml rifampicin.

730

731 **Molecular techniques and reagents**

732 *E. coli* strains used for molecular cloning and conjugation, and plasmids used or created during
733 this work are listed in Supplementary File 2. *E. coli* were typically grown in 5ml LB liquid media
734 at 30°C or 37°C with shaking in the presence of appropriate antibiotic selection to maintain
735 plasmids. For propagation on solid media, LB agar was used. Antibiotic concentrations used
736 were as follows: 100µg/ml ampicillin, 20µg/ml chloramphenicol, 10µg/ml gentamicin, and
737 10µg/ml tetracycline. Supplementary File 3 lists all DNA primers used for polymerase chain

738 reactions (PCR), which are organized based on their “Wiles Primer” (WP) number. Unless
739 specifically noted, standard molecular techniques were applied and reagents were used
740 according to manufacturer instructions. Restriction enzymes and other molecular biology
741 reagents for PCR and nucleic acid modifications were obtained from New England BioLabs
742 (Ipswich, MA). Various kits for plasmid and PCR amplicon purification were obtained from
743 Zymo Research (Irvine, CA). The Wizard Genomic DNA Purification Kit (Promega, Madison,
744 WI) was used for isolating bacterial genomic DNA. DNA oligonucleotides for PCR were
745 synthesized by Integrated DNA Technologies (Coralville, IA). Sanger sequencing was done by
746 Sequetech (Mountain View, CA). Custom gene synthesis was done by GenScript (Piscataway,
747 NJ). A Leica MZ10 F fluorescence stereomicroscope with 1.0x, 1.6x, and 2.0x objectives and
748 Leica DFC365 FX camera were used for screening and imaging fluorescent bacterial colonies
749 (Leica, Wetzlar, Germany). Images were captured and processed using standard Leica
750 Application Suite software and ImageJ⁶². Nucleotide sequences of 16S rRNA genes used for
751 phylogenetic analysis are provided in Supplementary File 4, and were obtained via “The
752 Integrated Microbial Genomes & Microbiome Samples” (IMG/M) website
753 (<https://img.jgi.doe.gov/m/>)⁶³ or the RNAmmer web tool⁶⁴. 16S rRNA sequences were aligned
754 using Clustal Omega⁶⁵ and an unrooted phylogenetic tree was drawn using the Phylodendron
755 web tool (<http://iubio.bio.indiana.edu:7131/treeapp/treeprint-form.html>).

756

757 **Plasmid construction**

758 The plasmid-based tools developed in this work have been deposited at Addgene (Cambridge,
759 MA), along with their sequences (<https://www.addgene.org/>). Supplementary File 5 contains
760 annotated nucleotide sequences of select genetic parts that were used to build plasmids and

761 gene expression scaffolds. Details on how plasmids were specifically constructed are provided
762 in Supplementary File 6.

763

764 **Domestication-free Tn7 tagging using pTn7xTS and pTn7xKS**

765 A detailed Tn7 tagging protocol based on pTn7xTS and pTn7xKS—which includes
766 optimization and troubleshooting steps, and notes on strain-specific procedures—is provided in
767 Supplementary File 7. Generally, and as outlined in Figure 2, triparental conjugation was
768 performed between a single target bacterial strain, an *E. coli* SM10 donor strain carrying the
769 transposase-containing pTNS2 helper plasmid, and an *E. coli* SM10 donor strain carrying
770 either a pTn7xTS or pTn7xKS domestication-free tagging vector. Prior to mating, bacteria were
771 prepared by subculturing them to an approximate optical density of 0.4–0.6 at 600nm in LB
772 media with required antibiotics and at the appropriate growth temperature. Cells were then
773 combined 1:1:1 (500 μ l each), washed once by centrifugation and aspiration in 1ml LB media or
774 0.7% NaCl, and suspended in a final 25 μ l volume of the same media used for washing. Next,
775 the concentrated mating mixture was transferred to a 25mm-wide 0.45 μ m filter disc (EMD
776 Millipore, Billerica MA; product #HAWP02500) that had been placed on top of a TSA plate.
777 Once the mating mixture dried, the plate was incubated at 30°C for 3–5h. After incubation, the
778 filter disc was placed in 1ml 0.7% NaCl within a 50ml conical tube and bacteria were dislodged
779 by vortexing and pipetting. In cases where a pTn7xTS-based vector was used, 100 μ l of the
780 bacterial suspension was spread onto a TSA plate containing gentamicin and incubated
781 overnight at 37°C to select for recombinants. To ensure the recovery of low frequency
782 recombinants, the remaining 900 μ l of the suspension was pelleted by centrifugation,
783 suspended in 100 μ l 0.7% NaCl, and plated in the same way. In cases where a pTn7xKS-

784 based vector was used, 100 μ l of the bacterial suspension was spread onto a TSA plate
785 containing gentamicin and 1mM isopropyl- β -D-thiogalactoside (IPTG), and incubated overnight
786 at 30°C. The remaining 900 μ l was prepared as above, plated on TSA with gentamicin and
787 IPTG, and incubated at 30°C.

788 The following day, putative recombinant target bacteria were colony-purified by
789 streaking on TSA without antibiotic selection at 30°C. Of note, when recombinant bacteria are
790 tagged with a gene encoding a fluorescent protein, performing colony-purification in the
791 absence of antibiotic selection followed by visual screening of fluorescence is a convenient
792 way to verify that the Tn7 transposon has chromosomally integrated and the tagging vector
793 has been lost. Purified clones were picked, cultured in LB media containing gentamicin, and
794 genotyped by PCR to verify correct insertion of the Tn7 transposon at the *attTn7* site. The
795 universal primer WP11, which anneals within the Tn7R region of the Tn7 transposon, was
796 used with a species-specific primer that anneals to an adjacent chromosomal sequence within
797 the 3' end of the *glmS* gene to generate a small (~250bp) amplicon if the transposon is
798 present. Species-specific primers used were as follows: *Vibrio* ZOR0018, WP50; *Vibrio*
799 ZOR0035, WP51; *Vibrio* ZOR0036, WP12; *Vibrio* ZWU0020, WP12; *Aeromonas* ZOR0001,
800 WP49; *Aeromonas* ZOR0002, WP52; *Pseudomonas* ZWU0006, WP256; *Acinetobacter*
801 ZOR0008, WP259; *Enterobacter* ZOR0014, WP257; *Plesiomonas* ZOR0011, WP260; *E. coli*
802 HS, WP150; *Aeromonas* HM21, WP49; *Shewanella* MR-1, WP48.

803

804 **Generation of markerless deletions via allelic exchange using pAX1 and pAX2**

805 A detailed protocol for carrying out allelic exchange using pAX1 and pAX2—which includes
806 optimization and troubleshooting steps, and notes on strain-specific procedures—is provided in

807 Supplementary File 8. Briefly, and as summarized in Figure 4, allelic exchange cassettes for
808 mediating markerless deletion of target genetic loci (i.e., the *pomAB* locus of *Vibrio* ZWU0020
809 and *Aeromonas* ZOR0001) were generated through splice by overlap extension and inserted
810 into a pAX-based vector. Next, diparental conjugation was performed between a single target
811 bacterial strain (i.e., *Vibrio* ZWU0020 or *Aeromonas* ZOR0001) and an *E. coli* SM10 donor
812 strain carrying the assembled allelic exchange vector. Prior to mating, bacteria were prepared
813 by subculturing them to an approximate optical density of 0.4–0.6 at 600nm in LB media with
814 required antibiotics and at the appropriate growth temperature. Cells were then combined 1:1
815 (750 μ l each), washed once by centrifugation and aspiration in 1ml LB media or 0.7% NaCl,
816 and suspended in a final 25 μ l volume of the same media used for washing. Next, the
817 concentrated mating mixture was transferred to a 25mm-wide 0.45 μ m filter disc that had been
818 placed on top of a TSA plate. Once the mating mixture dried, the plate was incubated at 30°C
819 for 3–5h. After incubation, the filter disc was placed in 1ml 0.7% NaCl within a 50ml conical
820 tube and bacteria were dislodged by vortexing and pipetting. For the generation of *Vibrio*
821 ZWU0020 $\Delta pomAB$, which employed a pAX1-related vector, 100 μ l of the bacterial suspension
822 was spread onto a TSA plate containing gentamicin and incubated overnight at 37°C to select
823 for merodiploids. The remaining 900 μ l of the suspension was pelleted by centrifugation,
824 suspended in 100 μ l 0.7% NaCl, and plated in the same way to ensure recovery of rare
825 recombinants. For the generation of *Aeromonas* ZOR0001 $\Delta pomAB$, which employed a pAX2-
826 based vector, 100 μ l of the bacterial suspension was spread onto a TSA plate containing
827 gentamicin and 10ng/ml anhydrotetracycline (aTc), and incubated overnight at 30°C. The
828 remaining 900 μ l was prepared as above, plated on TSA with gentamicin and aTc, and
829 incubated at 30°C.

830 The following day, colonies of putative merodiploid target bacteria that were expressing
831 the GFP tracker were purified by streaking on TSA without antibiotic selection at 30°C. This
832 purification step also served to verify that the allelic exchange vector had integrated into the
833 chromosome. Purified clones were picked, cultured in LB media containing gentamicin to
834 maintain their merodiploid state, and archived as a frozen stock. To screen for second
835 recombination events, merodiploids were cultured overnight in LB media without antibiotic
836 selection and spread onto several TSA plates, again without antibiotic selection, at a density
837 that allowed ~100–200 discrete colonies to form. Colonies exhibiting partial or complete loss of
838 GFP expression were purified by streaking on TSA at 30°C. Putative mutants were screened
839 and genotyped by PCR using primers that flanked the modified locus, which produced two
840 differently sized amplicons that represented the wild-type and mutant alleles. Primers WP163
841 and WP164 were used to genotype *Vibrio* ZWU0020 $\Delta pomAB$ mutants and primers WP192
842 and WP195 were used to genotype *Aeromonas* ZOR0001 $\Delta pomAB$ mutants.

843

844 **In vitro growth measurements**

845 In vitro growth of bacterial strains was assessed using the FLUOstar Omega microplate reader
846 (BMG LABTECH, Offenburg, Germany). Prior to growth measurements, bacteria were grown
847 overnight in 5ml LB media at 30°C with shaking. Cultures were diluted 1:100 into fresh LB
848 media and dispensed in quadruplicate (i.e., four technical replicates) (200 μ l/ well) into a sterile
849 96 well clear flat bottom tissue culture-treated microplate (Corning, Corning, NY; product
850 #3585). Absorbance measurements at 600nm were then recorded every 30 minutes for 16
851 hours (or until stationary phase) at 30°C with shaking. Growth measurements were repeated at

852 least two independent times for each strain (i.e., two biological replicates) with consistent
853 results. Data were exported and graphed using GraphPad Prism 6 software.

854

855 **Swim motility assays**

856 Prior to the assessment of swimming motility, bacteria were grown overnight in 5ml LB media
857 at 30°C with shaking. 1ml of bacterial culture was then washed by centrifuging cells at 7,000xg
858 for 2 minutes, aspirating media, and suspending in 1ml 0.7% NaCl. This centrifugation/
859 aspiration wash step was repeated once more and bacteria were suspended in a final volume
860 of 1ml 0.7% NaCl. 1µl of washed bacterial culture was then inoculated into a TSA plate
861 containing 0.2% agar (30g/L tryptic soy broth and 2g/L bacto agar). Swim plates were
862 incubated at 30°C for 5–7h and imaged on a Gel Doc XR+ Imaging System (Bio-Rad,
863 Hercules, CA). Motility assays were repeated at least two independent times (i.e., two
864 biological replicates) with consistent results.

865

866 **Spot tests**

867 *E. coli* SM10 donor cells carrying vectors that contain temperature and/or kill switch-based
868 post-conjugation counterselection systems were grown overnight in LB media with required
869 antibiotics and at the appropriate growth temperatures. For assessing temperature-based
870 counterselection, ten-fold serial dilutions were made on TSA plates containing gentamicin and
871 incubated overnight at 30°C or 37°C. For assessing kill switch-based counterselection, ten-fold
872 serial dilutions were made on TSA plates containing gentamicin +/- 1mM IPTG (in the case of
873 pTn7xKS) or 10ng/ml aTc (in the case of pAX2) and incubated overnight at 30°C. Plates were
874 imaged on a Bio-Rad Gel Doc XR+ Imaging System. All spot tests were performed at least two

875 independent times (i.e., two biological replicates), each including at least two technical
876 replicates, with consistent results.

877

878 **Live Imaging**

879 Live larval zebrafish were imaged using a home-built light sheet fluorescence microscope
880 described in detail elsewhere^{49,66}. In brief, a thin sheet of laser light is obtained by rapidly
881 scanning the excitation beam with a galvanometer mirror. Fluorescence emission is captured
882 by an objective lens mounted perpendicular to the sheet. 3D images are obtained by
883 translating the sample along the detection axis. The entire volume of the intestine
884 (approximately 1200x300x150 microns) is imaged in four sub-regions that are computationally
885 registered after acquisition. Total acquisition time of a single intestine is less than 1 min with 1-
886 micron steps between planes. For all images, the exposure time was 30ms and the excitation
887 laser power was 5mW prior to entering the imaging chamber.

888

889 **Image Analysis**

890 Bacterial abundances and locations were estimated using the analysis pipeline described in⁴⁹.
891 In brief, we identify individual cells in 3D using a wavelet filtering-based algorithm⁶⁷ and identify
892 multicellular aggregates using a graph-cut segmentation algorithm⁶⁸. The number of cells in an
893 aggregate is estimated by dividing the total aggregate intensity by the average intensity of
894 individual bacteria. Individuals detected within an aggregate are discarded. One-dimensional
895 population distributions are obtained by dividing the intestine into 5-micron bins constructed
896 down the length of the intestine along a manually drawn line and assigning the centroid of
897 each detected object to a bin. Global population centers are computed as the center of mass

898 of this 1D distribution. Of note, this analysis pipeline was originally developed and optimized
899 for a different strain not imaged here⁴⁹ and its performance on the 8 present strains has not
900 been rigorously assessed. Based on manual inspection and analysis, we estimate an
901 uncertainty of at most 10% for the planktonic fraction and for the population center of mass,
902 which is certainly more than adequate to detect the global trends we report.

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916 **COMPETING INTERESTS**

917 The authors declare that the tools and methods described in this work are the subject of an
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L152

l153 **FIGURE SUPPLEMENT AND SUPPLEMENTARY MOVIE LEGENDS**

l154 **Figure 2-Figure Supplement 1.** Spot tests demonstrating temperature-based
l155 counterselection of *E. coli* SM10 donor cells using the Tn7-tagging vector pTn7xTS. Ten-fold
l156 serial dilutions of *E. coli* SM10 carrying either the control vector pUC18R6KT-mini-Tn7T-GM
l157 (pTW56) or its temperature-sensitive derivative pTn7xTS were plated on tryptic soy agar
l158 containing 10µg/ml gentamicin and cultivated overnight at 30°C or 37°C. Functional
l159 differences between each vector are highlighted in vector maps. *ori_{R6K}*, *pir*-dependent origin of
l160 replication; *ori₁₀₁/repA101^{ts}*, temperature-sensitive origin of replication.

l161

l162 **Figure 2-Figure Supplement 2.** Spot tests demonstrating kill switch-based counterselection of
l163 *E. coli* SM10 donor cells using the Tn7-tagging vector pTn7xKS. **(A)** Kill switch design.
l164 Expression of the three toxin-encoding genes *hokB*, *ghoT*, and *tisB*—which impair ATP
l165 synthesis—is controlled by the LacI-repressible promoter P_{tac} (O+). “O+” indicates presence of
l166 the lac operator sequence in the promoter. Tight regulation of toxin genes is achieved by using
l167 the P_{lacIQ} promoter variant to control transcription of *lacI*, which drives ten-fold higher
l168 expression over native P_{lacI}. The allolactose analogue isopropyl-β-D-thiogalactoside (IPTG) is
l169 used for kill switch induction. **(B)** Ten-fold serial dilutions of *E. coli* SM10, carrying pUC18T-
l170 mini-Tn7T-GM (pTW54) derivatives that either contain a kill switch with no toxin genes
l171 (pTW88), three toxin genes (pTn7xKS), or a single toxin gene (pTW98), were plated on tryptic
l172 soy agar containing 10µg/ml gentamicin +/- 1mM IPTG and cultivated overnight at 30°C.
l173 Functional differences between each vector are highlighted in vector maps. *ori_{ColE1}*, high copy
l174 number origin of replication.

l175

L176 **Figure 3-Figure Supplement 1.** Design features of expression scaffolds and modernized Tn7-
L177 tagging vectors. **(A)** The previously described high-retention pGEN-mcs vector was chosen for
L178 maintaining, modifying, and prototyping expression scaffolds. Scaffolds encoding either sfGFP
L179 (pXS-sfGFP, green circle), dTomato (pXS-dTomato, red circle), or mPlum (pXS-mPlum, purple
L180 circle) were built into the multiple cloning site (mcs). Scaffold orientations relative to vector are
L181 noted. As in Figure 3, a scaffold diagram highlights the interchangeable sequence motifs and
L182 cyan arrowheads below mark the locations of restriction sites for making modifications.
L183 Presence of green, red, or purple circles next to restriction enzyme names indicate the
L184 availability of that site within each respective vector. *It should be noted that the AgeI enzyme
L185 cuts 190bp into the 15A origin of replication. Other pGEN-mcs features: *ori*_{15A}, broad host
L186 range origin of replication; T1, transcriptional terminator; *par*, partitioning system from pSC101;
L187 *amp*^R, ampicillin resistance gene; *hok/sok*, toxin/antitoxin system; *parRM*, centromere-like
L188 partitioning system. **(B)** Expression scaffolds were subcloned into the mcs of the temperature-
L189 sensitive Tn7-tagging vector pTn7xTS (described in Figure 2A). The relative orientation of the
L190 scaffolds is noted. Restriction site availability for each vector encoding either GFP (pTn7xTS-
L191 sfGFP, green circle), dTomato (pTn7xTS-dTomato, red circle), or mPlum (pTn7xTS-mPlum,
L192 purple circle) is outlined as in **A**. **(C)** Expression scaffolds were subcloned into the mcs of the
L193 kill switch containing Tn7-tagging vector pTn7xKS (described in Figure 2C). The relative
L194 orientation of the scaffolds is noted. Restriction site availability for each vector encoding either
L195 GFP (pTn7xKS-sfGFP, green circle), dTomato (pTn7xKS-dTomato, red circle), or mPlum
L196 (pTn7xKS-mPlum, purple circle) is outlined as in **A**.
L197

L198 **Figure 3-Figure Supplement 2.** Domestication-free tagging of *Vibrio* ZWU0020. **(A)** Diagram
L199 shows the conserved *attTn7* insertion site for Tn7 (Tn) downstream of the *glmS* gene within
L200 *Vibrio* ZWU0020's chromosome (chr). Cargo comprises one of three genes encoding either
L201 sfGFP, dTomato, or mPlum. "T" denotes transcriptional terminators; unch., uncharacterized.
L202 **(B)** PCR-confirmation of Tn7 insertion variants carrying different fluorescent markers made
L203 using temperature and kill switch-based tagging vectors (i.e., pTn7xTS and pTn7xKS,
L204 respectively). Black arrows above gene diagrams mark primer annealing sites in addition to the
L205 expected amplicon size for successful insertion events. Representative DNA gels showing
L206 genotyping results are provided to the right of each diagram. Wild-type ZWU0020 (WT)
L207 produces no amplicon because the transposon is not present. **(C)** Plotted is the average
L208 optical density at 600nm (OD_{600}) vs. time (hours) of WT ZWU0020, and its undomesticated
L209 fluorescently tagged derivatives (*attTn7::sfGFP*, *attTn7::dTomato*, and *attTn7::mPlum*), during
L210 shaking growth in LB broth at 30°C. Range bars are based on four technical replicates. **(D)**
L211 Swim motility WT ZWU0020 and the indicated fluorescently tagged variants from **C** in 0.2%
L212 tryptic soy agar at 30°C.

L213

L214 **Figure 5-Figure Supplement 1.** Design features of customizable allelic exchange vectors. **(A)**
L215 Molecular scaffolds that hold antibiotic resistance genes, a merodiploid (MD) tracker, and a
L216 domestication-free kill switch system are partitioned by restriction sites (cyan arrowheads). A
L217 *Sma*I restriction site, which produces blunt ends when cleaved, serves as a flexible point of
L218 entry for cloned allelic exchange cassettes. Full vector assemblies are shown for **(B)** pAX1 and
L219 **(C)** pAX2. The hashed line within each vector schematic highlights the scaffold regions shown
L220 to the right, which list the locations of available restriction sites for engineering and provide

L221 element details. Terminators T_0 and T_1 prevent transcriptional read-through of the Smal
L222 cloning site and interference of kill switch elements. *ori*₁₀₁/*repA*101^{ts}, temperature-sensitive
L223 origin of replication; *oriT*, origin of transfer; *gent*^R, gentamicin resistance gene; *clm*^R,
L224 chloramphenicol resistance gene; *amp*^R, ampicillin resistance gene; P_{gent} , *gent* promoter; P_{clm} ,
L225 *clm* promoter; P_{CP25} , synthetic constitutive promoter; GFP^{mut3.1}, GFP variant; *tisB*, *ghoT*, and
L226 *hokB* encode toxic peptides; *tetR* encodes the repressor TetR; P_{TetO} , synthetic TetR-
L227 repressible promoter; P_{tac} (O-), synthetic constitutive promoter without lac operator sequence.

L228

L229 **Figure 5-Figure Supplement 2.** Spot tests demonstrating temperature and kill switch-based
L230 counterselection of *E. coli* SM10 donor cells using the allelic exchange vectors pAX1 and
L231 pAX2. Ten-fold serial dilutions of *E. coli* SM10 carrying either pAX1 or pAX2 were plated on
L232 tryptic soy agar (TSA) containing 10µg/ml gentamicin and cultivated overnight at 30°C or 37°C.
L233 Results obtained with SM10/pTW56, which are also presented in Figure 2-Figure Supplement
L234 1, are provided as a reference control. Functional differences between each vector are
L235 highlighted. *ori*_{R6K}, *pir*-dependent origin of replication; *ori*₁₀₁/*repA*101^{ts}, temperature-sensitive
L236 origin of replication. **(B)** Ten-fold serial dilutions of *E. coli* SM10 carrying either pTW56 or pAX2
L237 were plated on TSA containing 10µg/ml gentamicin +/- 10ng/ml anhydrotetracycline (aTc) and
L238 cultivated overnight at 30°C or 37°C. As in **A**, functional differences between each vector are
L239 highlighted in vector maps. *tisB*, *ghoT*, and *hokB* encode toxic peptides; *tetR* encodes the
L240 repressor TetR.

L241

L242 **Figure 6-Figure Supplement 1.** Additional information related to the engineering of the *Vibrio*
L243 ZWU0020 $\Delta pomAB$ deletion mutant. **(A)** *Vibrio* ZWU0020 *pomAB* merodiploids robustly

L244 express GFP. After outgrowth and plating on nonselective media, mosaic merodiploid colonies
L245 exhibiting loss of GFP and thus, loss of the allelic exchange vector, can be isolated. Images
L246 labeled “GFP” show GFP fluorescence and “darkfield” show total colony structure. **(B)** Plotted
L247 is the average optical density at 600nm (OD₆₀₀) vs. time (hours) of WT ZWU0020 and its
L248 $\Delta pomAB$ derivative during shaking growth in LB broth at 30°C. Range bars are based on four
L249 technical replicates.

L250

L251 **Figure 6-Figure Supplement 2.** Markerless deletion of *pomAB* in *Aeromonas veronii* using
L252 the allelic exchange vector pAX2. **(A)** Top: wild-type *pomAB* locus in *Aeromonas veronii*
L253 ZOR0001. Bottom: result of markerless *pomAB* deletion fuses the start codon of *pomA* to the
L254 stop codon of *pomB*. Black arrows mark approximate primer annealing sites for genotyping
L255 and the size of each amplification product is indicated. **(B)** *Aeromonas* ZOR0001 *pomAB*
L256 merodiploids robustly express GFP. After outgrowth and plating on nonselective media,
L257 mosaic merodiploid colonies exhibiting loss of GFP, and thus loss of the allelic exchange
L258 vector, can be isolated. Images labeled “GFP” show GFP fluorescence and “darkfield” show
L259 total colony structure. **(C)** Agarose gel showing PCR-based genotyping of wild-type (WT),
L260 merodiploid (MD), and four putative $\Delta pomAB$ (Δ) mutants. To verify the dual functionality of
L261 pAX2, two merodiploids were isolated using either its temperature (T) or kill switch (K) donor
L262 cell counterselection systems. Putative $\Delta pomAB$ mutants were derived from “K” merodiploids.
L263 Migration distances of WT and mutant alleles are indicated. Mutant 3 is an example of a wild-
L264 type revertant, whereas 1, 2, and 4 are $\Delta pomAB$ mutants. **(D)** Swim motility of WT ZOR0001
L265 and the four putative $\Delta pomAB$ mutants from **C** in 0.2% tryptic soy agar at 30°C. The attenuated
L266 swimming phenotype of mutants 1, 2, and 4 corroborates genotyping results. Of note,

L267 *Aeromonas* ZOR0001 encodes multiple lateral flagellar systems in addition to one polar
L268 flagellum; therefore, partial loss of motility is expected. (E) Plotted is the average optical
L269 density at 600nm (OD₆₀₀) vs. time (hours) of WT ZOR0001 and $\Delta pomAB$ mutant 1 during
L270 shaking growth in LB broth at 30°C. Range bars are based on four technical replicates.

L271

L272 **Figure 6-Figure Supplement 3.** Examples of various patterns of GFP loss observed within
L273 merodiploid colonies. *Vibrio* ZWU0020 (top row) and *Aeromonas* ZOR0001 (bottom row)
L274 merodiploid cells were plated on non-selective tryptic soy agar. After overnight growth,
L275 colonies were screened for loss of GFP, which indicates that a second recombination event
L276 has occurred. White arrowheads mark GFP-negative patches.

L277

L278 **Figure 7-Figure Supplement 1.** Additional images showing intestinal colonization patterns
L279 and growth modes of zebrafish symbionts. (A) Cartoon diagram of a 5-day old larval zebrafish.
L280 Purple dashed box outlines region imaged in C. (B) Diagram shows the boundaries of the bulb,
L281 mid-gut, and distal gut within the larval intestine. (C) Maximum intensity projections of 3D
L282 image stacks acquired by light sheet fluorescence microscopy for indicated bacterial strains.
L283 Orange dotted outline marks the intestine in each image. Scale bar: 200µm.

L284

L285 **Figure 7-Figure Supplement 2.** Summary of traits exhibited by bacterial symbionts within the
L286 larval zebrafish intestine.

L287

L288 **Figure 7-Figure Supplement 3.** Phylogenetic relatedness and summary of motility
L289 phenotypes. Shown is an unrooted phylogenetic tree generated using full-length nucleotide

L290 sequences of the 16S rRNA gene from all strains manipulated in this study. Strains used for
L291 live imaging are in bold black type and symbols denote motility phenotypes.

L292

L293 **Figure 7-Figure Supplement 4.** Motility of select strains in soft agar. Indicated strains were
L294 inoculated in 0.2% tryptic soy agar and incubated at 30°C for ~5h. **Pseudomonas* ZWU0006
L295 exhibits a delay in swimming and advances after a 24h incubation period.

L296

L297 **Supplementary Movie 1.** Example of *Vibrio* ZWU0020 growth mode and behavior within the
L298 zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal bulb of a 5-
L299 day old larval zebrafish colonized with a 100:1 mixture of *Vibrio* ZWU0020 expressing
L300 dTomato (left panel) or sfGFP (right panel). The GFP-tagged subpopulation highlights the
L301 highly motile and planktonic nature of this strain within the intestine. Scale bars: 50µm.

L302

L303 **Supplementary Movie 2.** Example of *Enterobacter* ZOR0014 growth mode and behavior
L304 within the zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal
L305 bulb of a 5-day old larval zebrafish colonized with *Enterobacter* ZOR0014 expressing sfGFP. A
L306 small portion of the *Enterobacter* mid-gut population can be seen fluxing into the bulb due to
L307 peristaltic contractions. Non-motile cells are evident along with larger multicellular aggregates.
L308 Scale bar: 50µm.

L309

L310 **Supplementary Movie 3.** Example of *Aeromonas* ZOR0001 growth mode and behavior within
L311 the zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal mid-gut
L312 of a 5-day old larval zebrafish colonized with *Aeromonas* ZOR0001 expressing sfGFP. Non-

L313 motile cells and small aggregates can be seen experiencing flux due to peristaltic contractions.

L314 Scale bar: 50 μ m.

L315

L316 **Supplementary Movie 4.** Example of *Aeromonas* ZOR0002 growth mode and behavior within

L317 the zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal bulb of a

L318 5-day old larval zebrafish colonized with *Aeromonas* ZOR0002 expressing sfGFP. Non-motile

L319 cells and small aggregates can be observed. Scale bar: 50 μ m.

L320

L321 **Supplementary Movie 5.** Example of *Vibrio* ZOR0036 growth mode and behavior within the

L322 zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal bulb of a 5-

L323 day old larval zebrafish colonized with *Vibrio* ZOR0036 expressing sfGFP. Highly motile cells

L324 as well as large multicellular aggregates can be observed. Scale bar: 50 μ m.

L325

L326 **Supplementary Movie 6.** Example of *Plesiomonas* ZOR0011 growth mode and behavior

L327 within the zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal

L328 bulb of a 5-day old larval zebrafish colonized with *Plesiomonas* ZOR0011 expressing sfGFP.

L329 Motile cells as well as large multicellular aggregates can be observed. Notably, the swim

L330 speed of *Plesiomonas* cells appears to be more moderate compared to *Vibrio* or

L331 *Pseudomonas* strains. Scale bar: 50 μ m.

L332

L333 **Supplementary Movie 7.** Example of *Pseudomonas* ZWU0006 growth mode and behavior

L334 within the zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal

L335 bulb of a 5-day old larval zebrafish colonized with *Pseudomonas* ZWU0006 expressing sfGFP.

L336 Highly motile cells as well as large multicellular aggregates can be observed. Scale bar: 50 μ m.

L337

L338 **Supplementary Movie 8.** Example of *Vibrio* ZWU0020 $\Delta pomAB$ growth mode and behavior

L339 within the zebrafish gut. Movie depicts live imaging of a single optical plane in the intestinal

L340 bulb of a 5-day old larval zebrafish colonized with *Vibrio* ZWU0020 $\Delta pomAB$ expressing

L341 dTomato. Non-motile cells and small aggregates can be observed. Scale bar: 50 μ m.

L342 **SUPPLEMENTARY AND SOURCE DATA FILE LEGENDS**

L343 **Supplementary File 1.** Wild and Recombinant Bacteria.

L344 **Supplementary File 2.** 16S rRNA Nucleotide Sequences.

L345 **Supplementary File 3.** *E. coli* Strains and Plasmids.

L346 **Supplementary File 4.** Primers.

L347 **Supplementary File 5.** Nucleotide Sequences of Select Genetic Parts.

L348 **Supplementary File 6.** Plasmid Construction.

L349 **Supplementary File 7.** Protocol: Tn7 tagging with pTn7xTS and pTn7xKS

L350 **Supplementary File 8.** Protocol: Allelic exchange with pAX1 and pAX2

L351 **Figure 1-Source Data 1.** Raw in vitro growth data for Figure 1A

L352 **Figure 3-Source Data 1.** Raw in vitro growth data for Figure 3-Figure Supplement 2C

L353 **Figure 6-Source Data 1.** Raw in vitro growth data for Figure 6-Figure Supplement 1B

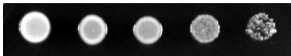
L354 **Figure 6-Source Data 2.** Raw in vitro growth data for Figure 6-Figure Supplement 2E

L355 **Figure 8-Source Data 1.** Values plotted in Figure 8

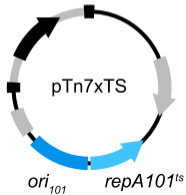
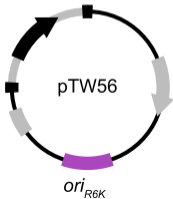
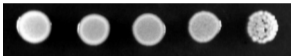
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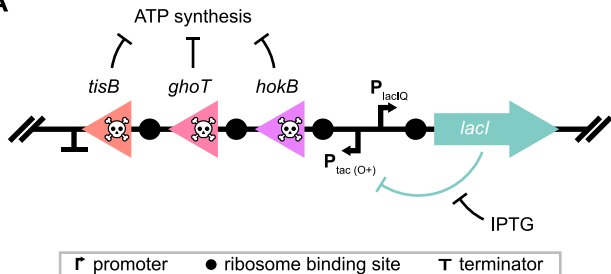
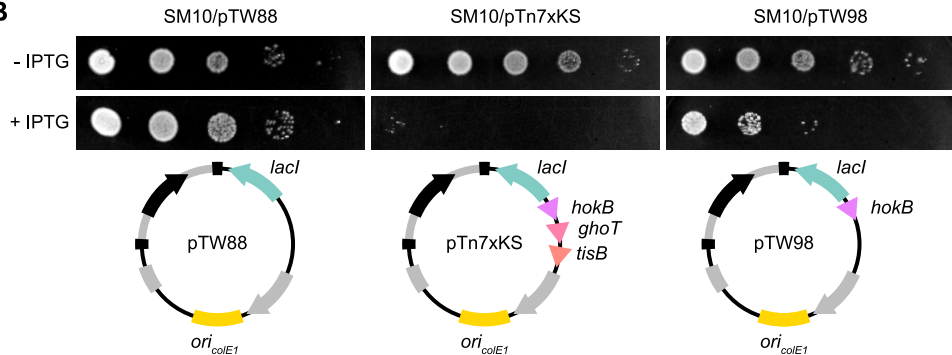
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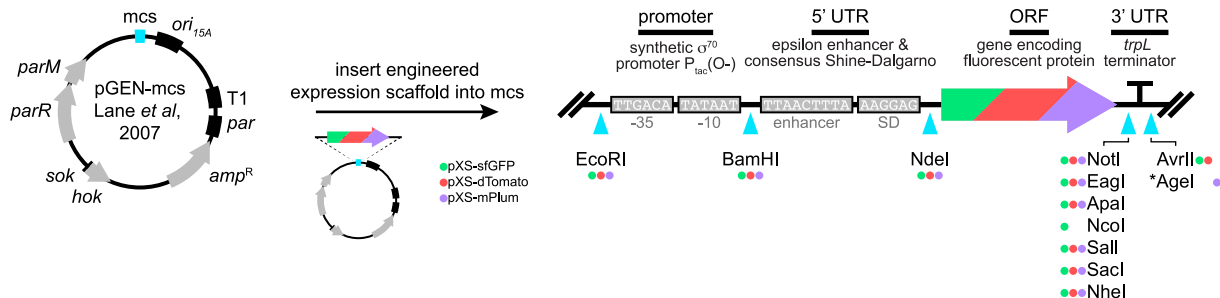
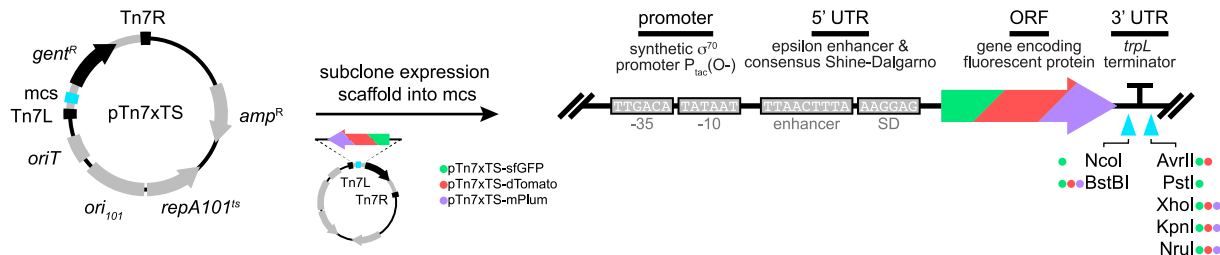
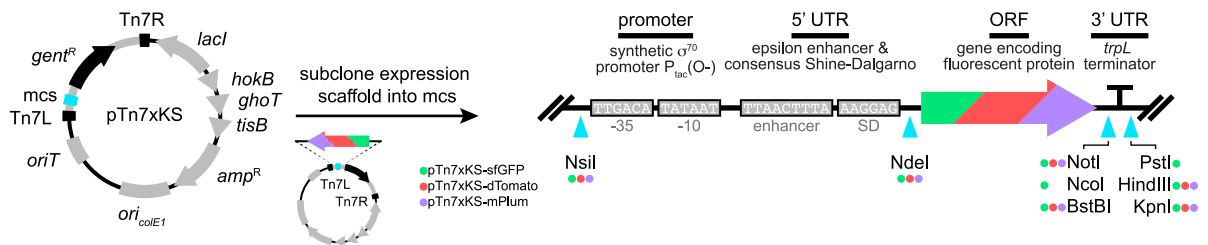
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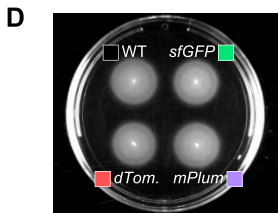
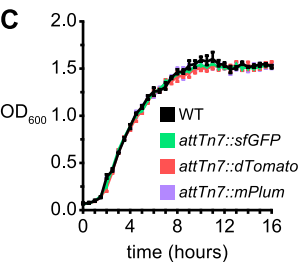
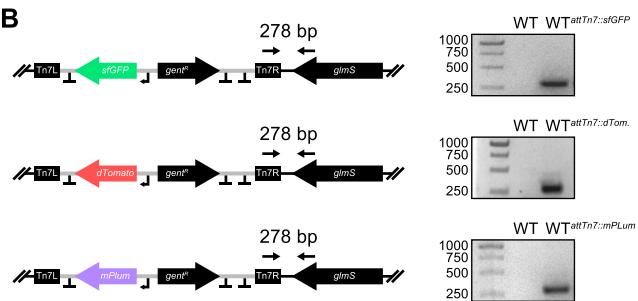
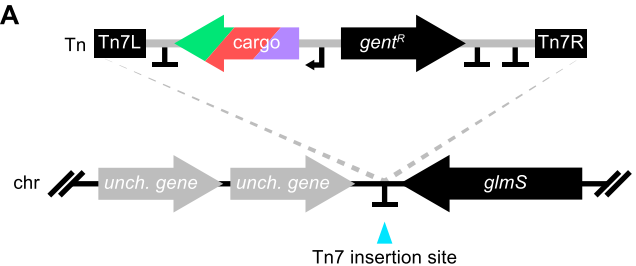


37°C

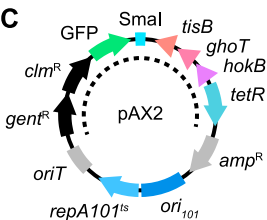
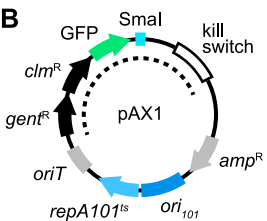
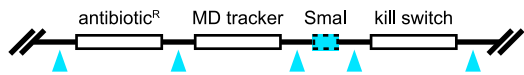


A**B**

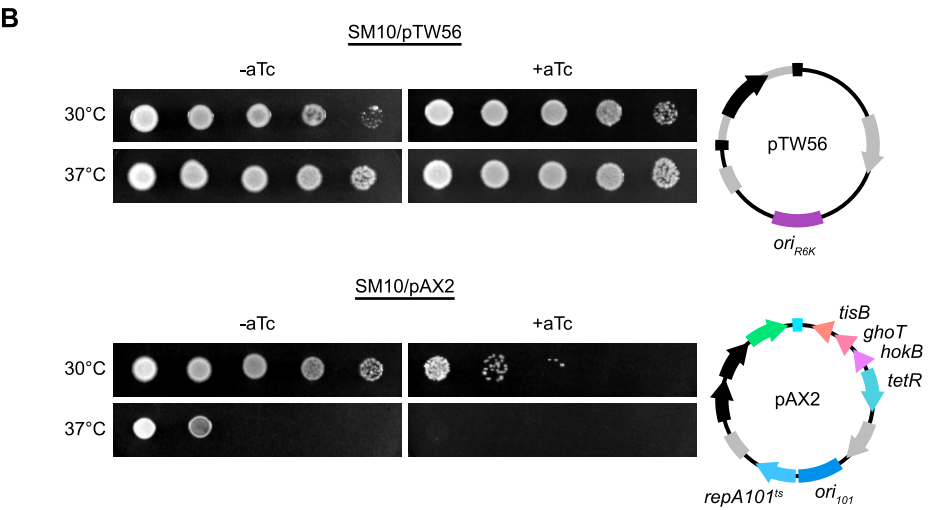
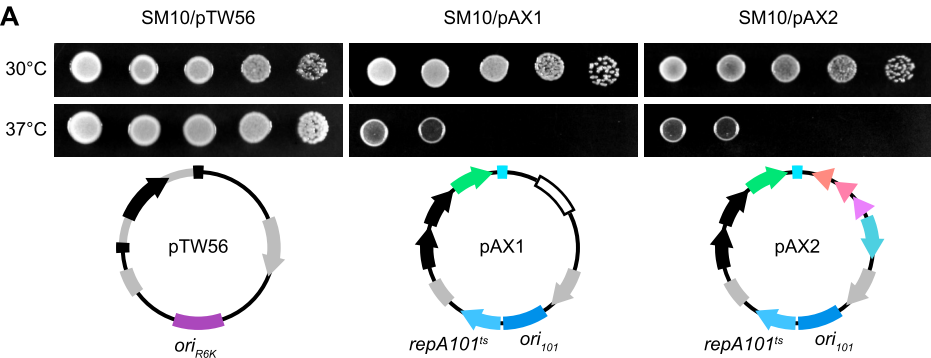
ApGEN-based Expression Scaffolds**B**Tn7-tagging Vectors with Temperature-based Counterselection**C**Tn7-tagging Vectors with Kill Switch-based Counterselection



A design overview of allelic exchange vector scaffolds

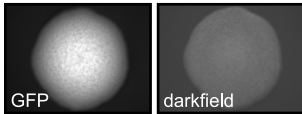
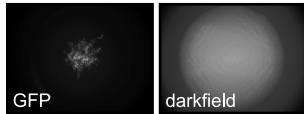
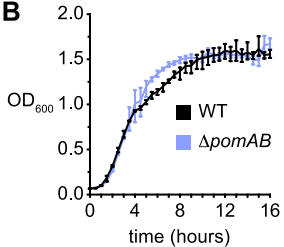


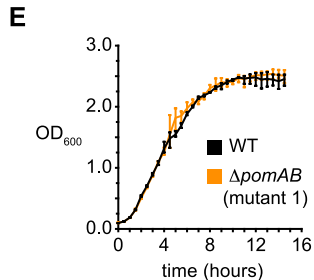
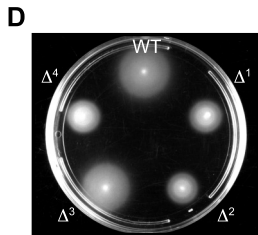
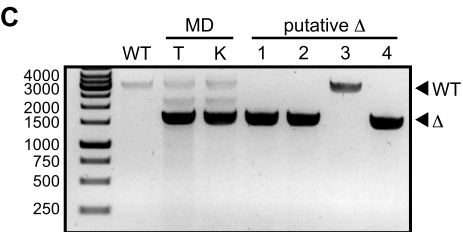
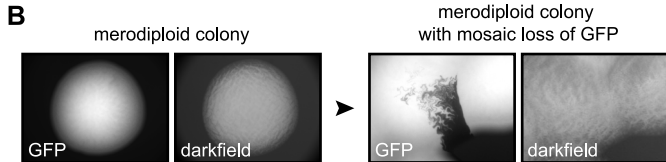
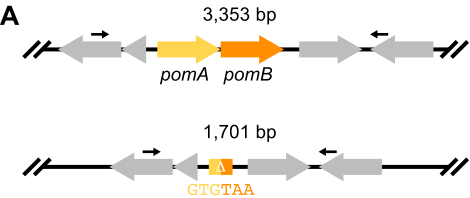
▭ promoter ● ribosome binding site T terminator



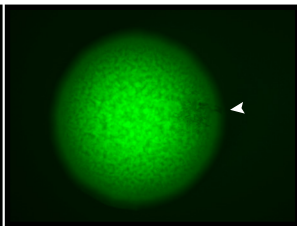
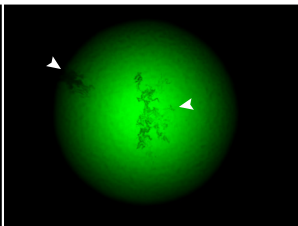
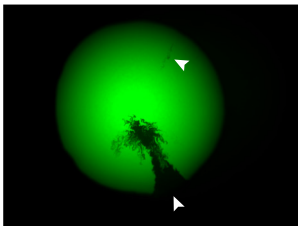
A

merodiploid colony

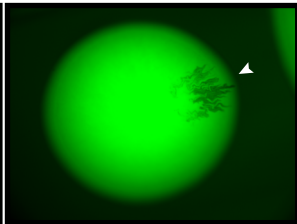
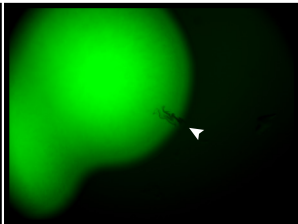
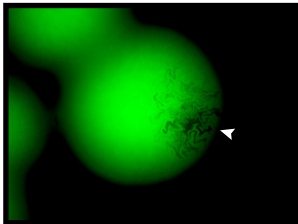
merodiploid colony
with mosaic loss of GFP**B**



Vibrio
ZWU0020



Aeromonas
ZOR0001



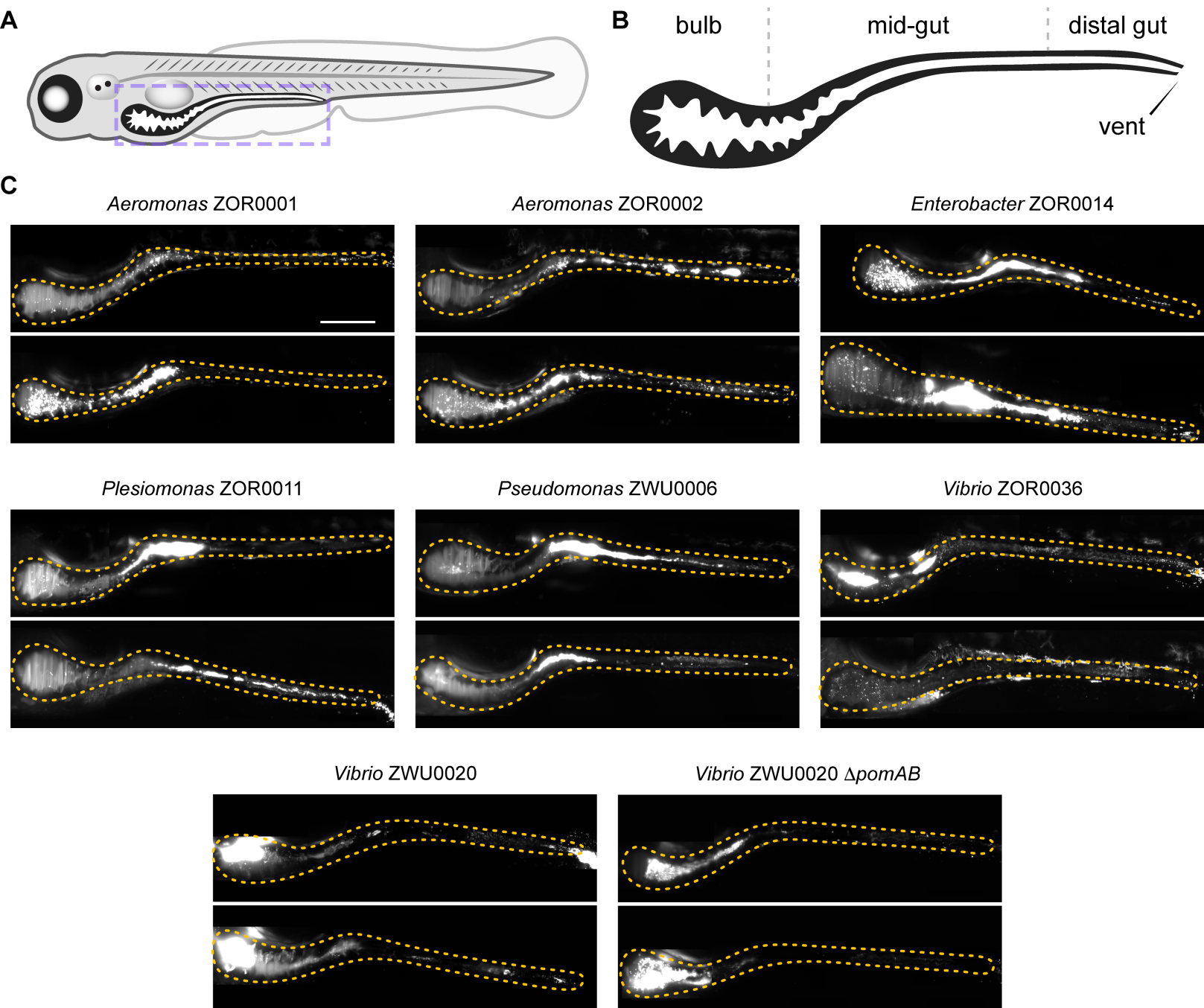
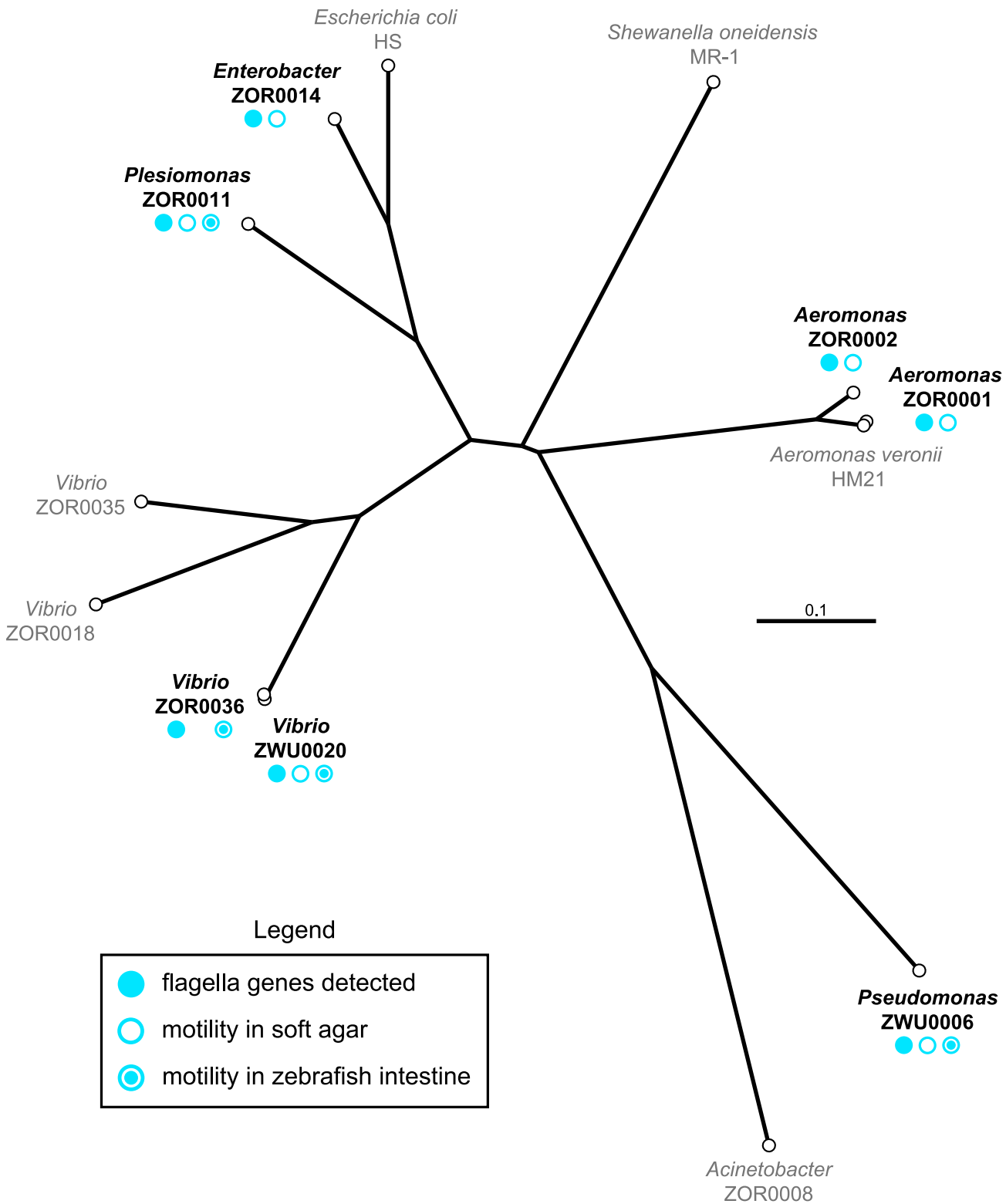


Figure 7-Figure Supplement 2.

Summary of traits exhibited by bacterial symbionts within the larval zebrafish intestine.

Strain	Median Abundance ^a	Dominant Growth Mode	Motile Individuals
<i>Aeromonas</i> ZOR0001	7.54×10^3	Aggregated	No
<i>Aeromonas</i> ZOR0002	5.41×10^3	Aggregated	No
<i>Enterobacter</i> ZOR0014	1.87×10^4	Aggregated	No
<i>Plesiomonas</i> ZOR0011	2.39×10^4	Aggregated	Yes
<i>Pseudomonas</i> ZWU0006	1.19×10^4	Aggregated	Yes
<i>Vibrio</i> ZOR0036	2.66×10^3	Mixed	Yes
<i>Vibrio</i> ZWU0020	5.02×10^4	Planktonic	Yes
ZWU0020 $\Delta pomAB$	8.17×10^3	Mixed	No

^aPopulation abundances were derived from three animals per strain and are based on segmentation of 3D images.



Vibrio
ZWU0020

Enterobacter
ZOR0014

Vibrio
ZOR0036

Plesiomonas
ZOR0011

Aeromonas
ZOR0001

**Pseudomonas*
ZWU0006

Aeromonas
ZOR0002

