

# Synergistic coevolution accelerates genome evolution

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## Abstract

Ecological interactions are key drivers of evolutionary change. Although it is well-documented that antagonistic coevolution can accelerate molecular evolution, the evolutionary consequences of synergistic coevolution remain poorly understood. Here we show experimentally that also synergistic coevolution can speed up the rate of molecular evolution. Pairs of auxotrophic genotypes of the bacterium *Escherichia coli*, whose growth depended on a reciprocal exchange of amino acids, were experimentally coevolved, and compared to two control groups of independently growing cells. Coevolution drove the rapid emergence of a strong metabolic cooperation that correlated with a significantly increased number of mutations in coevolved auxotrophs as compared to monoculture controls. These results demonstrate that synergistic coevolution can cause rapid evolution that in the long run may drive diversification of mutualistically interacting species.

## One-Sentence Summary

Synergistic coevolution among obligate mutualists increases the rate of molecular evolution relative to independent types.

## 37 Main Text

38 Identifying the factors that drive molecular change in the genomes of organisms is  
39 one of the most central goals of evolutionary biology (1, 2). Past research suggests  
40 that biotic interactions are fundamental for determining the evolution of organisms in  
41 nature (3, 4). Individuals displaying phenotypes that are advantageous in the context  
42 of a certain ecological interaction will be favoured by natural selection, while others  
43 lacking the corresponding alleles will be selected against (5). In cases where  
44 selection pressures are reciprocal, ecological interactions even set off coevolutionary  
45 dynamics of adaptation and counter-adaptation that can lead to perpetually  
46 escalating arms-races among groups of interacting individuals (6). As a  
47 consequence, ecological interactions do not only strongly affect the Darwinian fitness  
48 of the organisms involved, but also drive the molecular evolution of their genomes.

49 This causal link is well-established for antagonistic interactions, such as between  
50 parasites and their hosts (7, 8) or predators and their prey (9). For both of these  
51 cases, the so-called Red Queen hypothesis predicts adaptations that increase the  
52 fitness of one of the two interacting partners should simultaneously decrease the  
53 fitness of its corresponding counterpart (10–13). From this process results a strong  
54 evolutionary pressure that forces both sides to constantly evolve new adaptive  
55 phenotypes in order to survive and reproduce. Both comparative (14, 15) and  
56 experimental studies (7, 16, 17) corroborate that antagonistic coevolution increases  
57 the rate of molecular evolution, particularly affecting specific loci that are driving the  
58 interaction (14, 15, 18).

59 In contrast, little is known on how mutualistic coevolution affects the genomes of  
60 both interacting partners. Even though it is clear that engaging in mutualistic  
61 interactions can drastically affect the fitness of the organisms involved (19), the  
62 question how mutualistic cooperation affects the speed of molecular evolution,  
63 remains unanswered.

64 Two possibilities are conceivable. First and analogous to antagonistic coevolution,  
65 also in synergistic interactions individuals may be faced with a constantly evolving  
66 partner and, thus, experience the need to continuously respond to the accruing  
67 changes. In this case, synergistic coevolution would speed up molecular evolution.  
68 Alternatively, interacting partners may converge to a point of evolutionary stasis (20,  
69 21), at which a unilateral change in one of the two parties is going to negatively affect  
70 the interaction as a whole. Under these conditions, stabilizing selection should  
71 maintain phenotypes of mutualists over extended periods of time, which, as a  
72 consequence, should decelerate the rate of molecular evolution.

73 Comparative studies analysing this issue by focusing on one side of a mutualistic  
74 association found evidence for an increased rate of molecular evolution in mutualistic  
75 species as compared to closely related non-mutualists existing outside the interaction  
76 (22–24). However, a direct empirical test of how synergistic coevolution affects the  
77 rate of molecular evolution in two interacting partners is lacking.

78 Here, we address this issue using experimental evolution of replicated populations  
79 of the bacterium *Escherichia coli*. Serially propagating two auxotrophic genotypes of  
80 this species (i.e.  $\Delta tyrA$  and  $\Delta trpB$ ) resulted in the rapid evolution of a costly metabolic

81 cooperation (25). Only 150 generations of coevolution transformed the initial  
82 consortia, in which both strains could only grow when they reciprocally exchanged  
83 essential amino acids, into a truly cooperative interaction, in which both parties  
84 started to actively invest costly resources to benefit their corresponding counterpart.  
85 This evolutionary transition was enabled by the formation of multicellular clusters  
86 among auxotrophic genotypes, which not only facilitated an exchange of amino acids  
87 between interaction partners (26, 27), but also immediately rewarded an increased  
88 cooperative investment of either party by positive fitness feedbacks (25). Importantly,  
89 these patterns were neither observed in auxotrophs that were cultivated individually  
90 in the presence of the required amino acid, nor in populations of metabolically  
91 autonomous (i.e. prototrophic) wild type cells, which were both propagated the same  
92 way as cocultured auxotrophs. This experimental design afforded the separation of  
93 effects resulting from the adaptation to the abiotic environment from those stemming  
94 from an adaptation to the cocultured partner.

95 After the evolution experiment, whole-population samples and individually isolated  
96 strains of all three experimental groups (i.e. (i) monocultures of prototrophic wild type  
97 (WT), (ii) monocultures of auxotrophic genotypes (M), and (iii) cocultures of  
98 auxotrophic genotypes (C)) were subjected to high-coverage, second-generation  
99 whole-genome re-sequencing to determine the number and identity of mutations that  
100 arose in the course of the evolution experiment. For this, derived genomes of all  
101 three treatment-groups were compared to the genomes of their corresponding  
102 evolutionary ancestor.

103 In this analysis, coevolved auxotrophs showed significantly increased numbers of  
104 mutations as compared to the two control groups (Fig. 1).

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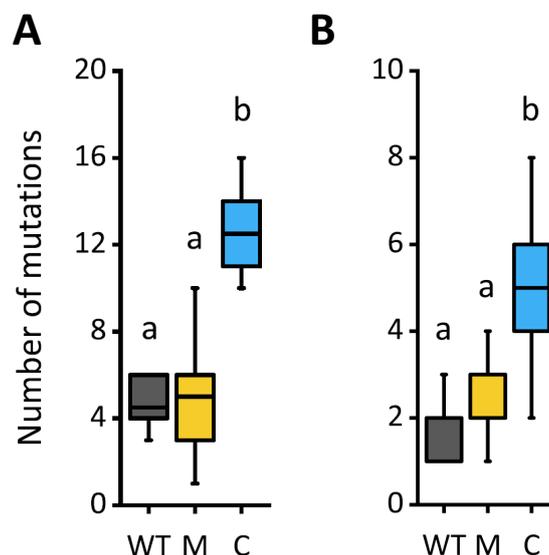
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117 **Fig. 1 Coevolved auxotrophs accumulated more mutations than control groups.** Absolute  
118 numbers of mutations of derived populations of wild type (WT), auxotrophic monocultures (M), and  
119 cocultured auxotrophs (C). Comparison of experimental groups was performed on the level of  
120 (A) whole population samples (Bonferroni posthoc test:  $P < 0.05$ ; WT:  $n=6$ , M:  $n=11$ , C:  $n=6$ ) and  
121 (B) samples of isolated clones (Bonferroni posthoc test:  $P < 0.05$ ; WT:  $n=10$ , M:  $n=20$ , C:  $n=31$ ).  
122 Different letters indicate significant differences between groups.

123 This was true on both the strain- and the population-level: median numbers ( $\pm$  95%  
124 confidence interval) of mutations detected in coculture samples were more than twice  
125 as high as the ones detected in both control groups (i.e. population-level: C:  $12.5 \pm$   
126  $1.6$ , WT:  $4.5 \pm 1.8$ , M:  $5.0 \pm 1.4$ ; strain-level: C:  $5.0 \pm 0.5$ , WT:  $2.0 \pm 0.5$ , M:  $2.0 \pm$   
127  $0.9$ ). This observation is consistent with the interpretation that synergistic coevolution  
128 in auxotrophic cocultures accelerated the rate of molecular evolution relative to  
129 control populations that were able to grow independently. Additionally, statistically  
130 analysing clonal samples showed that the obligate interaction had a significant effect  
131 on the accumulated number of mutations (univariate linear model:  $P < 0.05$ ,  
132 observed power: 0.85, WT:  $n=10$ , M:  $n=20$ , C:  $n=31$ ).

133 To analyse the degree of divergence among derived genomes, gene-level  
134 distance trees were constructed by comparing the spectrum of mutations detected  
135 within samples. These trees revealed on both the level of isolated strains (Fig. 2) and  
136 entire populations (Fig. S1) that coevolved auxotrophs showed much higher levels of  
137 genetic divergence than the two control groups. This pattern was further corroborated  
138 by comparing pairwise distances based on the branch lengths among groups of  
139 isolated strains, which were significantly increased in coculture-derived samples  
140 relative to the ones of control groups (Dunnett T3 posthoc test:  $P < 0.05$ ). Strikingly,  
141 auxotrophic cocultures formed independent branches in the tree, which were clearly  
142 distinct from the ones of control groups (Figs. 2 and S1). Depending on their

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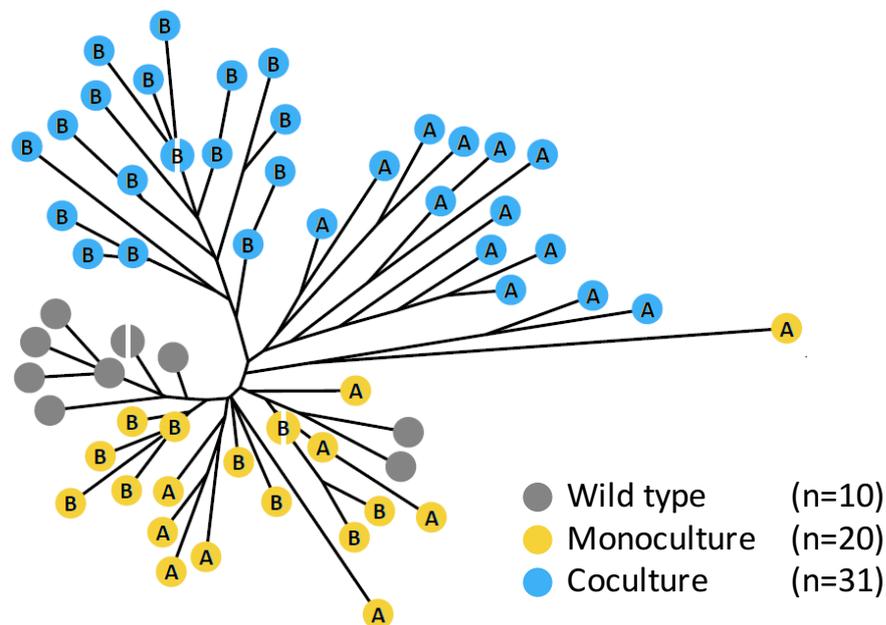
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159 **Fig. 2 Coevolved auxotrophs showed an increased partner-specific divergence relative to**  
160 **control groups.** Distance tree of isolated strains (A = tyrosine auxotroph, B = tryptophan auxotroph)  
161 that is based on the neighbour-joining analysis of a gene-level genotypic matrix (i.e. genes carrying a  
162 mutation were treated as alleles different to the wild type background). Split nodes indicate two  
163 samples mapped to the same position.

164 auxotrophy-causing mutation, the two different cocultured genotypes (i.e.  $\Delta tyrA$  and  
165  $\Delta trpB$ ) even segregated into completely disparate branches, which suggests  
166 specialisation in the context of the mutually beneficial interaction. In contrast, this  
167 pattern was not observed in control groups. Here, derived populations of the  
168 prototrophic wild type and monocultures of the two auxotrophs formed intermixed  
169 branches without a clear genotypic separation. These findings strongly suggest (i)  
170 general differences in the spectrum of mutations between auxotrophic cocultures and  
171 control groups, (ii) highly divergent evolutionary trajectories between the two  
172 cooperating partners within a mutualistic consortium, and (iii) increased levels of  
173 parallel evolution in samples from cocultures relative to control groups.

174 To further investigate whether the three treatment groups indeed differed in their  
175 degree of parallel evolution, the Jaccard index was calculated on the level of mutated  
176 genes for clonal samples for all possible comparisons within and between  
177 experimental groups (Fig. 3). Because growth of the two cocultured auxotrophic  
178 genotypes was limited by the availability of the required amino acid, the main route to  
179 increase in fitness was a mutation that enhanced growth of the other auxotrophic  
180 genotypes that were part of the same cluster. In this way, cooperative mutants  
181 automatically received more of the required amino acid in return (i.e. a positive  
182 feedback loop (25)). Hence, due to strong selection pressures that likely acted on  
183 cocultures of auxotrophs, analysed genomes should display a high degree of parallel  
184 evolution between replicate populations. In contrast, monocultures of auxotrophs and  
185 prototrophic wild type cells grew autonomously. Thus, the fitness effect of any  
186 mutation that improved a strain's growth and survival in the current abiotic  
187 environment had to be strong enough in order to persist against and potentially  
188 outcompete other genotypes. Given the comparably short duration of the selection  
189 experiment (i.e.  $\sim 153$  generations), a much lower degree of parallel evolution was  
190 expected for these samples. Testing this hypothesis indeed revealed that coevolved  
191 auxotrophs showed a significantly increased degree of parallel evolution as  
192 compared to the two control groups (Fig. 3). This pattern emerged on a population-  
193 level (Fig. 3A) as well as when genotypes of a similar auxotrophy were compared  
194 between monoculture and coculture conditions (Fig. 3B). The generally increased  
195 degree of parallel evolution in cocultured auxotrophs indicates that these populations  
196 were likely subject to increased selection pressures.

197 In contrast, the degree of parallel evolution between groups of samples was  
198 generally low (Fig. 3) with very few mutational targets being shared between groups  
199 (Fig. S2). Of all 117 mutated genes identified on a population-level, only one gene  
200 was found to be mutated in all three experimental groups (Fig. S2A). Also, comparing  
201 the mutational spectra of auxotrophs evolved under monoculture and coculture  
202 conditions revealed that very few mutated genes were shared between groups  
203 ( $\Delta tyrA$ : 3%,  $\Delta trpB$ : 0%, Fig. S2B). Moreover, analysing the mutational data confirmed  
204 the previously observed high degree of divergence between cocultured auxotrophs  
205 (Fig. 2): of all 74 mutated genes identified in coevolved genotypes, only a single gene  
206 was found to be mutated in both of these two groups, indicating that their  
207 evolutionary trajectories were completely different. Based on these data, we  
208 conclude that strong selection resulting from a synergistic coevolution is indeed

209 causal for both the observed divergence in evolutionary trajectories as well as the  
210 accelerated accumulation of mutations.

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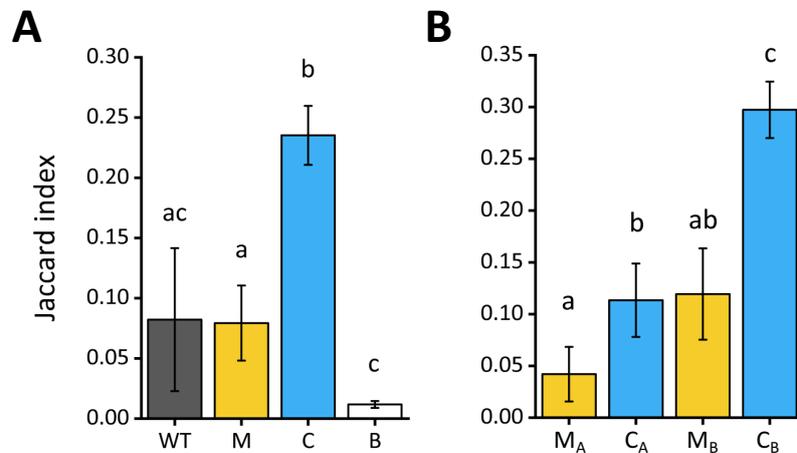
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222 **Fig. 3 Coevolved auxotrophs show an increased degree of parallel evolution relative to control**  
223 **groups.** Degree of parallel evolution within and between experimental groups based on mutated  
224 genes in sequenced isolates is given as mean Jaccard indices ( $\pm$  95% confidence interval).  
225 (A) Comparison between populations of wild type (WT, n=45), monocultures of auxotrophs (M,  
226 n=110), cocultures of auxotrophs (C, n=231), and all experimental groups (B, n=1567). Different  
227 letters indicate significant differences between group (Dunnnett T3 posthoc test:  $P < 0.05$ ).  
228 (B) Comparison between isolates of monocultures (M<sub>A</sub>, n=11; M<sub>B</sub>, n=11) and cocultures (C<sub>A</sub>: n=  
229 13, C<sub>B</sub>: n=18) on the level of the two individual auxotrophies (A: tyrosine auxotrophy ( $\Delta$ *tyrA*); B:  
230 tryptophan auxotrophy ( $\Delta$ *trpB*)). Different letters indicate significant differences between groups  
231 (Dunnnett T3 posthoc test:  $P < 0.01$ ).

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233 Parallel evolution among replicate lineages is a strong signature of selection  
234 favouring the corresponding mutations (28). Particularly high levels of parallel  
235 evolution were for instance observed in tryptophan-auxotrophic isolates from  
236 cocultures: *ompF*, which encodes the outer membrane porin F (OmpF), was found to  
237 be mutated in all ten replicate populations. The detected mutations replaced amino  
238 acids with longer side chains (i.e. tyrosine, glutamic acid, aspartic acid), which face  
239 into the pore-channel, with shorter ones (i.e. alanine, glycine, serine, and cysteine)  
240 (see respective SNPs in Supplemental Table 1), thus potentially increasing  
241 permeability for exchanged amino acids (29, 30). Moreover, 82% of all analysed  
242 samples (14 out of 17) carrying mutations in *ompF* were also found to have a  
243 mutation in *lrp*. The majority of these mutations caused a frameshift (i.e. ~57%), thus  
244 rendering the leucine-responsive regulatory protein (Lrp) non-functional. Importantly,  
245 knockouts of the global regulator Lrp exhibit a selective advantage during stationary  
246 phase, which resembles the amino acid starvation experienced by cocultured  
247 auxotrophs in our selection experiment. Interestingly, a side-effect of deleting *lrp* is  
248 also a reduced ability to transport aromatic amino acids such as tyrosine and  
249 tryptophan (31), which could explain the strong selection for increased OmpF-  
250 permeability in these mutants.

251 In the case of the cocultured tyrosine auxotrophs, the spectrum of detected  
252 mutations was more diverse and thus less similar between strains (Fig. 3B).  
253 Nevertheless, also here, striking examples for parallel evolution were observed: In  
254 five out of ten populations, a IS5-mediated 10-11 kB deletion was detected within the  
255 exact same region comprising 12 genes, amongst them *sspA*. The benefit of lacking  
256 the functional stringent starvation protein A (SspA) is particularly interesting, given  
257 that a frameshift mutation within *sspA* was additionally detected in another  
258 population. In contrast to genotypes with non-functional Lrp, strains lacking SspA  
259 exhibit reduced viability during prolonged stationary phase (32) by for instance  
260 increased sensitivity to acidification (33). This detrimental effect needs to be  
261 compensated by a yet unknown mechanism. One advantage originating from this  
262 mutation is hypermotility due to released H-NS expression (33, 34), which potentially  
263 increases the likelihood to encounter compatible partners for cross-feeding, when not  
264 being part of a multicellular cluster. This effect was likely particularly important, when  
265 cocultures experienced low cell densities after being transferred into fresh culture  
266 medium during the evolution experiment.

267 Our results show that mutualistic coevolution increases the rate of molecular  
268 change in genomes of synergistically interacting organisms. However, an alternative  
269 explanation to account for the observed pattern could simply be demographic  
270 differences among experimental groups. Indeed, at the beginning of the evolution  
271 experiment, auxotrophic cocultures grew much slower and to a much lower density  
272 than populations of the two control treatments (25). However, until the end of the  
273 evolution experiment, cocultured auxotrophs managed to reduce their generation  
274 time as well as to increase their growth rate and the final population density they  
275 achieved at the end of a growth cycle. Eventually, coevolved auxotrophs even  
276 overhauled auxotrophic monocultures regarding these fitness parameters to finally  
277 reach levels that were statistically indistinguishable from the ones of monocultures of  
278 prototrophic controls (25). Nevertheless, genetic drift in populations of a small size  
279 could have led to a genome-wide accumulation of non-adaptive genomic changes,  
280 ultimately giving rise to an increased rate of molecular evolution. However, several  
281 observations rule out this possibility. First, genotypes of cocultured auxotrophs  
282 formed specific branches in the distance tree that were clearly distinct from the ones  
283 of control groups (Fig. 2). Second, genomes of coevolved auxotrophs showed the  
284 highest level of parallel evolution in comparison to the other experimental groups  
285 (Fig. 3A). Third, the vast majority of mutations detected in coevolved auxotrophs  
286 were either non-synonymous (clones: 35,8%, populations: 31,2%), non-sense  
287 (clones: 9,9%, populations: 10,4%), or insertions as well as deletions (clones: 38,9%,  
288 populations: 42,9%) (Supplemental Table 1), suggesting these are adaptive changes.  
289 None of these observations would be expected if only demographic differences  
290 caused the increased rate of molecular evolution.

291 A feature that was critical for the evolution of mutualistic cooperation in our  
292 experiment was the formation of multicellular clusters that consisted of both types of  
293 auxotrophs (25). These aggregates most likely enhanced the exchange of amino  
294 acids between cells in the otherwise shaken liquid environment (26, 27). Under these  
295 conditions, the fitness of a given bacterial cell within a cluster is limited by the supply

296 of adaptive mutations that, for example, enhance production levels of the exchanged  
297 amino acid. The emergence of such mutations has been shown to cause a positive  
298 fitness feedback, in which an increased cooperative investment by one cell is  
299 immediately rewarded: enhancing the growth of the corresponding other partner  
300 automatically results in an increased supply of the required amino acid in return.  
301 Multicellular clusters, in which these mutations arise, will increase in frequency and  
302 therefore be transferred to the next growth cycle with a higher probability than  
303 clusters lacking these mutations. Thus, competition between clusters that differ in  
304 their mutational makeup, can likely explain the enhanced rate of evolution observed  
305 in coevolved auxotrophs. This powerful mechanism can therefore account for the  
306 increased rate of molecular evolution of mutualistically interacting individuals  
307 observed in our study. Interestingly, this situation is strikingly similar to the one  
308 mutualistic interactions face in nature. Also here, mutations that enhance growth and  
309 reproduction of the whole mutualistic consortia will likely be favoured over consortia  
310 lacking these mutations.

311 Our work identifies coevolution between two mutualistically interacting genotypes  
312 as a powerful mechanism to drive molecular change. Strong synergistic benefits  
313 resulting from the ecological interaction among individuals set off an evolutionary  
314 dynamic, in which strains have to continuously evolve novel adaptive mutations in  
315 order to persist against other competing groups. Reciprocal selection pressures  
316 emerging from coevolutionary interactions may therefore be causally involved in  
317 generating the bewildering diversity in form and function one can observe in  
318 mutualistically interacting species.

319

## 320 **Materials and Methods**

### 321 *Bacterial strains*

322 We used *Escherichia coli* BW25113 (35) as the wild type (WT), which was genetically  
323 modified by P1 phage transduction (36). Derived auxotrophic genotypes contained  
324 an in-frame replacement of the targeted amino acid biosynthesis gene (i.e. *trpB* or  
325 *tyrA*) with a kanamycin cassette. To allow discrimination of different genotypes on  
326 agar plates, the phenotypic marker genes *araDAB* (derived from *E. coli* REL607 (37))  
327 and *lacZ* (derived from *E. coli* MG1655 (38)) were additionally introduced into WT  
328 and auxotrophic strains by P1 transduction. As a result, one set of strains carried the  
329 functional alleles for arabinose utilization and  $\beta$ -galactosidase, which appears blue on  
330 TA-Agar (39) supplemented with 0.1 mM IPTG (Isopropyl  $\beta$ -D-1-  
331 thiogalactopyranoside) and 50  $\mu\text{g ml}^{-1}$  X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-  
332 galactopyranoside), while the other set of WT phenotypes appears red.

333

### 334 *Culture conditions*

335 In all experiments, minimal medium for *Azospirillum brasilense* (MMAB) (40) with  
336 0.5 % glucose instead of malate and without biotin was used as culture medium.  
337 Cultures were incubated under shaking conditions at 30 °C and 225 rpm. Only  
338 monocultures of auxotrophic genotypes were supplemented with tryptophan or

339 tyrosine (150  $\mu$ M for precultures and 50  $\mu$ M for the evolution experiment). To start an  
340 experiment, bacterial strains were freshly streaked from cryo-stocks on LB agar  
341 plates and incubated for 18-24 h at 30 °C. Individual colonies were used as biological  
342 replicates to inoculate each 1 ml MMAB of overnight preculture, which were set to an  
343 optical density (OD<sub>600nm</sub>) of 2 the next day. Respective aliquots of these cultures  
344 were subsequently used to inoculate 4 ml MMAB medium with an initial OD<sub>600nm</sub> of  
345 0.005. In the case of auxotrophic cocultures, each genotype was inoculated with an  
346 initial OD<sub>600nm</sub> of 0.0025.

347

### 348 *Evolution experiment*

349 Complementary auxotrophic strains (*E. coli*  $\Delta$ *trpB::kan araDAB lacZ*, and *E. coli*  
350  $\Delta$ *tyrA::kan ara-  $\Delta$ lacZ* or the reverse combination of phenotypic labelling) were  
351 combined in cocultures to generate a synthetically designed obligate byproduct  
352 interaction. To determine effects of genotypic background (prototrophy or  
353 auxotrophy, and presence or absence of phenotypic marker genes) on the  
354 accumulation of mutations, two control groups contained monocultures of utilized  
355 genotypes. Six biological replicates of each generated genotype were used to start  
356 the evolution experiment, adding up to twelve monocultures of WT, twelve cocultures  
357 of auxotrophs, and 24 monocultures of auxotrophic genotypes (i.e. 12 of each type).  
358 Populations were initially transferred every seven days for a total of five transfers,  
359 which was followed by 15 transfers every three days, adding up to a total of 80 days  
360 or approximately 153 bacterial generations. At the end of each cycle, optical  
361 densities were determined in 200  $\mu$ l culture in microtiter plates by spectrophotometry  
362 in a plate reader (Spectramax M5, Applied Biosystems, United States) and 20  $\mu$ l of  
363 culture were transferred into 4 ml of fresh MMAB-medium. Depending on the cycle-  
364 length, glycerol stocks (20% glycerol) were prepared each six or seven days and  
365 stored at -80 °C. Cocultures were regularly tested for revertant phenotypes that  
366 showed prototrophic growth (i.e. were capable to grow on MMAB-Agar without amino  
367 acid supplementation). Two out of twelve cocultures were excluded from further  
368 analysis due to the evolution of prototrophic phenotypes. Accordingly, also the  
369 matching biological replicates in auxotrophic monocultures were excluded from  
370 further analysis. In addition, one replicate of *E. coli* Tyr<sup>-</sup> Ara<sup>-</sup> Lac<sup>-</sup> from monocultures  
371 was excluded due to contamination. Terminal populations were spread on modified  
372 TA agar plates to isolate evolved clones based on colour and colony morphology for  
373 whole-genome resequencing. Phenotypic diversity was observed in eight out of ten  
374 cocultures, and three out of 19 monocultures of auxotrophs, while all WT  
375 monocultures remained phenotypically homogeneous. Isolates were stored at -80 °C  
376 until further analysis.

377

### 378 *Genome resequencing and analysis*

379 Evolved populations were sequenced on the level of isolated clones and on the  
380 metagenome-level. For this, isolates from terminal populations were incubated in LB  
381 medium and whole populations in the respective native minimal medium until the  
382 maximum optical density was reached. Genomic DNA was extracted using the

383 Epicentre MasterPure™ Complete DNA & RNA purification kit (MC85200, Biozym  
384 Scientific, Germany). Further steps were performed by the Max Planck Genome  
385 Centre Cologne, Germany (<https://mpgc.mpipz.mpg.de/home/>): Quality control of  
386 samples was performed on Genomic DNA ScreenTape Analysis® using TapeStation  
387 Analysis Software A.02.01 (Agilent Technologies, USA), followed by TruSeq-  
388 compatible library preparation. Clonal samples were sequenced on the Illumina  
389 HiSeq2500 platform in 100-bp paired-end mode for all WT and coculture samples  
390 and in 150-bp paired-end mode for samples from auxotrophic monocultures.  
391 CASAVA (v1.8.2) (41) and bcl2fastq2 (v.2.18.0.12) (42) were used for basecalling  
392 and demultiplexing. We used cutadapt (v1.9.1) (43) for trimming Illumina sequencing  
393 adapters with a minimal overlap of 12. PhiX sequences were identified using bowtie  
394 (v1.9.1) (44) and BMAP (v37.28) (45). We discarded reads with fewer than 15  
395 nucleotides. Only complete read pairs were kept. Observed coverage was  
396 approximately 75-fold at quality scores above 30. Sequencing was successfully  
397 performed for 65 clonal samples in total. Numbers of sequenced clones depended on  
398 the number of observed morphotypes on agar plates (see above). Further analysis of  
399 mutations revealed two cases of clones from the same population of cocultures to  
400 exhibit identical genome sequences. To avoid pseudoreplication, the corresponding  
401 pairs were treated as one. Besides genotypes isolated from derived populations of  
402 WT (n = 10), monocultures (n = 22, with  $\Delta trpB$ : n=11 and  $\Delta tyrA$ : n=11), and  
403 cocultures of auxotrophs (n = 31, with  $\Delta trpB$ : n = 18 and  $\Delta tyrA$ : n = 13), also the six  
404 ancestral genotypes were sequenced to identify mutations that were already present  
405 at the beginning of the evolution experiment. Metagenomes were sequenced on the  
406 Illumina HiSeq3000 platform using 150-bp single-end mode. To allow quantitative  
407 comparison of accumulated mutations without sampling bias, whole populations were  
408 analysed from WT populations (n = 6) as well as of populations of auxotrophic  
409 monocultures (n = 12, with  $\Delta trpB$ : n=6 and  $\Delta tyrA$ : n=6) and their corresponding  
410 cocultures (n = 6). Observed coverage was approximately 1,250-fold at quality  
411 scores above 30. Mapping of reads on the published reference genome of  
412 *Escherichia coli* BW25113 (CP009273\_1) (46) and identification of mutations was  
413 performed using the BRESEQ-pipeline (47, 48). For population samples, the  
414 polymorphism mode with the “Polymorphic Read Alignment (RA) Evidence” option “--  
415 polymorphism-minimum-coverage-each-strand” was set to 40. Identified mutations  
416 and evidence for new junctions (“Unassigned new junction evidence”) were  
417 rechecked by verifying individual reads to sufficiently indicate the presence of the  
418 mutations and to only map once onto the genome, especially when using the  
419 polymorphism mode. If reads indicating a certain mutation were confirmed to map  
420 elsewhere in the reference genome with 100% homology by using NCBI nucleotide  
421 BLAST (10.1093/nar/gkn201), as in the case of e.g. the highly homologous tRNA-  
422 encoding genes, respective mutations were rated as false-positives and excluded  
423 from further analysis. Absolute numbers of mutations were determined by counting  
424 each mutation regardless of size or structure as a single event. Complex mutations  
425 were first resolved in clonal samples as described for BRESEQ (48) and further used  
426 to successfully resolve all detected complex mutations in population samples by  
427 confirming identical architecture (for instance reads to be tiled at identical positions).

428 One population of auxotrophs that evolved in monoculture carried a non-sense  
429 mutation in the gene *mutS*. Inactivation of *mutS* is well-known to cause a  
430 hypermutator phenotype that rapidly accumulates increased numbers of single  
431 nucleotide polymorphisms (SNPs) (49). These *mutS*-induced mutations have mainly  
432 neutral or deleterious effects (50). Since this study aimed at analysing the effect of  
433 synergistic coevolution on the rate of genomic evolution, the corresponding  
434 population as well as the respective isolates were excluded from further analysis.

435

#### 436 *Quantification of parallel evolution*

437 The Jaccard Index (J) was calculated to estimate parallel evolution at the level of  
438 shared mutated genes between samples (51). J-values range between zero and one  
439 with lower values indicating that fewer mutations occurred simultaneously in the  
440 compared samples and larger numbers pointing to an increased similarity between  
441 samples. J was calculated for all possible combinations within individually sequenced  
442 isolates and within whole population samples, excluding comparisons with the same  
443 sample.

444

#### 445 *Distance trees*

446 Divergent evolution between experimental groups was analysed with a standard  
447 neighbour-joining method and GrapeTree (52) was used for visualisation. All  
448 identified mutations were summarized in a genotyping matrix that indicated whether a  
449 given gene within each sample was either mutated or showed the WT allele.  
450 Complex mutations that affected more than one gene were treated as single alleles  
451 as well, since these mutations were single evolutionary events that separate a  
452 mutant from another lineage.

453

454

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601 Visualization: DP

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603 Project administration: CK

604 Supervision: CK

605 Writing – original draft: DP, CK

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### 609 **Competing interests**

610 The authors declare no competing interests.

611

612

### 613 **Data and materials availability**

614 Sequencing data will be made publicly available by uploading it to a data repository  
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616

617

### 618 **Supplementary Materials**

619 Figs. S1 to S2

620 Tables S1 to S2

621