1 Vertical transmission at the pathogen-symbiont interface: *Serratia symbiotica* and aphids

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3 Julie Perreau[a,b], Devki J. Patel[a], Hanna Anderson[a], Gerald P. Maeda[a], Katherine M. Elston[b],

4 Jeffrey E. Barrick[b], Nancy A. Moran[a]

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6 [a] Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712

7 [b] Department of Molecular Biosciences, University of Texas at Austin, Austin, TX 78712

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11 # Address correspondence to Julie Perreau, jperreau@utexas.edu

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Abstract

Many insects possess beneficial bacterial symbionts that occupy specialized host cells and are maternally transmitted. As a consequence of their host-restricted lifestyle, these symbionts often possess reduced genomes and cannot be cultured outside hosts, limiting their study. The bacterial species *Serratia symbiotica* was originally described by noncultured strains that live as mutualistic symbionts of aphids and are vertically transmitted through transovarial endocytosis within the mother's body. More recently, culturable strains of *S. symbiotica* were discovered that retain a larger set of ancestral *Serratia* genes, are gut pathogens in aphid hosts, and are principally transmitted via a fecal-oral route. We find that these culturable strains, when injected into pea aphids, replicate in the hemolymph and are pathogenic. Unexpectedly, they are also capable of maternal transmission via transovarial endocytosis: using GFP-tagged strains, we observe that pathogenic *S. symbiotica*, but not *Escherichia coli*, are endocytosed into early embryos. Furthermore, pathogenic *S. symbiotica* strains are compartmentalized into specialized aphid cells in a similar fashion to mutualistic *S. symbiotica* strains during later stages of embryonic development. Thus, cultured, pathogenic strains of *S. symbiotica* have the latent capacity to transition to lifestyles as mutualistic symbionts of aphid hosts. This capacity is blocked by pathogenicity: their hosts die before infected progeny are born. To transition into stably inherited symbionts, culturable *S. symbiotica* strains may need to adapt to regulate their titer, limit their pathogenicity, and/or provide benefits to aphids that outweigh their cost.

Keywords: Secondary symbiont, *Buchnera aphidicola*, bacteriocyte, sheath cells

38 **Importance**

39 Insects have evolved various mechanisms to reliably transmit their beneficial bacterial symbionts
40 to the next generation. Sap-sucking insects, including aphids, transmit symbionts by endocytosis
41 of the symbiont into cells of the early embryo within the mother's body. Experimental studies of
42 this process are hampered by the inability to culture or genetically manipulate host-restricted,
43 symbiotic bacteria. *Serratia symbiotica* is a bacterial species that includes strains ranging from
44 obligate, heritable symbionts to culturable gut pathogens. We demonstrate that culturable *S.*
45 *symbiotica* strains, that are aphid gut pathogens, can be maternally transmitted by endocytosis.
46 Cultured *S. symbiotica* therefore possess a latent capacity for evolving a host-restricted lifestyle
47 and can be used to understand the transition from pathogenicity to beneficial symbiosis.
48

Introduction

Host-associated bacteria can be placed on a continuum ranging from parasitic to mutualistic. Mutualistic symbionts often arise from pathogenic ancestors and rarely revert back to pathogenicity (1). This one-way transition is expected when vertical transmission, usually from mother to offspring, replaces horizontal transmission as the dominant route of new infection, causing symbionts to benefit from host reproduction and thereby to face strong selection for avirulence (2–4). In insects, the reliable vertical transmission of mutualistic symbionts can be accomplished by mechanisms that are external, including the placement of symbionts or symbiont-containing capsules on the egg surface, or internal, via transovarial transmission (5, 6).

Transovarial transmission, the transfer of maternal symbionts to eggs or embryos within the mother's body, is common in obligate symbioses where symbionts occupy special host cells (bacteriocytes) within the body cavity (7). Individual bacteria may be transferred, as in aphids, cicadas, leafhoppers, cockroaches, and bedbugs, or entire bacteriocytes may be transferred, as in whiteflies (7–10). In ancient symbioses with exclusively maternal transmission, hosts appear to control transmission, as the symbionts involved often possess reduced genomes devoid of pathogenicity factors that would allow them to invade host cells (11). However, host mechanisms for transmission may depend on bacterial factors for inter-partner recognition, and thereby limit which bacterial species or strains can make the transition from pathogenicity to commensal or mutualistic symbioses.

Aphids (Hemiptera: Sternorrhyncha: Aphidoidea) are a clade of roughly 5,000 species that feed exclusively on nutrient-poor plant sap and depend on the primary bacterial symbiont *Buchnera aphidicola* for biosynthesis of essential amino acids missing from their diet (12–16). *B. aphidicola* have been transovarially transmitted in aphids for over 160 million years (17).

72 They occupy bacteriocytes, possess highly reduced genomes, and cannot persist outside of their
73 hosts (11, 18). In addition to *B. aphidicola*, many aphids also host secondary symbionts, such as
74 *Serratia symbiotica, Candidatus* Hamiltonella defensa, *Candidatus* Regiella insecticola, and
75 *Candidatus* Fukatsuia symbiotica (19–22). Due to their more recent associations with aphids,
76 secondary symbionts provide an opportunity to understand the transition to host-restricted
77 lifestyles.

78 *S. symbiotica* strains have evolved diverse associations with aphids. They range from
79 pathogens and facultative mutualists to obligate mutualists that co-reside with *B. aphidicola* (23–
80 32). The genome sizes and gene contents of *S. symbiotica* strains reflect this variation in lifestyle,
81 ranging from larger genomes similar to those of free-living *Serratia* (31, 33), to highly reduced
82 genomes similar to those of *B. aphidicola* and other obligate symbionts (24, 26, 27, 32). The first
83 descriptions of *S. symbiotica*, initially named Pea Aphid Secondary Symbiont (PASS) or the "R-
84 type" symbiont, were of strains that occupied secondary bacteriocytes, sheath cells, and
85 hemolymph (34, 19). Unlike *B. aphidicola,* these *S. symbiotica* strains are not required by their
86 hosts; they provide secondary benefits, such as protection against heat stress (23, 35), and against
87 parasitoid wasps (36). However, similar to *B. aphidicola,* they are host-restricted and vertically
88 transmitted by a transovarial route (8, 37, 38). A detailed study showed that a mutualistic *S.*
89 *symbiotica* strain, present in the hemolymph, migrates to early embryos and is endocytosed with
90 *B. aphidicola* into the syncytium, i.e., a specialized, multinucleated cell of the early embryo (8).
91 In the pea aphid, *B. aphidicola* and *S. symbiotica* are later segregated into distinct bacteriocytes.

92 Recently, strains of pathogenic *S. symbiotica* have been discovered living in the guts of
93 *Aphis* species collected in Belgium and Tunisia (25, 29–31). These strains are hypothesized to
94 resemble ancestors of facultative and co-obligate *S. symbiotica* strains (39, 40). In contrast to

95 previously studied *S. symbiotica* strains, their primary transmission route appears to be
96 horizontal, through honeydew (feces) and host plant phloem (39, 40). Also, in contrast to
97 previously studied strains, these *S. symbiotica* can be cultured axenically. *S. symbiotica* CWBI-
98 2.3^{T}, from the black bean aphid (*Aphis fabae*) (25), retains a larger gene set and larger genome
99 (3.58 Mb) (33) than do facultative *S. symbiotica* strains Tucson and IS (2.78 and 2.82 Mb,
100 respectively) (41, 42).

101 In this work, we investigate whether cultured *S. symbiotica* strains are capable of vertical
102 transmission similar to facultative or obligate symbionts. We isolated a new *S. symbiotica* strain,
103 HB1, that shares many features with CWBI-2.3^{T} but is notably less pathogenic. We examined the
104 capacity of each of these strains to colonize hemolymph of the pea aphid (*Acyrthosiphon pisum*)
105 and access embryos through the transovarial route described for *B. aphidicola* (8). Both strains
106 are endocytosed into embryos, but pathogenic strains kill their hosts before the birth of offspring
107 infected by the transovarial route. Using GFP-tagged strains, we addressed whether transovarial
108 transmission is open to any bacterial cell that comes into contact with the embryo, or whether
109 this process involves specific partner recognition. We find that *Escherichia coli* cannot colonize
110 embryos, despite achieving a high titer within hosts. Thus, the endocytosis step required for
111 transovarial transmission limits the taxonomic range of bacteria that can readily evolve to
112 become aphid symbionts.

113

114 Results

115 Pathogenic *S. symbiotica* form a distinct group closely related to mutualistic strains

116 We examined the evolutionary relationships of all *S. symbiotica* with publicly available complete
117 genome sequences. To this list, we added a recently isolated and sequenced strain, designated *S.*

118 *symbiotica* HB1, from the melon aphid (*Aphis gossypii*). Our phylogenetic analysis was based on
119 176 shared orthologous genes and was rooted with outgroups that included other *Serratia* and
120 more distant *Enterobacterales* species (Figure 1, Figure S1, Table S1). *S. symbiotica* is split into
121 two clades, as previously reported (43). Clade A is comprised of strains that act as pathogens,
122 mutualists, or co-obligate symbionts in aphids from across the family Aphididae, while clade B
123 is composed of strains that live only as co-obligate symbionts in aphids of the subfamily
124 Lachninae. Clade A strains possess a range of genome sizes (1.54–3.58 Mb), GC content (47.9–
125 52.5%), host species, and lifestyles. Within clade A, the cultured, gut pathogen strains (CWBI-
126 2.3^{T}, HB1, Apa8A1, and 24.1) form a clade closely related to vertically transmitted mutualist
127 strains (Tucson, IS, MCAR-56S, AURT-53S). Although average nucleotide identities are high
128 (96.0–98.6%) across these strains (Table S2), the gut pathogen strains retain larger genomes
129 (3.09–3.58 Mb) and more *Serratia*-specific marker genes than non-pathogenic, maternally
130 transmitted *S. symbiotica* (0.65–2.82 Mb) (Figure 1, Table S1). Together, these observations
131 suggest that both lifestyles have recently emerged from a common, presumably pathogenic
132 ancestor.
133
134 **Cultured *S. symbiotica* strains are pathogenic when injected into pea aphid hemolymph**
135 Based on previous studies, *S. symbiotica* CWBI-2.3^{T} acts as a gut pathogen in its original host,
136 the black bean aphid (40), but appears to be less pathogenic in the gut of pea aphids (44). In both
137 hosts, *S. symbiotica* CWBI-2.3^{T} appears to be restricted to the gut and is not observed infecting
138 hemolymph. To determine whether *S. symbiotica* CWBI-2.3^{T} and HB1 can persist and act as
139 pathogens in the hemolymph of pea aphids, and to determine if these strains are capable of
140 vertical transmission to offspring, we injected fourth instar pea aphids with tagged strains CWBI-

141 2.3^{T}-GFP or HB1-GFP. Then, we tracked aphid survival, vertical transmission rates, and
142 bacterial titer over time. For comparison, we simultaneously performed injections of *S.*
143 *marcescens* Db11, a known insect pathogen (45), injections of hemolymph from pea aphids
144 infected with facultative, vertically transmitted *S. symbiotica* Tucson (41), and injections of
145 buffer as a negative control. We found that *S. symbiotica* CWBI-2.3^{T}-GFP and HB1-GFP act as
146 pathogens in pea aphid hemolymph. The survival rates of aphids injected with *S. symbiotica*
147 CWBI-2.3^{T}-GFP, *S. symbiotica* HB1-GFP, or *S. marcescens* Db11 were much lower than those
148 of aphids injected with buffer or with *S. symbiotica* Tucson ($P < 0.001$, Cox Proportional
149 Hazards Model) (Figure 2A).

150 Typically, *S. symbiotica* CWBI-2.3^{T} infects by a fecal-oral route or from plants (40). To
151 determine if *S. symbiotica* CWBI-2.3^{T}-GFP and HB1-GFP can be transmitted to offspring via the
152 transovarial route, as for mutualistic symbiotic strains, we collected newborn offspring of
153 surviving aphids every 24 hours and screened them for *S. symbiotica* by plating aphids and
154 noting presence or absence of colony-forming units (CFU). As transovarial transmission of
155 symbionts occurs early in embryonic development, there is a delay between injection and the
156 birth of infected offspring (34). To determine when after injection we should begin to observe
157 offspring infected by transovarial transmission, we injected mutualistic *S. symbiotica* Tucson and
158 monitored its transmission by sampling offspring and using PCR to screen for the presence of *S.*
159 *symbiotica*. Transmission of *S. symbiotica* Tucson was identified in newborn offspring starting at
160 10 days post-injection (DPI) (Figure 2B). The proportion of infected offspring per mother
161 increased over time, reaching 100% for most mothers by 15 DPI (Figure 2B). In contrast to
162 aphids injected with *S. symbiotica* Tucson, aphids injected with *S. symbiotica* CWBI-2.3^{T} did not
163 survive past 9 DPI (Figure 2A), and aphids injected with *S. symbiotica* HB1 showed reduced

164 fecundity and produced only a few, non-viable offspring after 9 DPI. Therefore, successful
165 vertical transmission of these strains appears to be limited by their pathogenicity.
166 We hypothesized that the virulence of *S. symbiotica* CWBI-2.3^T-GFP and HB1-GFP was
167 related to their titer in hemolymph. To test this hypothesis, aphids were sacrificed every 24 hours
168 to measure the CFU per aphid. *S. symbiotica* CWBI-2.3^T-GFP and HB1-GFP grow exponentially
169 within aphids, plateauing at ~10^9 CFU per aphid by 5-6 DPI (Figure 2C). The significant
170 difference in the growth rate of CWBI-2.3^T and HB1 at 3 and 4 DPI may explain the difference
171 in aphid survival rate across these treatment groups (Figure 2A). As *S. symbiotica* Tucson is not
172 culturable, qPCR was used to compare titers of CWBI-2.3^T-GFP and HB1-GFP to those reached
173 by Tucson at 5 DPI, and to the titers at which other mutualist *S. symbiotica* strains persist in pea
174 aphids reared in the lab (Figure 2D). Relative titer was calculated as the copy number of a single-
175 copy *Serratia* gene (*dnaK*) normalized by a single-copy pea aphid gene (*ef1a*). Both *S.*
176 *symbiotica* CWBI-2.3^T-GFP and HB1-GFP reach higher titers than *S. symbiotica* Tucson at 5
177 DPI (Figure 2D). The titer of *S. symbiotica* Tucson at 5 DPI is comparable to the steady-state
178 titer of *S. symbiotica* strains maintained in naturally infected, clonal pea aphids established as
179 laboratory lines (Figure 2D).
180
181 **Cultured *S. symbiotica* strains are capable of colonizing *Ac pisum* embryos**
182 Intergenerational transmission of *S. symbiotica* CWBI-2.3^T and HB1 was prevented because the
183 pathogenicity of these strains killed mothers before any infected embryos could be born. In order
184 to determine whether they were, nevertheless, capable of transovarial transmission, we injected
185 aphids with *S. symbiotica* CWBI-2.3^T, dissected ovarioles at 7 DPI, and used fluorescence *in-situ*
186 hybridization to visualize its infection pattern relative to the primary symbiont *B. aphidicola*

187 during embryonic development. To compare these results to the transmission pattern of a
188 mutualist strain, we injected aphids with hemolymph from pea aphids infected with *S. symbiotica*
189 Tucson (Figure 3A-D).

190 Embryos injected with *S. symbiotica* Tucson display regular growth and development,
191 reaching 400 µm in length by the time of katatrepsis (37) (Figure 3A). As previously described,
192 *B. aphidicola* and mutualistic *S. symbiotica* are transmitted to the syncytium of stage 7 blastula
193 from hemolymph via an endocytic process (8, 37, 46). *S. symbiotica* Tucson appears to be unable
194 to infect embryos at later developmental stages, as embryos that were beyond stage 7 at the time
195 of injection lack *S. symbiotica* (Figure 3B). This observation is supported by the absence of *S.*
196 *symbiotica* in offspring born the first 10 days after injection (Figure 2B) (34). Those embryos
197 exposed to *S. symbiotica* Tucson at stage 7 possess *S. symbiotica* in sheath cells, but Tucson does
198 not invade primary bacteriocytes that contain *B. aphidicola* (Figure 3C). The endocytosis of *S.*
199 *symbiotica* Tucson into stage 7 embryos occurs at the posterior end of the embryo (Figure 3D),
200 as previously described for the closely related strain *S. symbiotica* IS (8).

201 Despite its pathogenicity, the infection pattern of CWBI-2.3^T is similar to that of the non-
202 pathogenic Tucson strain (Figures 3E, 3F). Cells of CWBI-2.3^T are attached to the embryonic
203 surface but do not infect embryos that are beyond stage 7 (Figure 3G). Following infection,
204 CWBI-2.3^T is packaged similarly to the Tucson strain; both are sorted into sheath cells and
205 cannot colonize the primary bacteriocytes that house *B. aphidicola* (Figures 3C, 3H). CWBI-2.3^T
206 is endocytosed into early embryos with *B. aphidicola* (Figure 3I), as is *S. symbiotica* HB1
207 (Movie S1). Remarkably, at 7 DPI, CWBI-2.3^T greatly outnumbers *B. aphidicola* in the syncytial
208 cell (Figure 3I). In contrast to *S. symbiotica* Tucson, infection with cultured *S. symbiotica* CWBI-

209 2.3^{T} stunts embryonic growth, though it does not prevent progression through characteristic
210 developmental stages (Figures 3E, 3F).
211
212 **Transovarial transmission is a specific capability of *S. symbiotica* strains**
213 To determine if endocytosis is selective at the level of bacterial species, we tested the
214 transmission capability of *E. coli* K-12 strain BW25113. *E. coli* is related to *B. aphidicola, S.*
215 *symbiotica,* and several other mutualistic symbionts of aphids which are all within
216 *Enterobacterales* (20). We chose strain BW25113 because it can infect the gut and hemolymph
217 of pea aphids and kills aphids a few days post infection (47). We created the tagged *E. coli* strain
218 BW25113-GFP and injected it into fourth instar aphids as described above. For comparison, we
219 injected *S. symbiotica* CWBI-2.3^{T}-GFP into a separate set of fourth instar aphids. *E. coli*
220 BW25113-GFP forms a robust infection in pea aphid hemolymph, reaching comparable titers to
221 *S. symbiotica* CWBI-2.3^{T}-GFP at 5 DPI (Figure 4A). We dissected single ovarioles from ten
222 aphids in each treatment group at 5 DPI and observed early embryos to determine infection
223 status. Using this approach, *S. symbiotica* CWBI-2.3^{T}-GFP could be seen infecting early
224 embryos (Figures 4B, 4C, Movies S2, S3). In contrast, *E. coli* BW25113-GFP attaches to the
225 embryonic surface, sometimes coating the entire exterior of the embryo, but was never observed
226 endocytosing into embryos (Figures 4D, 4E, Movies S4, S5).
227
228 **Discussion**
229 Transovarial transmission is a key feature of many insect-bacterial symbioses wherein bacteria
230 provide a benefit to their host. This transmission route is linked to irreversible bacterial
231 transitions from pathogenicity to mutualism (2). However, many relationships that rely on

232 transovarial transmission are ancient, and their early stages cannot be experimentally
233 recapitulated, leaving unanswered if and how pathogenic bacteria access this transmission route.
234 Focusing on secondary symbionts may be more useful for understanding these early transitions,
235 as some secondary symbionts or their close relatives are culturable, genetically tractable, and can
236 be removed from or introduced to hosts without dramatically compromising host fitness (48–50).
237 For example, *Sodalis praecaptivus,* a close relative to *Sodalis* species found as host-restricted
238 symbionts across diverse insects, uses quorum sensing to attenuate virulence and gain access to
239 vertical transmission in a non-native host, the tsetse fly (51). The bacterial species *S. symbiotica*
240 was first known as a vertically transmitted mutualist, but pathogenic strains were subsequently
241 cultured from aphids collected in Europe and Africa (25, 29–31), and, in this study, in North
242 America. These strains have provided a new opportunity to dissect the early steps involved in the
243 transition to a host-restricted lifestyle. Knowing that vertical transmission is a key to this
244 transition, we aimed to determine whether culturable, pathogenic *S. symbiotica* could access this
245 pathway and what, if any, limitations are faced by *S. symbiotica* in this transition.
246 Cultured *S. symbiotica* strains are close relatives to non-culturable, vertically transmitted
247 mutualists. However, several lines of evidence suggest these strains lack a history of maternal
248 transmission in aphids. For one, persistent vertical transmission generally leads to the irreversible
249 loss of genes such that they can no longer access a free-living or pathogenic lifestyle (52).
250 Relative to vertically transmitted strains, CWBI-2.3^{T}, HB1, Apa8A1, and 24.1 are culturable,
251 maintain larger genomes, and possess more ancestral genes common to free-living *Serratia*
252 (Figure 1). Second, these strains do not appear to undergo vertical transmission in natural
253 infections. Following ingestion by black bean or pea aphids, *S. symbiotica* CWBI-2.3^{T} is not
254 subsequently detected in hemolymph or embryos, but is present in the gut and in honeydew,

255 suggesting that the dominant route of transmission for this strain is fecal-oral (29, 39, 44). We
256 injected CWBI-2.3^T and HB1 into hemolymph to determine if they are capable of vertical
257 transmission in pea aphids. Vertical transmission is theorized to be the primary force driving
258 permanent bacterial transitions from pathogenicity to mutualism, but to do so, vertical
259 transmission must precede mutualism (2). That pathogenic *S. symbiotica* CWBI-2.3^T and HB1
260 are endocytosed into the syncytial cell of early embryos along with *B. aphidicola* provides
261 empirical evidence for the precedence of vertical transmission in this system. Together, these
262 results suggest that the aphid gut has served as an access point for environmental or plant-
263 associated *Serratia* to infect aphids and that gut pathogenicity was an ancestral lifestyle for
264 strains that are now intracellular and mutualistic (29).

265 *S. symbiotica* is common to natural populations of pea aphids (34, 53), and previous 16S
266 rRNA gene surveys have identified *S. symbiotica* in aphid tribes from across the Aphidoidea (29,
267 54). However, pathogenic and mutualistic strains have near-identical 16S rRNA sequences, so it
268 is unclear how many cases represent *S. symbiotica* pathogens. To date, pathogenic strains have
269 been cultured only from *Aphis* species. However, *S. symbiotica* CWBI-2.3^T can horizontally
270 transmit across aphids feeding on the same plant (39), and can infect the guts of alternative aphid
271 species, including the pea aphid (44), suggesting that pathogenic *S. symbiotica* may be more
272 widespread across aphid genera in nature. The global distribution of *Aphis*-associated strains,
273 along with their ability to transmit using the same transovarial route as *B. aphidicola,* suggests
274 that related gut-associated strains may serve as a source pool for the evolution of commensal or
275 mutualistic strains. Mutualism may have arisen several times independently in *S. symbiotica,*
276 and, along with the subsequent horizontal transfer, would contribute to the phylogenetic
277 discordance between facultative symbionts and their aphid hosts (54). The acquisition and

278 replacement of secondary symbionts has occurred during the evolution of many ancient insect-
279 microbe symbioses and may help hosts to escape the "evolutionary rabbit hole" of dependence
280 on a primary symbiont that has become an ineffective mutualist due to genome decay (55, 56).
281 Transovarial transmission in aphids occurs by bacterial endocytosis into the syncytial cell of
282 early embryos (8). What host and bacterial factors are involved in this pathway are unclear, but
283 insights may be gained from other systems in which hosts are genetically tractable. In
284 *Drosophila,* knockout of yolk proteins or the Yolkless receptor results in reduced localization to
285 and/or endocytosis of *Spiroplasma* in embryos, suggesting that the vitellogenin pathway is
286 involved in transovarial transmission (57). While parthenogenetic aphids do not undergo
287 vitellogenesis or produce visible yolk, it is possible that similar receptor-mediated processes are
288 used for *B. aphidicola* and *S. symbiotica* transmission, and that specific bacterial ligands are
289 required. If this is the case, *S. symbiotica* strains that normally live in the aphid gut appear to
290 possess the requisite molecular determinants, as they display an innate potential for endocytosis
291 into embryos. Furthermore, the inability of *E. coli* BW25113 to endocytose into the syncytial cell
292 of embryos suggests that the endocytic step of transovarial transmission contributes to selectivity
293 in this system. While many bacterial taxa occasionally infect pea aphids (30, 58), few are found
294 as long-term mutualists. The primary symbiont, *B. aphidicola*, is stably maintained in most aphid
295 lineages, though rare replacements exist (e.g., (59)). Additionally, few species are found as
296 secondary symbionts*,* and most are members of the *Enterobacterales*, including *S. symbiotica,*
297 *Ca.* H. defensa*, Ca.* R. insecticola*, Ca.* F. symbiotica*,* and *Arsenophonus,* and, less commonly
298 other bacterial groups, including *Wolbachia, Rickettsia*, and *Spiroplasma* (21, 22).
299 Aphids that are co-infected with *B. aphidicola* and mutualistic secondary symbionts possess
300 several known mechanisms that limit the competition between these bacteria. For one, hosts can

301 sort symbionts into distinct cell types, with *B. aphidicola* in primary bacteriocytes and *S.*
302 *symbiotica* relegated to secondary bacteriocytes and sheath cells (60). Despite its pathogenicity,
303 we observed that CWBI-2.3^T is not able to invade primary bacteriocytes with *B. aphidicola* and
304 is compartmentalized into sheath cells in a similar manner as mutualistic strains (Figure 3). Pea
305 aphid genotypes may vary in their ability to associate with secondary symbionts. In contrast to
306 the results obtained with *Acyrthosiphon pisum* LSR1 in our study, when the facultative, host-
307 restricted strain *S. symbiotica* IS was transferred to *Acyrthosiphon pisum* AIST, it showed a
308 disordered localization, invading primary bacteriocytes with *B. aphidicola* (61). In these cases, *S.*
309 *symbiotica* IS was trapped in primary bacteriocytes, unable to exocytose during transmission (8).
310 The specific exocytosis of *B. aphidicola* also likely plays a role in limiting competition between
311 *B. aphidicola* and secondary symbionts across multiple generations.
312 The vertical transmission of CWBI-2.3^T and HB1 in pea aphids is limited by their virulence
313 in hemolymph, as infected aphids do not live long enough to birth offspring infected by
314 transovarial transmission. Both CWBI-2.3^T and HB1 are more pathogenic in hemolymph than
315 facultative *S. symbiotica* Tucson, but also notably far less pathogenic than *S. marcescens* Db11.
316 Possibly, adaptation to the gut selects for reduced *Serratia* virulence by allowing *Serratia* the
317 time to form a robust gut infection and transmit to other aphids, including offspring, via
318 honeydew (40). The virulence of CWBI-2.3^T and HB1 coincides with unregulated titer, as both
319 of these strains attain 100 to 1,000-fold higher titers relative to mutualistic *S. symbiotica* when
320 injected into hemolymph. This enormous difference in titer, and the constancy of the low titers
321 observed for the symbiotic strains, suggests that mutualistic *S. symbiotica* growth is regulated.
322 The regulation of virulence and titer is important in the establishment of vertical transmission.
323 Self-regulation of both titer and virulence through quorum sensing has been demonstrated in

324 *Sodalis praecaptivus,* and allows this species to establish vertically transmitted infections in
325 weevil and tsetse fly hosts (51, 62). Whether mutualistic *S. symbiotica* have relied on similar
326 mechanisms to establish persistent vertical transmission in aphids is unclear. Alternatively, *S.*
327 *symbiotica* virulence may be attenuated by the loss of one or several key virulence factors before
328 the establishment of vertical transmission. The experimental tractability of these strains will
329 allow for future investigations focused on these attenuation mechanisms and the role of vertical
330 transmission in the transition to a host-restricted lifestyle in aphids.
331

332 ## Materials and Methods

333 **Additional details are provided in SI Appendix.**
334

335 **Isolation and culture of *S. symbiotica.*** S. *symbiotica* strain CWBI-2.3^T (DSM 23270) was
336 obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures and was
337 grown on tryptic soy agar (TSA) plates at 27 °C (25). *S. symbiotica* strain HB1 was isolated from
338 the melon aphid (*Aphis gossypii*), collected in August 2018 from HausBar Farms in Austin,
339 Texas. Details are provided in SI Appendix.
340

341 ***S. symbiotica* HB1 genome sequencing.** *S. symbiotica* HB1 was grown in TSB at room
342 temperature, harvested at an OD_{600} ~ 1.0, and DNA was extracted with the DNeasy Blood &
343 Tissue Kit (Qiagen). A paired-end sequencing library with dual barcodes was prepared using the
344 Illumina Nextera XT DNA kit, and sequencing was performed on an Illumina iSeq 100. Raw
345 reads were trimmed using Trimmomatic (63) and assembled using the SPAdes algorithm (64) via
346 Unicycler (65). Genome contamination and completeness were assessed using CheckM (66).

347

348 **Phylogenetic analysis.** *S. symbiotica* and outgroup genomes used for phylogenetic analysis are
349 listed in Table S1. Genomes were downloaded from the NCBI Assembly Database on March 2,
350 2020. All outgroup genomes were filtered for completeness > 95% and contamination < 5%
351 using CheckM (66). Annotations were obtained using Prokka (67), and 176 single-copy
352 orthologs were identified by OrthoFinder (68). These single-copy orthologs were aligned with
353 MAFFT (69), trimmed using a BLOSUM62 matrix in BMGE (70), and concatenated using an in-
354 house script, producing an alignment with 56,881 total amino acid positions. A tree was
355 constructed by maximum likelihood with a JTT+R10 model and 100 bootstraps, using IQ-Tree
356 (71). The complete phylogeny is available as Figure S1. The presence of *Serratia* marker genes
357 was determined using CheckM with the *Serratia* marker gene set provided with CheckM. The
358 average nucleotide identity for *S. symbiotica* genomes was calculated using FastANI (72).
359

360 **Chromosomal integration of sfGFP.** Superfolder GFP (sfGFP) was integrated into the
361 chromosomes of *S. symbiotica* CWBI-2.3^{T}, *S. symbiotica* HB1, and *E. coli* BW25113 through
362 mini-Tn7 tagging, as described by Choi *et al.* (73). Details are provided in SI Appendix.
363

364 **Tracking aphid survival, fecundity, and transmission after injection with *S. marcescens, S.***
365 ***symbiotica,* and injection buffer.** Fourth instar pea aphids were injected with *S. marcescens*
366 Db11*,* recombinant *S. symbiotica* CWBI-2.3^{T}-GFP, recombinant *S. symbiotica* HB1-GFP,
367 hemolymph from pea aphids infected with *S. symbiotica* Tucson, or injection buffer, as described
368 above. Every 24 h, survival was recorded, offspring were collected, and surviving adults were

369 moved to a fresh dish. Adults were collected at death or at the end of the experiment at 15 DPI
370 and screened for the presence or absence of *S. symbiotica*. Details are provided in SI Appendix.
371
372 **Bacterial titer by spot-plating and quantitative PCR (qPCR).** Fourth instar pea aphids were
373 injected with recombinant *S. symbiotica* CWBI-2.3^{T}-GFP, recombinant *S. symbiotica* HB1-GFP,
374 or with hemolymph from pea aphids infected with *S. symbiotica* Tucson, as described above. At
375 24 hours, aphids were transferred in sets of 15 to seedlings of *V. faba* and stored under long-day
376 conditions (16H light, 8H dark) in incubators held at constant 20 °C. At each timepoint, aphids
377 were collected in separate tubes, surface sterilized in 10% bleach for 1 minute, rinsed in
378 deionized water for 1 min, then crushed and resuspended in 100 μL PBS. For aphids injected
379 with culturable *S. symbiotica* CWBI-2.3^{T}-GFP or HB1-GFP, 50 μL of this homogenate was used
380 for spot plating and 50 μL frozen for DNA extraction and qPCR. For aphids injected with *S.*
381 *symbiotica* Tucson, all 100 μL of homogenate was frozen and used for DNA extraction and
382 qPCR. Details are provided in SI Appendix.
383
384 **Statistical analyses.** All statistical analyses and graphing were performed in the R programming
385 language (version 3.6.3) (74). Survival rates for each treatment group were visualized as Kaplan-
386 Meier survival curves, and comparisons of rates across treatment groups were performed using
387 the Cox Proportional Hazards Model. Bacterial titers across treatment groups were compared
388 using Kruskal-Wallis analysis of variance, followed by Dunn's multiple comparisons test.
389
390 **Fluorescence *in-situ* hybridization (FISH) microscopy.** Fourth instar pea aphids were injected
391 with wild-type *S. symbiotica* CWBI-2.3^{T}, wild-type *S. symbiotica* HB1, or with hemolymph from

392 pea aphids infected with *S. symbiotica* Tucson, as described above. Embryos were dissected at 4
393 DPI (Movie S1) or 7 DPI (Figure 3) in 70% ethanol. FISH was performed as in Koga *et al.* (8)
394 with slight modifications. Details are provided in SI Appendix.
395
396 **Live imaging of *E. coli* and *S. symbiotica* in pea aphids.** For live imaging, fourth instar
397 *Acyrthosiphon pisum* LSR1 were injected with recombinant *S. symbiotica* CWBI-2.3^{T}-GFP or
398 with recombinant *E. coli* BW25113-GFP, as described above. At 24 hours, aphids were
399 transferred in sets of 15 to seedlings of *V. faba* and stored under long-day conditions (16H light,
400 8H dark) in incubators held at constant 20 °C. At 5 DPI, a subset of aphids from each treatment
401 group were used to obtain titer counts via spot-plating, as described above, and remaining aphids
402 were dissected in TC-100 insect medium. Single ovarioles from ten infected aphids per treatment
403 group were observed under a Zeiss LSM 710 confocal microscope.
404

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416

417 **Data availability**

418 This Whole Genome Shotgun project for *S. symbiotica* HB1 has been deposited at
419 DDBJ/ENA/GenBank under the accession JACBGK000000000. The version described in this
420 paper is version JACBGK010000000.
421

422 **Competing interests**

423 The authors declare that they have no competing interests.
424

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429

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621

622 **Figure Legends**

623

624 **Figure 1.** Phylogeny and gene content evolution of *S. symbiotica*. (A) Maximum likelihood
625 phylogeny of *S. symbiotica,* based on concatenation of 176 single-copy orthologs (56,881 amino
626 acid positions) shared across *S. symbiotica,* other *Serratia* species, *E. coli, Yersinia pestis,* and
627 *Salmonella enterica*. Genomes used for the phylogeny and relevant references for *S. symbiotica*
628 genome features are listed in Table S1. Bootstrap values are indicated with symbols on nodes.
629 The complete phylogeny is presented in Figure S1.

630

631 **Figure 2.** Infection dynamics of *S. symbiotica* strains CWBI-2.3^{T} and HB1 injected into
632 hemolymph of fourth instar pea aphids. (A) Survival curves of pea aphids injected with cultured
633 *S. marcescens* Db11, recombinant *S. symbiotica* CWBI-2.3^{T}-GFP, or recombinant *S. symbiotica*
634 HB1-GFP, with hemolymph from *S. symbiotica* Tucson, or with injection buffer. *** $P < 0.001$,
635 Cox Proportional Hazards Model. (B) Fecundity and transmission of *S. symbiotica* for adults

636 injected with *S. symbiotica* Tucson. Boxplots in gray depict the total offspring, including infected
637 and uninfected offspring, per adult. Boxplots in orange depict total offspring infected with *S.*
638 *symbiotica* Tucson per adult. Filled curves represent a LOESS regression and display the
639 increase in offspring infected with *S. symbiotica* Tucson from 10 DPI to 15 DPI. (C) Bacterial
640 titer of recombinant *S. symbiotica* strains CWBI-2.3^{T}-GFP and HB1-GFP, obtained by spot-
641 plating dilutions from whole aphids. ** $p < 0.01$, Wilcoxon rank-sum test. (D) Relative titer of *S.*
642 *symbiotica* across treatment groups at 5 DPI (left) and for 7-day-old pea aphids from lab-reared
643 clonal lines that are naturally infected with vertically transmitted *S. symbiotica* strains,
644 established from collections in Austin in 2014 (Austin), Tucson in 2019 (Tuc19), Tucson in 1999
645 (Tucson), and Wisconsin in 2017 (WIG5) (right). Relative titer was calculated as the copy
646 number of *S. symbiotica dnaK* normalized by copy number of the single copy *Acyrthosiphon*
647 *pisum* gene *ef1a*. CWBI-2.3^{T} and HB1 samples used for qPCR are the same as those used to
648 determine bacterial titer by spot-plating. Letters a and b denote two groups of strains that have
649 significantly different titers from one another and indistinguishable titers within groups at $\alpha =$
650 0.01 by the Kruskal-Wallis test followed by Dunn's multiple comparison test.
651
652 **Figure 3.** Transovarial transmission of the pathogenic strain *S. symbiotica* CWBI-2.3^{T} and the
653 mutualistic strain *S. symbiotica* Tucson in stage 7 embryos of pea aphids. (A) *S. symbiotica*
654 Tucson infection across embryonic stages at 7 DPI. (B) *S. symbiotica* Tucson does not infect
655 embryos that are beyond stage 7 of development at the time of injection, as indicated by a lack of
656 *S. symbiotica* Tucson in late-stage embryos. (C) In infected, mid-stage embryos, *S. symbiotica*
657 Tucson cannot infect primary bacteriocytes that contain *B. aphidicola* but does infect sheath
658 cells. (D) *S. symbiotica* Tucson enters the syncytium of an early-stage blastula with *B. aphidicola*

659 but is underrepresented relative to *B. aphidicola* during this infection. (E-F) *S. symbiotica*
660 CWBI-2.3^T infection across embryonic stages at 7 DPI. Embryos depicted in (E) and (F) derive
661 from the same ovariole but were separated during dissection. Infection with this strain results in
662 reduced embryonic growth, as indicated by scale bars. (G) *S. symbiotica* CWBI-2.3^T does not
663 infect embryos that are beyond stage 7 of development at the time of injection, as indicated by a
664 lack of *S. symbiotica* CWBI-2.3^T in late-stage embryos. (H) In infected, mid-stage embryos, *S.*
665 *symbiotica* CWBI-2.3^T cannot infect primary bacteriocytes that contain *B. aphidicola* but does
666 infect sheath cells. (I) *S. symbiotica* CWBI-2.3^T enters the syncytium of an early-stage blastula
667 with *B. aphidicola* and is overrepresented relative to *B. aphidicola* during this infection. Blue,
668 red, and green signal are used for host nuclei, *S. symbiotica,* and *B. aphidicola,* respectively.
669
670 **Figure 4.** Ability of *S. symbiotica* CWBI-2.3^T but not *E. coli* BW25113 to achieve transovarial
671 transmission to stage 7 embryos of pea aphids. (A) Recombinant *S. symbiotica* CWBI-2.3^T-GFP
672 and *E. coli* BW25113-GFP reach comparable titers 5 DPI, as determined by spot-plating. (B, C)
673 Recombinant *S. symbiotica* CWBI-2.3^T-GFP enters the posterior region of stage 7 blastula.
674 Embryos were dissected and visualized at 5 DPI. Dotted lines outline *S. symbiotica* present
675 within the embryo. (D, E) Recombinant *E. coli* BW25113-GFP does not enter into stage 7
676 blastula. Embryos were dissected and visualized at 5 DPI. Arrows indicate bacteria attached to
677 the embryonic surface. Scale bars represent 20 μM.
678

679 # Supplementary Figures, Tables, and Movies

680

681 **Figure S1.** Full phylogeny of *S. symbiotica* and outgroup. Maximum likelihood phylogeny with
682 a JTT+R10 model and 100 bootstraps, based on concatenation of 176 single-copy orthologs
683 (56,881 amino acid positions) shared across *S. symbiotica, Serratia*, *E. coli, Yersinia pestis,* and
684 *Salmonella enterica*. Full list of genomes used for the phylogeny is provided in Table S1.
685
686 **Table S1.** Accession numbers, genome information, and references for analyzed *S. symbiotica*
687 strains (Figures 1, S1).
688
689 **Table S2.** Average nucleotide identities for pairwise combinations of *S. symbiotica* strains.
690
691 **Movie S1.** Z-stack showing *S. symbiotica* HB1 entering the syncytium of a stage 7 embryo with
692 *B. aphidicola* at 4 DPI. Blue, red, and green signal are used for host nuclei, *S. symbiotica,* and *B.*
693 *aphidicola,* respectively.
694
695 **Movie S2.** Z-stack showing *S. symbiotica* CWBI-2.3^{T}-GFP entering a live stage 7 embryo. Z-
696 stack corresponds to the embryo depicted in Figure 4B.
697
698 **Movie S3.** Z-stack showing *S. symbiotica* CWBI-2.3^{T}-GFP entering a live stage 7 embryo. Z-
699 stack corresponds to the embryo depicted in Figure 4C.
700
701 **Movie S4.** Z-stack showing *E. coli* BW25113-GFP surrounding, but not entering, a live stage 7
702 embryo. Z-stack corresponds to the embryo depicted in Figure 4D.
703

704 **Movie S5.** Z-stack showing *E. coli* BW25113-GFP surrounding, but not entering, a live stage 7

705 embryo. Z-stack corresponds to the embryo depicted in Figure 4E.

706

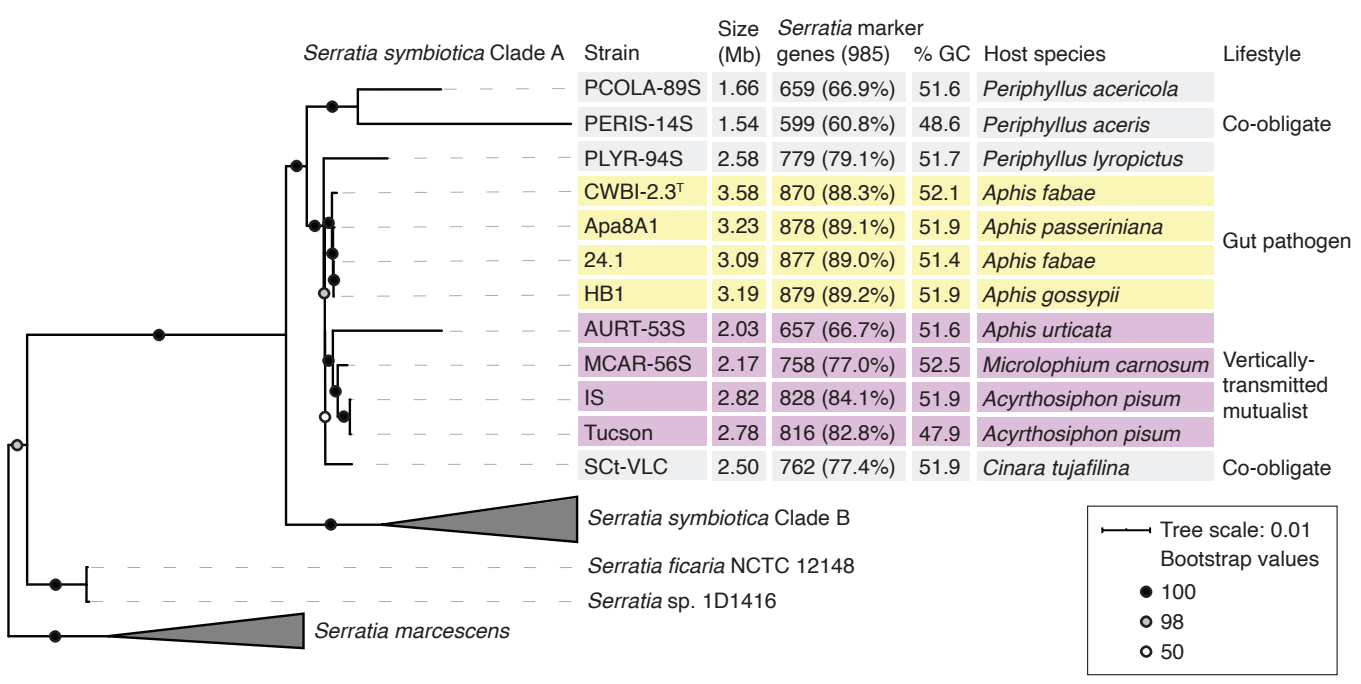

Figure 1. Phylogeny and gene content evolution of *S. symbiotica*. (A) Maximum likelihood phylogeny of *S. symbiotica*, based on concatenation of 176 single-copy orthologs (56,881 amino acid positions) shared across *S. symbiotica*, other *Serratia* species, *E. coli*, *Yersinia pestis*, and *Salmonella enterica*. Genomes used for the phylogeny and relevant references for *S. symbiotica* genome features are listed in Table S1. Bootstrap values are indicated with symbols on nodes. The complete phylogeny is presented in Figure S1.

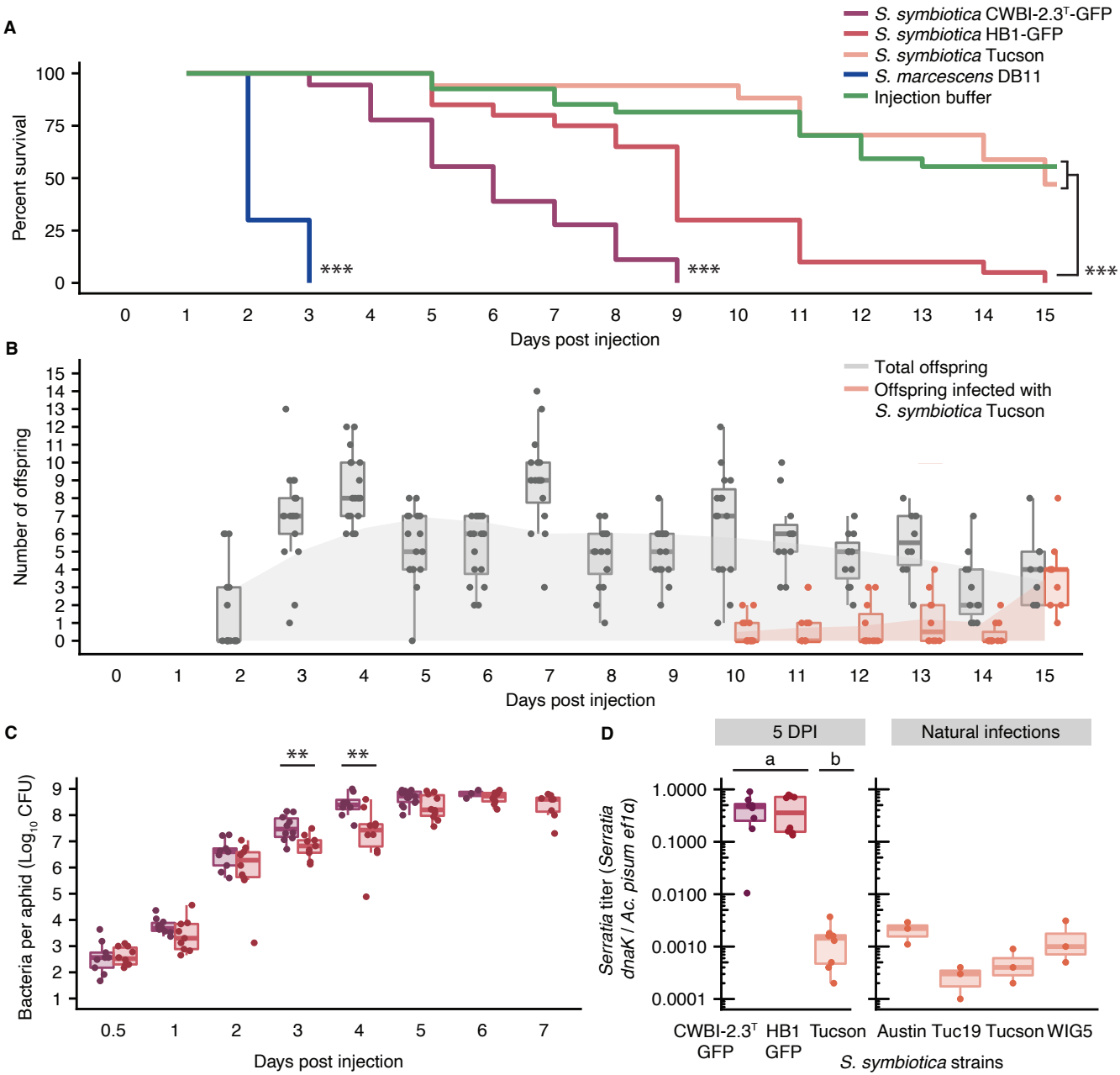

Figure 2. Infection dynamics of *S. symbiotica* strains CWBI-2.3^T and HB1 injected into hemolymph of fourth instar pea aphids. (A) Survival curves of pea aphids injected with cultured *S. marcescens* Db11, recombinant *S. symbiotica* CWBI-2.3^T-GFP, or recombinant *S. symbiotica* HB1-GFP, with hemolymph from *S. symbiotica* Tucson, or with injection buffer. *** $P < 0.001$, Cox Proportional Hazards Model. (B) Fecundity and transmission of *S. symbiotica* for adults injected with *S. symbiotica* Tucson. Boxplots in gray depict the total offspring, including infected and uninfected offspring, per adult. Boxplots in orange depict total offspring infected with *S. symbiotica* Tucson per adult. Filled curves represent a LOESS regression and display the increase in offspring infected with *S. symbiotica* Tucson from 10 DPI to 15 DPI. (C) Bacterial titer of recombinant *S. symbiotica* strains CWBI-2.3^T-GFP and HB1-GFP, obtained by spot-plating dilutions from whole aphids. ** $p < 0.01$, Wilcoxon rank-sum test. (D) Relative titer of *S. symbiotica* across treatment groups at 5 DPI (left) and for 7-day-old pea aphids from lab-reared clonal lines that are naturally infected with vertically transmitted *S. symbiotica* strains, established from collections in Austin in 2014 (Austin), Tucson in 2019 (Tuc19), Tucson in 1999 (Tucson), and Wisconsin in 2017 (WIG5) (right). Relative titer was calculated as the copy number of *S. symbiotica dnaK* normalized by copy number of the single copy *Acyrthosiphon pisum* gene *ef1a*. CWBI-2.3^T and HB1 samples used for qPCR are the same as those used to determine bacterial titer by spot-plating. Letters a and b denote two groups of strains that have significantly different titers from one another and indistinguishable titers within groups at $\alpha = 0.01$ by the Kruskal-Wallis test followed by Dunn's multiple comparison test.

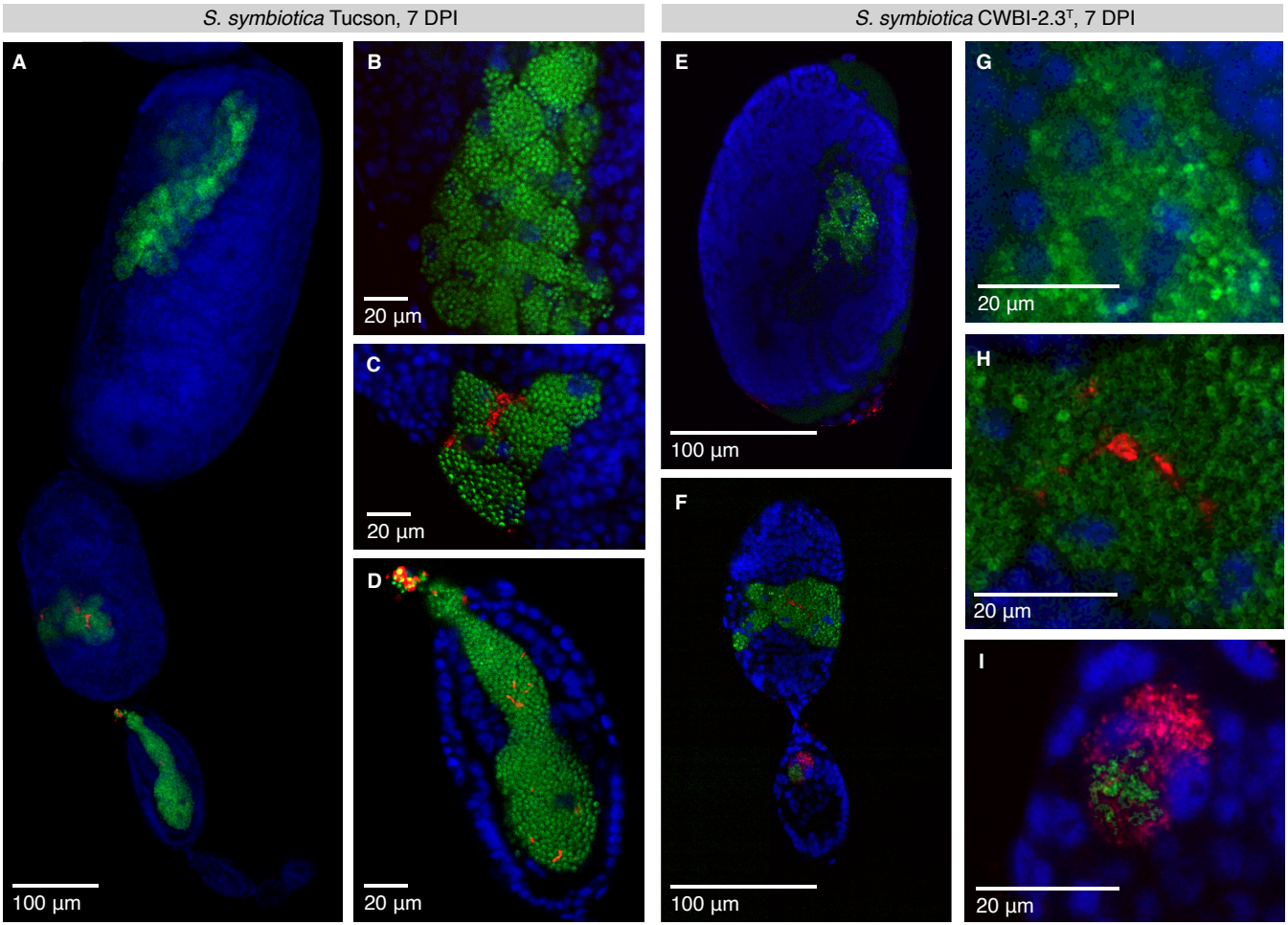

Figure 3. Transovarial transmission of the pathogenic strain *S. symbiotica* CWBI-2.3^T and the mutualistic strain *S. symbiotica* Tucson in stage 7 embryos of pea aphids. (A) *S. symbiotica* Tucson infection across embryonic stages at 7 DPI. (B) *S. symbiotica* Tucson does not infect embryos that are beyond stage 7 of development at the time of injection, as indicated by a lack of *S. symbiotica* Tucson in late-stage embryos. (C) In infected, mid-stage embryos, *S. symbiotica* Tucson cannot infect primary bacteriocytes that contain *B. aphidicola* but does infect sheath cells. (D) *S. symbiotica* Tucson enters the syncytium of an early-stage blastula with *B. aphidicola* but is underrepresented relative to *B. aphidicola* during this infection. (E-F) *S. symbiotica* CWBI-2.3^T infection across embryonic stages at 7 DPI. Embryos depicted in (E) and (F) derive from the same ovariole but were separated during dissection. Infection with this strain results in reduced embryonic growth, as indicated by scale bars. (G) *S. symbiotica* CWBI-2.3^T does not infect embryos that are beyond stage 7 of development at the time of injection, as indicated by a lack of *S. symbiotica* CWBI-2.3^T in late-stage embryos. (H) In infected, mid-stage embryos, *S. symbiotica* CWBI-2.3^T cannot infect primary bacteriocytes that contain *B. aphidicola* but does infect sheath cells. (I) *S. symbiotica* CWBI-2.3^T enters the syncytium of an early-stage blastula with *B. aphidicola* and is overrepresented relative to *B. aphidicola* during this infection. Blue, red, and green signal are used for host nuclei, *S. symbiotica*, and *B. aphidicola*, respectively.

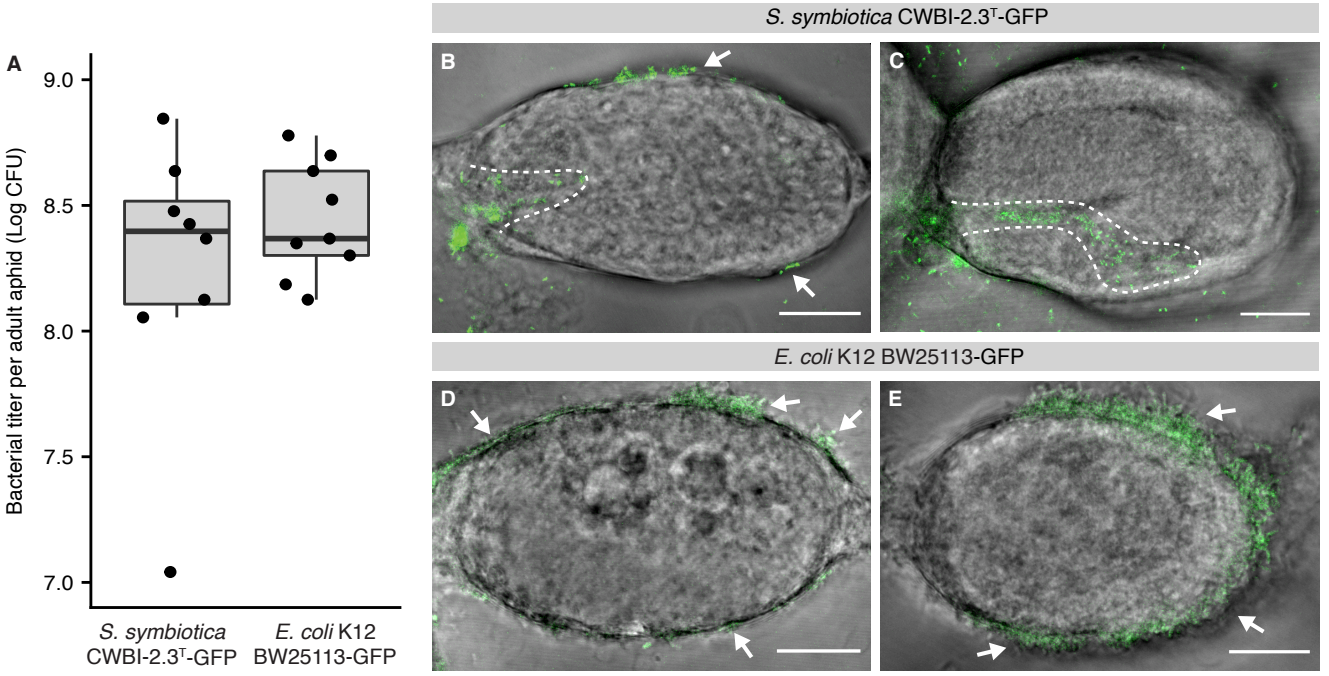

Figure 4. Ability of *S. symbiotica* CWBI-2.3^{T} but not *E. coli* BW25113 to achieve transovarial transmission to stage 7 embryos of pea aphids. (A) Recombinant *S. symbiotica* CWBI-2.3^{T}-GFP and *E. coli* BW25113-GFP reach comparable titers 5 DPI, as determined by spot-plating. (B, C) Recombinant *S. symbiotica* CWBI-2.3^{T}-GFP enters the posterior region of stage 7 blastula. Embryos were dissected and visualized at 5 DPI. Dotted lines outline *S. symbiotica* present within the embryo. (D, E) Recombinant *E. coli* BW25113-GFP does not enter into stage 7 blastula. Embryos were dissected and visualized at 5 DPI. Arrows indicate bacteria attached to the embryonic surface. Scale bars represent 20 µM.