Genomic signatures of honey bee association in an acetic acid symbiont

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Running head: Evolutionary signatures in an acetic acid symbiont

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ABSTRACT

Honey bee queens are central to the success and productivity of their colonies; queens are the only reproductive members of the colony, and therefore queen longevity and fecundity can directly impact overall colony health. Recent declines in the health of the honey bee have startled researchers and lay people alike as honey bees are the most important pollinators in agriculture. Honey bees are important pollinators of many major crops and add billions of dollars annually to the US economy through their services. One factor that may influence queen and colony health is the microbial community. Although honey bee worker guts have a characteristic community of bee-specific microbes, the honey bee queen digestive tracts are colonized predominantly by a single acetic acid bacterium: *Parasaccharibacter apium.* This bacterium is related to flower-associated microbes such as *Saccharibacter floricola,* and initial phylogenetic analyses placed it as sister to these environmental bacteria. We used comparative genomics of multiple bee-associated strains and the flower-associated *Saccharibacter* to identify genomic changes associated with the ecological transition to bee association. We identified several genomic differences in the bee-associated strains, including a complete CRISPR/Cas system. Many of the changes we note here are predicted to confer upon them the ability to survive in royal jelly and defend themselves against mobile elements, including phages. Our results are a first step towards identifying potential benefits provided by the honey bee queen microbiota to the matriarch of the colony.

IMPORTANCE

The health of the world's most important agricultural pollinator, the honey bee, depends on the health of the matriarchs: honey bee queens. These queens are colonized by a specific microbe not found in associated workers but instead, present in the diet given to queen bees. Here we identified genomic signatures of the transition to bee association in this microbe, showing that the queen-associated microbe has adapted to the acids present in honey and royal jelly through the acquisition of acid resistance genes and an enzyme important in de-acidification. Our results highlight potential benefits provided to the queen by her microbial associates.

INTRODUCTION

The honey bee (*Apis mellifera*) is extremely important economically because of the pollination services it provides to numerous agricultural crops. As a result, there is increasing interest in determining how the microbiome supports and influences bee function. While a honey bee colony is made up of bees with diverse roles, or castes, the majority of studies on bee microbiomes have focused on workers specifically. The microbial community of worker bees consists of eight to ten core bacterial species (1-6). The characterization of these groups led to speculation about their role in honey bee health and whether or not they provision nutrients (5) or assist in the breakdown of plant-derived carbohydrates, as is the case in other insect-microbe interactions (7-9). There has also been speculation as to the role of the microbiome in resistance to pathogens, as microbial communities have been shown to protect the bumble bee (*Bombus terristris*) from the parasite *Crithidia bombi* (10). Honey bee-associated microbes interact with each other in diverse ways both *in vitro* and *in vivo*,

67 suggesting that they may interact syntrophically within workers (2, 11). While these studies
68 focused on honey bee workers are intriguing, it is surprising that only recently was the
69 microbiome of queen bees throughout development recently characterized (12).

70 Interestingly, the microbial community associated with queen bees is vastly different
71 than associated workers and is comprised of a large percentage of acetic acid bacteria, a group
72 of bacteria present only at very small percentages in workers. One of the primary bacteria that
73 differentiate queens from workers is the recently described *Parasaccharibacter apium* (13). *P.*
74 *apium* is in the family *Acetobacteraceae* and occupies defined niches within the hive, including:
75 queen guts, nurse hypopharyngeal glands, nurse crops, and royal jelly, and is only rarely found
76 outside of these areas (14, 15). Evidence suggests that it might play a role in protecting
77 developing larvae and queens from pathogens such as *Nosema* (13, 16). Given that *P. apium*
78 makes up a large proportion of the queen gut microbiome, it is possible that it plays an
79 important role in queen nutrition, protection from pathogens, and possibly modulating queen
80 fertility, fecundity, and longevity (17).

81 *P. apium* is part of a clade of acetic acid bacteria (AAB, a group within the family
82 *Acetobacteraceae*) that contains both free-living and bee-associated members. Comparative
83 genomics, then, can give us insights into the changes associated with the transition to bee-
84 association in this clade. This comparison can also help elucidate what sets *P. apium* apart from
85 closely related species and the role(s) it might be playing in the hive environment. To that end,
86 we used the genomes of four recently announced *P. apium* strains (18), an unpublished *P.*
87 *apium* genome assembly, as well as four genomes of the closely-related genus *Saccharibacter*
88 (19-21), and a genome of the bumblebee symbiont, *Bombella intestini* (22), to begin to tease

apart the unique capabilities of *P. apium*. Insights gained here could prove critical in determining the factors responsible for maintaining queen health in colonies and could ultimately lead to the development of interventions to improve queen health and mitigate the detrimental impacts of queen failure on this economically critical species.

RESULTS

Acetic acid bacteria phylogeny

To robustly determine where the *Parasaccharibacter apium* strains and *Saccharibacter* spp. are placed among the AAB, we constructed a maximum likelihood phylogeny using 16S rRNA sequences derived from each of the 17 AAB genera. Our final tree largely agrees with previously published ABB phylogenies (Figure 1) (23-26). Bootstrap support along the backbone of the tree is low, likely owing to the inclusion of sequences from multiple strains of the same species with little sequence divergence. Sequences were largely grouped into monophyletic clades by genus, with one exception; the genus *Gluconacetobacter* is split into a paraphyletic grade, with each branch dominated by a different *Gluconacetobacter* species (clades 1-5, Figure 1A). *Parasaccharibacter* plus *Saccharibacter* comes out as sister to the *Gluconobacter* clade, and the clade encompassing all three of these genera (clade 6, Figure 1A) is sister to *Acetobacter*. Within the *Parasaccharibacter*/*Saccharibacter* clade, the flower-associated *S. floricola* is sister to the remaining clade (clade 7, Figure 1B), comprised of a mix of taxonomic classifications from *Parasaccharibacter*, *Saccharibacter*, and *Bombella* species. The inconsistent taxonomic nomenclature of the microbes within this clade is typified by the *Parasaccharibacter* classification, which is applied to one *P. apium* strain (AS1), which clusters with a handful of

uncharacterized *Acetobacteraceae* bacteria (clade 8, Figure 1B), to the exclusion of all the other *P. apium* and *Saccharibacter* strains. It should be noted that, while the genome for *P. apium* AS1 is on GenBank, little else is known about this strain. Further taxonomic ambiguity is highlighted by the presence of a member of a completely separate genus, *Bombella apis,* grouped squarely among *P. apium* and *Saccharibacter* strains, indicating that it is likely a member of one of those two genera. However, branch lengths within this clade are generally short, especially among the main *Parasaccharibacter*/*Saccharibacter* clade (clade 9, Figure 1B), making it difficult to resolve exact relationships.

Core ortholog phylogeny of Parasaccharibacter apium *and* Saccharibacter *strains*

We used OrthoMCL (v2.0.9 (27)) to define clusters of orthologous genes (COGs) using the *Parasaccharibacter*, *Saccharibacter*, and *Bombella* genomes listed in Table 1; *Gluconobacter oxydans* H24 was used as an outgroup (see Supplementary Information for more detail; Table S1). To better resolve the phylogenetic relationships between *P. apium*, *Saccharibacter* spp., and *Bombella intestini*, we constructed a second maximum likelihood phylogeny using aligned and concatenated amino acid sequences of the 1,259 single-copy COGs. This robustly supported amino acid phylogeny broadly agrees with our previously constructed 16S phylogeny (Figure 2). In the core ortholog tree, *B. intestini* interrupts the monophyly of honey bee-associated *Saccharibacter* spp plus *P. apium*. Notably, this tree groups *B. intestini* more closely to the majority of the *P. apium* strains, while *P. apium* AS1 is more distantly related. Similar to the 16S tree, we again see quite short branch lengths within the bee-associated AAB species,

132 particularly among those in clade D, which includes all *P. apium* and *Saccharibacter* genomes
133 except *P. apium* AS1 and *S. floricola* (Figure 2).
134 Given the discrepancy between nomenclature and phylogeny and the short branch
135 lengths, we calculated genome-wide Average Nucleotide Identity (gANI) and aligned fraction
136 (AF) to clarify species relationships. All pairwise comparisons between genomes in clade D met
137 both gANI and AF thresholds for being considered the same species (namely, gANI > 96.5 and
138 AF > 0.6 (28)), while no other genome pairs reach both thresholds (Figures S1 and S2). Because
139 of the genetic similarity within clade D and phylogenetic distinction from the rest of the
140 genomes in this tree, the remainder of our analyses focus largely on this clade; for clarity we
141 will refer to this clade as the '*Parasaccharibacter* clade' from here forward.
142
143 *Signatures of bee association in the P. apium genomes*
144 To identify genes associated with the transition to bee association, we identified COGs
145 that contained at least one gene from each bee-associated AAB genome (*Parasaccharibacter*
146 clade, *B. intestini*, and *P. apium* AS1) and were also missing in *S. floricola*. There were a total of
147 1,286 COGs containing at least one gene from each of the aforementioned genomes, but only
148 89 were also missing in *S. floricola*. We determined the putative functions of these genes using
149 the *P. apium* reference genome representative for each COG (Table S2). It should be noted that
150 all annotations discussed from here forward are putative and require further functional
151 characterization.
152 Several bee-associated unique genes stood out as particularly interesting, the first being
153 gluconolactonase. Lactonases, such as gluconolactonase, reversibly catalyze the hydrolysis of

lactones (such as gluconolactone) to the hydroxyl acid form (such as gluconic acid). Gluconolactone is found in both honey and royal jelly and is thought to be partially responsible for the antibacterial properties of both compounds (29). In water, this compound can be hydrolyzed into gluconic acid, acidifying the environment and preventing bacterial growth (30-32). The presence of this gene capable of reversing this acidification – at least locally – may explain how *Parasaccharibacter* is able to thrive in the presence of royal jelly (13). BLAST searches of the metatranscriptomes and metagenomes of bacteria in the "core" honey bee microbiome (33) resulted in zero hits, indicating that none of the "core" microbiome members possess a homolog of this gene. The presence of gluconolactonase may help explain the unique distribution of *Parasaccharibacter* within the hive. Another bee-associated unique gene is an HdeD family acid-resistance protein, which in *E. coli* participates in resistance to acids at high cell densities (34). The presence of this gene in *P. apium* may indicate an adaptation to living in low pH environments – such as the queen bee digestive tract or royal jelly (35).

An AI-2 E family transporter was identified as unique to the bee-associated AAB. AI-2 is an auto-inducer responsible for activating cascades associated with quorum sensing. While *P. apium* does not contain any AI-2 synthesis genes, the presence of an AI-2 E family transporter indicates that it may be responding to exogenous AI-2 produced by other bacteria, possibly in a competitive interaction. Bolstering the competition hypothesis is the presence of fusaric acid resistance (FUSC) genes in the *P. apium* genomes. Fusaric acid and its analogs can be quorum sensing inhibitors (36), so the presence of FUSC genes might be an adaptation that allows *P. apium* to evade quorum sensing inhibition attempts by other microbes. Alternatively, these FUSC genes may play a role in competition with fungal species. Fusaric acid is produced by

176 several species of fungus and is antibacterial (37). Therefore, the FUSC genes may play a role in
177 *P. apium*'s protection of honey bee larvae and queens from infection with *Nosema* pathogens
178 (16).

179 An invasion-associated locus B (Ialb) protein was identified as present in bee-associated
180 AAB, but absent in *S. floricola*. In *Bartonella bacilliformis*, *ialb* mutants are impaired in their
181 ability to colonize human erythrocytes, suggesting a role for this protein in eukaryotic cell
182 invasion (38). While it is not clear whether *P. apium* is ever intracellular, the presence of *ialb*
183 suggests that it may have this capability.

184 The final set of genes of particular interest in this analysis is a complete Type I-E
185 CRISPR/Cas cassette. To determine if this CRISPR/Cas cassette was active, we annotated the
186 genomes for the presence of CRISPR arrays, and found that all of the genomes that have this
187 CRISPR/Cas cassette contain multiple CRISPR arrays. It is possible that these CRISPR arrays were
188 present in the most recent common ancestor of the *Parasaccharibacter* clade and have simply
189 been maintained in these current genomes; if that were the case, we would expect the spacers
190 in these CRISPR arrays to be highly similar between all strains. However, if these arrays are part
191 of an active CRISPR/Cas system, we would expect the spacers to differ from strain to strain,
192 reflecting unique challenges encountered by each strain. To rule out the possibility that these
193 arrays are ancestral, we aligned each spacer sequence from a given genome to all other spacer
194 sequences from the other genomes and calculated the percent identity. The minimum best
195 intergenomic match for any spacer was 40%, while the maximum was just 65% identical over
196 the length of the spacer, indicating that the spacer sequences are unique from genome to
197 genome and the CRISPR/Cas systems identified here are likely active.

198 *B. intestini* was isolated from a bumble bee gut, so we also looked at genes that were
199 unique to this bacterium to determine whether there are any obvious signatures of bumble bee
200 association in its genome. There were a total of 65 genes that were unique to *B. intestini*,
201 including a complete type IV secretion system (T4SS) and several genes involved in antibiotic
202 production or resistance. Putative annotations of these 65 genes are in Table S3.
203
204 *Identification of horizontally transferred gene regions*
205 Horizontal transfer of DNA between unrelated bacteria is a commonly known
206 mechanism by which bacteria can acquire new traits and adapt to novel environments (39-42).
207 We identified two regions of phage origin, one in *S. floricola* and one in the *P. apium* reference
208 genome (Figure 3; see Supplementary Information for more detail). Movement and insertion of
209 bacteriophage sequences in a genome can have profound effects on the evolution of that
210 genome (43-45) and future work will determine whether these phage are lytic and what their
211 host ranges may be. To determine whether the bacteria in the *Parasaccharibacter* clade have
212 undergone other potential horizontal gene transfer (HGT) events, we determined the spatial
213 distribution of genes of particular interest (e.g. clade-specific, species-specific, or strain-specific
214 genes) across the bacterial genomes (Figure 3). Some of the genes specific to different clades
215 occur in clusters, an indication that they may have originated elsewhere and been horizontally
216 inherited as a chunk of contiguous DNA. We then looked for anomalies in sequence
217 composition (%GC) and phylogeny to determine whether they were putatively horizontally
218 transferred. Using this combination of methods, we identified a total of five HGT regions in the

219 *Parasaccharibacter* clade, which we have numbered 1-5 (see Table S4 for %GC and lineage
220 probability index (LPI)-difference deviations for each gene in each HGT).

221 HGT1 (Figure 4A) is present in all genomes in the *Parasaccharibacter* clade, and contains
222 10 genes, although *P. apium* C6 is missing one of the genes (the second-to-last gene at the 3'
223 end of the HGT, annotated as an ABC transport auxiliary component). The three most 5' genes
224 show homology to YfaP (an uncharacterized conserved protein), SrfB (part of the surfactin
225 antibiotic synthesis machinery), and an uncharacterized bacterial virulence factor. The genes in
226 the 3' half of this HGT contain a number of domains involved in membrane transport. We
227 hypothesize that the two halves of this HGT work together to synthesize and export antibiotics
228 as a form of defense or regulation of competing bacteria. Lending support to the hypothesis
229 that this HGT is involved in defense or immunity is the fact that a CRISPR array lies immediately
230 5' of this HGT in each genome (Table S5). Bacterial defense mechanisms tend to occur in
231 clusters of "defense islands" (46), so the presence of this CRISPR array is perhaps a further
232 indication of this HGTs role in bacterial immunity.

233 HGTs 2 and 3 (Figure 4B and 4C) are restricted solely to the *P. apium* reference genome
234 and are both bacterial restriction-modification (R-M) systems. Bacterial R-M systems are a
235 defense against invading DNA (i.e. bacteriophage). They act by methylating host DNA at specific
236 sites; invading DNA with the same recognition site will be un-methylated, recognized as foreign,
237 and targeted for degradation (47). HGT2 contains 6 genes, which make up the core components
238 of a bacterial (R-M) system. Interestingly, the domain architecture in this R-M system has been
239 recognized as a precursor to eukaryotic defenses against transposable elements (48). HGT3
240 (Figure 4C) consists of 3 genes comprising 5 domains; the 5'-most gene consists of a predicted

241 restriction-modification DNA methylase coupled to a specificity domain, the middle gene is
242 predicted to be an XhoI restriction enzyme, and the 3'-most gene is a PHP phosphoesterase
243 coupled to a RecN DNA repair ATPase. Taken together, it appears that HGTs 2 and 3 are
244 responsible for recognition of and defense against foreign DNA.

245 HGT4 (Figure 4D) is present in all *P. apium* genomes in the *Parasaccharibacter* clade and
246 contains 3 genes: two GDP-D-mannose dehydratases (GMD) and an O-linked N-
247 acetylglucosamine transferase (OGT). GMD plays a role in the metabolism of mannose and
248 fructose, sugars commonly found in nectar (49). The presence of GMD in *P. apium* genomes
249 might allow for the consumption of nectar or nectar components by these bacteria. OGT, on
250 the other hand, plays a role in post-translational modification of thousands of identified
251 proteins (50). However, while OGT-mediated post-translational modification is common in
252 eukaryotes, it is far more rare in bacteria (51). To date, only a handful of prokaryotic OGTs have
253 been identified, and the targets of these OGTs remain unclear (52, 53). Given the role OGTs
254 play in eukaryotic post-translational modification and the fact that many bacterial effector
255 proteins show homology to eukaryotic proteins (54), it is possible that the presence of OGT in *P.*
256 *apium* represents a pathway for host-microbe interaction and symbiont-mediated protein
257 modification.

258 HGT5 (Figure 4E) is unique to the *P. apium* strains A29, B8, and C6, all strains that had
259 been isolated from honey bee larvae. Like HGTs 1-3, HGT5 contains genes that may play a role
260 in protection against foreign DNA. There are four genes in the 5' section of HGT5, three of
261 which are kinases, and the fourth contains a SAD/SRA domain in its 5' end, and an HNH
262 endonuclease domain in its 3' end. In bacteria, the SAD/SRA domain is often found associated

with an HNH domain (55) and it is thought that the two domains act together to recognize and cleave foreign DNA (56). The 3' section of HGT5 consists of a conjugative relaxase, a TraG/TraD family ATPase (a coupling protein involved in bacterial conjugation and/or T4SS), a homolog of the pyocin activator protein PrtN, a homolog of a yeast RNA polymerase I subunit, and two additional genes with no annotations. The presence of a PrtN homolog is particularly interesting, as in *Pseudomonas aeruginosa* pyocins are antibacterial agents, often acting to depolarize the membrane of target cells (57),(58). Interestingly, one of the two unannotated genes in the 3' region of HGT5 shows weak homology to a phage shock protein, which are proteins involved in the response to stress that may weaken the energy status of the cell (59). This protein, then, may play a part in immunity to membrane depolarization. Given the presence in HGT5 of: an HNH endonuclease coupled to a SAD/SRA domain, a conjugative relaxase, a TraG/TraD family ATPase, a pyocin activator protein, and a protein with at least some homology to a phage shock protein, we hypothesize that it may play a role in pathogenesis or defense.

DISCUSSION

Here, we used the genomes of five *P. apium* strains, four *Saccharibacter* strains, and the closely related *B. intestini* to gain insight into the genomic changes associated with the transition to honey bee symbiosis in this group. We note several genomic differences – some of which were horizontally acquired – between bee-associated bacteria and the flower-associated *S. floricola* that may have allowed for the expansion of *P. apium* into previously unoccupied niches within the honey bee colony. These differences can be classified as changes that

introduce: 1) novel metabolic capabilities, 2) defense and/or virulence mechanisms, and 3) mechanisms for interaction with other microbes and/or the host.

Metabolic genes identified here include gluconolactonase, which may allow for the de-acidification of royal jelly (29-32), and two copies of GMD, a gene that plays a role in the metabolism of mannose and fructose, components of nectar and honey (49). Distinct defense and/or virulence mechanisms were identified, including: a functional CRISPR/Cas system, two R-M systems, and an HGT with some homology to known virulence mechanisms. Interestingly, the R-M systems were identified in the only genome in the clade that also contains a phage sequence (the *P. apium* reference sequence). Restriction modification systems, like phages, can act as selfish genetic elements (60), so their presence in this genome may indicate that it was historically more permissive to invading DNA. These R-M systems may also have been coopted by the prophage to prevent super-infection with additional phages (61).

Genes involved in the interaction with other microbes and/or the host that we identified include: an AI-2 family transporter, fusaric acid resistance genes, *ialb*, and *ogt*. Given that *P. apium* does not encode any of the canonical genes for the production of quorum-sensing molecules, it seems likely that *P. apium* is responding to exogenous AI-2 (and/or fusaric acid and its analogs) produced by other members of the bee microbiome (62). The *ialb* and *ogt* genes provide routes for interaction with the host, as *ialb* may play a role in eukaryotic cell invasion (38) and *ogt* is known to modify thousands of eukaryotic proteins (50). Taken together, we hypothesize that the novel combination of metabolic, quorum-sensing, defense/virulence, and eukaryotic interaction genes in the *Parasaccharibacter* clade genomes allowed for the

utilization of a unique food source and protection from an onslaught of previously un-encountered challenges and facilitated the transition to honey bee association in this clade.

P. apium has been shown to benefit honey bee larval development and provide protection against *Nosema* (16). Some of the genes identified here, while allowing *P. apium* to transition to honey bee symbiosis, may also be related to its ability to protect the bee host from infection with *Nosema* or other pathogens. If indeed these genes are responsible for the transition to, and maintenance of, honey bee symbiosis, we would expect to see a modified evolutionary trajectory relative to those genes not involved in the symbiosis. We currently lack sufficient sampling of non-bee-associated bacteria in this clade to do such analyses; however, future studies addressing this question should allow for further elucidation of the genes involved in the transition to honey bee association. Those analyses, coupled with functional characterization of the genes of interest identified here, should lay the foundation for the development of beneficial intervention strategies in this economically critical insect.

METHODS

Acetic acid bacteria phylogeny

To determine the placement of *Parasaccharibacter* and *Saccharibacter* among the AAB, we downloaded all 16S rRNA sequences from the Silva database (63-65) that met the following criteria: 1) from a species belonging to one of the seventeen genera of AAB (26), 2) length at least 1200 bases, and 3) sequence quality >90. Additionally, the 16S sequence for *Rhodopila globiformis* – which is in the family *Acetobacteraceae* but is not part of AAB – was included as an outgroup. Given the close relation to *Saccharibacter floricola*, 16S sequences for *Bombella*

328 *intestini* (66) and *Bombella sp.* MRM1 (*Bombella apis*)(67) were included. We BLASTed the
329 *Saccharibacter floricolca* 16S sequence against the *Parasaccharibacter* and *Saccharibacter*
330 genomes (Table 1) to pull out their respective 16S sequences for use in this phylogeny. All
331 sequences were aligned using the SINA aligner (68); parameters used were set using the --auto
332 option. A maximum likelihood phylogeny was constructed using RAxML with the GTRGAMMA
333 substitution model and 1000 bootstrap replicates (v8.2.11, (69)). The final tree was visualized
334 using FigTree (v1.4.2, http://tree.bio.ed.ac.uk/software/figtree/).
335
336 *Orthology analysis*
337 To facilitate downstream analyses, we clustered genes from all genomes in Table 1 –
338 plus *Gluconobacter oxydans* H24 as an outgroup – into clusters of orthologous genes (COGs)
339 using OrthoMCL (v.2.0.9, (27)). Amino acid sequences were downloaded from NCBI and
340 clustering was performed using default OrthoMCL parameters. These clusters were then
341 classified as single-copy orthologs (defined as containing exactly one representative from each
342 genome), variable (defined as missing a representative from at least one genome and having
343 varying numbers of representatives from each of the other genomes), multi-copy ortholog
344 (containing at least one representative from each genome, but multiple copies from at least
345 one genome), or genome-specific (containing at least two genes that all came from the same
346 genome) using an in-house Perl script.
347
348
349

350 Parasaccharibacter *and* Saccharibacter *core ortholog phylogeny*

351 We constructed a phylogeny using concatenated amino acid alignments of all single-
352 copy COGs. The amino acid sequences were aligned using the MAFFT L-INS-I algorithm (v7.310,
353 (70)), and alignments were then concatenated, and used to construct a maximum likelihood
354 phylogeny using RAxML with substitution model PROTGAMMALGF and 1000 bootstrap
355 replicates (v8.2.11, (69)). The final tree was visualized using FigTree (v1.4.2,
356 http://tree.bio.ed.ac.uk/software/figtree/).

357

358 *Calculation of genomic similarity*

359 To determine relatedness and species assignment, we calculated genome-wide Average
360 Nucleotide Identity (gANI) and aligned fraction (AF) for each pairwise comparison using
361 ANIcalculator (28). Predicted transcript sequences for each pairwise comparison were passed to
362 the software, which output gANI and AF in each direction for the pairwise comparison. As gANI
363 and AF can vary depending on the direction of comparison due to differences in genome length,
364 we report the average of the pairwise calculations in each direction.

365

366 *Synteny analysis*

367 We used Mauve (71, 72) to determine the syntenic regions between the
368 *Parasaccharibacter apium* and *Saccharibacter* spp. genomes. The *Parasaccharibacter apium*
369 reference genome is resolved to a single chromosome, so it was used as the reference
370 sequence in Mauve's "move contigs" tool, and the likely order and orientation of contigs in the
371 other genomes was determined. To facilitate downstream analyses, the output of Mauve's

372 "move contigs" tool was used to order, orient, and concatenate contigs into single pseudo-
373 chromosomes for each genome. Structural rearrangements were then visualized using Mauve's
374 built-in graphical interface.
375
376 *Annotation of CRISPR arrays and phage sequences*
377 Pseudo-chromosomes for each genome were uploaded to CRISPRFinder to determine
378 location and sequence of CRISPR arrays (73). We used an in-house Perl script to determine the
379 maximum intergenomic percent identity of spacer sequences. We used PHAge Search Tool
380 Enhanced Release (PHASTER) (74, 75) to define phage-like regions. Any region determined to be
381 "questionable" or "intact" by PHASTER that also appeared as an insertion in the host genome in
382 our synteny analysis was labeled as likely to be of phage origin.
383
384 *Determination of bee-associated bacteria-specific orthologs*
385 We identified all COGs that contained at least one gene from each genome of bee-
386 associated bacteria and no genes from *S. floricola.* We then took the *P. apium* reference
387 genome representative for each of these COGs and got KEGG annotations for as many as
388 possible using BlastKOALA (76). For those genes that we were not able to get KEGG
389 annotations, we used NCBI's BLAST to aid in determining potential function of these bee-
390 associated bacteria-specific genes. This list of genes and their potential functions was then
391 manually curated to hypothesize genes that may have allowed for the transition to bee-
392 association.
393

394 *Analysis of horizontal gene transfers*

395 To determine whether or not genes in any of the *Parasaccharibacter* and *Saccharibacter*
396 genomes arrived via horizontal transfer, we employed a combination of sequence-composition,
397 phylogenetic, and synteny approaches. We mapped genes of particular interest (e.g. genes
398 unique to certain clades, species, or strains) to their locations on the linear pseudo-
399 chromosomes constructed during synteny analysis. Additionally, we calculated the %GC for
400 each gene. We then determined how many standard deviations each gene was from the
401 genome-wide mean %GC. The third prong of this analysis involved identifying genes that were
402 phylogenetically aberrant. To do this, we used Darkhorse (77) to calculate the lineage
403 probability index (LPI) twice for each gene, once including BLAST hits to *Parasaccharibacter* and
404 *Saccharibacter* subject sequences, and once excluding such hits. In doing so, genes that are
405 likely to be horizontally transferred will have a larger discrepancy between LPI values than
406 genes that were vertically inherited (see supplemental methods for details). We then identified
407 regions as likely to be HGTs if they met the following criteria: 1) a block of at least three
408 syntenic genes that show interesting phylogenetic distributions (e.g. unique to clade, species,
409 or strain) where 2) a majority of genes in the region are at least 1 standard deviation from the
410 mean %GC or LPI difference (or both).

411

412 *Domain annotation of genes of interest*

413 We used HHpred (https://toolkit.tuebingen.mpg.de/#/tools/hhpred, (78)) to determine
414 domain architecture and gain an understanding of potential function of the genes in each HGT.
415 For genes of interest that were part of a COG, all members of the COG were first aligned using

the MAFFT L-INS-I algorithm (v7.310, (70)). These multiple sequence alignments (or single amino acid sequences in the case of strain-unique genes) were then uploaded to HHpred's online tool and homology was determined using HMMs in the COG_KOG_v1.0, Pfam-A_v31.0, and SMART_v6.0 databases; only domains scoring above 60% probability are discussed here. Gene models for each region of interest were then constructed and visualized using the HHpred results and in-house R scripts. HGT5 occurs at the junction of two contigs in the linear pseudo-chromosomes we constructed. The abutting ends of each contig have annotations for partial pseudogenes, such that when they are joined a complete gene is created. We BLASTed the nucleotide sequence of this gene against the NCBI nr database to determine a putative function.

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Table 1. Genome names, accession number, and isolation sources for complete genomes used in these analyses. *Parasaccharibacter apium* G7_7_3c is the *P. apium* reference genome.

Genome	GenBank accession number	Isolation source
Parasaccharibacter apium G7_7_3c	GCA_002079945.1	*Apis mellifera* hindgut(18)
Parasaccharibacter apium A29	GCA_002917995.1	*Apis mellifera* larva(18)
Parasaccharibacter apium B8	GCA_002917945.1	*Apis mellifera* larva(18)
Parasaccharibacter apium C6	GCA_002917985.1	*Apis mellifera* larva(18)
Parasaccharibacter apium AS1	GCA_002592045.1	*Apis melliera* larva
Saccharibacter sp. AM169	GCA_000723565.1	*Apis mellifera* stomach(20)
Saccharibacter sp. M18	GCA_002150105.1	*Apis mellifera* stomach(21)
Saccharibacter sp. 3.A.1	GCA_002150125.1	Honey(21)
Saccharibacter floricola	GCA_000378165.1	Flower(19)
Bombella intestini	GCA_002003665.1	*Bombus lapidarius* crop(22)
Gluconobacter oxydans H24	GCA_000311765.1	Industrial sample(79)

FIGURES AND LEGENDS

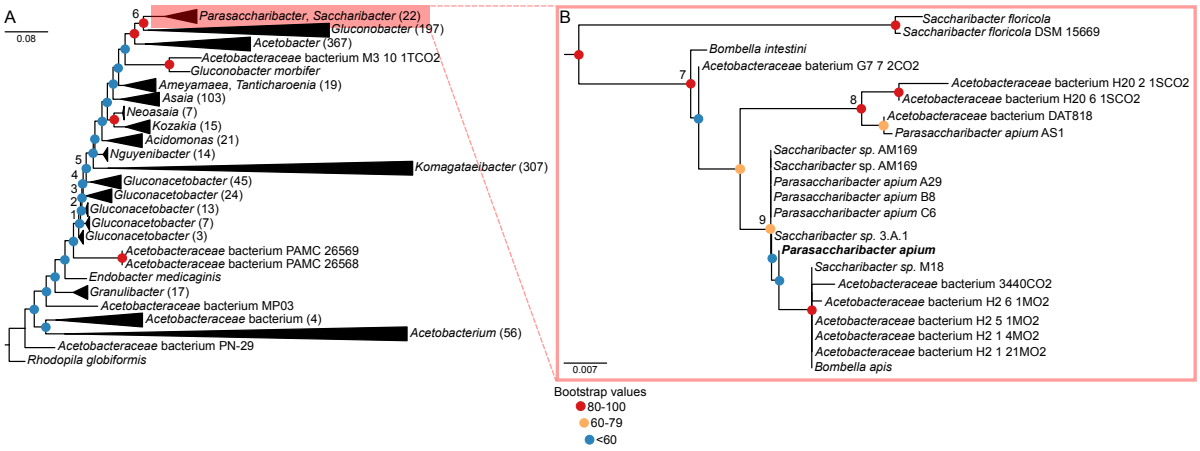


Figure 1. Maximum likelihood phylogenetic tree of Acetic Acid Bacteria constructed from full-length 16S rRNA sequences. Bootstrap scores are indicated at each node, and numbers at certain nodes are for reference in the main text. A) Full tree of all 17 genera of Acetic Acid Bacteria. Clades were collapsed based on genus. Genera that make up at least 40% of the sequences in a clade are listed, except for the highlighted clade. Numbers in parentheses represent the total number of sequences in each collapsed clade (Supplemental File S3 for full tree). B) Zoomed-in look at the *Parasaccharibacter*/*Saccharibacter* clade. The bolded *Parasaccharibacter apium* represents the reference *P. apium* sequence.

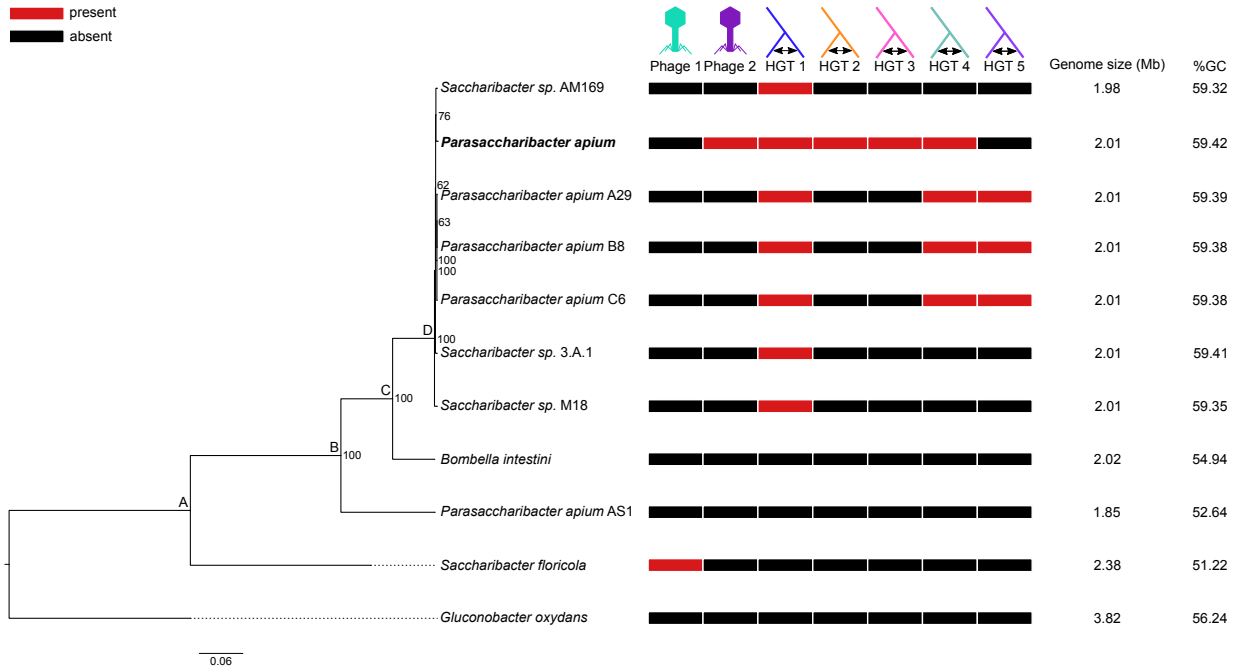
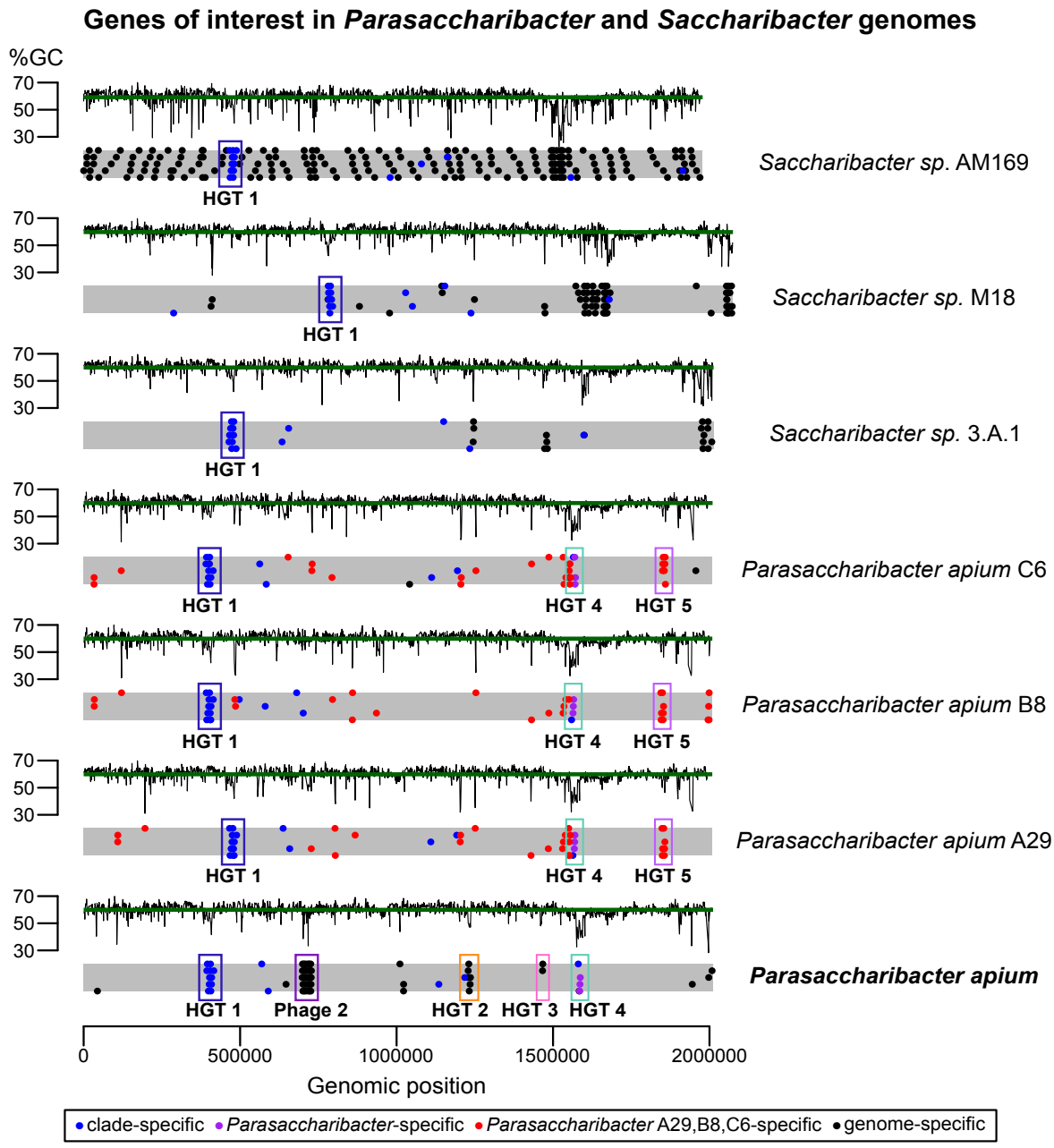


Figure 2. Maximum likelihood phylogenetic tree of the *Parasaccharibacter*/*Saccharibacter* clade constructed from concatenated amino acid alignment of 1259 single copy orthologous genes. Bootstrap scores are indicated at each node. Letters at certain nodes are for reference in the main text. Colored boxes represent the presence (red) or absence (black) of each of 7 genomic regions of interest. Genome size and %GC are also displayed. Bolded *Parasaccharibacter apium* represents the reference *P. apium* sequence.



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657 **Figure 3.** Genomic locations of genes of interest in *Parasaccharibacter* and *Saccharibacter*
658 genomes. Each gray bar is a representation of the genome, with each dot representing the
659 location of a gene in each of four categories (see legend). Regions of interest mentioned in the
660 text are highlighted and labeled. %GC for every gene is plotted above each genome
661 representation, with the green line indicating the genome-wide average %GC. Bolded
662 *Parasaccharibacter apium* represents the *P. apium* reference sequence.
663

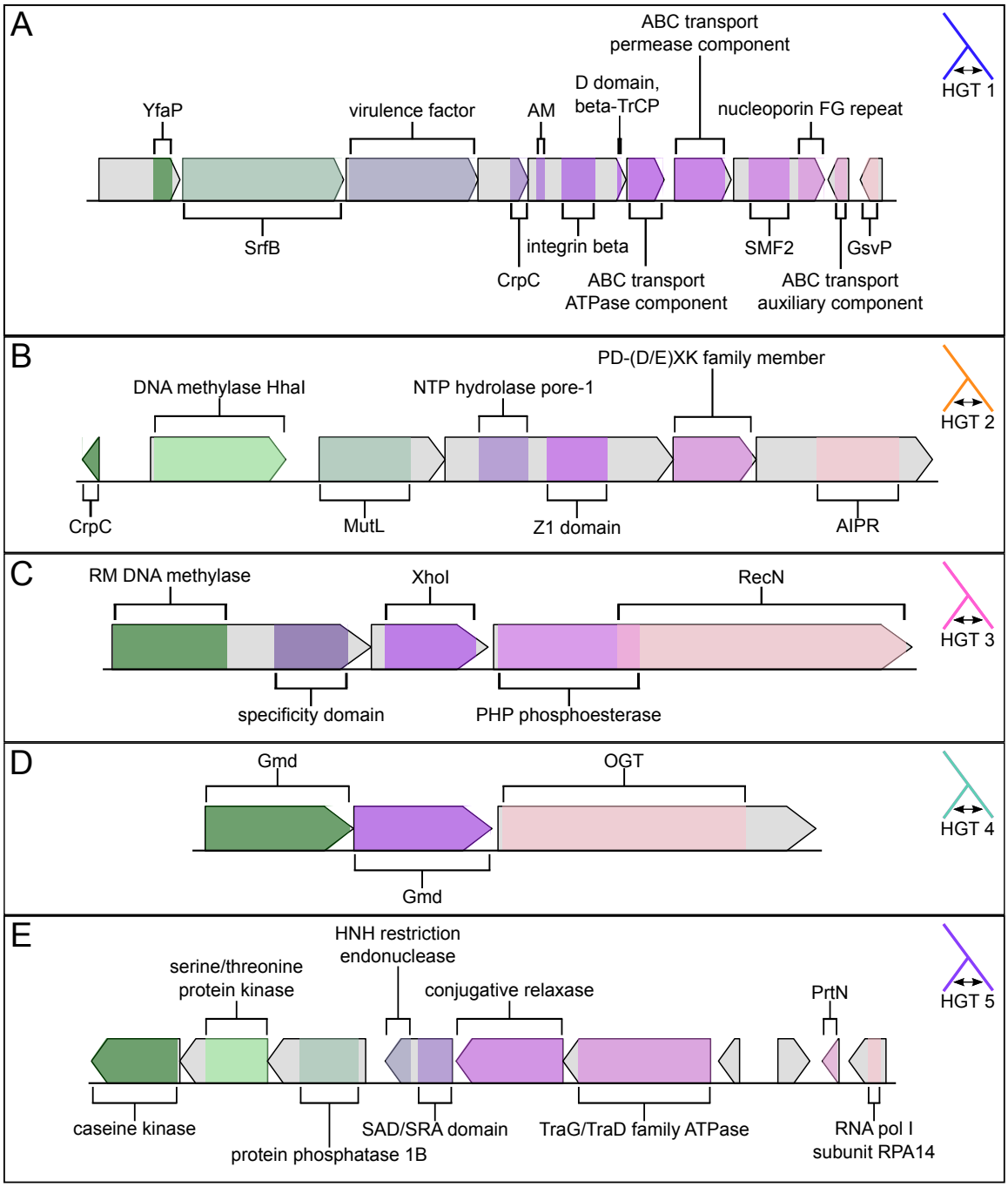

Figure 4. Gene models for each of 5 genomic regions of interest. Gene models are drawn to scale within each panel, but not across panels. A) HGT1. Abbreviations are: CrpC: cysteine rich protein C, AM: automated matches, SMF2: sulfatase modifying factor 2, GsvP: gas vesicle protein C. B) HGT2. Abbreviations are: CrpC: cysteine rich protein C, AIPR: abortive infection phage resistance protein. C) HGT3. Abbreviations are: RM: restriction-modification. D) HGT 4. Abbreviations are: Gmd: GDP-D-mannose dehydratase, OGT: O-linked N-acetylglucosamine transferase OGT. E) HGT5. Abbreviations are: SAD/SRA: SET and Ring finger Associated, PrtN: pyocin activator protein.

673 **SUPPLEMENTAL FIGURES AND TABLE LEGENDS**

674 **Table S1.** OrthoMCL clusters and gene counts for each type of COG in each genome.

675 **Table S2.** Accession number, annotation source, annotation score, and putative annotation for

676 each of the bee-specific genes identified.

677 **Table S3.** Accession number, annotation source, annotation score, and putative annotation for

678 each of the *Bombella intestini* genes identified.

679 **Table S4.** %GC and LPI-difference standard deviations for each gene in each genome harbing

680 each HGT.

681 **Table S5.** Positions and spacer counts for each CRISPR array identified in the *Parasaccharibacter*

682 clade genomes.

683 **Figure S1.** Pairwise genome-wide average nucleotide identity (gANI) for all genomes analyzed.

684 **Figure S2.** Pairwise aligned fraction (AF) for all genomes analyzed.

685 **Supplemental file S1.** Supplemental methods and results.

686 **Supplemental file S2.** BLAST results for genes present in phage 1, present in *Saccharibacter*

687 *floricola.*

688 **Supplemental file S3.** BLAST results for genes present in phage 2, present in the

689 *Parasaccharibacter apium* reference sequence.

690 **Supplemental file S4.** Newick format file for complete 16S rRNA gene tree (Figure 1).