Genome evolution following an ecological shift in nectar-dwelling *Acinetobacter*

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Abstract

The bacterial genus *Acinetobacter* includes species found in environmental habitats like soil and water, as well as species adapted to be host-associated or pathogenic. High genetic diversity may allow for this habitat flexibility, but the specific genes underlying switches between habitats are poorly understood. One lineage of *Acinetobacter* has undergone a substantial habitat change by evolving from a presumed soil-dwelling ancestral state to thrive in floral nectar. Here we compared the genomes of floral-dwelling and pollinator-associated *Acinetobacter*, including newly described species, with genomes from relatives found in other environments to determine the genomic changes associated with this ecological shift. Following one evolutionary origin of floral nectar adaptation, nectar-dwelling *Acinetobacter* species have undergone reduction in genome size compared to relatives and have experienced dynamic gene gains and losses as they diversified. We found changes in gene content underlying carbohydrate metabolism and nitrogen metabolism, which we predict to be beneficial in nectar environments. Gene losses follow a pattern consistent with genome streamlining, whereas gains appear to result from both evolutionary divergence and horizontal gene transfer. Most notably, nectar-dwelling *Acinetobacter* acquired the ability to degrade pectin from plant pathogens and the genes underlying this ability have duplicated and are under selection within the clade. We hypothesize that this ability was a key trait for adaptation to floral nectar, as it could improve access to nutrients in the nutritionally unbalanced habitat of nectar. These results identify the genomic changes and traits coinciding with a dramatic habitat switch from soil to floral nectar.

Introduction

The gammaproteobacteria genus *Acinetobacter* is diverse and includes (1–3). These taxa inhabit a broad range of environments, including soil and water (1–3). Some *Acinetobacter* lineages have also evolved to be host-associated or animal pathogens, with a notable example being the recently emerged human pathogen *Acinetobacter baumannii* (4). Strains in the genus are phenotypically and genetically diverse and frequently adapt to new ecological niches (5, 6). However, few direct connections have been made between specific genomic changes and ecological transitions within *Acinetobacter*, or in bacteria more broadly (7). One poorly characterized habitat transition within the genus *Acinetobacter* is adaptation for growth in floral nectar. Several *Acinetobacter* species found in floral nectar appear to be most closely related to soil-dwelling relatives (4, 8). Nectar represents a significant environmental shift compared to soil habitats, likely with different selective pressures. Genomic comparisons between *Acinetobacter* adapted to floral nectar versus other habitats can uncover how bacteria evolve to new environments and which genetic traits facilitate major ecological switches.

The high genetic diversity and genomic plasticity within *Acinetobacter* may be driven by mechanisms facilitating horizontal gene transfer (HGT), including competence for natural transformation (9–12), conjugative abilities, and prevalent mobile elements such as plasmids, prophage, and insertion sequences (8, 13, 14). Horizontally acquired genomic islands are common observed throughout the *Acinetobacter* genus (15, 16) and can contain genes conferring beneficial phenotypes like antibiotic resistance and plasmid mobilization (17, 18). HGT is a source of evolutionary novelty in bacteria (19), but other sources of genetic diversity can also be important, such as error-prone polymerases in *A. baumannii* (4, 8, 20), or gene duplication followed by divergence (21). Gene duplication can also potentially lead to increased gene

expression, allowing for enhanced nutrient acquisition, temperature stress tolerance, and overall resistance to antibiotics and pesticides (22), but it is unclear how broadly important this mechanism is for bacterial adaptation.

Genetic novelty in bacteria can allow for the evolution of new traits and subsequent exploitation of new niches (23–25). In some cases, specific genetic elements have been linked to habitat-specific fitness. For instance, *A. baumannii* has resistance islands with mobile genetic elements and antibiotic resistant genes, allowing for persistence in hospital settings (6, 26). Antibiotic resistance is a common example of a novel trait resulting from a specific environmental selective pressure because it is easily observable and important in well studied pathogen systems. Other traits that are relevant in natural systems have occasionally been connected to ecological changes in bacteria (27, 28), but such connections can be difficult to infer. In other cases, traits that are linked to success in a specific environment may be known, but not their genetic basis. For instance, the ability to access nutrients from pollen is a unique and potentially beneficial trait in floral nectar-dwelling *Acinetobacter*, but how this trait was gained is unknown (29).

Floral nectar is a nutritional reward produced by flowers to attract pollinating animals. It is high in carbohydrates; the sugar content in floral nectar can reach 90% of nectar dry weight (30, 31) and it is a resource for microbes as well (32, 33). However, floral nectar habitats create several stresses for microbes, including limitation of nutrients other than sugar (34–38). Although nectar contains amino acids and lipids (39), it can contain limiting amounts of nitrogen for some microbes (40, 41). These factors make nectar a selective environment and can lead to strong priority effects where early arriving microbes prevent subsequent colonization of flowers (40, 42).

93 Culture dependent and independent methods have revealed diverse microbes that thrive
94 in these conditions (43–49). The genus *Acinetobacter* makes up a high proportion of bacterial
95 taxa in floral nectar and is prevalent and readily cultured from nectar environments (44, 50).
96 *Acinetobacter* is also frequently found associated with floral visitors. For example, *Acinetobacter*
97 *apis* was isolated from the gut of the western honey bee, *Apis mellifera*, and bee pollen
98 provisions and nests sometimes include *Acinetobacter* (51–54). However, it is unknown whether
99 *Acinetobacter* found with pollinators are nectar-dwelling species being dispersed by floral
100 visitors, or if they are specific associates of pollinators. For ease here, we refer to strains isolated
101 from both nectar and floral visitors as nectar-dwelling strains.
102 Previous phylogenomic analysis of *Acinetobacter* isolates from nectar and bees found
103 that they were closely related to soil-dwelling species (50). This previous work suggested one
104 evolutionary origin of nectar-dwelling/bee association but did not assess evolutionary patterns
105 within this lineage. We do so here, with and additional 15 genomes from nectar-dwelling
106 isolates. These include the genomes of three previously described species, *A. apis*, *A. boissieri*,
107 and *A. nectaris* (45, 51), newly sequenced *A. nectaris* isolates, and newly sequenced genomes of
108 three recently described species, *A. pollinis*, *A. rathckeae*, and *A. baretiae* (50). For comparison,
109 we included genomes from *A. brisouii*, which is isolated from soil and water and was previously
110 found to be the closest relative of *A. nectaris*, as well as those from eight other environmental
111 *Acinetobacter* species. We hypothesized that the switch to floral nectar from soil would
112 drastically change the selective pressures experienced by this *Acinetobacter* lineage, leading to
113 changes in gene content. We used comparative genomics to understand which genes may
114 become redundant or beneficial for bacteria in floral nectar, and to identify acquired genes that
115 may have facilitated this environmental switch.

116

117 **Results and Discussion**

118 *Phylogeny and genome characteristics of nectar-dwelling* Acinetobacter

119 To understand the evolutionary history of nectar-dwelling *Acinetobacter*, we constructed
120 a phylogenomic tree using genomes of *Acinetobacter* isolates from floral nectar and floral
121 visitors. The isolates collected from floral nectar and pollinators form a clade, with a bootstrap
122 support of 100, separate from soil, water, and animal dwelling *Acinetobacter* species (Fig. 1).
123 This confirms that there is one known evolutionary origin of a nectar-dwelling within
124 *Acinetobacter* and that this group evolved from a presumed soil-dwelling ancestor. The six
125 species in the nectar clade appear to not be isolated from environments outside of floral nectar or
126 pollinators, based on 16S rRNA sequence comparisons to GenBank databases (50). Multiple of
127 these species, *A. nectaris*, *A. boissieri*, and *A. pollinis*, are abundant and common in floral nectar
128 from locations worldwide, and our isolates came from both North America and Europe (45, 50,
129 55, 56). This suggests that the clade is specialized for growth in floral nectar and/or associated
130 with pollinators and is widely found in these habitats (44, 57, 58).

131 We used genomic comparisons between nectar-dwelling *Acinetobacter* and relatives
132 living in distinct environments to uncover genomic patterns associated with nectar specialization.
133 Relative to species found in other environments, species in the nectar-specialized *Acinetobacter*
134 clade have smaller genomes and lower numbers of protein coding genes (Table 1). There is a
135 significant difference between genome sizes within the nectar-specialist clade, 2.38-2.75 Mb,
136 and the environmental clade, 2.90-4.88 Mb (p-value=0.00023). Across genomes of nectar-
137 dwelling strains vs. environmental strains, nectar specialists have 243-977 fewer protein coding
138 genes, a 10-30% reduction in proteins. Genomic reduction can occur for various reasons.

139 Genome streamlining is common for species living in stable, nutrient poor conditions such as
140 some soil and marine habitats (59–62), and is thought to be driven by selection and facilitated
141 when bacteria have large effective population sizes (59, 63–65). Some environmental stresses
142 may also promote genome streamlining due to selection (66, 67). Gene loss can also be
143 degenerative and result from genetic drift, with extreme examples occurring in bacteria that are
144 host-restricted and experience frequent population bottlenecks (68–71).

145 Although nectar-dwelling bacteria may experience population bottlenecks due to the
146 transient nature of the floral environment, we do not find strong evidence for genetic drift as is
147 seen in host-restricted bacteria; such species often show high evolutionary rates, high rates of
148 pseudogenes, and low genome GC content (70, 72). Evolutionary rate tests in PAML found no
149 support for a faster rate in nectar-dwelling *Acinetobacter*, compared to the null hypothesis of a
150 global clock across the nectar and environmental *Acinetobacter* phylogeny (69). Similarly, we
151 did not find evidence of genomic degeneration in the form of pseudogenes, as the number of
152 pseudogenes detected in nectar-dwelling *Acinetobacter* ranged from 125-294, while
153 environmental *Acinetobacter* had a similar range of 187-283 and other *Acinetobacter* species fall
154 within this range as well (Table 1) (73, 74). The only pattern found that is potentially supportive
155 of genetic drift is that nectar isolates have slightly lower percent GC compositions relative to
156 soil-dwelling species (Table 1). We therefore hypothesize that the habitat switch from soil to
157 nectar altered selective pressures on gene content in nectar dwelling species and allowed for the
158 loss of some functions through genome streamlining. To further investigate this, we sought to
159 define the gene content and functional capacities of nectar-dwelling species compared to soil-
160 dwelling relatives.
161

162 *Gene content evolution with the switch to nectar*

163 To determine content of predicted proteins among *Acinetobacter* clades, we performed an
164 ortholog clustering analysis to identify shared orthologs, recent paralogs, and unique genes (75)
165 (Table S1). This analysis resulted in 7,334 orthologs in total identified across all genomes, 1,076
166 of which were present across all genomes. Environmental strains had 5,558 orthologs in total,
167 with 2,921 orthologs only found in environmental species, and 111 environmental specific
168 orthologs shared across these species. The nectar-dwelling clade had 4,413 total orthologs, with
169 1,776 orthologs only found in nectar-dwelling species, and 53 of these nectar specific orthologs
170 found in all nectar clade strains.

171 To trace gene gain and loss events within the nectar-specialist clade, we performed a
172 maximum likelihood ancestral state reconstruction analysis. Overall, there have been dynamic
173 gene gains across the evolution of the group. Substantial ortholog gain events occurred at the
174 ancestral node for the nectar-dwelling clade, the ancestral nodes to the species *A. pollinis, A.*
175 *rathckeae,* and *A. baretiae,* and at additional ancestral nodes (Fig.1, nodes 1, 2, 5, 6, 8 and 9).
176 Gene gains were significantly higher than gene losses throughout the nectar-dwelling clade, with
177 gains of 209-345 orthologs at tips and nodes (Fig. 1, Fig. S1). Numbers of gene loss events
178 generally increased closer to the tips of the tree (Fig. 1, Fig. S1), suggesting that genome
179 streamlining may be a relatively recent process in the clade. Given that the nectar-dwelling
180 genomes are smaller in total size than the genomes of relatives (Table 1), these patterns suggests
181 that some gene gains identified in this analysis are the result of divergence leading to novel
182 orthologs rather than horizontal acquisition of new genes alone. Selective pressures from
183 environmental changes can increase processes like mutation rate, leading to diversification in

184 orthologs (76). Here, adaptation to the novel nectar environment may have led to increased gene
185 divergence.

186 Gene gains and losses occurred across diverse functional categories in the nectar-
187 dwelling clade (Fig. 2). We investigated specific changes in gene content in functional
188 categories, with the largest differences between nectar-dwelling species and environmental
189 relatives occurring in the categories of metabolism of aromatic compounds, nitrogen metabolism,
190 and carbohydrate metabolism.

191 The number of genes involved in metabolism of aromatic compounds (Fig. 2, Fig. S2A)
192 varied between nectar and environmental strains. Although the difference in total genes was not
193 significant, 48 out of 67 genes in this category were present in at least one environmental
194 *Acinetobacter* species, but completely absent in the nectar clade (Table S1). All but one of the
195 remaining genes were present in both clades, but rarely common across all nectar-dwelling
196 strains. Several of the genes for aromatic compound metabolism that are present in nectar-
197 dwelling *Acinetobacter* strains are involved in benzoate metabolism, an aromatic compound that
198 has been shown to be released by plants (77). The decrease in genes in this category suggests that
199 nectar-dwelling *Acinetobacter* encounter a limited diversity of aromatic compounds compared to
200 species in other environments.

201 Gene content differences in nectar-dwelling strains compared to environmental relatives
202 also suggest that shifts in nitrogen and amino acid metabolism strategies accompanied the shift to
203 nectar dwelling. Genes involved in amino acid metabolism were significantly higher in nectar-
204 dwelling strains (p-value=0.003). This difference was mainly due to an excess of genes for the
205 transport of certain nitrogen sources, including putrescine, proline, glycine, serine, alanine,
206 arginine, ornithine, methionine, leucine, glutamate, and aspartate (Table S1). In contrast,

207 nitrogen metabolism genes were reduced by about half in nectar dwelling *Acinetobacter*
208 compared to environmental species, although the difference was not significant (Table S1).
209 Specifically, nectar-dwelling species genomes are missing glutamine synthases, glutamate
210 synthases and ammonium transporters. Many of these are genes for which multiple orthologs are
211 present in environmental *Acinetobacter* and only one has been retained in nectar-dwelling
212 strains. Floral nectar is low in nitrogen relative to carbon (31, 78), and the ability to assimilate
213 nitrogen sources has been linked to competition and growth in floral nectar in both yeasts (79)
214 and *Acinetobacter* (80, 81). A shift towards more transport systems for nitrogen sources could be
215 driven by selection for nitrogen scavenging.

216 Genes involved in carbohydrate metabolism showed an overall decrease in nectar-
217 dwelling strains (Fig.2), however this overall decrease was not statistically significant (p-
218 value=0.089). The loss in carbohydrate genes was driven by the subcategories of central
219 carbohydrate metabolism and significantly by genes in the category of organic acid metabolism,
220 (p-value=2.2e-16) (Fig. S2B). Other subcategories, notably monosaccharide metabolism, showed
221 a significant gain in genes (p-value=3.022e-16) (Fig. S2B). Compared to environmental habitats,
222 floral nectar provides an abundance of simple carbohydrates, including fructose, sucrose, and
223 glucose (78, 82). Many of the nectar-dwelling species can assimilate fructose, and some can
224 assimilate glucose and sucrose (50, 80). In comparison, non-nectar dwelling species such as *A.*
225 *baylyi*, are often unable to utilize fructose, sucrose, or glucose as sole carbon sources, but can
226 metabolize other diverse carbon sources (83–85). In support of these observations, we found that
227 nectar-dwelling species tend to have more genes for metabolizing and transporting simple sugars
228 (Table S1). For example, all nectar dwelling species have the gene coding for 1-
229 phosphofructokinase (EC 2.7.1.56), which is part of the fructose and mannose metabolism

230 pathway, whereas the genomes of environmental strains were less likely to have this gene.
231 Nectar-dwelling strain genomes were also more likely to have the gene encoding xylanase,
232 which is responsible for the breakdown of the common plant polysaccharide xylan to xylose (86,
233 87). These genes may be more beneficial for nectar-dwellers than soil-dwelling *Acinetobacter*.
234 In keeping with overall changes in carbohydrate metabolism in nectar, we found that
235 phosphotransferase system (PTS) genes specific to nectar sugars are more common in nectar-
236 dwelling strains compared to environmental strains (Table 2). PTS genes are a common method
237 for bacteria to transport sugars into cells via a phosphorylation cascade (88, 89). PTS can also be
238 involved in sensing and regulation of physiological processes related to sugar, such as
239 carbohydrate active enzymes, chemotaxis, and biofilm formation (90). These multicomponent
240 systems are specific to distinct molecules including fructose, mannitol, sucrose, and glucose. The
241 sucrose-specific PTS enzyme complex (EIIABC) is present within *A. apis*, *A. boisseri*, *A.*
242 *rathckeae*, and *A. baretiae* strains, but absent from all environmental strains, as well as *A.*
243 *nectaris* and *A. pollinis*. Fructose-specific EIIABC complexes were found in all nectar-dwelling
244 strains and only three environmental species, *A. lwoffii*, *A. baylyii*, and *A. brisouii*. This pattern
245 suggests that PTS enzymes may have been beneficial for making the ecological switch to high
246 sugar environments. Overall, these findings show a shift in carbohydrate metabolism in keeping
247 with growth in nectar, although we found mainly subtle differences in gene content.
248 Because of the difference in sugars available to environmental versus nectar-dwelling
249 *Acinetobacter*, we further investigated their carbohydrate metabolic capabilities by comparing
250 carbohydrate active enzymes identified using the CAZY database (91, 92). Genomes of
251 environmental species contain significantly more genes in the glycosyl transferase (GT) family
252 (p-value = 0.001), with an average of 23 genes per environmental species genome and 15 per

253 nectar dweller species genome (Fig. S3). This difference is mainly driven by enzymes in the GT2
254 and GT4 families. The role of these specific genes within *Acinetobacter* species is unclear, but
255 generally enzymes in these families are involved in the synthesis of cell wall, capsular, and
256 extracellular biofilm polysaccharides (93–96), suggesting that some of these functions may be
257 different in nectar-dwelling *Acinetobacter*. We note, however, that several biofilm formation
258 genes are found in the nectar-dwelling strains, including *pgaABCD* genes of the poly-β-1,6-*N*-
259 acetyl-D-glucosamine (PGA) operon responsible for the maintenance of biofilm stability (97). Of
260 the 15 nectar-dwelling strains, nine have the complete PGA operon, while four out of the 11
261 environmental strains have the full operon. Of the individual genes in the operon *A. pollinis*
262 strains have 2-6 times the copies of *pgaB* genes, which contain binding domains critical for
263 export of PGA (98), suggesting biofilm formation as an important trait in nectar-dwelling
264 environments.

265 In contrast to the decrease in GT enzymes, nectar-dwellers contain significantly more
266 genes in glycoside hydrolase (GH) families (p-value=8.152e-8), averaging 15 enzymes per
267 species compared to approximately 13 genes per environmental species (Fig. S3). This pattern
268 was mainly driven by genes in the GH 28 family, which are involved in the breakdown of the
269 polygalacturonic acid backbone of pectin (99). Pectin is a major component of plant cells walls,
270 and we hypothesize, as discussed below, that the ability to degrade this polysaccharide may be
271 beneficial in floral nectar.

272

273 *Novel functions in nectar*

274 We investigated which genes had been likely gained by HGT in nectar-dwelling
275 *Acinetobacter*, with the hypothesis that such genes may confer novel functions. We found

276 genomic islands within all members of the nectar-dwelling clade and some strains also contained
277 intact prophages within their genomes. Gene count from genomic islands ranged from 111 – 352
278 genes with approximately 50% annotated as "hypothetical" and the remainder involved in
279 plasmid or transposon mobilization, phage replication, or Type1 secretion components.
280 Mobilization genes were present in genomic islands within the nectar dwelling clade suggesting
281 that movement of genomic material is facilitated by mobile elements like plasmids (Table S2).
282 Among known functions within genomic islands, we found genes coding for pectin
283 degradation enzymes, specifically PL1 family pectin lyases and GH28 family
284 polygalacturnonases. All species in the nectar-dwelling clade contain at least one of these genes,
285 with several species possessing multiple copies of genes associated with the degradation of
286 pectin (Table S3). Pectin is a recalcitrant polysaccharide that provides structural stability in plant
287 cell walls and the outer layers of pollen grains (100). Among bacteria, enzymes for degrading
288 pectin are commonly found in necrotrophic plant pathogens, which use them to digest plant
289 tissue (101). These enzymes are notably absent among all *Acinetobacter* genomes in GenBank,
290 with the exception of the orthologs found in the nectar clade (Table S3). Sequences in GenBank
291 with the highest similarity to nectar-clade orthologs of PL1 and GH28 genes are found outside of
292 the genus in plant pathogens such as *Pectobacterium*, *Erwinia* and *Dickeya* (Fig. 3A-B). This
293 pattern supports that these genes were acquired by nectar-dwelling *Acinetobacter* by HGT, likely
294 from a necrotrophic plant pathogen in the Enterobacterales.
295 Tracing the pattern of gains in pectin degradation genes onto the phylogeny of nectar-
296 dwelling *Acinetobacter* suggests that at least one copy each of the pectin lyase and
297 polygalacturonase genes were present in the common ancestor of the nectar-clade *Acinetobacter*
298 species analyzed here (Fig. 3A-B). In several strains these two orthologs are located next to each

299 other on the chromosome, so they may have been gained together in one event. As seen in the
300 gene trees for the pectin lyase and polygalacturonase orthologs, we determined that these genes
301 experienced multiple duplication events with paralogs sister to each other (Fig. 3A-B). These
302 duplications occurred within the species *Acinetobacter pollinis*, which contains six copies of
303 polygalacturonase and three copies of pectin lyase (within the strain SCC477 as an example).
304 Additional horizontal transfers, losses, or duplication events may have occurred within the nectar
305 clade, as some species have multiple copies within a gene tree (*A. boissieri*, Fig. 3A) or
306 differences in topology between gene trees and species trees (*A. apis*, Fig. 3B). Some of these
307 copies are on contigs that are likely from plasmids (based on increased read depth and the
308 presence of plasmid replication genes), which may have facilitated duplication and transfers of
309 these genes. Duplication was more common for the polygalacturonase than the pectin lyase
310 genes, and the polygalacturonase genes were also the only example of multiple copies outside of
311 the *A. pollinis* (Fig. 3A).

312 The fact that these genes have been maintained, and even duplicated, within the nectar-
313 dwelling clade suggests that they may serve an important ecological role for these bacteria. In
314 support of this, amino acid substitutions in several of pectin degrading enzyme protein sequences
315 show signatures of positive selection (Table S4). Positive selection was detected at the nodes and
316 tips of the polygalacturonase gene tree (Fig. 3), particularly for *A. pollinis* (nine sites) and *A. apis*
317 (seven sites) orthologs (Table S4). The high number of duplication events of these genes in *A.*
318 *pollinis*, together with signatures of positive selection, suggests that pectin degrading enzymes
319 may be functionally diversifying in this species. Both of these enzymes cleave linkages in the
320 polygalacturonic acid backbone of pectin (102). Necrotrophic plant pathogens typically have

321 diverse copies of these enzymes, with slight variations in catalytic ability, in order to effectively
322 degrade pectin (102–104). Potentially, this pattern is convergently evolving in *A. pollinis*.
323 To investigate the potential function of the amino acids under selection in *Acinetobacter*
324 polygalacturonase enzymes, we generated predicted protein structures of representative orthologs
325 from *A. pollinis* strain FNA3 and the *A. apis* type strain (105). The polygalacturonase binding
326 site contains four highly conserved motifs NTD, G/QDD, G/SHG and RIK. NTD and RIK are
327 highly conserved catalytic regions while G/QDD and G/SHG are substrate binding regions (106).
328 In nectar strain orthologs all conserved regions were present (NTD, GDD, GHG, RIK) but no
329 sites under selection were near these active sites (Fig. 4). It is therefore unclear from their
330 location on the protein structure how sites under selection might impact protein function.
331 It is not known how much nectar-dwelling *Acinetobacter* interact with major sources of
332 pectin in plant tissue, but to our knowledge they have not been observed to infect plants.
333 However, microbes in nectar are regularly interact with pollen grains, which are introduced into
334 nectar by pollinator activity (107). In fact, some *Acinetobacter* strains can cause pollen grains to
335 burst open or pseudogerminate (29). This ability is beneficial for *Acinetobacter*, as it is
336 associated with increased growth in nectar when pollen is present (29). Floral nectar has been
337 shown to be nitrogen limiting for both yeasts and bacteria (80, 81), so the ability to access
338 nitrogen from pollen within nectar could increase microbial fitness. However, pollen is protected
339 by a resistant exine layer and is difficult to degrade (108). Pectin is an essential component of
340 pollen cell walls and pollen tubes (109), and pectin degrading enzymes have been hypothesized
341 to be involved in pollen breakdown by bacterial gut symbionts of honey bees (110). We
342 hypothesize that the pectin degradation enzymes in *Acinetobacter* could be involved in accessing
343 nutrients from pollen, which could explain the apparent importance of genes coding for such

344 enzymes in the clade. In support of this, we find that most of the GH28 and PL1 proteins in
345 *Acinetobacter* have secretion signals (Fig. 4B-D, Fig. S4B-D), similar to secreted pectin
346 degrading enzymes in *Pectobacterium* (102), suggesting that they should act extracellularly.
347 Furthermore, we find the most selection on these genes within *A. pollinis* and *A. apis*. The former
348 shows strong impacts on pollen bursting and pseudogermination (29) and the latter was isolated
349 from honey bees and is likely to encounter pollen regularly. We speculate that the ability to
350 degrade pectin could be a key trait allowing *Acinetobacter* to thrive in nectar and in association
351 with pollinators.
352
353 *Conclusions*
354 In this study we show that the ecological change from soil-dwelling to nectar-dwelling
355 led to genomic reduction, possibly due to genome streamlining, a phenomenon common among
356 bacteria living in oligotrophic habitats (66). Even with this streamlining, nectar-dwelling species
357 had higher levels of gene gain events compared to gene losses. Many of the gain gains occur at
358 early branching nodes within the nectar clade, and the nectar clade had an increase in the number
359 of genes involved in monosacharride metabolism and sugar transport, likely due to the high
360 sugar environment of nectar (78). We also found changes in nitrogen and amino acids
361 metabolism genes suggesting a switch towards nitrogen scavenging in comparison to
362 environmental *Acinetobacter*, consistent with nitrogen limitation in floral nectar (55). Nectar-
363 dwelling *Acinetobacter* species have also acquired pectin degrading enzymes, presumably
364 through HGT, from plant pathogens. We found duplication, diversification, and positive
365 selection within pectin degrading genes, supporting our hypothesis that these genes may provide
366 an important ecological function. Overall, we find that genome evolution from gene loss,

367 diversification, and HGT may have all contributed to the *Acinetobacter* habitat switch to floral
368 nectar.
369
370 **Materials and Methods**
371
372
373 *Phylogenetic analyses*
374 A phylogenomic species tree was inferred using 26 *Acinetobacter* genomes and
375 *Pseudomonas syringae* pv. *tomato* strain DC3000 as an outgroup (Table 1). These included
376 sequences from nine environmental *Acinetobacter* species, and three previously published
377 nectar-dwelling and pollinator associated *Acinetobacter* genomes (45, 51) obtained from
378 GenBank. We did not include strains from the clades containing the animal pathogens *A.*
379 *baumannii* or *A. parvus*, as previous work found these groups to have undergone distinct
380 evolutionary changes compared to soil-dwelling relatives (4). Our recently published nectar-
381 dwelling *Acinetobacter* genomes (see (50) for isolation information) were assembled using
382 Discovar *de novo* (111), checked for completeness using CheckM (112), and annotated using the
383 RAST Server (112) Nucleotide sequences from previously published *Acinetobacter* genomes
384 were downloaded from GenBank and annotated in RAST for consistency. Protein sequences
385 were used in the PhyloPhlAn 3.0 pipeline (114) to determine conserved proteins within
386 *Acinetobacter* genomes. PhyloPhlAn identified 399 conserved proteins and their nucleotide
387 sequences were used for phylogenetic tree reconstruction. Nucleotide sequences for the
388 identified protein sequences were aligned using MAFFT (115). Maximum likelihood trees were
389 reconstructed using IQ-Tree (116) with bootstrapping set to 1000 and a symmetric substitution
390 model (117). Welch's t-test was used to determine the significance between genome size from
391 the nectar clade and environmental clade.

392 Gene trees were reconstructed using polygalacturonase and pectin lyase genes from the
393 *Acinetobacter* genomes. Outgroups were selected by using nectar-dwelling *Acinetobacter* spp.
394 pectin lyase and polygalacturonase genes as BLAST queries in GenBank. We found that all of
395 the best BLAST hits for these genes were from necrotrophic plant pathogens so we included the
396 most similar representative sequences in the analyses. *Acinetobacter* polygalacturonase and
397 pectin lyase genes were identified in our newly sequenced genomes by RAST annotation,
398 BLAST of the genomes using plant pathogen orthologs as queries, and comparison with the
399 CAZy database to confirm that we had identified all orthologs. Genes were aligned using
400 MAFFT (115) and were used for maximum likelihood phylogenetic inference in IQ-Tree using
401 the TIM3 substitution model which was selected using model finder in IQ-Tree.
402
403 *Ortholog analyses*
404 Orthologous protein sequence clustering was conducted using OrthoMCL (75) using the
405 following parameters: mode = 1, inflation = 2, pi cutoff = 50. To determine the ancestral state of
406 orthologs across the *Acinetobacter* species tree, the software package Count was used (118)
407 implementing Wagner Parsimony. This analysis determines the genes gained and lost at each
408 node of the *Acinetobacter* phylogenomic tree minimizing state changes and assuming all
409 character states are reversible (119). To estimate the number of pseudogenes present within the
410 genomes, we used the program Pseudofinder (120). The algorithm identifies pseudogenes from
411 Genbank files by analyzing average coding sequence (CDS) length, fragmented CDS, and
412 intergenic pseudogenes and alignment lengths are compared against homologs identified by
413 blastp hits from a reference protein database (121). The following parameters were used to
414 predict potential intergenic, fragmented, truncated, and long pseudogenes: intergenic length = 30,

415 length pseudo = 0.65, shared hits = 0.5, hitcap = 15, intergenic threshold = 0.3. Plasmids were
416 assembled using unfiltered paired end 2 x 250 Illumina sequencing reads (50) and the
417 plasmidSPAdes assembly tool (122). Plasmids less than 1000 bp were excluded from final
418 plasmid count. Fisher's exact test was used to calculate all significance test between orthologs
419 from the nectar groups and the environmental group.
420
421 *Selection and protein analyses*
422 dN/dS (ω) values were estimated for the polygalacturonase and pectin lyase coding
423 region alignments and IQ gene tree phylogenies using codeml in the PAML v4.4 package, with
424 gaps included (123). For each branch and node in the phylogenies, a likelihood-ratio test for
425 positive selection was performed to compare nested branch-site models (Model A_{null} versus
426 Model A) (124) (Table S4).
427 The AI protein prediction software, Alphafold, was used to predict protein structure of
428 pectin degradation enzymes. The Alphafold algorithm is a neural network that generates a
429 multiple sequence alignment (MSA) from the query protein sequence provided and extracts
430 evolutionary information to generate protein predictions (105). A web version of Alphafold was
431 used for these predictions (125). In order to determine if amino acid sites under selection were
432 functionally important, we predicted the structure of polygalacturonase genes identified to be
433 under positive selection from the PAML analysis, specifically genes from *Acinetobacter pollinis*
434 strain FNA3 (GenBank locus tags I2F29_RS12745 and I2F29_RS12925), *Acinetobacter apis*
435 (CFY84_RS01715), and *Phaseolibacter flectens* (L871_RS0110380). Three-dimensional protein
436 predictions were edited using ChimeraX to highlight sites under selection (126).
437

438 **Data availability**

439 All data is available in the supplemental materials or NCBI databases. Genomic assemblies are
440 available in GenBank under accessions VTDP00000000, VTDO00000000, VTDM00000000,
441 VTDL00000000, JAEQDL000000000, JAERJC000000000, JAERJB000000000,
442 JAEQDM000000000, VTDS00000000, VTDT00000000, VTDR00000000, and
443 VTDQ00000000. Sequence reads are available in the SRA under accessions SRR26518995-
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445

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453 performed analyses. Writing was collaborative between V.A.S., T.A.H., and T.R., with
454 comments from L.J.B.

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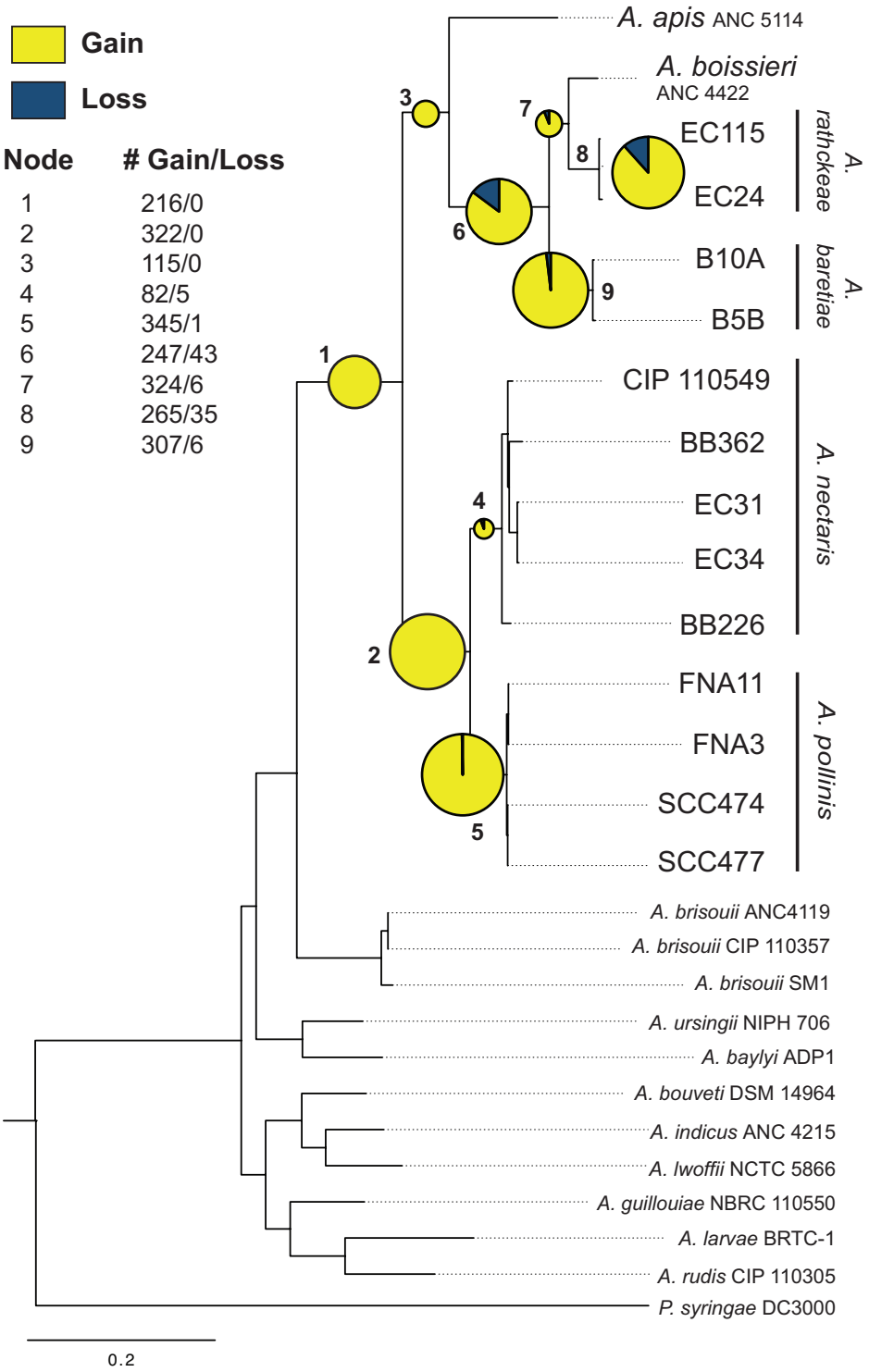

Figure 1. *Acinetobacter* species maximum likelihood phylogenomic tree based on 399 conserved protein sequences. All nodes have a bootstrap value of 100. Ancestral state reconstruction of ortholog gains and losses at nodes within the nectar-dwelling clade are shown. Nodes are labelled numerically and a pie chart at each node shows the proportion of ortholog gains (yellow) versus losses (blue). Pie size is scaled by total number of gain and loss events.

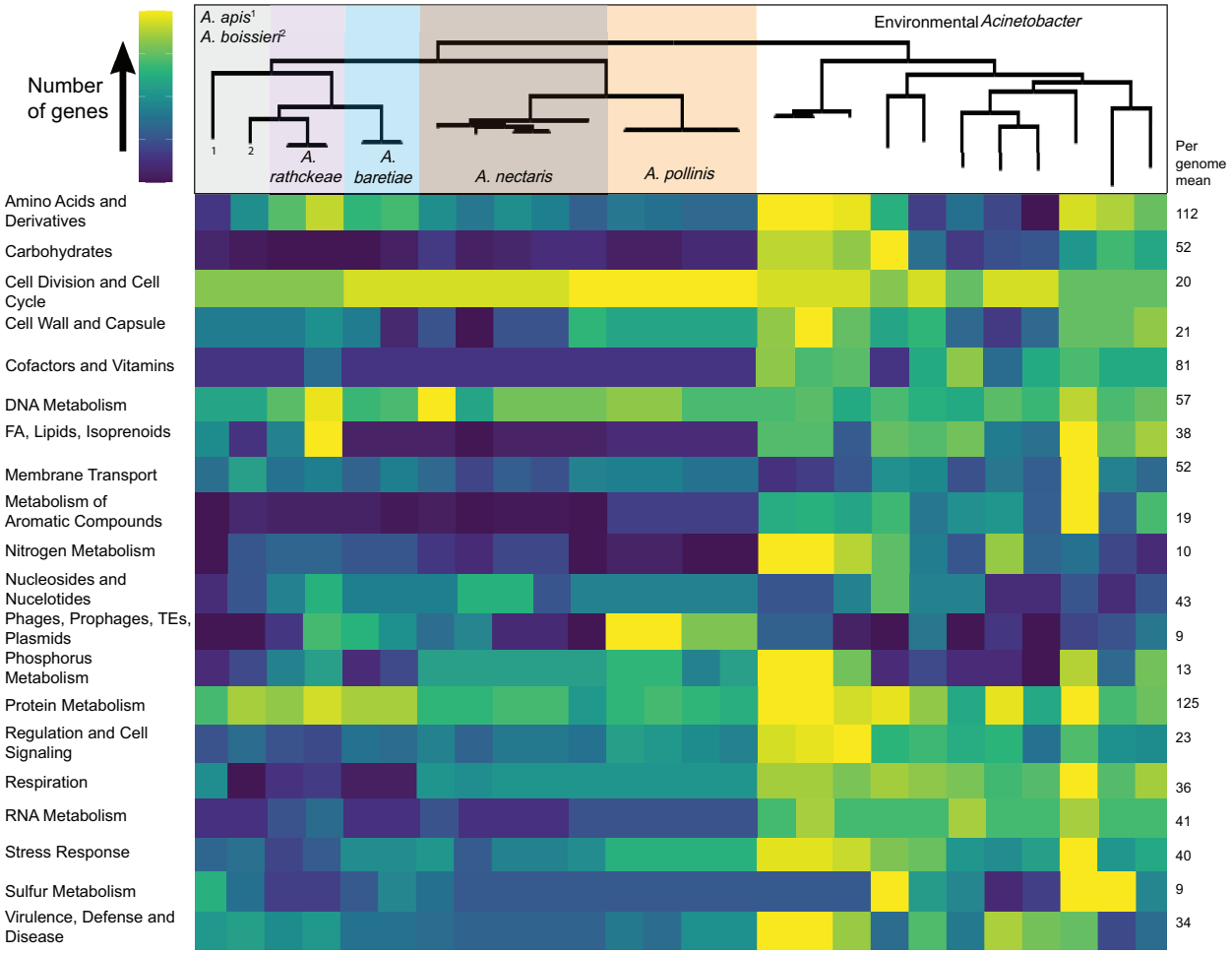

Figure 2: Heatmap showing number of orthologs across functional categories from nectar-dwelling and environmental *Acinetobacter* strains. Category assignments are based on RAST annotations (FA = fatty acid metabolism, TE = transposable elements), and a phylogenomic species tree (Figure 1) is shown. Each row is colored independently based on the variance of gene content within the category. A color scale with yellow indicating the highest number of genes and deep blue indicating the lowest number of genes is used. Genes of unknown function or categories with <6 mean orthologs per genome are excluded. The mean number of orthologs within a genome for each category is given.

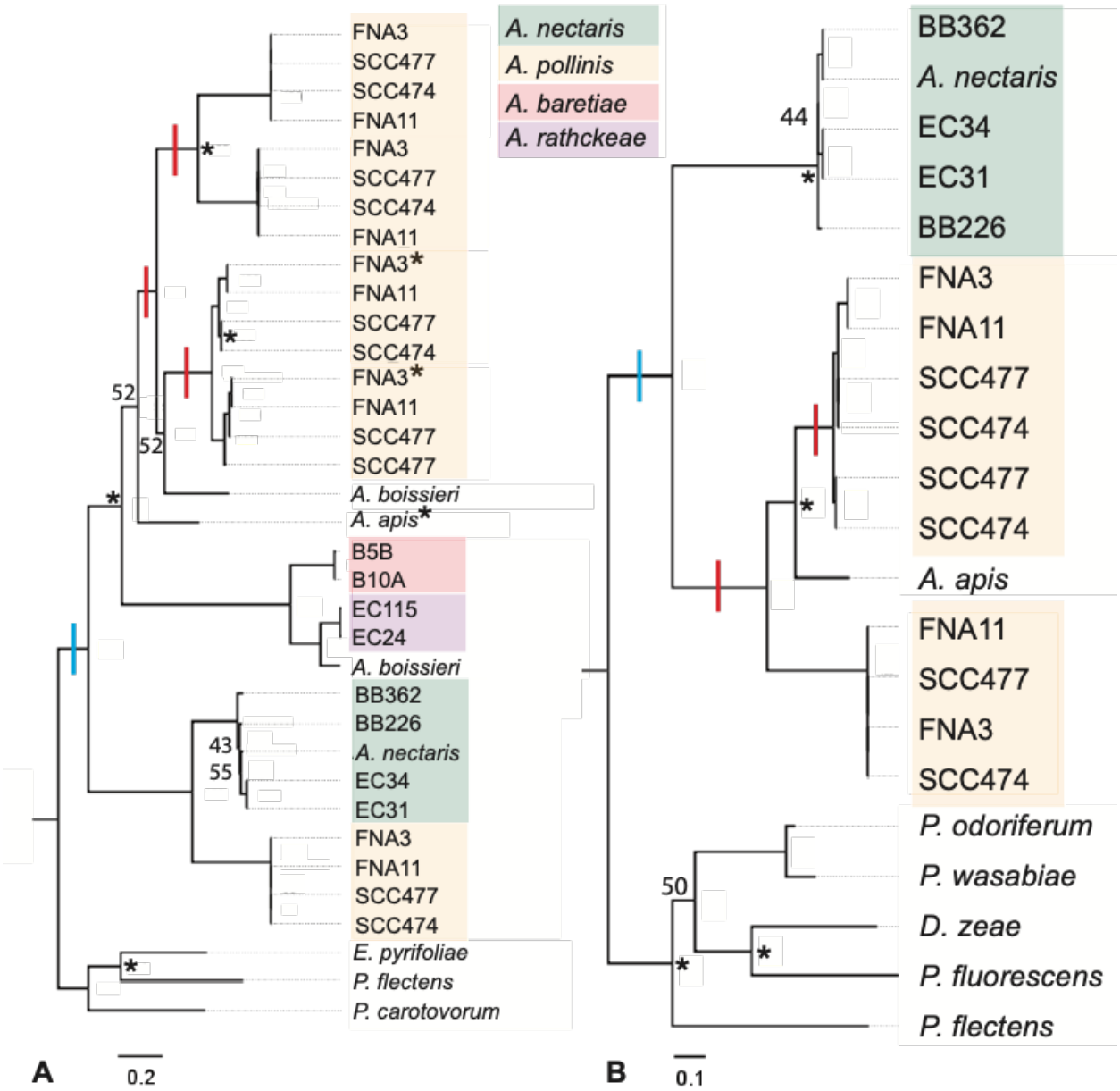

Figure 3. Maximum likelihood phylogenetic trees of A) the polygalacturonase gene, and B) the pectin lyase gene from nectar-dwelling *Acinetobacter* strains and the most closely related orthologs from plant pathogens. Red bars indicate likely duplication events and blue bars indicated horizontal gene transfer events. Bootstrap values below 80 are displayed at nodes. Asterisks show nodes and tips with significant positive selection. Orthologs from outside of *Acinetobacter* are from plant pathogens (*Erwinia pyrifoliae* (GenBank accession CP023567), *Pseudomonas flectans* (JAEE01000003), *Pectobacterium carotovorum* (CP001657), *Pectobacterium odoriferum* (MTAN01000008), *Pectobacterium wasabiae* (CP015750), *Dickeya zeae* (CP006929), and *Pseudomonas fluorescens* (OV986001)). Outgroup orthologs represent the best BLAST hits (>50% amino acid sequence identity, one ortholog per species) in GenBank databases.

863 Table 1. Genome characteristics of nectar-dwelling Acinetobacter strains and comparison
864 environmental strains. Type strains are designated.

Strain	Accession	Pseudo-genes	Genome Size (Mb)	G+C Content (%)	Protein Coding Genes	Proteins assigned to COGs
A. apis ANC 5114[T]	FZLN00000000	125	2.41	38.3	2232	1985
A. boissieri ANC 4422 [T]	NZ_FMYL00000000	182	2.69	38.0	2554	2378
A. rathckeae EC115	VTDP00000000	192	2.62	39.2	2438	2388
A. rathckeae EC24	VTDO00000000	214	2.75	39.3	2565	2531
A. baretiae B10A [T]	VTDM00000000	234	2.59	37.5	2531	2402
A. baretiae B5B	VTDL00000000	253	2.70	37.5	2629	2486
A. nectaris CIP 110549 [T]	NZ_AYER00000000	189	2.67	36.7	2569	2458
A. nectaris BB362	JAEQDL000000000	249	2.38	36.9	2269	2173
A. nectaris EC31	JAERJC000000000	200	2.42	36.7	2312	2303
A. nectaris EC34	JAERJB000000000	212	2.39	36.8	2288	2284
A. nectaris BB226	JAEQDM000000000	255	2.58	36.7	2421	2312
A. pollinis FNA11	VTDS00000000	245	2.67	36.6	2621	2599
A. pollinis FNA3	VTDT00000000	248	2.67	36.6	2615	2575
A. pollinis SCC474	VTDR00000000	294	2.75	36.7	2623	2605
A. pollinis SCC477 [T]	VTDQ00000000	262	2.75	36.7	2634	2609
A. brisouii ANC4119	NZ_APPR00000000	187	3.11	41.7	3036	3021
A. brisouii CIP 110357 = DSM18516	NZ_AYEU00000000	184	3.09	41.6	3025	3003
A. brisouii SM1	NZ_JZRE00000000	189	2.99	41.8	2913	2651
A. baylyi ADP1		222	3.6	40.4	3376	2897
A. ursingii NIPH 706	NZ_APQB00000000	234	3.49	40	3468	2920
A. bouveti DSM 14964 = CIP 107468	NZ_APQD00000000	198	3.37	40	3468	2657
A. indicus ANC 4215	ATGH01000001	238	3.17	45.4	3064	2632
A. lwoffii NCTC 5866 = CIP 64.10 = NIPH 512	NZ_AYHO00000000	268	3.21	43	3107	2635
A. guillouiae NBRC 110550	NZ_APPJ00000000	258	4.88	38.1	4648	3362
A. larvae BRTC-1	NZ_CP016895	283	3.74	41.6	3453	2598
A. rudis CIP110305	ATGI00000000	241	4	39.1	3748	2849

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869 Table 2. Presence and absence of phosphotransferase system (PTS) genes within *Acinetobacter*
870 strains and the isolation environment for each strain (citations provided where available).

Strain	None	PTS fructose-specific EIIABC complexes	PTS sucrose-specific EIIBC complexes	Strain environment (source citation)

A. apis ANC5114		1	1	Honey bee gut (51)
A. boissieri ANC4422		1	1	Floral nectar (45)
EC115		1	1	Floral nectar (50)
EC24		1	1	Floral nectar (50)
B10A		1	1	Honey bee gut (50)
B5B		1		Honey bee mouth (50)
A. nectaris CIP110549		1		Floral nectar (45)
BB362		1		Floral nectar (50)
EC31		1		Floral nectar (50)
EC34		1		Floral nectar (50)
BB226		1		Floral nectar (50)
FNA11		1		Floral nectar (50)
FNA3		1		Floral nectar (50)
SCC474		1		Floral nectar (50)
SCC477		1		Floral nectar (50)
A. brisouii ANC4119		1		Peat (127)
A. brisouii DSM189516=CIP 110357		1		Unknown
A. brisouii ANC 3789=SM1		1		River in Endau-Rompin National Park
A. baylyi CIP107474		1		Activated sludge plant (12)
A. ursingii NIPH706	1			Human blood
A. bouveti CIP110357	1			Unknown
A. indicus ANC4215	1			Unknown
A. lwoffii CIP64.10		1		Unknown
A. guillouiae NIPH991	1			Human ear swab
A. larvae BRTC1	1			Moth larvae (128)
A. rudis CIP110305	1			Raw milk and wastewater (129)

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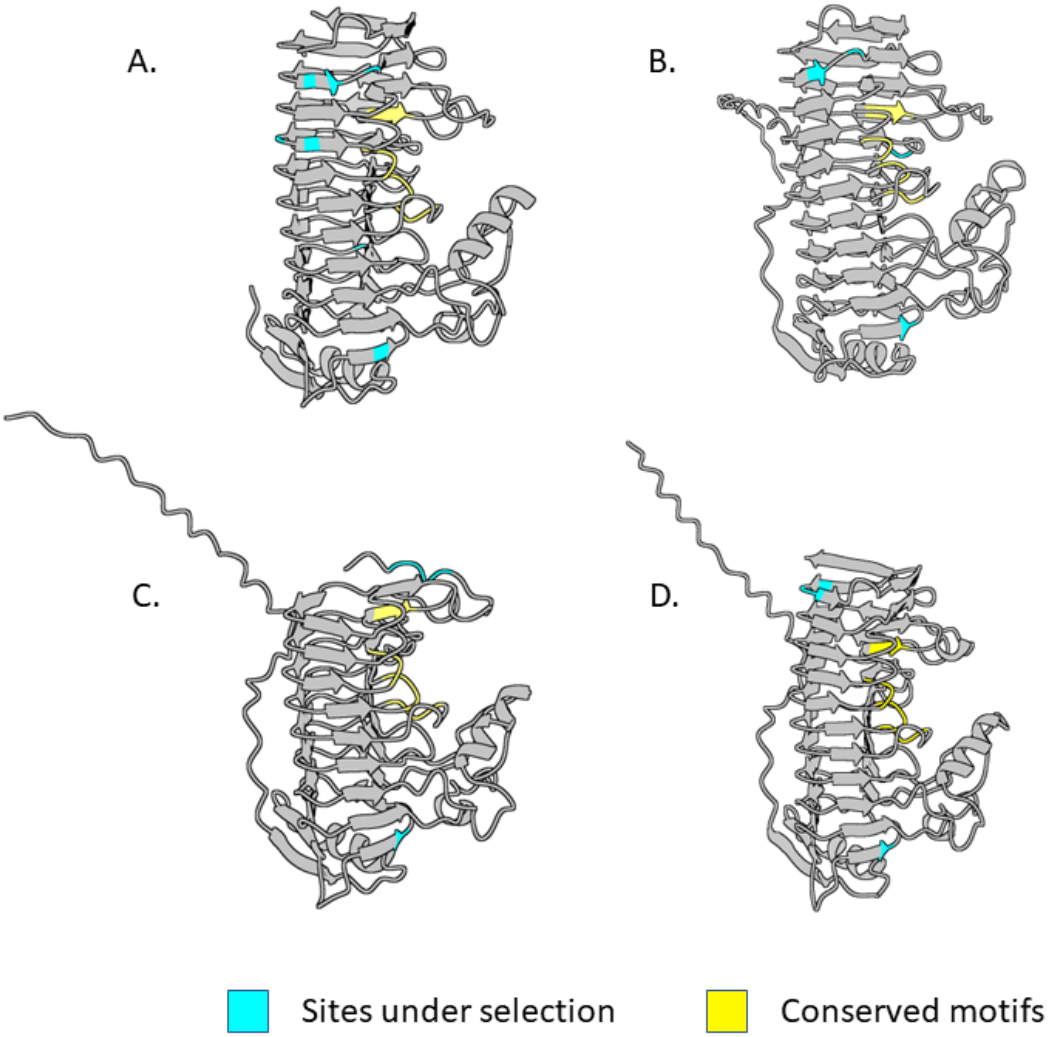

Figure 4: Alphafold protein predictions of polygalacturonase genes from *Acinetobacter apis* (A), *Acinetobacter pollinis* FNA3 (B-C), and *Phaseolibacter flectens* (D). Conserved motifs and sites under selection are highlighted blue and yellow respectively. Secretion signal tags are present in B-D. These extend off the protein to the left in C-D, but backwards in B. B is rotated in Fig. S2 to show the secretion signal.