Reciprocal cross-feeding between bacteria can limit the emergence of metabolic dependencies

Short title: Cross-feeding can promote gene retention

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ABSTRACT

Cross-feeding is prevalent in microbial communities. Through time, cross-feeding is thought to enrich for loss-of-function mutations, thereby creating or reinforcing dependencies between community members. However, few studies have compared how cross-feeding affects the evolutionary trajectory of partners compared to monoculture conditions. Here we compared mutations that were differentially enriched in bacterial monocultures versus cocultures pairing phototrophic *Rhodopseudomonas palustris* with fermentative *Escherichia coli* in an obligate cross-feeding relationship based on the exchange of nitrogen and carbon for 650-800 generations. Opposite trends for the number of differentially enriched mutations were observed for each species; *R. palustris* accumulated more unique mutations in monoculture whereas *E. coli* accumulated more unique mutations in coculture. Contrary to expectations, the emergence of additional dependencies was more apparent for both species in monoculture, even though additional layers of cross-feeding involving iron and adenine were present in coculture. We reasoned that iron and adenine cross-feeding occurred at levels sufficient to repress gene expression in the recipient, thereby promoting gene retention by lowering gene cost. Thus, the influence of cross-feeding on evolutionary trajectories can vary with organisms and conditions, and there are situations where cross-feeding can limit, rather than promote, emergent dependencies.

IMPORTANCE

Bacteria commonly engage in cross-feeding where nutrients are transferred between neighbors. Cross-feeding is thought to alleviate energy expenditures for genes whose role can be met by cross-fed nutrients, leading to eventual gene loss. However, few examples have been documented, especially in comparison to monocultures where bacteria are grown without a cross-feeding partner. Here we grew cocultures pairing phototrophic *Rhodopseudomonas palustris* with fermentative *Escherichia coli* alongside corresponding monocultures for 650-800 generations. While coculture conditions required obligate exchange of nitrogen and carbon, additional cross-feeding of adenine and iron likely occurred. Contrary to expectations, iron and uncharacterized dependencies emerged in monocultures but not in cocultures. Low expression for iron scavenging and adenine synthesis genes in cocultures suggested that cross-feeding repressed gene expression, thereby lowering gene cost. Thus, whereas there are likely cases where cross-feeding makes costly genes dispensable, there are also cases where cross-feeding lowers gene cost, thereby promoting gene retention.

INTRODUCTION

Microbial metabolism shapes local chemical environments in ways that elicit regulated responses from neighboring cells, or even select for adaptive mutations. In some cases, extracellular metabolites can be used as nutrients by neighboring cells, establishing cross-feeding (1). Cross-feeding can lead to obligate dependencies through adaptive loss-of-function (LOF) mutations, when the benefit of exploiting an extracellular metabolite outweighs the cost of retaining a biosynthetic gene (2). When the recipient benefits at the expense of the producer, the recipient can be labeled a 'cheater' but when the recipient does not detrimentally affect the producer, the recipient can be labeled a 'beneficiary' (2, 3). The latter is described in the Black Queen Hypothesis (BQH), wherein cross-feeding provides a cost-benefit advantage that promotes adaptive gene-loss (2). The BQH is commonly used to explain the prevalence of auxotrophs, which cannot synthesize one or more essential nutrients (4, 5). Indeed, several groups observed that auxotrophs with biosynthetic gene deletions have a fitness advantage over their prototrophic counterparts when corresponding biosynthetic precursors were provided (6-10).

Whereas cross-feeding can promote adaptive gene loss, gene loss is also potentially constrained during cross-feeding by several factors. For example, the energetic costs of N_2 fixation, converting N_2 into 2 NH_4^+ via nitrogenase, was predicted to be under pressure for gene loss because NH_4^+ can escape and potentially enable higher growth rate for recipients (2). However, we found that growth rates supported by leaked NH_4^+ did not exceed those possible via N_2-fixation due to low NH_4^+ availability relative to N_2, even when bacteria were engineered to excrete NH_4^+ (11). Moreover, as has been documented with cheaters (12-14), nutrient availability can repress gene expression and thereby lower gene cost (4, 13, 15); most of a gene's cost comes from its expression (16-20). Thus, repressing gene expression could have similar cost-savings as a LOF mutation and thereby decrease selective pressure for LOF mutants.

Previously we engineered cocultures of phototrophic *R. palustris* and fermentative *E. coli* to study the mechanisms and evolution of obligate cross-feeding (21-25). Using anoxic conditions, *E. coli* ferments glucose into organic acids that provide essential carbon to *R. palustris* while *R. palustris* converts N_2 into NH_4^+, providing essential nitrogen to *E. coli* (21). The two species are not believed to interact in nature, providing an opportunity to observe how the relationship evolves without pre-adaptation (22). Whereas the coculture was engineered to rely on obligate NH_4^+ cross-feeding, other interaction layers exist. For example, we discovered that *R. palustris* externalizes enough adenine to support an *E. coli* purine auxotroph population (26, 27). How such secondary interactions affect the evolutionary trajectory of each species is unknown.

Here we compared differential mutation accumulation in *E. coli* and *R. palustris* monocultures versus cocultures for >600 generations. The number of differentially enriched mutations in monocultures versus cocultures was opposite for each organism. For several mutations, we performed a preliminary investigation to gain insights into why they were differentially enriched. In some cases, we correlated a lack of

103 dependency emergence in coculture to inhibitory effects of cross-feeding on gene
104 expression. Our results offer diverse insights into how cross-feeding influences
105 evolutionary trajectories and caution against general expectations for dependencies
106 through LOF mutations.
107
108 **RESULTS AND DISCUSSION**
109
110 ***R. palustris* and *E. coli* can coexist for hundreds of generations.** We established
111 long-term, batch, anaerobic (i) monocultures of phototrophic, N_2-fixing, NH_4^+-excreting
112 *R. palustris* NifA* (CGA676) (28), supplied with a mixture of organic acid salts (neutral
113 pH), ethanol, and bicarbonate to mimic *E. coli* fermentation products, (ii) monocultures
114 of fermentative *E. coli* PFM2, a MG1655 derivative with repaired *rpoS* and *rph* genes
115 (29, 30), supplied with 10 mM glucose and 10 mM NH_4Cl, and (iii) cocultures supplied
116 with 25 mM glucose (Fig 1A). All cultures were constantly illuminated, and 2.5% v/v was
117 transferred every seven days. All conditions included an exponential growth phase but
118 only monocultures experienced a nutrient-limited stationary phase. In cocultures,
119 glucose and consumable organic acids remained at the time of transfer and likely
120 supported growth, however slow due to the low pH. Ultimately, all conditions were
121 serially transferred for 650 – 803 generations (~5 generations per transfer; Fig 1).
122
123 We sequenced gDNA from populations at several timepoints. We then compared
124 mutations that were differentially enriched in monoculture versus coculture by applying
125 following criteria: (i) exclude synonymous mutations, (ii) no exact mutation in the
126 opposing monoculture or coculture condition, (iii) observed in the last two sequenced
127 timepoints, (iv) with a frequency >0.1 in the last timepoint. These criteria resulted in a
128 list of 167 mutations from *R. palustris* monocultures, 31 from *E. coli* monocultures, 43
129 from *R. palustris* in cocultures, and 132 from *E. coli* in cocultures (Fig 2A, Table S1-5).
130 These counts include redundancy, where some mutations were observed in multiple
131 lines. In all conditions, most mutations were nonsynonymous, except for *E. coli*
132 monocultures, where most mutations were small insertions/deletions (Fig 2B). Mutations
133 due to mobile genetic elements were only observed in *E. coli* (Fig 2B). To narrow our
134 focus on positive selection, we added a fifth criteria of parallelism where the same gene
135 or intergenic region must be mutated in at least two lineages.
136
137 **Differentially enriched *R. palustris* mutations mostly occurred in monocultures.**
138 Most differentially mutated *R. palustris* genes and intergenic regions occurred in
139 monocultures: 19 in monoculture vs 5 in coculture (Fig 3). We considered that the
140 higher mutation frequency in monoculture could be due to a *mutS,* encoding a mismatch
141 repair protein; lines 2 and 7 had a *mutS* mutations that met our criteria and most other
142 lines had a low frequency *mutS* mutation at some point during the experiment.
143 However, the total number of differentially-enriched mutations was similar across *R.*
144 *palustris* monoculture lines (range: 8-23; Table S1) with only 6 of 21 mutations in line 2
145 and 11 of 23 mutations in line 7 potentially attributed to *mutS*, since they occurred at a
146 lower frequency than the *mutS* mutation (*mutS* mutation frequency was 13 and 22% in
147 lines 2 and 7, respectively; Table S1). Thus, *mutS* mutations did not likely drive many of
148 the other mutations.
149

150 Several differentially mutated genes in coculture also acquired different mutations in
151 monoculture. Among these was *ppsR2*, encoding an O_2-responsive regulator of genes
152 for phototrophic growth. *ppsR2* is commonly mutated in *R. palustris* because they
153 provide an advantage under low-light conditions, which occurs at high cell densities
154 (31). Other genes that were commonly mutated in monoculture and coculture were
155 *cbbM*, encoding the Calvin cycle type II Rubisco and *cbbR*, encoding a Calvin cycle
156 regulator. It is unclear why mutations were enriched in these genes, but it is possible
157 that they are associated with the role of CO_2 fixation in electron balance during
158 photoheterotrophic growth or electron distribution between CO_2 and N_2 fixation (28, 32).
159 We did not investigate Calvin cycle gene mutations further herein.
160
161 ***R. palustris* monocultures evolved to utilize H_2.** Both species produce H_2, *E. coli* via
162 fermentation and *R. palustris* as a coproduct of nitrogenase activity (33). Curiously, H_2
163 levels decreased through serial transfers in *R. palustris* monocultures but not in
164 cocultures (Fig 4A, B). A commonly mutated gene in *R. palustris* monocultures was
165 *hoxJ,* which encodes a sensor kinase that governs H_2-oxidizing hydrogenase by
166 repressing the transcriptional activator HoxA (Fig 4C). Normally, HoxJ repression of
167 HoxA is relieved when HupUV senses H_2. However, the *R. palustris* ancestor had a
168 *hupV* mutation, causing HoxJ to constitutively repress HoxA (34). Deleting HoxJ was
169 previously shown to lead to hydrogenase activity (34).
170
171 Based on the above, we hypothesized that our *hoxJ* mutants also permitted H_2
172 oxidation. To test this hypothesis, we introduced a 5-bp *hoxJ* deletion, observed in 7
173 monoculture lines, into the NifA* ancestor. Indeed, the mutant did not accumulate H_2,
174 (Fig 4D). Thus, we reason that *hoxJ* mutations that were enriched in monoculture
175 allowed *R. palustris* to use H_2 as an electron source.
176
177 We questioned why *hoxJ* mutants were only enriched in monoculture. Previously,
178 transposon disruption of *hoxJ* in monoculture led to a fitness benefit in stationary phase
179 (35). The benefit likely stemmed from H_2-mediated CO_2 fixation after organic substrates
180 were exhausted; strains with WT *hoxJ* could not grow after this point (35). We first
181 asked whether there is something about coculture conditions that generally prevents H_2
182 oxidation. When we repaired the *hupV* mutation (*hupV*+) or deleted *hoxJ*, no H_2
183 accumulated in either monoculture or coculture, indicating that *R. palustris* hydrogenase
184 can be activated in coculture (Fig 4E). Therefore, something must have prevented
185 enrichment of metabolically active *hoxJ* mutants in coculture.
186
187 We considered two possibilities that could explain the lack of *hoxJ* mutant enrichment in
188 coculture. First, consumable organic acids, which remained available at the time of
189 transfer in cocultures, could have allowed the general *R. palustris* population to remain
190 competitive against *hoxJ* mutants, preventing their enrichment. A second possibility is
191 that *E. coli* outcompeted *R. palustris* for nickel, which is required for hydrogenase
192 activity. Since our media used for serial transfers lacked a nickel supplement, nickel
193 must have come as a contaminant in other media components, and its availability was
194 likely low. Although the absence of H_2 in cocultures with Δ*hoxJ* or *hupV*+ mutants
195 indicates that *R. palustris* can compete against *E. coli* for nickel, this might only be true

196 when the H_2-oxidizing *R. palustris* population is large. An emergent, low frequency, H_2-
197 oxidizing *R. palustris* subpopulation might instead be outcompeted by *E. coli* for nickel.
198
199 ***R. palustris* siderophore gene mutations are correlated with expression level.** A
200 frequently mutated gene cluster in *R. palustris* monocultures was RPA2382-90, for
201 siderophore synthesis (Fig 3, Fig 5A, B). We speculate that these were LOF mutations,
202 giving rise to 'cheaters' that scavenged siderophores from a siderophore-producing
203 subpopulation. However, we questioned why *R. palustris* siderophore mutants were not
204 observed in cocultures.
205
206 We hypothesized that siderophore gene mutations were not enriched in coculture
207 because *E. coli* facilitated *R. palustris* iron acquisition, thus decreasing the need for *R.*
208 *palustris* to synthesize siderophores. The situation would be analogous to that observed
209 for *P. aeruginosa*, which produces siderophores only when needed, due to their cost.
210 High *P. aeruginosa* siderophore production in low-iron conditions led to a higher
211 frequency of siderophore-deficient mutants compared to iron-rich conditions where
212 siderophore production was low (12-14). In support of this hypothesis, we previously
213 saw that *R. palustris* siderophore gene transcripts were low in coculture with *E. coli*
214 MG1655, relative to monocultures (23). We verified that this trend was also true for *R.*
215 *palustris* in coculture with *E. coli* PFM2; RT-qPCR analysis showed that the RPA2390
216 transcript level in coculture was 20% of that in monoculture (Fig 5C). Adding soluble
217 iron to monocultures decreased RPA2390 expression to 3% of that observed without
218 added iron, suggesting that the low expression in coculture was in response to higher
219 iron availability (Fig 5C). Thus, iron must be scarce in *R. palustris* monocultures,
220 inducing siderophore production, whereas in coculture, *E. coli* somehow increases iron
221 availability, repressing *R. palustris* siderophore synthesis.
222
223 The mechanism of increased iron availability for *R. palustris* in coculture deserves
224 investigation beyond the scope of the current study. However, we suspect that *R.*
225 *palustris* can use *E. coli* siderophores. Others have speculated that *R. palustris* uses
226 foreign siderophores because its genome encodes multiple siderophore transporters but
227 only one synthesis cluster (36, 37). In support of this hypothesis, the *E. coli* siderophore
228 *entF* transcript level was 3.3-fold higher in coculture than in monoculture (Fig. 5D),
229 perhaps in response to iron loss to *R. palustris*
230
231 **Other *R. palustris* mutations with unknown phenotypes.** Other genes that were
232 mutated in monoculture include a methylmalonyl-CoA mutase encoded by RPA1837, a
233 possible flagellin encoded by RPA2297 that is outside the flagellar gene cluster, and a
234 hypothetical protein encoded by RPA4084 (6 of 10 lines).
235
236 One of the few *R. palustris* genes that was differentially mutated in at least 5 coculture
237 lines was RPA3297, annotated to encode a urea or short-chain amino acid transporter.
238 Each mutation was an insertion or deletion that should cause a frame shift and thus
239 they are all likely LOF mutations. This transporter was previously observed to have
240 elevated protein levels in coculture compared to monoculture (23). We speculate that

241 this elevated expression was disadvantageous, but the function of the gene product
242 remains unknown.
243
244 Two *R. palustris* coculture lines also had a common intergenic mutation between *hbdA*
245 and *etfA*. These genes are likely involved in fatty acid degradation and might be
246 commonly regulated by LiuR (38). The effect of this mutation is unknown.
247
248 **Differentially enriched *E. coli* mutations mostly occurred in cocultures.** The
249 number of differentially enriched mutations in *E. coli* were oppositely skewed than for *R.*
250 *palustris* (Fig 2). The same pattern held for differentially mutated *E. coli* genes and
251 intergenic regions in at least two lineages per condition: 6 were mutated in monoculture
252 vs 19 in coculture (Fig 6). Similar observations of cocultures leading to more mutations
253 were made by another group who paired *E. coli* auxotrophs in reciprocal cross-feeding
254 cocultures versus monocultures (39).
255
256 ***E. coli* mutations in coculture reflect adaptation to nitrogen starvation.** In
257 coculture, the *E. coli* growth rate is limited to ~20% of maximum due to the rate at which
258 it receives NH_4^+ from *R. palustris* (21, 22). As in previous long-term cocultures pairing a
259 different *R. palustris* NifA* strain with *E. coli* MG1655 (22), several *E. coli* mutations in
260 coculture suggested adaptation to this low-nitrogen condition. For example, mutations
261 were commonly observed in *glnGL,* encoding the two-component system NtrBC that
262 controls nitrogen scavenging. Previously, we demonstrated that NtrBC is critical for *E.*
263 *coli* survival in the low-nitrogen coculture environment (22, 23). Other mutations in *glnK*
264 and *amtB*, which respectively encode a nitrogen-responsive regulatory protein and an
265 NH_4^+ transporter, likely also help with nitrogen acquisition in coculture (Fig 6); we also
266 previously demonstrated the importance of *E. coli* AmtB in coculture (24).
267
268 ***E. coli* purine transporter mutants are enriched in coculture.** We designed our
269 cocultures to be based on the reciprocal exchange of fermentation products and NH_4^+
270 (21). We later discovered that *R. palustris* also externalizes adenine that can be
271 consumed by *E. coli* (26, 27). Herein, *E. coli* exhibited several mutations in coculture
272 that could be in response to adenine availability. For example, mutations were enriched
273 upstream of *adeD (yicP)* encoding adenine deaminase (Fig 6). Insertion elements
274 upstream of *adeD* in the adenine permease gene *adeQ* (yicO) are known to activate
275 *adeD* expression (40). Mutations were also enriched in the adenine permease gene,
276 *adeP* (*purP*) (Fig 6). The *adeP* mutations were accompanied by a multi-gene
277 amplification that created up to 40 copies of *adeP* and neighboring genes (Fig 7A, B).
278 We revisited sequencing results from our previous long-term cocultures featuring *E. coli*
279 MG1655 (22) and found similar mutations upstream of *adeD* and amplification of the
280 same region (Fig 7C). Evolved *E. coli* monocultures did not show this amplification but
281 had up to 8-fold amplification in another region, with no obvious connection to adenine
282 (Fig S1).
283
284 We hypothesized that these mutations could lead to increased fitness in the presence of
285 adenine. We attempted to mimic a high *adeP* copy number via IPTG-induced *adeP*
286 expression from a plasmid. However, over-expressing *adeP* in PFM2 did not improve

287 growth in the presence of adenine over an empty vector control (Fig 7D, E). It is
288 possible that *adeP* amplification is only advantageous in the context of the surrounding
289 amplified region or other enriched mutations.
290
291 To address an adenine-related fitness benefit more generally, we compared evolved *E.*
292 *coli* growth with and without adenine. Adenine benefited several coculture-evolved
293 PFM2 isolates, suggesting that some mutations were adaptive to adenine availability
294 (Fig 8). Although some monoculture isolates also responded positively to adenine,
295 including two possible auxotrophs, the trend was less pronounced; when isolates were
296 treated as replicates, the change in cell density with adenine versus without was
297 significantly higher for coculture-evolved PFM2 isolates but not for monoculture evolved
298 isolates (paired one-tail t-test, $p = 0.002$ versus 0.14, respectively).
299
300 **Strain-specific adenine toxicity likely influences *E. coli* evolution.** Adenine
301 availability in coculture might also explain differential enrichment of mutations in
302 previous cocultures featuring *E. coli* MG1655 (22). MG1655 is inhibited by adenine due
303 to an *rph* mutation that disrupts *pyrE* regulation (41), leading to suboptimal precursor
304 distribution between purine and pyrimidine synthesis in the presence of adenine (42).
305 The *rph* mutation is repaired in *E. coli* PFM2 (29, 30). Through serial transfers, PFM2
306 mutations in *rph* and purine and pyrimidine synthesis genes were rare in monoculture
307 and coculture (Fig 9A). In contrast, MG1655 populations (only evolved in coculture)
308 contained a high frequency of *rph* mutations and a low frequency of purine synthesis
309 gene (*pur*) mutations (Fig 9B). We hypothesized that MG1655 *rph* and purine
310 biosynthesis mutations alleviated adenine toxicity. Indeed, repairing the ancestral *rph*
311 mutation or deleting *purH*, rendering MG1655 a purine auxotroph, alleviated alanine
312 toxicity (Fig 9C).
313
314 **Coculture adenine availability did not enrich for adenine auxotrophs.** Given that *de*
315 *novo* purine synthesis is energetically costly, we expected to observe *E. coli* purine
316 synthesis LOF mutations in cocultures where adenine is available from *R. palustris*;
317 adenine is amply available in long-term cocultures (43). However, no purine
318 biosynthesis gene mutations were observed in cocultures (Fig 9A), nor were adenine
319 auxotrophs observed among 40 coculture isolates screened (Fig 8); only four
320 monoculture isolates in lines 18 and 20 were possibly adenine auxotrophs (Fig 8).
321
322 The BQH predicts that cross-feeding will lead to LOF mutations only when the benefit of
323 cross-feeding outweighs the cost of maintaining the gene (2). To directly test whether
324 adenine auxotrophs would have an advantage in coculture, we compared PFM2 growth
325 versus that of a *ΔpurH* mutant in a minimal medium with and without adenine. The
326 *ΔpurH* mutant required adenine for growth, but despite the availability of adenine, the
327 *ΔpurH* mutant did not grow faster than the PFM2 parent (Fig 10A, B). We also directly
328 compared the fitness of PFM2 versus a *ΔpurH* mutant in coculture with *R. palustris*
329 NifA* (CGA676). We used a range of initial frequencies so that we could simultaneously
330 test for possible coexistence by mutual invasion, where an equilibrium frequency can be
331 extrapolated from the x-intercept by linear regression analysis (11, 44-46). Although
332 there was poor linear correlation in the invasion-from-rare assay, the *ΔpurH* mutant

333 tended to decrease in frequency relative to parent in cocultures, where adenine is
334 available (Fig 10C). Thus, our results suggest that a *ΔpurH* mutant does not have a
335 competitive advantage over PFM2 in the presence of adenine, thus explaining why
336 PFM2 adenine auxotrophs were not enriched in cocultures.
337
338 **Adenine represses *E. coli* purine synthesis gene expression.** One reason why
339 PFM2 purine auxotrophs were not enriched in coculture could be if adenine availability
340 repressed gene expression, thereby decreasing gene cost. This would be analogous to
341 the situation described above for *R. palustris* siderophore genes. In support of this
342 hypothesis, we previously saw that *E. coli* MG1655 had lower *pur* transcript levels in
343 coculture (23). We verified that the same is true for PFM2 by RT-qPCR quantification of
344 *purH* transcript; coculture PFM2 *purH* levels were 20% of those in monoculture (Fig
345 11A). Adding adenine to monocultures resulted in a *purH* transcript level that was 12%
346 of that observed without adenine, suggesting that the low expression in coculture was
347 due to adenine availability (Fig 11A).
348
349 *R. palustris* also had less *purH* transcript in coculture, 50% of that in monoculture (Fig
350 11B). It is unclear how *E. coli* would influence *R. palustris purH* transcript levels.
351 Regardless, it is curious that adenine availability combined with *purH* expression in
352 monoculture did not lead to *R. palustris pur* LOF mutations (Table S1) nor any purine
353 auxotrophs (Fig 12). We speculate that *R. palustris* lacks adenine uptake mechanisms,
354 which would also help explain extracellular adenine accumulation (26, 43); poor uptake
355 mechanisms can be an important factor in extracellular metabolite accumulation (47-
356 49).
357
358 ***E. coli* monocultures enrich for auxotrophs.** Whereas cocultures did not enrich for *E.*
359 *coli* auxotrophs, several evolved monoculture PFM2 isolates could not grow in a
360 minimal medium (Fig 8). Enrichment of auxotrophs in monoculture could suggest the
361 emergence of cross-feeding between *E. coli* subpopulations that were not available in
362 coculture, perhaps due to competition from an *R. palustris* population that is an order-of-
363 magnitude larger (21, 22). Although we did not investigate these possible *E. coli*
364 monoculture cross-feeding relationships further, we speculate that a source of the
365 auxotrophies could be mutations in *rpoC*, encoding the RNA polymerase β'-subunit.
366 RpoC mutations are known to result in polyauxotrophs (50).
367
368 Curiously, we did not see a similar emergence of *R. palustris* auxotrophs (Fig 12). *E.*
369 *coli* and other diverse bacteria are known to support, or quickly evolve to support,
370 auxotrophic strains via cross-feeding (51-55). It is possible that *R. palustris* cannot
371 support diverse auxotrophs beyond those that require purines (26).
372
373 **Other *E. coli* mutations with unknown phenotypes.** Other differentially enriched *E.*
374 *coli* mutations include those in *aceE* that evolved in monocultures (Fig 6). *aceE* encodes
375 a pyruvate dehydrogenase subunit, which is important for respiration but is generally not
376 involved in *E. coli* fermentative metabolism (56). It is possible that expression of *aceE* is
377 sub-optimally high under the high growth rates supported in monoculture conditions
378 (23). Other mutations enriched in monoculture occurred in genes for post-translational

379 regulation of the TCA cycle enzyme isocitrate dehydrogenase (*aceK*), ribosome
380 modification (*rluC*), a vitamin C transporter (*ulaA/B*), and a deoxyribose salvage enzyme
381 (*deoB*).
382
383 Several genes mutated in coculture might impact biofilm formation and acid sensitivity.
384 We speculate that these mutations could help *E. coli* associate with *R. palustris* and/or
385 *E. coli* cross-feeding partners and potentially respond to fermentative acid stress. For
386 example, *csgD/B* is involved in curlin-based biofilm formation (57), which is regulated in
387 part by *cpxRP* genes that were also mutated (58). *ycgV* encodes an outer membrane
388 protein that might be involved in biofilm formation and *fimH/A* are involved in fimbriae-
389 mediated adhesion (57). Mutations in *yifE* (*maoP*)/*hdfR*, might also affect biofilm
390 formation. MaoP was noted to affect biofilm formation in addition to its role in
391 chromosome positioning and segregation (59). HdfR might have a regulatory link to the
392 transition between motile and sessile lifestyles (60).
393
394 HdfR might also be linked to acid resistance by regulating amino acid decarboxylation
395 genes encoded by *gltBD (61).* Mutations in *hdfR* were also observed in cocultures with
396 MG1655 (22). Mutations were also observed in *cspC*/*yobF*, which encode for proteins
397 that respond to acid and other stressors (62). Some of the genes associated with the
398 amplified region in cocultures might also be linked to acid resistance such as *mnmE*
399 (63) and *yidZ* (64).
400
401 **Concluding remarks.** Long-term serial transfers of *R. palustris* and *E. coli*
402 monocultures and cocultures differentially enriched for mutations. More differentially
403 enriched mutations were observed in *R. palustris* monocultures compared to cocultures,
404 and vice-versa for *E. coli*. Our results suggest that the extent to which cross-feeding
405 influences the evolutionary landscape depends on the organisms and conditions.
406
407 A generalization from our observations is that monocultures enriched for dependencies
408 more than cocultures. Against expectations for cross-feeding to promote LOF mutations
409 in a recipient related to synthesis of a cross-fed metabolite, *R. palustris* siderophore
410 mutants and unknown *E. coli* auxotrophs were only enriched in monoculture. We reason
411 that adenine and siderophore LOF mutants were not observed in coculture because
412 cross-feeding generated metabolite levels that were sufficient to repress gene
413 expression, thereby lowering gene cost; when gene cost is minimized, selective
414 pressure for LOF mutations is also minimized, thus promoting gene retention. Our
415 results suggest that the effects of cross-feeding on gene loss versus retention can be
416 more nuanced than is often assumed.
417
418 **MATERIALS AND METHODS**

419 **Bacterial strains.** *R. palustris* CGA676 is derived from CGA0092 (65) and carries a
420 *nifA** mutation that causes NH_4^+ excretion under N_2-fixing conditions (21, 28). *R.*
421 *palustris* mutants were made by homologous recombination using constructs in the
422 suicide vector pJQ200SK (66). All primers are in Table S6. pJQhoxJΔ5bp was made by
423 amplifying 1.4 kb from an evolved isolate from line A2 using primers JLM32 and JLM33,
424 digesting with BamHI and XhoI sites, and then ligating into pJQ200SK that had been

425 digested with the same enzymes. The ligation reaction was used to transform NEB10β
426 for plasmid verification by PCR and Sanger sequencing. pJQhoxJΔ5bp was then used
427 to transform *E. coli* S17-1 by electroporation. In each case, transformed *E. coli* was
428 recovered on LB agar with gentamycin (30 µg ml^{-1}). *E. coli* S17-1 with pJQ-ΔhoxJ and
429 pJQ-hupV were gifts from C. S. Harwood (67).
430
431 *R. palustris nifA* hoxJ*Δ5bp (CGA4065), *nifA** Δ*hoxJ* (CGA4050) and *nifA* hupV*+
432 (CGA4062) were then made by introducing pJQhoxJΔ5bp, pJQ-ΔhoxJ, or pJQ-hupV,
433 respectively, into CGA676 by conjugation with an *E. coli* S17-1 donor as described (67).
434 Single recombinants were recovered on photosynthetic medium (PM) agar (68, 69) with
435 10 mM succinate and 100 µg ml^{-1} gentamycin. Double recombinants were recovered by
436 counterselection on PM succinate plates with 10% sucrose, screened for gentamycin
437 sensitivity, and then verified by PCR amplification and/or Sanger sequencing.
438
439 *E. coli* PFM2 was a gift from P. Foster (29, 30). The Δ*purH* mutant was made via
440 lambda Red recombination (70) using constructs amplified from the Δ*purH*::km^R KEIO
441 mutant JW3970 (71) using primers YCC29 and YCC30. FLP-mediated excision was
442 used to remove the km resistance cassette (70). All mutants were verified by PCR
443 amplification and Sanger sequencing.
444
445 **Growth conditions.** Colonies were recovered from 25% glycerol -80 °C frozen stocks
446 by streaking to lysogeny agar (*E. coli*) or PM agar with 10 mM succinate. Single
447 colonies were then used to inoculate starter cultures. Anoxic media in test tubes were
448 prepared by bubbling N_2 through 10 ml of media in 27-ml anaerobic test tubes, then
449 sealing with rubber stoppers and aluminum crimps prior to autoclaving. Monocultures
450 and cocultures were grown horizontally at 30°C with light from a 45 W halogen bulb
451 (430 lumens) and shaking at 150 rpm in minimal M9-derived coculture medium (MDC)
452 (21) with either (i) *E. coli* monocultures: 10 mM glucose, 10 mM NH_4Cl, and cation
453 solution (100X stock: 100 mM $MgSO_4$ and 10 mM $CaCl_2$); *R. palustris* monocultures: 20
454 mM sodium acetate and 10 mM NH_4Cl; or cocultures: 25 mM glucose and cation
455 solution. Cultures with plasmid-carrying strains were also supplemented with 100 µg/ml
456 gentamycin or 25 µg/ml chloramphenicol as appropriate. Starter cultures were
457 inoculated with single colonies. *R. palustris* starter cultures were grown in MDC with 20
458 mM acetate and 10 mM NH_4Cl. *E. coli* starter cultures were grown aerobically in
459 lysogeny broth, with 30 µg/ml kanamycin (km) when appropriate. *E. coli* starter cultures
460 were washed twice in 1 ml MDC prior to inoculating test cultures or bioassays.
461 Cocultures were inoculated with 0.1 ml each of *R. palustris* and *E. coli* to an initial
462 optical density (OD_{660}) of ~0.003 each.
463
464 Cultures in 96-well plates were treated similarly except that oxic solutions were used to
465 prepare 0.2 ml volumes in each well. Anoxic conditions were achieved by sealing plates
466 inside a BD GasPak EZ large incubation container with anaerobic sachets.
467
468 **Experimental evolution.** Founder monocultures of *E. coli* PFM2 and *R. palustris* NifA*
469 CGA676 were each grown from a single colony in anoxic MDC with either 10 mM
470 glucose, cation solution, and 3 mM NH_4Cl for PFM2 or 20 mM sodium acetate for

471 CGA676. A single founder monoculture was then used to inoculate 10 monocultures
472 and 10 cocultures. All cultures were grown horizontally without shaking at 30°C with
473 light in MDC. PFM2 monocultures were supplemented with 10 mM glucose, cation
474 solution, 25 mM NaCl, and 2.3 mM NH_4Cl. These cultures were started later after
475 finding that 25 mM glucose led to periodic extinction due to prolonged exposure to
476 acidic fermentation products. As a result, *E. coli* monocultures were transferred less
477 times than for other conditions. No data from monocultures with 25 mM glucose were
478 presented herein. CGA676 monocultures were supplemented with 25 mM glucose,
479 cation solution, 8 mM disodium succinate, 7.3 mM sodium acetate, 0.25 mM sodium
480 formate, 1.4 mM sodium lactate, and 6.3 mM ethanol. Cocultures were supplemented
481 with 25 mM glucose, cation solution, and 25 mM NaCl. Acidification was not a problem
482 despite using 25 mM glucose because *R. palustris* consumes enough organic acids to
483 dampen the pH drop (21). Every 7 days, cultures were vortexed and 0.25 ml was
484 transferred to fresh medium. About every 5 transfers, stocks were diluted 1:1 with 50%
485 glycerol and stored at -80ºC. Separately, cell pellets from 1 ml samples were frozen for
486 gDNA extraction.
487
488 **Analytical procedures.** Cell densities were measured via turbidity (OD_{660}) using a
489 Genesys 20 visible spectrophotometer (Thermo-Fisher). H_2 was measured using a
490 Shimadzu GC-2014 gas chromatograph with a thermal conductivity detector as
491 described by sampling 0.1 ml of headspace using a gas-tight syringe (72). Statistical
492 analyses were performed using Graphpad Prism (v10).
493
494 **Competition assays.** Competition assays were conducted in an invasion-from-rare
495 format to consider coexistence by the mutual invasion criterion, where each population
496 can increase when rare (44, 46). Cocultures were started from various initial
497 frequencies (targeting 0.01 – 0.99) of each *E. coli* strain (WT vs Δ*purH* or WT vs
498 Δ*purH*::km^R) for a total initial cell density of ~10^6 colony forming units (CFU) / ml. *R.*
499 *palustris* CGA676 was inoculated to an initial density of ~10^6 CFU / ml. Frequencies
500 were determined upon inoculation and after 5 days. WT and Δ*purH* mutants were
501 distinguished by counting CFUs on M9 agar with cations and 25 mM glucose and on LB
502 agar and then determining Δ*purH* populations from the difference. When Δ*purH*::km^R
503 mutants were used LB agar included km to allow for direct determination of population
504 size. Change in frequency = (*E. coli* Δ*purH* / (*E. coli* WT + *E. coli* Δ*purH*))$_{final}$ – (*E. coli*
505 Δ*purH* / (*E. coli* WT + *E. coli* Δ*purH*))$_{initial}$ (45).
506
507 **Genome sequencing and mutation analysis.** gDNA was purified from cells using a
508 Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions. Lysis
509 was facilitated after resuspension by adding proteinase K (50 µg/ml final), and
510 incubating at 56°C for 10 min. RNaseA (4 µl, Promega) was then added and the lysate
511 was incubated for 2 min before proceeding.
512
513 DNA fragment libraries were made using a NextFlex Bioo Rapid DNA kit and libraries
514 were sequenced using Illumina NextSeq 500 150x150 paired-end runs by the IU Center
515 for Genomics and Bioinformatics. Paired-end reads were pre-processed for quality with
516 cutadapt 3.4 (73) with the following options: -a AGATCGGAAGAGC -A

517 AGATCGGAAGAGC ; -q 15,10; -u 6. Mutations were called using breseq v. 0.32.0 on
518 polymorphism mode (74). *E. coli* monoculture population sequences were mapped to
519 the MG1655 genome (accession NC_000913); *R. palustris* monoculture population
520 sequences were mapped to a concatenated reference genome consisting of the
521 CGA009 chromosome (accession BX571963), and its plasmid pRPA (accession
522 BX571964). Co-culture sequences were mapped to a concatenation of the *E. coli* and
523 *R. palustris* reference genomes. Polymorphisms that co-occurred in both the
524 monoculture and co-culture datasets were filtered and maintained as a subset to enrich
525 for the most informative variants representing treatment differences (Table S1-4). This
526 filtering step also removed sequence differences between the reference sequences and
527 those of the experimental strains used. Variants were prioritized as mutations of interest
528 if they were detectable at the final two sequencing timepoints and co-occurred across
529 multiple populations in the same locus. All mutations can be found in Tables S1-4.
530 Locus tag conversions can be found in Table S5.
531
532 **Reverse transcription quantitative real-time PCR (RT-qPCR).** Cultures received 100
533 μM adenine or ammonium ferric [iron(III)] citrate as indicated. Cultures were harvested
534 in exponential phase at 0.6-0.8 OD_{660} except for *E. coli* monocultures (+/- adenine
535 experiment), which were harvested at 0.3-0.4 OD_{660} to avoid adenine depletion.
536 Cultures were chilled on ice and pelleted by centrifugation. Lysis, RNA purification, and
537 cDNA generation were performed exactly as described (26). Standard curves were
538 generated using gDNA. Transcripts were quantified as described (26) using the
539 appropriate primers (Table S6) with a Mastercycler ep *realplex* real-time PCR system
540 (Eppendorf). Data was analyzed by *realplex* software using Noiseband. Specificities
541 were validated by melting curves and by the presence of a single band on an agarose
542 gel.
543
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545
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555
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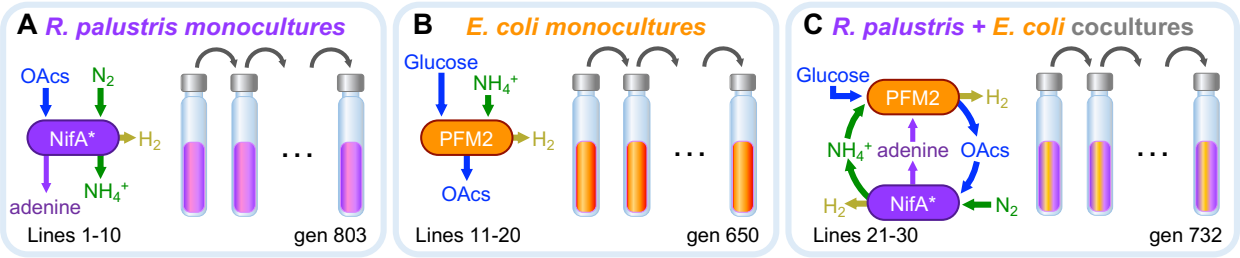
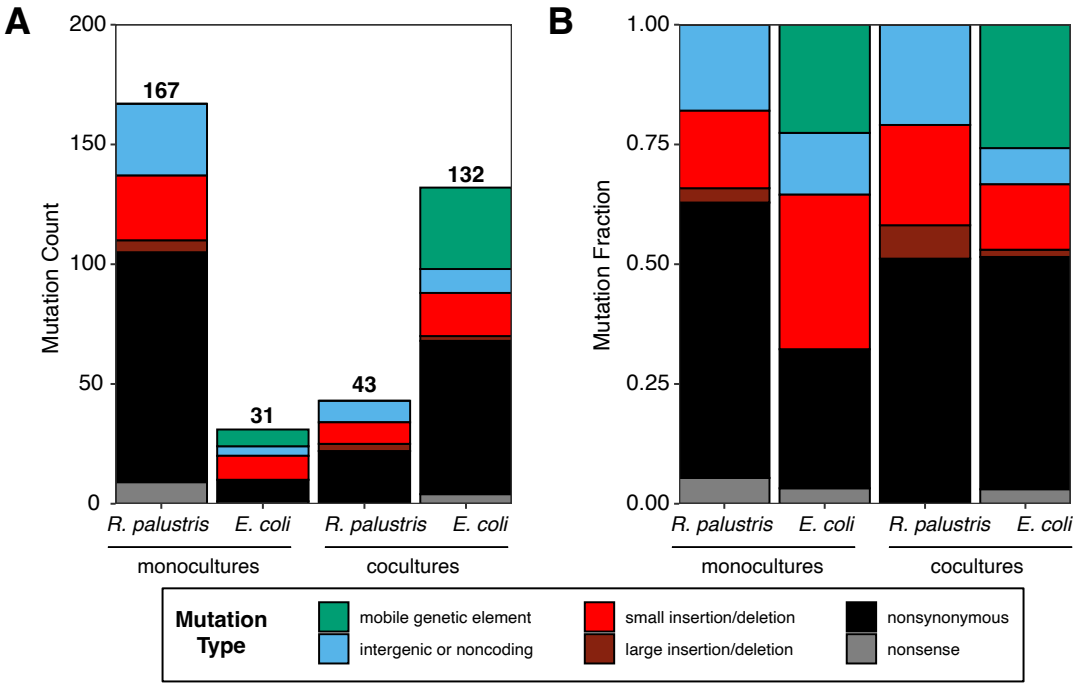
740

741 **Figures and legends**
742



743
744
745 **Fig 1.** Long-term anaerobic monoculture and coculture conditions. All cultures were laid
746 flat without shaking and were constantly illuminated. **(A)** Phototrophic *R. palustris* NifA*
747 (CGA676) was fed a mixture of organic acids (OAcs), ethanol, and $NaHCO_3$ to mimic
748 carbon sources available from *E. coli* fermentation products. The NifA* mutation causes
749 NH_4^+ excretion during N_2 fixation. H_2 is also produced as a co-product of N_2 fixation. *R.*
750 *palustris* also excretes adenine independent of the NifA* mutation. **(B)** *E. coli* PFM2 was
751 fed 10 mM NH_4Cl and 10 mM glucose, which it fermented to a mixture of OAcs, ethanol,
752 CO_2, and H_2. **(C)** Cocultures were fed 25 mM glucose. Growth of each species is
753 dependent on the exchange of NH_4^+ and OAcs.
754



755
756 **Fig 2. Differentially enriched mutations in long-term monocultures and**
757 **cocultures.** Mutation counts and fractions are the sum of mutations across all 10 lines
758 in each condition. To be counted, mutations had to meet the following criteria: (i)
759 exclude synonymous mutations, (ii) no exact mutation in the opposing monoculture or
760 coculture condition, (iii) observed in the last two sequenced timepoints, (*Rp*
761 monoculture, generations 781 and 803; *Ec* monoculture, generations 500 and 650;
762 coculture, generations 655 and 732), (iv) with a frequency >0.1 in the last timepoint.

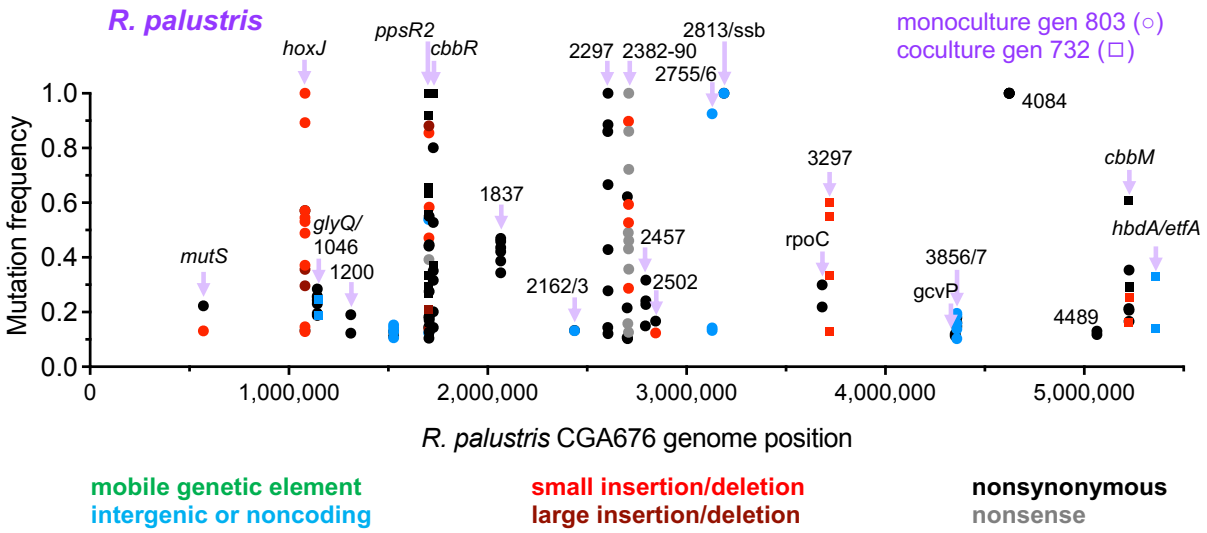


Fig 3. Mutations enriched in *R. palustris* NifA* in long-term monocultures and cocultures. Each point represents a mutation in a single lineage. Mutations are shown if they are not a synonymous mutation and were observed: (i) exclude synonymous mutations, (ii) no exact mutation in the opposing monoculture or coculture condition, (iii) observed in the last two sequenced timepoints, (*Rp* monoculture, gen 781 and 803; *Rp* coculture, gen 655 and 732), (iv) with a frequency >0.1 in the last timepoint, and (v) the same gene or intergenic region was mutated in at least two lines for a given condition. Generation: gen.

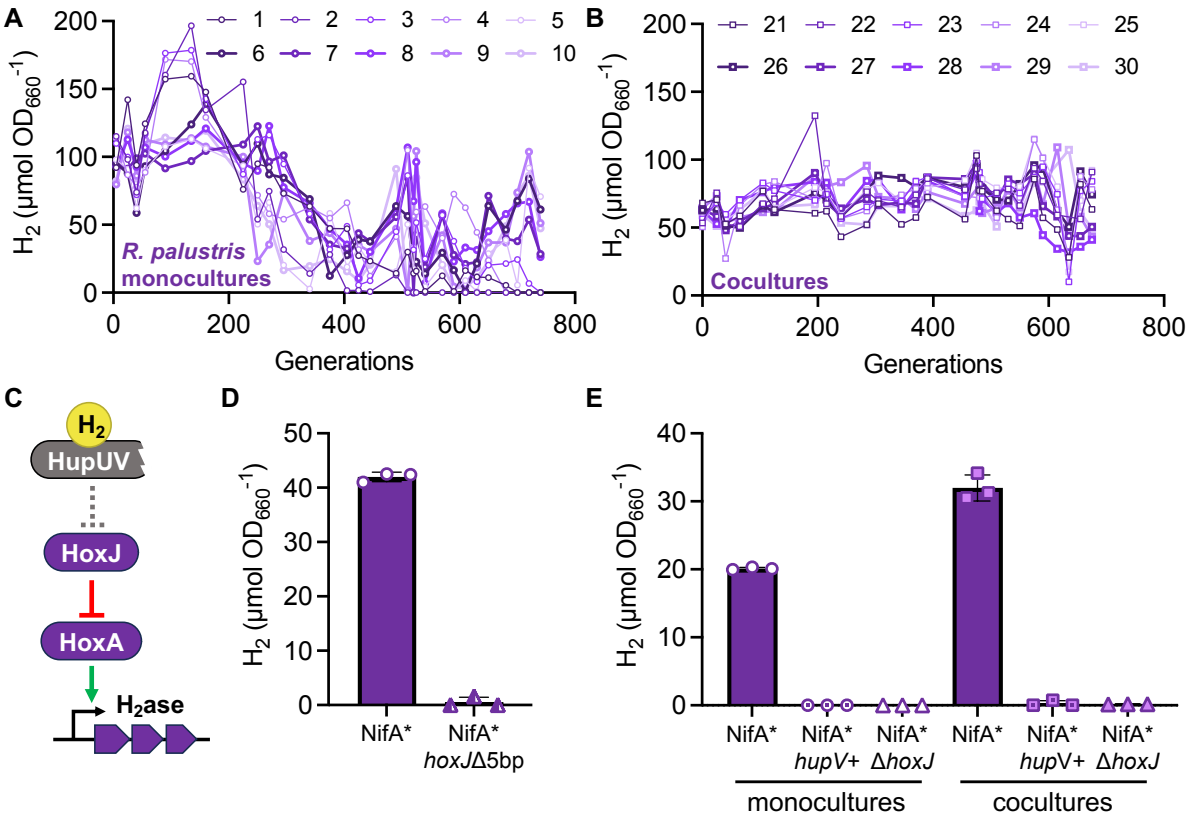


Fig 4. *R. palustris* monocultures evolve H_2 utilization. H_2 was quantified in 7-day-old cultures at selected time points for *R. palustris* monoculture lines 1-10 **(A)** and coculture lines 21-30 **(B)**. **(C)** *R. palustris* NifA* (CGA676) contains a *hupV* mutation that prevents the H_2 sensor HupUV from repressing HoxJ. HoxJ then constitutively represses the transcriptional activator of hydrogenase (H_2ase) genes, HoxA. **(D)** Stationary phase H_2 in the NifA* ancestor without and with a 5-bp *hoxJ* deletion commonly observed in evolved monocultures. Cultures were grown in the same MDC medium used for monoculture serial transfers plus 1 µM $NiSO_4$. **(E)** Stationary phase H_2 levels in the NifA* ancestor without or with *hupV* repaired (*hupV*+) or *hoxJ* deleted. Monocultures were grown in MDC with 20 mM acetate. Cocultures were grown in same media used for coculture serial transfers. **(D, E)** Bars, mean ± SD; n=3.

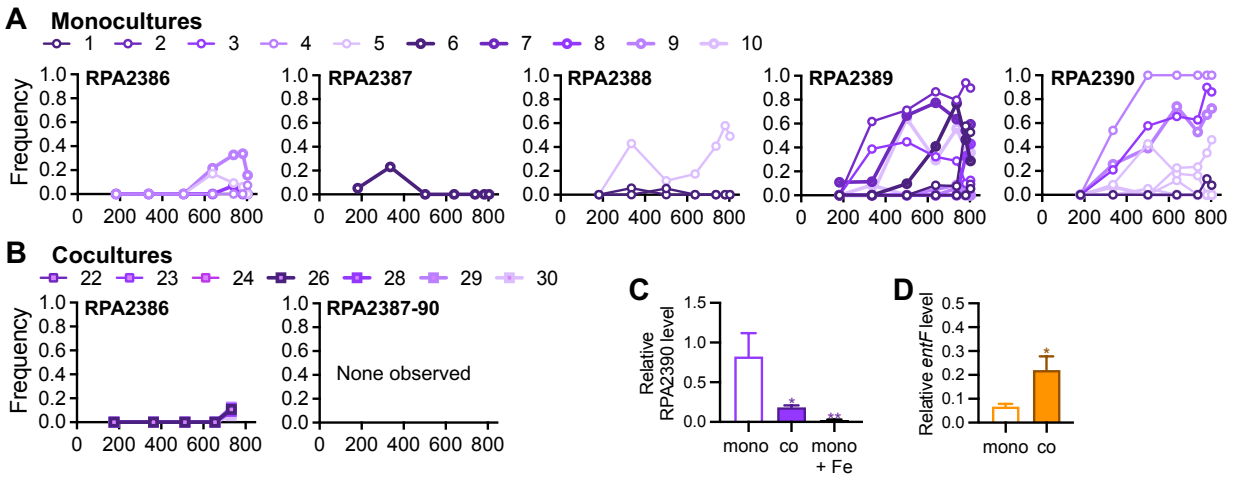


Fig 5. Siderophore gene loss is prevalent in *R. palustris* monocultures where gene expression is high. A, B. *R. palustris* siderophore gene (RPA2386-90) mutation frequencies in monoculture (**A**) and coculture lines (**B**). No mutations were observed in lines 21, 25, and 27. Repeated lines with the same symbols in a given graph indicate different mutations in the same gene. **C, D.** RT-qPCR quantification of siderophore synthesis genes in *R. palustris* (**C**; RPA2390 relative to *fixJ*) and in *E. coli* (**D**; *entF* relative to *hcaT*). Values are the mean ± SD, n = 4. Statistically significant differences from the monoculture (mono) condition for each strain were determined using an unpaired two-tail t-test; *, $p < 0.05$; **, $p < 0.01$; co, coculture; Fe, ammonium ferric citrate.

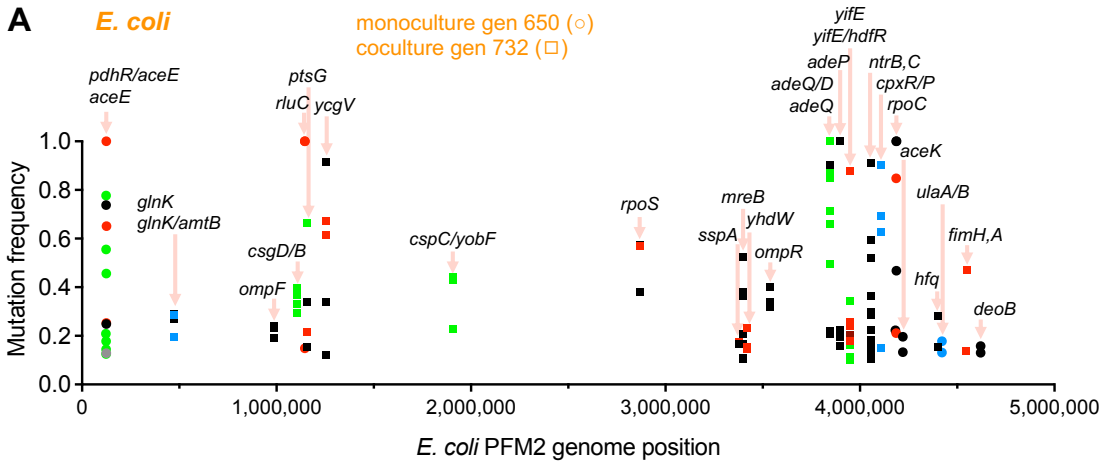


Fig 6. Mutations enriched in *E. coli* PFM2 in long-term monocultures and cocultures. Each point represents a mutation in a single lineage. Mutations are shown if they are not a synonymous mutation and were observed: (i) exclude synonymous mutations, (ii) no exact mutation in the opposing monoculture or coculture condition, (iii) observed in the last two sequenced timepoints, (*Ec* monoculture, gen 500 and 650; *Ec* coculture, gen 655 and 732), (iv) with a frequency >0.1 in the last timepoint, and (v) the same gene or intergenic region was mutated in at least two lines for a given condition. Generation: gen.

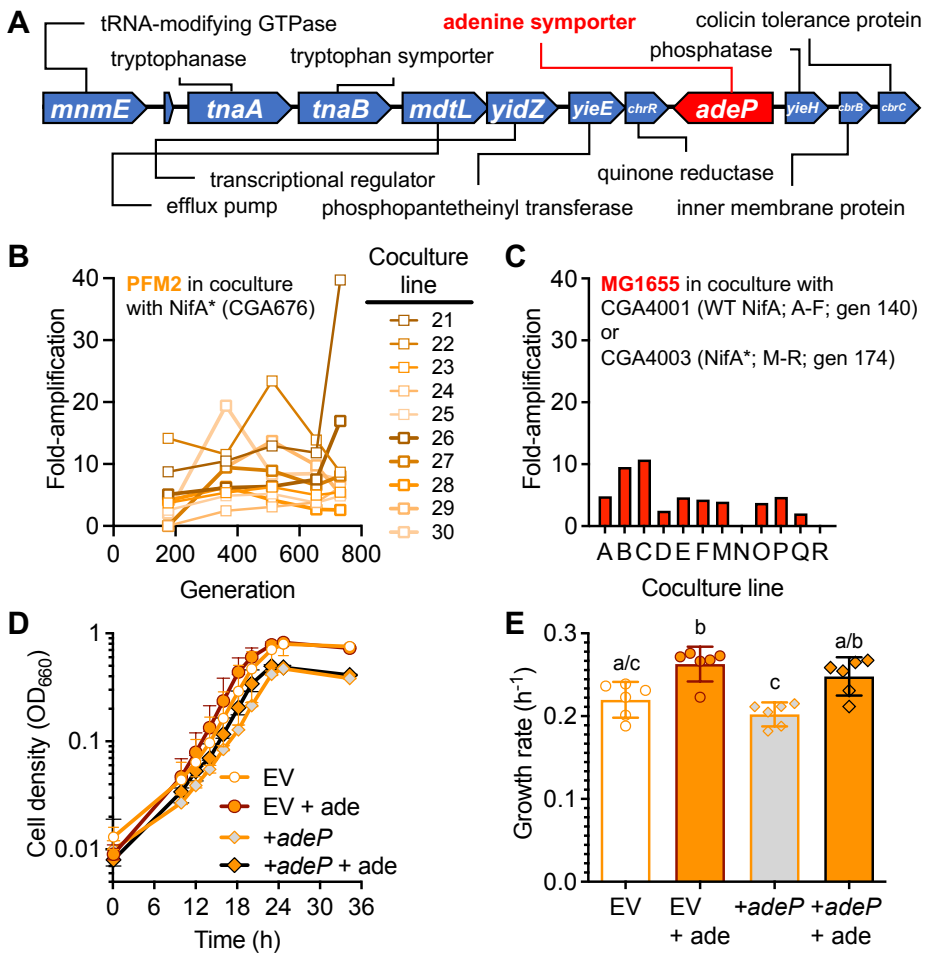


Fig 7. Amplification of *adeP* alone does not benefit PFM2 in the presence of adenine. A. Region of amplified *E. coli* genes observed in MG1655 and PFM2 cocultures but not in monocultures. The boundaries of the amplification differed between lineages but always contained these genes. **B, C.** Level of amplification observed in MG1655 in coculture (**B**) and in PFM2 in coculture (**C**). **D.** Effect of over-expressing *adeP* on PFM2 growth in monocultures with and without 35 μM adenine (ade). Over-expression was achieved by expressing *adeP* from the plasmid pCA24N with 30 μM IPTG. Data points are mean ± 95% C.I.; n=3. EV, empty vector. **E.** Growth rates from the same conditions as panel **D**; Bars, mean ± 95% C.I.; n=6, combining two separate experiments. Different letters indicate statistical differences (p <0.05) from a one-way ANOVA with Tukey's multiple comparisons test.

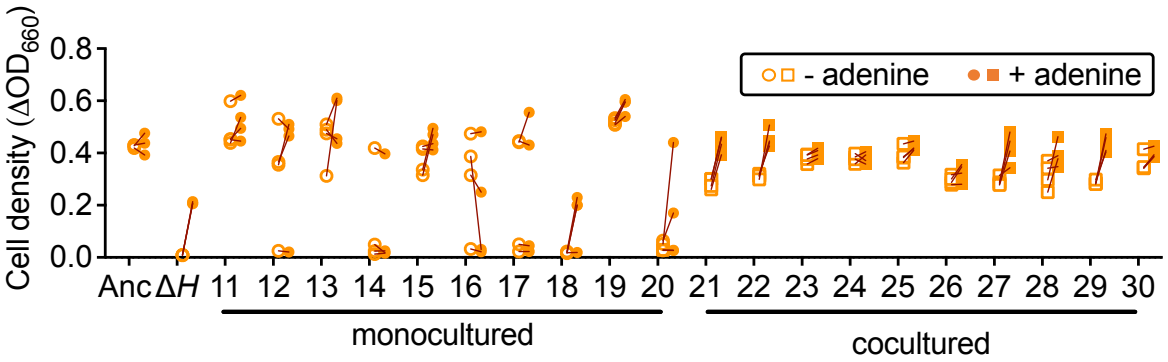


Fig 8. *E. coli* adenine auxotrophs are not prevalent in long-term cocultures. Randomly chosen evolved *E. coli* isolates (generation 650; n=4) were grown overnight in monoculture conditions with and without 50 μM adenine alongside the ancestral strain (Anc; PFM2) and a *ΔpurH* (*Δ*H) mutant (n=3). Each point represents a single measurement for a single isolate; replicate measurements were not made for any isolate. Each line connects measurements for the same isolate grown with and without adenine to facilitate comparisons.

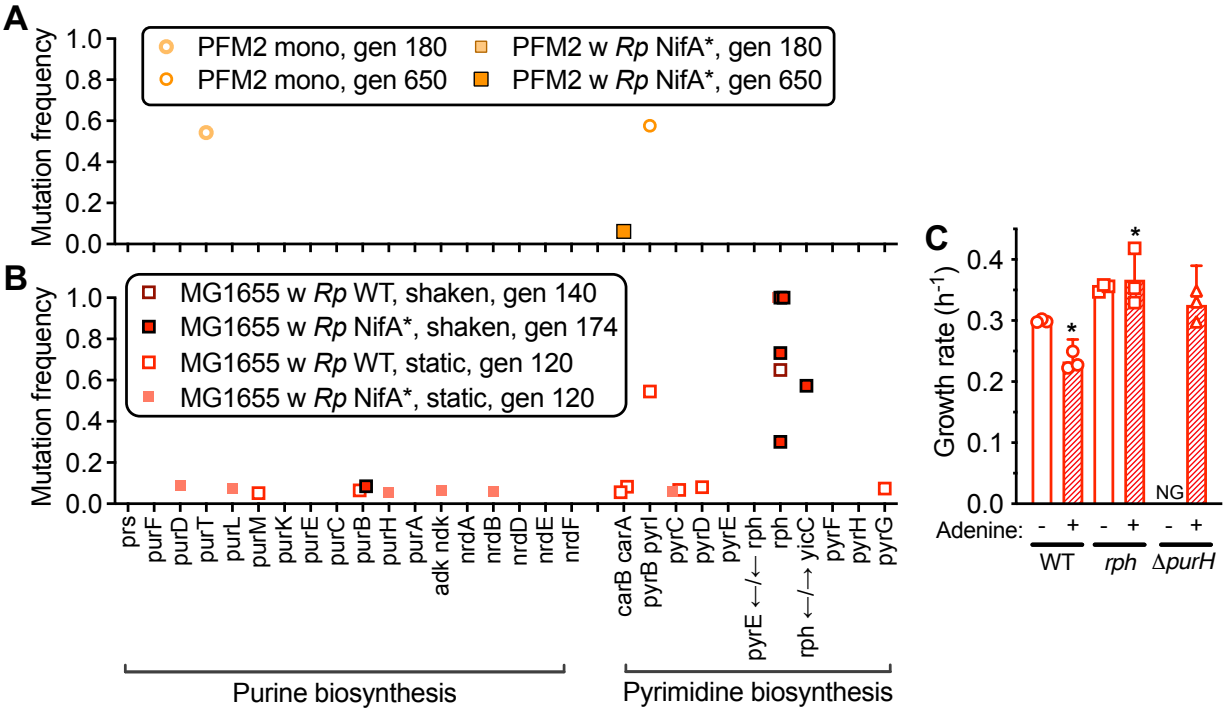


Fig 9. Mutations in genes for nucleotide synthesis are enriched in *E. coli* MG1655 but not PFM2. (A,B) Each point represents a mutation frequency in a given gene for a given line of *E. coli* PFM2 **(A)** or MG1655 **(B)**. **(C).** *E. coli* MG1655 adenine sensitivity can be relieved by repairing *rph* or deleting *purH*. Strains were grown in *E. coli* monoculture conditions without (-) or with (+) 35 μM adenine. *Statistical differences (p <0.05) compared to the wild-type (WT) conditions without adenine using a one-way ANOVA. NG, no growth.

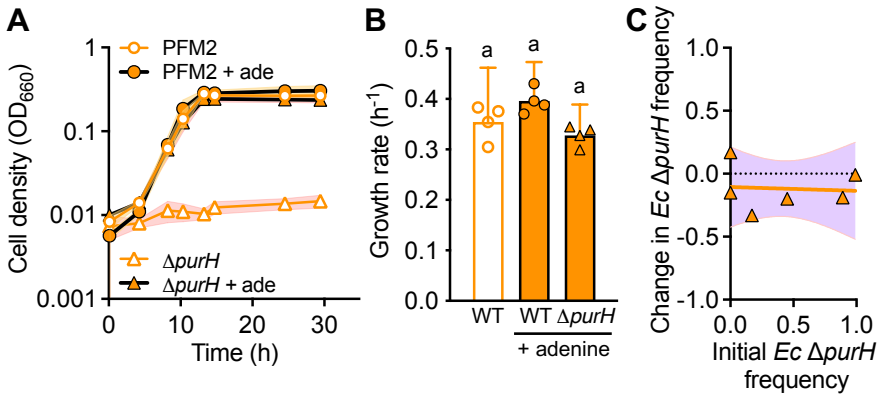


Fig 10. Adenine availability does not provide a fitness advantage to an engineered *E. coli* PFM2 purine auxotroph. A. Growth of WT *E. coli* PFM2 and its *ΔpurH* mutant in monoculture with and without 35 μM adenine (ade)**.** Points, mean ± SD as shading; n=3. **B.** Monoculture growth rates for WT PFM2 vs a corresponding *ΔpurH* mutant with and without 35 μM adenine. 'a,' indicates statistically similar values ($p > 0.01$) as determined using a one-way ANOVA with Tukey's multiple comparisons test. Bars, mean ± SD; n=3. **C.** Invasion-from-rare assay competing a *ΔpurH* mutant against its PFM2 parent in coculture with *R. palustris* CGA676 under N_2-fixing conditions where *R. palustris* excretes NH_4^+ and adenine. The orange line is the best fit from a linear regression analysis with 95% CI shaded in purple. Change in frequency = (*E. coli* Δ*purH* / (*E. coli* WT + *E. coli* Δ*purH*))$_{final}$ – (*E. coli* Δ*purH* / (*E. coli* WT + *E. coli* Δ*purH*))$_{initial}$. **B, C.** Each data point represents a measurement from a single biological replicate.

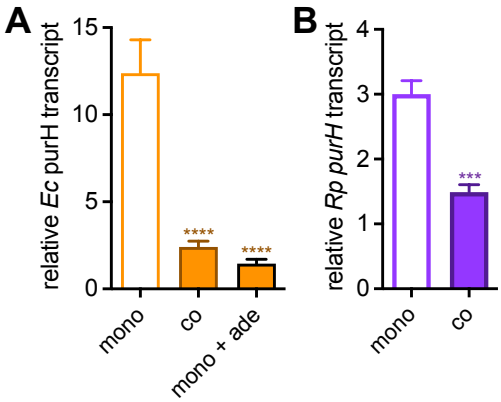


Fig 11. Purine biosynthesis genes are down-regulated in coculture. RT-qPCR quantification of *purH* transcript levels in *E. coli* PFM2 (**A**; relative to *hcaT*) and *R. palustris* CGA676 (**B**; relative to *fixJ*). 100 μM adenine (+ ade) was used to ensure that it was not used up before harvesting RNA. Bars, mean ± SD, n = 3-4. Statistically significant differences from the monoculture condition for each strain were determined using an unpaired two-tail t-test; ***, $p < 0.001$; ****, $p < 0.0001$.

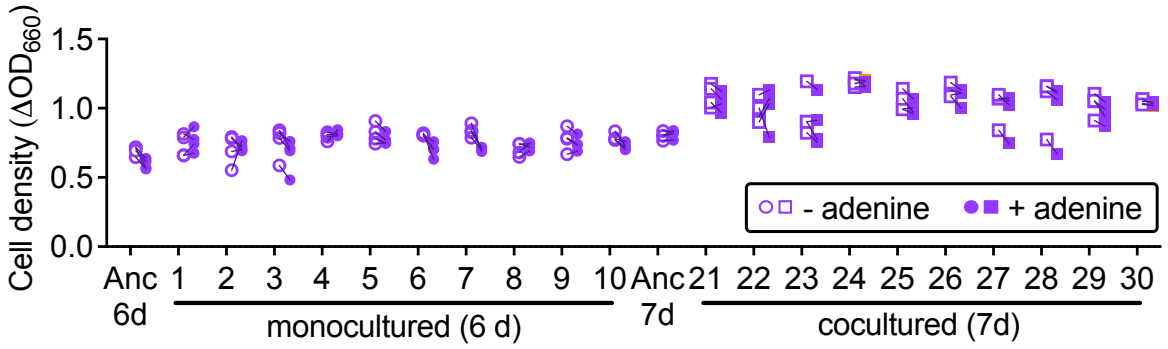


Fig 12. *R. palustris* auxotrophs are not prevalent in long-term cocultures. Randomly chosen evolved-*R. palustris* isolates (generation 650) were grown overnight in monoculture conditions with and without 50 μM adenine alongside the ancestral strain (Anc; CGA676) (n=4). Monoculture isolates were grown for 6 days and coculture isolates were grown for 7 days due to inclement weather, each with a corresponding ancestral control. Each point represents a single measurement for a single isolate; replicate measurements were not made for any isolate. Each line connects measurements for the same isolate grown with and without adenine to facilitate comparisons.