

1 **Metabolic coupling in bacteria**

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3 Shradha Shitut^{1,5}, Tobias Ahsendorf^{2,3}, Samay Pande^{1†}, Matthew Egbert⁴, Christian
4 Kost^{1,5*}

5
6 ¹ Experimental Ecology and Evolution Research Group, Department of Bioorganic
7 Chemistry, Max Planck Institute for Chemical Ecology, Jena, 07745, Germany

8
9 ² Deutsches Krebsforschungszentrum, Heidelberg, Baden-Württemberg, D-69120,
10 Germany

11
12 ³ Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA

13
14 ⁴ Department of Computer Science, University of Auckland, Auckland, 1010, New
15 Zealand

16
17 ⁵ Department of Ecology, School of Biology/Chemistry, University of Osnabrück,
18 Osnabrück, 49076, Germany

19
20 * Correspondence to: christiankost@gmail.com

21
22 †Current address- Department of evolutionary biology, Swiss Federal Institute of
23 Technology (ETH), Zurich, Switzerland

24 **ABSTRACT**

25 Symbiotic associations have radically shaped the diversity and complexity of life on
26 earth. Many known symbioses represent physiological fusions of previously
27 independent organisms, in which metabolites are traded between interacting partners in
28 intricate ways. The first steps leading to this tight entanglement, however, remain
29 unknown. Here we demonstrate that unidirectional cross-feeding of essential amino
30 acids between two bacterial cells can already couple their metabolisms in a source-sink-
31 like relationship. Auxotrophic recipients used intercellular nanotubes to derive amino
32 acids from other bacterial cells. Removal of cytoplasmic amino acids in this way
33 increased the amino acid production of donor cells by delaying feedback inhibition of the
34 corresponding amino acid biosynthetic pathway. Strikingly, even though donor cells
35 produced all the focal amino acids recipients required to grow, this additional metabolic
36 burden did not incur detectable fitness costs. Our results demonstrate that one loss-of-
37 function mutation is sufficient to couple the metabolic networks of two organisms, thus
38 resulting in a functional fusion of two previously independent individuals.

39

40 **KEY WORDS:** cross-feeding, nanotubes, source-sink mechanism, inter-cellular
41 coordination

42

43 **INTRODUCTION**

44 Life on Earth has produced a bewildering diversity of forms and physiologies.
45 Understanding the evolution of this complexity in organismal design is a fundamental
46 problem in biology. Major leaps in biological complexity have resulted from evolutionary

47 transitions, during which previously independent organisms were functionally integrated
48 to form a new, higher-level entity¹⁻³. Eminent examples of such symbiotic associations
49 involve transformative events such as the origin of the eukaryotic cell^{1,4,5} or the
50 emergence of plastids from a cyanobacterial progenitor⁶.

51 Selective advantages resulting from a cooperative division of labour among the
52 constituent lower-level units likely fuelled the emergence of these associations^{7,8}. By
53 interacting with individuals that feature novel traits, microorganisms could significantly
54 extend their metabolic repertoire⁹⁻¹¹. In this way, ecological strategies and evolutionary
55 trajectories became available to the newly emerged consortium that otherwise would be
56 inaccessible to individual organisms.

57 To function as a cohesive whole, the interacting partners need to coordinate their
58 cellular activities. In derived symbiotic systems, this usually involves a sophisticated
59 chemical communication between cells via an exchange of e.g. hormones¹², ions¹³, or
60 sugars¹⁴. However, it remains unclear how primitive symbiotic associations that lack
61 coevolved regulatory machinery can maintain their functional integrity. At early phases
62 of a symbiotic transition, the ability to coordinate functions among cells likely represents
63 a major hurdle that determines the evolutionary fate of the incipient symbiotic
64 association.

65 Here we used the experimental tractability of bacteria to study the simplest kind of a
66 metabolic interaction: the unidirectional transfer of metabolites from a producer to a
67 recipient cell. Our main goal was to identify whether two bacteria that engage in such a
68 one-way cross-feeding interaction, already display some primitive form of regulation to
69 coordinate their combined metabolism.

70 For this, we took advantage of a set of bacterial mutants that have been previously
71 used to study fitness consequences of obligate amino acid cross-feeding¹⁵. Deletion of
72 one biosynthetic gene rendered the growth of the resulting mutant (hereafter:
73 *auxotroph*) dependent on an external supply of amino acids, while deletion of another
74 gene caused an overproduction of one or more amino acids (hereafter: *overproducer*).
75 By combining both deletion alleles in one genetic background, ‘*cross-feeder*’ genotypes
76 were created, which reciprocally exchanged essential amino acids in coculture.
77 Surprisingly, coculturing two of these double-deletion mutants with complementary
78 amino acid requirements provided the cross-feeding consortium with a significant
79 growth advantage relative to the metabolically autonomous (i.e. prototrophic) wild type
80 cells – even when both types directly competed against each other¹⁵. This observation
81 suggested that cross-feeding genotypes benefitted from dividing their metabolic labour.
82 Moreover, loss of genes that are essentially involved in amino acid biosynthesis
83 triggered the formation of intercellular nanotubes, which auxotrophic bacteria used to
84 obtain cytoplasmic amino acids from other bacterial cells¹⁶. However, it remains unclear
85 how cross-feeding bacteria coordinate metabolite production and consumption despite
86 the lack of derived regulatory mechanisms.

87 We addressed this question using a unidirectional exchange of essential amino acids
88 between two genotypes of *Escherichia coli*. These one-way cross-feeding interactions
89 were established by matching amino acid donors with auxotrophic recipients that
90 obligately required the corresponding amino acid for growth. Utilizing genetically
91 engineered single gene deletion mutants for this purpose ruled out pre-existing traits
92 that arose as a consequence of a coevolutionary history among both interaction

93 partners. Moreover, a focus on unidirectional cross-feeding excluded confounding
94 effects that may occur in reciprocal interactions such as e.g. self-enhancing feedback
95 loops¹⁷. Taking advantage of intracellular reporter constructs allowed analysing both
96 internal amino acid pools as well as their production levels in real-time under *in-vivo*
97 conditions.

98 Our results show that the two bacterial genotypes exchange amino acids via
99 intercellular nanotubes. By lowering cytoplasmic amino acid-concentrations in donor
100 cells, auxotrophic recipients delayed the feed-back inhibition of the donor's biosynthetic
101 pathway, thus increasing overall production levels of the focal amino acid. In other
102 words, a nanotube-mediated exchange of cytoplasmic amino acids coupled the
103 metabolism of two interacting partners in a source-sink-like relationship. Our results
104 show the ease with which mechanisms emerge that regulate the metabolic exchange
105 between two symbiotic associates. By reducing conflicts of interests in this way, this
106 mechanism likely helps to stabilise incipient symbiotic associations, thus contributing to
107 the widespread distribution of metabolic cross-feeding interactions in nature.

108

109 **RESULTS**

110 **Construction and characterisation of uni-directional cross-feeding interactions**

111 To establish unidirectional cross-feeding interactions within *Escherichia coli*, five
112 different genotypes served as amino acid donors: Two single gene deletion mutants
113 (Δmdh and $\Delta nuoN$) that produce increased amounts of several different amino acids¹⁵,
114 two deletion mutants that produce increased amounts of either histidine or tryptophan
115 ($\Delta hisL$ and $\Delta trpR$)¹⁸, as well as unmanipulated *E. coli* WT cells. Three genotypes served

116 as recipients, which were auxotrophic for the amino acids histidine ($\Delta hisD$), lysine
117 ($\Delta lysR$), and tryptophan ($\Delta trpB$) (Fig. 1, Supplementary table 1) and thus essentially
118 required an external source of these metabolites to grow¹⁹.

119 As a first step, we quantified the amounts of amino acids the five donor strains
120 produced in monoculture during 24 hours of growth. Analysing culture supernatant and
121 cytoplasm of the focal donor populations using tools of analytical chemistry revealed
122 $\Delta nuoN$ produced significantly increased amounts of histidine, lysine, and tryptophan in
123 both fractions relative to the WT (Mann Whitney U-test: $P < 0.05$, $n=4$, Supplementary
124 figure 1), while the production levels of the Δmdh mutant did not differ significantly from
125 WT-levels (Mann Whitney U-test: $P > 0.05$, $n=4$, Supplementary figure 1). Similarly, both
126 the intra- and extracellular concentrations of tryptophan in the $\Delta trpR$ mutant were
127 significantly elevated over WT-levels (Mann Whitney U-test: $P < 0.05$, $n=4$,
128 Supplementary figure 1). In contrast, $\Delta hisL$ released twice as much of histidine into the
129 growth medium as was released by the WT (two sample Mann Whitney test: $P < 0.05$,
130 $n=4$, Supplementary figure 1), while it contained much lower levels of histidine in its
131 cytoplasm than the WT.

132

133 **Intercellular transfer of amino acids is contact-dependent**

134 Capitalizing on the set of well-characterised genotypes, we addressed the question
135 whether donor and recipient cells exchange amino acids in coculture and if so, whether
136 this interaction is contact-dependent. To this end, populations of donor and recipient
137 cells were cocultured in a device (i.e. *Nurmikko cell*), in which both partners can either
138 be grown together in the same compartment or separated by a filter membrane that

139 allows passage of small molecules, yet prevents direct interactions among bacterial
140 cells¹⁶. Inoculating donor and recipient strains in different combinations revealed in all
141 tested cases growth of auxotrophic recipients when they were not physically separated
142 from donors (Fig. 2A-C). Auxotrophic recipients grew significantly better when
143 cocultured with amino acid overproducers (Δmdh , $\Delta nuoN$, $\Delta hisL$, and $\Delta trpR$) than with
144 the WT (Dunnett's T3 post hoc test: $P < 0.05$, $n = 4$). However, physically separating donor
145 and recipient cells by introducing a filter membrane, effectively eliminated growth of
146 recipients in all cases. Surprisingly, this treatment did not affect growth of donor
147 populations (Fig. 2A-C). Three main insights result from this experiment: First,
148 producing the amino acids required by the auxotrophs for growth did not incur
149 detectable fitness costs to the donor strain (Dunnett's T3 post hoc test: $P > 0.05$, $n = 4$).
150 Second, the total productivity of the coculture involving amino acid overproducers as
151 donors (Δmdh , $\Delta nuoN$, $\Delta hisL$, and $\Delta trpR$) was significantly increased when cells were
152 cocultured in the same environment as compared to the situation when they were
153 physically separated by a filter membrane (Mann Whitney U-test: $P < 0.05$, $n = 4$, Fig. 2A-
154 C). Third, physical contact between donor and recipient cells was required for a transfer
155 of amino acids between cells.

156

157 **Cytoplasmic constituents are transferred from donor to recipient cells**

158 The observation that metabolite cross-feeding among cells was contact-dependent
159 suggested that separating cells with a physical barrier prevented the establishment of
160 structures required for amino acid exchange. A possible explanation for this could be
161 intercellular nanotubes, which would allow direct transfer of cytoplasmic amino acids

162 from donor to recipient cells¹⁶. This hypothesis was verified by differentially labelling the
163 cytoplasm of donor and recipient cells with plasmids that express either red or green
164 fluorescent proteins. Quantifying the proportion of recipient cells that contained both
165 cytoplasmic markers after 24 hours of growth in coculture using flow cytometry allowed
166 us to determine the exchange of cytoplasmic materials between cells under our
167 experimental conditions. Finding that all cocultures analysed comprised a significant
168 proportion of auxotrophic cells containing both fluorescent proteins simultaneously
169 confirmed that cytoplasmic materials such as protein and free amino acids have been
170 transferred from donor to recipient cells (Fig. 2D). However, it has been previously
171 shown that the presence of the amino acid, auxotrophic genotypes require for growth,
172 prevents the formation of nanotubes¹⁶. Uncoupling the obligate dependency by
173 supplementing the growth medium with saturating concentrations of the focal amino
174 acid provided no evidence for a significant increase in double-labelled auxotrophs (Fig.
175 2D), thus linking the establishment of these structures to the physiological requirement
176 for amino acid cross-feeding.

177

178 **Auxotrophic recipients derive amino acid from cocultured donor cells**

179 One hypothesis that could explain why recipients were able to grow in donor-recipient
180 cocultures (Fig. 2A-C) is that the physical contact between cells increased amino acid
181 production rates of donors. Amino acid production is energetically and metabolically
182 very costly to the bacterial cell²⁰⁻²². To minimize production costs, bacteria tightly
183 regulate their amino acid biosynthesis, for example by end product-mediated feedback
184 mechanisms that reduce production rates when cytoplasmic amino acid concentrations

185 exceed critical thresholds^{23,24}. In our case, recipient cells removed amino acids from the
186 cytoplasm of donors using nanotubes. This decrease in the cell-internal amino acid
187 pools could delay feedback inhibition in the donor cell, thus increasing its overall amino
188 acid production (Fig. 3). Quantifying the amount of free amino acids in the cytoplasm of
189 donor cells in both the absence and presence of an auxotrophic recipient would allow
190 testing the delayed-feedback inhibition hypothesis.

191 To determine cytoplasmic concentrations of free amino acids in real-time, we used
192 the lysine riboswitch as a cell-internal biosensor. When free lysine binds to the
193 riboswitch, it undergoes a conformational change, thus down-regulating expression of a
194 downstream reporter gene, in our case *gfp*²⁵. Introducing the plasmid-borne reporter
195 construct (hereafter: *Lys-riboswitch*, Supplementary figure 2) into the lysine auxotroph
196 Δ *lysR* and exposing the resulting cells to different concentrations of lysine validated the
197 utility of this biosensor: A strong negative correlation between the cells' cytoplasmic
198 amino acid concentrations as quantified via LC/MS/MS analysis of lysed cells and their
199 fluorescence emission ($r=-0.68$, $P=0.003$, Supplementary figure 3) corroborated that
200 this construct allowed indeed determining levels of free lysine in the cytoplasm of living
201 *E. coli* cells by simply quantifying their GFP emission.

202 Accordingly, introducing the lys-riboswitch into the lysine auxotrophic recipient
203 (Δ *lysR*) and growing the resulting strain in lysine-supplemented media revealed
204 consistently elevated levels of cytoplasmic lysine throughout the experiment (Fig. 4B).
205 In contrast, when the same recipient cells were grown in the absence of lysine, cell-
206 internal lysine levels were significantly reduced (FDR-corrected paired sample t-tests:
207 $P<0.005$, $n=4$, Fig. 4B), indicating amino acid starvation of auxotrophic cells.

208 Interestingly, when recipient cells were grown in the presence of one of the three donor
209 genotypes, their lysine levels resembled that of lysine-starved auxotrophs until 18 hours
210 of cocultivation, after which lysine levels increased back to the level of lysine-
211 supplemented cells (FDR-corrected paired sample t-tests: $P < 0.04$, $n = 4$, Fig. 4B). Prior
212 to these coculture experiments, auxotrophs had to be pre-cultured in lysine-containing
213 medium. Thus, the lysine levels measured in auxotrophs under coculture conditions
214 likely reflected the fact that these cells first used up internal residual lysine pools before
215 switching to other sources, in this case the cytoplasmic lysine of donor cells. Consistent
216 with this interpretation is the observation that the presence of donor cells that provided
217 this amino acid allowed lysine auxotrophs to grow (Fig. 4A). A strongly positive
218 correlation between the growth of lysine auxotrophs and their cell-internal lysine levels
219 corroborates that the lysine auxotrophic recipients obtained from cocultured donor cells
220 limited their growth ($r = 0.625$, $P = 0.003$, Supplementary figure 4).

221

222 **The presence of auxotrophic recipients increases cytoplasmic amino acid** 223 **concentrations in donor cells**

224 To test the delayed-feedback inhibition hypothesis, the lys-riboswitch was introduced
225 into the three donors WT, Δmdh , and $\Delta nuoN$. Each of these donor genotypes were then
226 grown in monoculture as well as in coculture with the lysine-auxotrophic strain $\Delta lysR$. In
227 these donor-recipient pairs only the donor contained the reporter plasmid.

228 The amino acid biosynthesis of WT cells is most stringently controlled, thus
229 preventing accumulation of free lysine in its cytoplasm. In contrast, the cytoplasm of the
230 $\Delta nuoN$ strain was characterized by generally increased amino acid levels

231 (Supplementary figure 1). Similarly, deletion of the malate dehydrogenase gene caused
232 an accumulation of citric acid cycle intermediates and thus a dysregulated amino acid
233 biosynthesis in the Δmdh mutant¹⁵. Hence, removing lysine from the cytoplasm of WT
234 cells is expected to trigger the strongest increase of cytoplasmic lysine levels. In
235 contrast, higher concentrations of lysine or its biochemical precursors in the cytoplasm
236 of the Δmdh and the $\Delta nuoN$ strain likely prevent a lowering of the lysine concentration
237 below the critical threshold that triggers a further production.

238 We tested these predictions by monitoring changes in intracellular lysine levels of
239 donor cells using the lys-riboswitch. In monocultures, lysine levels unveiled a steady
240 increase over time (Fig. 4C). This pattern, however, changed in the presence of the
241 auxotrophic recipient. When *E. coli* WT cells were used as donor, their cytoplasmic
242 lysine levels first increased significantly over the levels WT cells reached in monoculture
243 (FDR-corrected paired sample t-tests: $P < 0.03$, $n = 4$, Fig. 4C). After that lysine levels
244 dropped significantly before increasing back to monoculture levels (Fig. 4C). The
245 observed fluctuations in the lysine levels of the donor's cytoplasm are consistent with a
246 nanotube-mediated cell attachment that is contingent on the nutritional status of the
247 receiving cell. In contrast, when Δmdh and $\Delta nuoN$ were cocultured as donor strains
248 together with the auxotrophic recipient, their cytoplasmic lysine levels did not differ
249 significantly from the levels reached under monoculture conditions (Fig. 4C). Thus,
250 these observations are in line with the above expectations and confirm indeed that an
251 auxotroph-mediated removal of amino acids from the donor's cytoplasm was sufficient
252 to prompt an increased amino acid biosynthesis levels in donor cells. Conversely,
253 lysine-auxotrophic recipients displayed significantly increased lysine levels when

254 cocultured with one of the donor genotypes relative to lysine-starved monocultures.
255 Both observations together suggest a unidirectional transfer of amino acids from donor
256 to recipient cells that in turn results in an intercellular regulation of amino acid
257 biosynthesis. Hence, these findings concur with the delayed-feedback inhibition
258 hypothesis (Fig. 3).

259

260 **The presence of auxotrophic recipients increases transcription of biosynthesis** 261 **genes in donor cells**

262 Bacterial cells use feedback inhibition to maintain homeostasis of certain metabolites
263 in their cytoplasm. Once metabolite levels drop below a certain threshold, production
264 levels are increased to allow optimal growth^{26,27}. In the case of amino acid biosynthesis,
265 the promoter elements that control transcription of biosynthetic pathways are frequently
266 highly sensitive to intracellular levels of the synthesized amino acid²⁴, thus enhancing
267 transcription of the operon when the amino acid is scarce. As soon as amino acid
268 concentrations reach optimal levels, further transcription is blocked enzymatically²⁸ or
269 by direct binding of the amino acid to the operon²⁹.

270 Taking advantage of this principle, we employed plasmid-borne promoter-GFP-fusion
271 constructs (Supplementary figure 2) to identify transcriptional changes in amino acid
272 biosynthesis genes. These reporter constructs have been previously shown to
273 accurately measure promoter activity with a high temporal resolution³⁰. For analysing
274 the focal cross-feeding interactions, the fusion constructs for *hisL* and *trpL* were
275 selected, which sense the cytoplasmic concentration of histidine³¹ and tryptophan^{29,32},
276 respectively. Correlating GFP emission levels with the cytoplasmic concentration of the

277 corresponding amino acid as quantified chemically via LC/MS/MS revealed a
278 significantly negative relationship for both histidine ($r=-0.407$, $P<0.001$, Supplementary
279 figure 3) and tryptophan ($r=-0.237$, $P=0.038$, Supplementary figure 3), confirming the
280 link between transcription of metabolic genes and the cytoplasmic concentration of the
281 corresponding amino acids.

282 These promoter-GFP-fusion constructs were introduced into donor cells (i.e. WT,
283 Δmdh , $\Delta hisL$, and $\Delta trpR$), which were then cultivated for 24 hours in the absence or
284 presence of the $\Delta hisD$ or $\Delta trpB$ auxotrophic recipient cells. In line with expectations,
285 donor strains WT, $\Delta hisL$, and $\Delta trpR$ displayed a starkly increased transcription of the
286 respective biosynthetic operon in the presence of auxotrophic recipients as compared to
287 donors growing in monoculture (FDR-corrected paired t-tests: $P<0.05$, $n=4$, Fig. 5).
288 Together, these results demonstrate that the presence of auxotrophic recipients
289 significantly increased the amino acid production of donor cells. By withdrawing amino
290 acids from the cytoplasm of donor cells, auxotrophic recipients prompted donor cells to
291 readjust their amino acid levels by up-regulating the transcription of the corresponding
292 amino acid biosynthesis genes.

293

294 **DISCUSSION**

295 Our study demonstrates for the first time that the deletion of a single metabolic gene
296 from a bacterial genome can be sufficient to couple the metabolism of two previously
297 independent bacterial cells. Auxotrophic cells that had lost the ability to autonomously
298 produce a certain amino acid established intercellular nanotubes to derive the amino
299 acid they required for growth from other cells in the environment. Quantifying cell-

300 internal amino acid levels revealed a primitive form of intercellular regulation of amino
301 acid biosynthesis between donor and recipient cells in a source-sink-like manner. This
302 relationship emerged as a consequence of feedback-based control mechanisms in the
303 biosynthetic pathways of individual cells. The metabolic network of a cell provides and
304 maintains specific levels of the building block metabolites that are required for growth³³.
305 An excess or deficit of metabolites within cells can disturb the cell-internal equilibrium
306 and thus cause stress³⁴. Our results show how the removal of metabolites from the
307 donor's cytoplasm translates into increased production levels of the metabolite.
308 Strikingly, this source-sink-like relationship between donor and recipient did not impose
309 detectable fitness costs on the donor, but instead increased growth of the whole
310 bacterial consortium.

311 Obligate metabolic interactions are common in natural microbial communities^{35,36}.
312 When certain metabolites are sufficiently available in the environment, bacteria that lose
313 the ability to produce these metabolites autonomously (e.g. by a mutational deactivation
314 of the corresponding biosynthetic gene) gain a significant growth advantage of up to
315 30% relative to cells that produce these metabolites^{37,38}. As a consequence,
316 auxotrophic genotypes rapidly increase in frequency by deriving the focal metabolites
317 from both environmental sources and other cells in the vicinity. The results of our study
318 help to explain this tremendous fitness advantage: by selectively upregulating only
319 those biosynthetic pathways that enhance growth of the symbiotic consortium, cells only
320 invest resources into those metabolites that help the respective interaction partner to
321 grow. If the exchange is reciprocal, groups of cross-feeding cells gain a significant
322 fitness advantage relative to metabolically autonomous types, even when both parties

323 are directly competing against each other in the same environment¹⁵. Thus, the type of
324 intercellular regulation discovered in this study minimizes the amount of resources each
325 interaction partner needs to invest into the corresponding others. From this emerges a
326 metabolic division-of-labour, in which the benefit that participating cells gain is more,
327 than the costs incurred by the interaction. This effect reduces conflicts of interests within
328 consortia of cross-feeding cells, thus providing a mechanistic explanation for the
329 widespread distribution of this type of interaction in nature.

330 Nutritional stress or starvation in a cell is known to induce an aggregative lifestyle in
331 bacteria^{16,39,40}. In many cases, this physical contact is followed by an exchange of
332 cytoplasmic contents between interacting cells^{16,39,41}. Structurally similar connections
333 between cells are known to be involved in short- and long-distance communication in
334 many multicellular organisms^{42,43}. In both cases, networks of interacting cells are
335 challenged with the question of how to optimally organize transport within the network
336 such that all cells involved derive sufficient amounts of the traded signal or molecule.
337 While the intercellular communication within tissues of eukaryotic organisms is
338 notoriously difficult to study, our focal system provides a paradigmatic case to
339 experimentally study the constraints and rules that determine the assembly and
340 structure of intercellular communication networks. In this context, the results of our
341 study suggest that the distribution of metabolites within networks of interacting bacterial
342 cells mainly results from local interactions among neighbouring cells.

343 A metabolic relationship that is remarkably similar to the one studied here has been
344 described for the obligate association between aphids, *Acyrtosiphon pisum*, and their
345 endosymbiotic bacteria *Buchnera aphidicola*. In this system, the aphid host regulates

346 the amino acid production levels of its symbionts by changing its intracellular precursor
347 concentrations⁴⁴. This functional link is afforded by a mutational elimination of feedback
348 control in the corresponding biosynthetic pathway of the bacterial symbionts. Thus,
349 similar to the results of our study, manipulation of the biosynthetic pathway in the host
350 led to an efficient coupling of the metabolism of host and symbiont. An intimate
351 coordination such as this enabled the symbionts to function as an extension of the
352 host's metabolic network.

353 Our work highlights the ease, with which two previously independent organisms can
354 form a physiologically integrated whole: the mutational deactivation of a biosynthetic
355 gene is sufficient to trigger the establishment of this kind of metabolic interaction. Given
356 that a loss of seemingly essential biosynthetic genes is very common in bacteria³⁷ and
357 that a nanotube-mediated exchange of cytoplasmic materials is known to also occur
358 between different bacterial species¹⁶, it is well conceivable how a reductive genome
359 evolution of coevolving bacteria can result in the formation of a multicellular metabolic
360 network. Once a biosynthetic gene is lost, the resulting auxotrophic genotype is more
361 likely to lose additional genes than to regain the lost function via horizontal gene
362 transfer⁴⁵. Given that dividing metabolic labour in this way can be highly advantageous
363 for the interacting bacteria¹⁵ relative to metabolic autonomy, bacteria in their natural
364 environment may exist within networks of multiple bacterial cells that reciprocally
365 exchange essential metabolites rather than as functionally autonomous units.

366

367 **METHODS**

368 **Strains and plasmids used in the study**

369 *Escherichia coli* BW25113 was used as wild type, from which mutants that
370 overproduce amino acids (Δmdh , $\Delta nuoN$, $\Delta hisL$, and $\Delta trpR$) and mutants that are
371 auxotrophic for histidine ($\Delta hisD$), lysine ($\Delta lysR$), or tryptophan ($\Delta trpB$) were obtained by
372 a one-step gene inactivation method^{15,16} (supplementary table 1). Deletion alleles were
373 transferred from existing single gene deletion mutants (i.e. the Keio collection⁴⁶) into *E.*
374 *coli* BW25113 using the phage P1. The cytoplasm of all donor and recipient strains was
375 labelled by introducing one of the two plasmids pJBA24-*egfp* or pJBA24-*mCherry*. The
376 plasmids constitutively express the ampicillin resistance gene (*bla*) as well as either the
377 fluorescent protein EGFP (*egfp*) or mCherry (*mCherry*). Two reporter constructs were
378 used: (i) lys-riboswitch (pZE21-GFPaav-Lys) for measuring internal amino acid levels
379 (lysine) and (ii) promoter fusion plasmids (pUAA6-His and pUA66-Trp) for measuring
380 the transcriptional activity of the promoters *hisL* and *trpR* respectively (see
381 supplementary experimental procedures for plasmid construction and characterization
382 of reporter constructs).

383

384 **Culturing methods and general procedures**

385 Minimal media for *Azospirillum brasiliense* (MMAB)⁴⁷ without biotin and with fructose
386 (5 g l⁻¹) instead of malate as a carbon source served as the growth media in all
387 experiments. The required amino acids (histidine, lysine, and tryptophan) were
388 supplemented individually at a concentration of 100 μ M. Cultures were incubated at a
389 temperature of 30 °C and shaken at 220 rpm for all experiments. All strains were
390 precultured in replicates by picking single colonies from lysogeny broth (LB)⁴⁸ agar
391 plates and incubated for 18 hours. The next morning, precultures were diluted to an

392 optical density (OD) of 0.1 at 600 nm as determined by a Tecan Infinite F200 Pro
393 platereader (Tecan Group Ltd, Switzerland). 10 μ l of these precultures were inoculated
394 into 1 ml of MMAB. In case of cocultures, donor and recipient were mixed in a 1:1 ratio
395 by co-inoculating 5 μ l of each diluted preculture. To cultivate strains containing the lys-
396 riboswitch, ampicillin was added at a concentration of 100 μ g ml⁻¹ and kanamycin was
397 added at 50 μ g ml⁻¹ in case of strains containing the promoter-GFP-fusion constructs.
398 Anhydrotetracycline (aTc) (Biomol GmbH, Hamburg, Germany) was added at a
399 concentration of 42 ng ml⁻¹ to induce expression of the lys-riboswitch.

400

401 **Contact-dependent exchange of amino acids**

402 To determine if physical contact between cells is required for an exchange of amino
403 acids between donor and recipient cells, a previously described method was used¹⁶. In
404 brief, each donor (i.e. WT, Δmdh , $\Delta nuoN$, $\Delta hisL$, and $\Delta trpR$) was individually paired with
405 each recipient (i.e. $\Delta hisD$, $\Delta lysR$, and $\Delta trpB$) and every combination was inoculated
406 together into a Nurmikko cell that allows cultivation of both populations either together in
407 the same compartment or separated by a membrane filter (0.22 μ m, Pall Corporation,
408 Michigan, USA). The filter allows passage of free amino acids in the medium, but
409 prevents direct interaction between cells. After inoculating 4 ml of MMAB, the apparatus
410 was incubated for 24 h. Bacterial growth after 24 h was determined as colony forming
411 units (CFU) per ml culture volume by plating the serially-diluted culture on MMAB agar
412 plates that did or did not contain ampicillin or kanamycin for selection. The increase in
413 cell number was calculated as the logarithm of the difference between the CFU counts
414 determined at the onset (0 h) of the experiment and after 24 h. Each donor-recipient

415 combination was replicated 4-times for both experimental conditions (i.e. with and
416 without filter).

417

418 **Flow cytometric analysis of cytoplasmic protein transfer**

419 A previously established protocol was applied to identify a transfer of cytoplasmic
420 material from donor to recipient genotypes¹⁶. For this, pairs of donor and recipient cells
421 with differentially labeled cytoplasms (i.e. containing EGFP or mCherry) were co-
422 inoculated into 1 ml MMAB. At the beginning of the experiment (0 h) and after 24 h of
423 growth, the sample was analyzed in a Partec CyFlow Space flow cytometer (Partec,
424 Germany). In the flow cytometer, cells were excited at 488 nm with a blue solid-state
425 laser (20 mV) and at 561 nm with a yellow solid-state laser (100 mV). Green (*egfp*) and
426 red (*mCherry*) fluorescence emission was detected at 536 nm and 610 nm, respectively.
427 *E. coli* WT devoid of any plasmid was used as a non-fluorescent control. The number of
428 single- and double-labeled cells in a population was quantified at both time points. Data
429 analysis and acquisition was done using the FlowMax software (Partec GmbH,
430 Germany). The experiment was conducted by coculturing eGFP-labelled donor with
431 mCherry-labelled recipient genotypes and *vice versa* in all possible combinations (i.e.
432 each donor paired with each recipient, except in case of $\Delta hisL$ and $\Delta trpR$, which were
433 only paired with $\Delta hisD$ and $\Delta trpB$, respectively) for 24 h. Each combination was
434 replicated 4-times.

435

436 **Fluorescence measurement**

437 The fluorescence levels of cells containing the lys-riboswitch or the promoter-GFP-
438 fusion constructs were measured by transferring 200 μ l of the culture into a black 96-
439 microwell plate (Nunc, Denmark) and inserting the plate into a Tecan Infinite F200 Pro
440 platereader (Tecan Group Ltd, Switzerland). The plate was shaken for 5 seconds prior
441 to excitation at 488 nm followed by emission detection at 536 nm. Fluorescence values
442 were always recorded together with a cognate control measurement. In case of the lys-
443 riboswitch, the uninduced plasmid-containing culture served this purpose, while in case
444 of the promoter fusion constructs, the promoter-less plasmid (pUA66) was used as
445 control. See supplementary methods for plasmid reporter characterization and promoter
446 activity measurements.

447

448 **Statistical analysis**

449 Normal distribution of data was assessed using the Kolmogorov-Smirnov test and
450 data was considered to be normally distributed when $P > 0.05$. Homogeneity of
451 variances was determined using the Levene's test and variances were considered
452 homogenous if $P > 0.05$. One-way ANOVA followed by a Dunnett's T3 post hoc test was
453 used to compare growth differences in the contact-dependent growth analysis.
454 Differences in the fluorescence emission levels of donor cells in the presence and
455 absence of a recipient were assessed with paired sample t-tests. The same test was
456 used to compare the number of recipient (Δ lysR) CFUs at the start and at the end of the
457 coculture experiments to detect donor-enabled growth. The False Discovery Rate (FDR)
458 procedure of Benjamini et al. (2006) was applied to correct P values after multiple
459 testing. Pearson product moment correlation provided identification of the statistical

460 relationship between cytoplasmic amino acid levels and fluorescence emission as well
461 as between cytoplasmic lysine level and growth of the $\Delta lysR$ recipient.

462

463 **AUTHOR CONTRIBUTIONS**

464 SS and CK conceived the study, SS, CK, and SP designed the study. SS performed
465 all experiments. SS and CK interpreted and analyzed the data. TA generated some
466 plasmids for the study. SS and CK wrote the manuscript, all authors amended the
467 manuscript.

468

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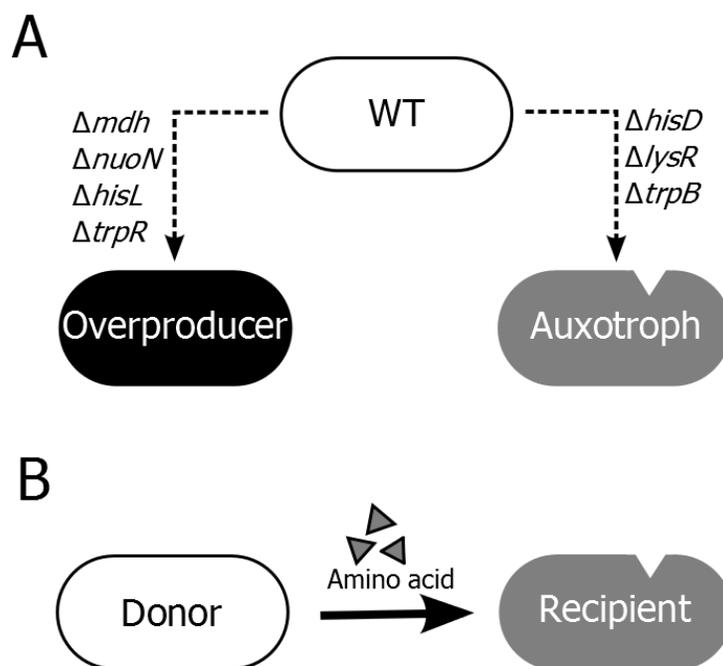
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613 **FIGURES**

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617 **Figure 1: Experimental system used.** (A) Design of genotypes. Single genes were
618 deleted from *E. coli* BW25113 wild type (WT) to generate mutants that produce
619 increased amounts of amino acids (overproducer, black) as well as mutants that
620 essentially require a certain amino acid to grow (auxotroph, grey). (B) Coculturing an
621 amino acid donor (i.e. WT or overproducer) together with an auxotrophic recipient
622 results in a one-way cross-feeding interaction that is obligate for the recipient, but not
623 the donor.

624

646 auxotrophic recipients (Rcpt, triangles) either together in the same compartment (-
647 Filter) or separated by a filter membrane (+ Filter) that allows passage of free amino
648 acids, but prevents direct physical contact among cells. Growth over 24 h was
649 determined as number of colony-forming units (CFU) per ml by subtracting the value at
650 0 h from the one reached at 24 h. Different letters indicate significant differences
651 (Dunnett's T3 post hoc test: $P < 0.05$, $n=4$). **(D)** Cells exchange cytoplasmic material. The
652 cytoplasm of donors and recipients were differentially labelled with the fluorescent
653 proteins EGFP and mCherry, respectively. Quantifying the proportion of double-labelled
654 auxotrophs containing both cytoplasmic markers after 0 h and 24 h of coculture allowed
655 assessing an exchange of cytoplasm between bacterial cells. The experiment was
656 conducted in the absence (grey triangles) and presence (black triangles) of the focal
657 amino acid (100 μM). Asterisks indicate significant differences (paired t-test: ** $P <$
658 0.001, * $P < 0.05$, $n=4$). In all cases, mean ($\pm 95\%$ confidence interval) are shown.

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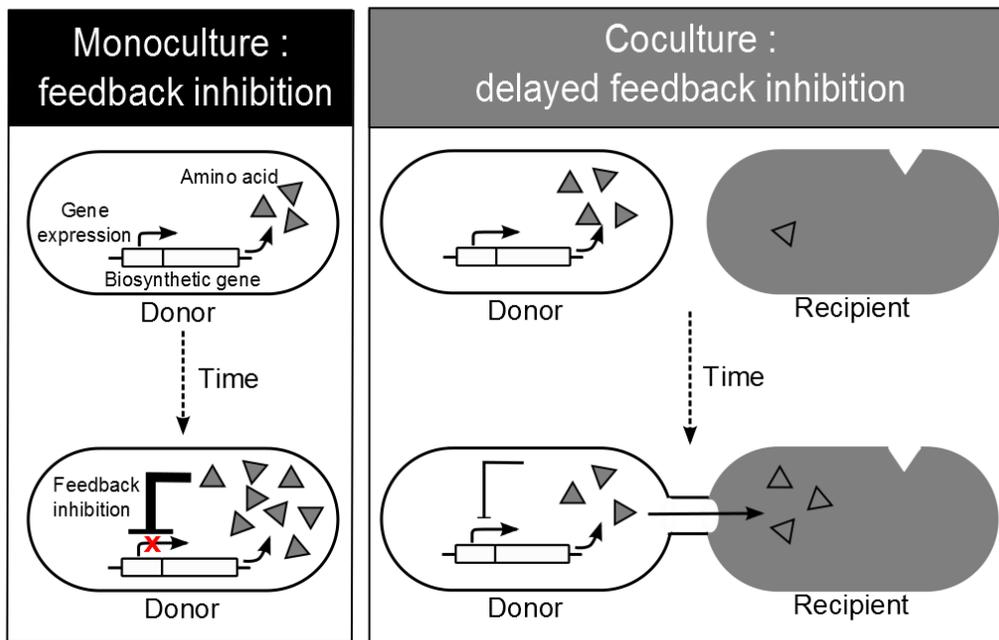
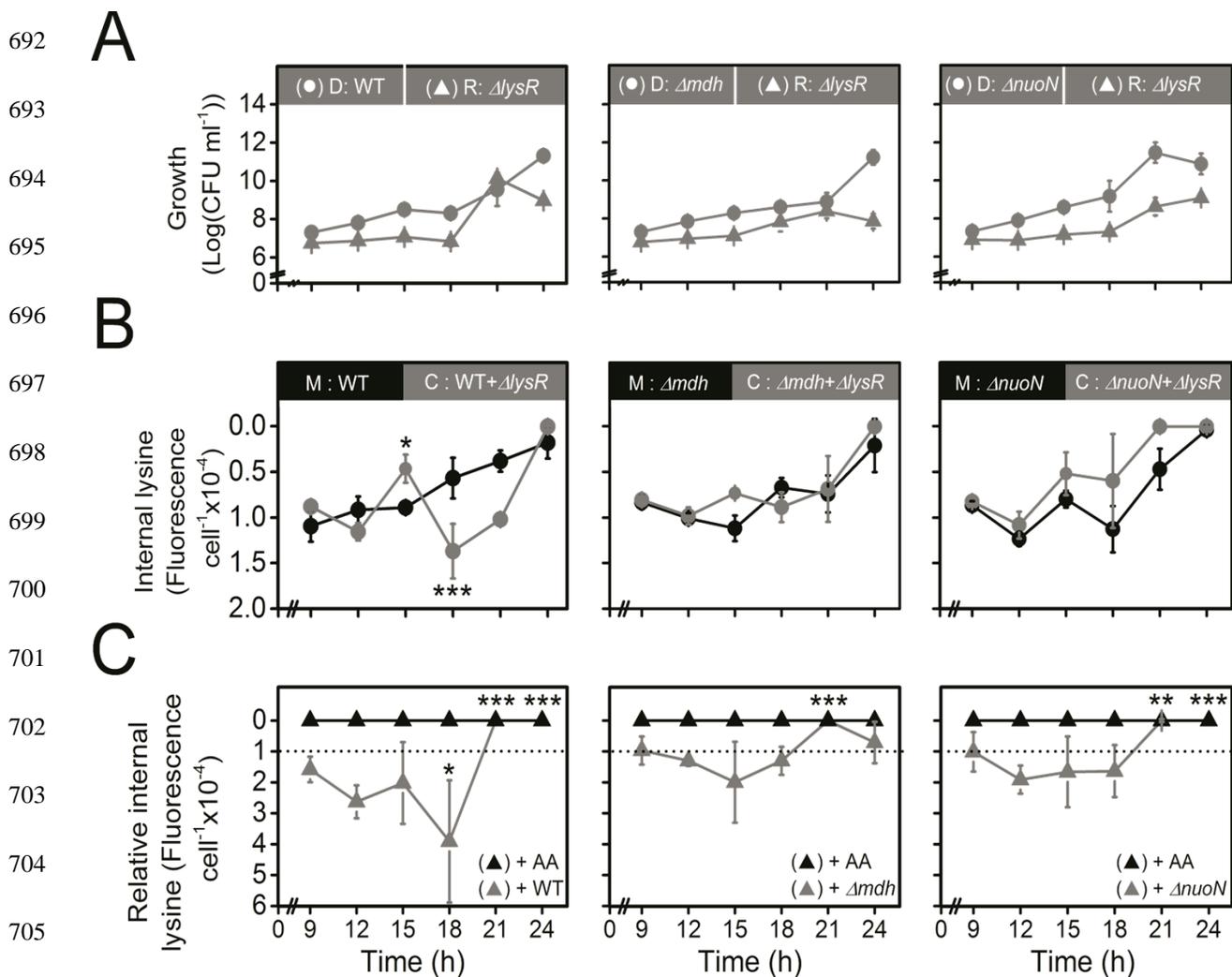


Figure 3: Delayed feedback inhibition hypothesis. In monoculture, amino acid concentrations in the cytoplasm of donor cells build up over time. When a certain concentration threshold is reached, these metabolites inhibit their own production by suppressing the expression of the corresponding amino acid biosynthesis genes (i.e. end product-mediated feedback inhibition). In coculture, auxotrophic recipients reduce cytoplasmic amino acid concentrations of donor cells. As a consequence, feedback inhibition of biosynthesis genes is delayed, thus resulting in an increased amino acid biosynthesis.



707 **Figure 4: The presence of auxotrophs increases cytoplasmic amino acid levels in**
 708 **donor cells. (A)** Growth of each partner in cocultures of donor (D, circles) and recipient
 709 (R, triangles) populations was determined as the number of colony-forming units (CFUs)
 710 ml⁻¹ over 24 h. **(B, C)** Cytoplasmic lysine levels were quantified by measuring GFP
 711 fluorescence emission from a cell-internal reporter and normalized per cell containing
 712 the reporter. Low fluorescence levels indicate high lysine levels (note the inverted y-
 713 axes). **(B)** Lysine levels in lysine-supplemented monocultures (+AA) and un-
 714 supplemented cocultures were measured relative to lysine-starved monocultures

715 (dashed line). In the presence of lysine, monocultures of the recipient (black triangles)
716 showed constantly increased cytoplasmic lysine levels. In coculture with the donor (grey
717 triangles), lysine levels in the recipient first declined and then increased back to the
718 level of the +AA condition. (C) In coculture with lysine-auxotrophic recipients,
719 cytoplasmic lysine levels of WT donor cells were significantly increased at 15 h of
720 growth and significantly decreased at 18 h of growth in coculture (C, grey circles)
721 relative to monoculture conditions (M, black circles). However, in case of the
722 overproducers Δmdh and $\Delta nuoN$, cell-internal lysine levels did not vary between mono-
723 and coculture conditions. In all cases, mean ($\pm 95\%$ confidence interval) are shown and
724 asterisks indicate the results of FDR-corrected paired sample t-tests (* $P < 0.05$,
725 ** $P < 0.01$, *** $P < 0.001$, $n=4$).

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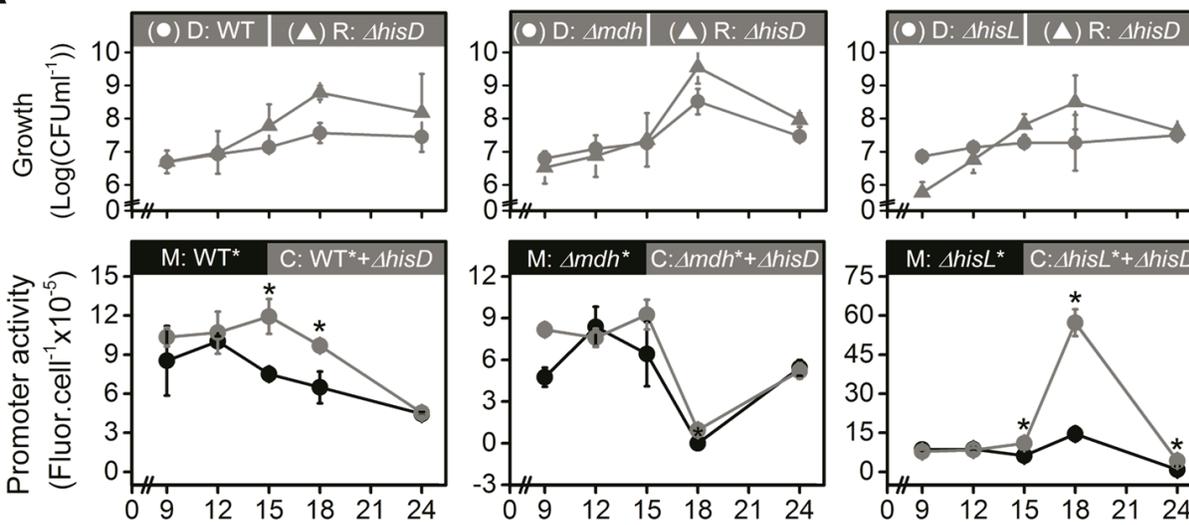
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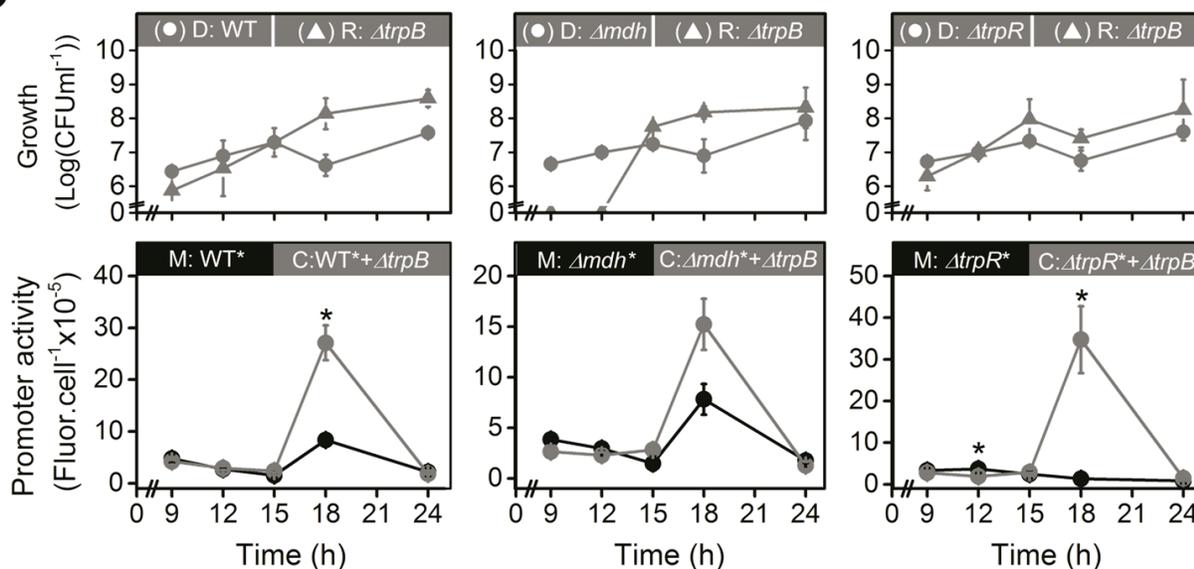
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736 **Figure 5: The presence of auxotrophs increases transcription of biosynthetic**

737 **genes in the donor. (A, B) Top panels depict growth of donor (D, circle) and recipient**

738 **(R, triangle) in coculture over time quantified as the number of colony-forming units**

739 **(CFU) per ml. Bottom panels show promoter activity of the donors' amino acid**

740 **biosynthesis gene in monoculture (M, black circles) and coculture with an auxotrophic**

741 **recipient (C, grey circles). Promoter activity was quantified as the emission of GFP**

742 fluorescence from a promoter-GFP-fusion construct and normalized per number of
743 donor cells (CFUs) containing the construct. Asterisks indicate significant differences of
744 the promoter activity of donor cells in mono- and coculture conditions (FDR-corrected
745 paired t-test: * $P < 0.05$, $n=4$). Populations of donor cells (D, circles) were grown in
746 monoculture or cultivated together with **(A)** the histidine auxotrophic recipient ($\Delta hisD$) or
747 **(B)** the tryptophan auxotrophic recipient ($\Delta trpB$). In all cases, mean ($\pm 95\%$ confidence
748 interval) are shown.