

1 **Metabolic dissimilarity determines the establishment of cross-feeding** 2 **interactions in bacteria**

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22 23 24 **Abstract**

25 The exchange of metabolites among different bacterial genotypes is key for
26 determining the structure and function of microbial communities. However, the factors
27 that govern the establishment of these cross-feeding interactions remain poorly
28 understood. While kin selection theory predicts that individuals should direct benefits
29 preferentially to close relatives, the potential benefits resulting from a metabolic
30 exchange may be larger for more distantly related species. Here we distinguish
31 between these two possibilities by performing pairwise cocultivation experiments
32 between auxotrophic recipients and 25 species of potential amino acid donors.
33 Auxotrophic recipients were able to grow in the vast majority of pairs tested (78%),
34 suggesting that metabolic cross-feeding interactions are readily established. Strikingly,
35 both the phylogenetic distance between donor and recipient as well as the dissimilarity
36 of their metabolic networks was positively associated with the growth of auxotrophic
37 recipients. Finally, this result was corroborated in an *in-silico* analysis of a co-growth
38 of species from a gut microbial community. Together, these findings suggest metabolic
39 cross-feeding interactions are more likely to establish between strains that are
40 metabolically more dissimilar. Thus, our work identifies a new rule of microbial
41 community assembly, which can help predict, understand, and manipulate natural and
42 synthetic microbial systems.

44 **Keywords**

45 Bacterial community, community assembly, cross-feeding, metabolic distance,
46 metabolite secretion, niche overlap, phylogenetic relatedness, public good.

47

48 **Significance**

49 Metabolic cross-feeding is critical for determining the structure and function of natural
50 microbial communities. However, the rules that determine the establishment of these
51 interactions remain poorly understood. Here we systematically analyze the propensity
52 of different bacterial species to engage in unidirectional cross-feeding interactions. Our
53 results reveal that synergistic growth was prevalent in the vast majority of cases
54 analyzed. Moreover, both phylogenetic and metabolic dissimilarity between donors
55 and recipients favored a successful establishment of metabolite exchange interactions.
56 This work identifies a new rule of microbial community assembly that can help predict,
57 understand, and manipulate microbial communities for diverse applications.

58

59 **Introduction**

60 Microorganisms are ubiquitous on our planet and are key for driving vital ecosystem
61 processes (1-3). They contribute significantly to the flow of elements in global
62 biogeochemical cycles (3, 4) and are also crucial for determining the fitness of plants
63 (5, 6) and animals (7, 8) including humans (9, 10). These vital functions are provided
64 by complex communities that frequently consist of hundreds or even thousands of
65 metabolically diverse strains and species (11, 12). However, the rules that determine
66 the assembly, function, and evolution of these microbial communities remain poorly
67 understood. Yet understanding the underlying governing principles is central to
68 microbial ecology and crucial for purposefully designing microbial consortia for
69 biotechnological (13) or medical applications (14, 15).

70 In recent years, both empirical and theoretical work increasingly suggests that the
71 exchange of essential metabolites among different bacterial genotypes is a key
72 process that can significantly affect growth (16, 17), composition (18), and the structure
73 of microbial communities (19). In those cases, one bacterial genotype releases a
74 molecule into the extracellular environment, which can be used by other cells in the
75 local vicinity. The released substances frequently include building block metabolites
76 such as amino acids (20, 21), vitamins (22, 23), or nucleotides (24) as well as
77 degradation products of complex polymers (19, 25). Even though these compounds
78 represent valuable nutritional resources, they are released as unavoidable byproducts
79 of bacterial physiology (26, 27) and metabolism (28) or due to leakage through the
80 bacterial membrane (29, 30). Consequently, the released compounds create a pool of
81 resources that can benefit both conspecifics and members of other species in the local
82 neighborhood (31-34). The beneficiaries include genotypes that opportunistically take
83 advantage of these metabolites as well as strains, whose survival essentially depends
84 on an external supply with the corresponding metabolite. These so-called *auxotrophic*

85 genotypes carry a mutation in a biosynthetic or regulatory gene, which renders the
86 resulting mutant unable to autonomously produce a certain metabolite such as an
87 amino acid, a vitamin, or a nucleotide. By utilizing metabolites that are produced by
88 another cell, a unidirectional cross-feeding interaction is generated. Auxotrophic
89 mutants that use compounds released by others frequently gain a significant fitness
90 advantage over prototrophic cells that produce the required metabolites by themselves
91 (35). Due to the tremendous benefits that can result from this process, cross-feeding
92 interactions are prevalent in all kinds of microbial ecosystems, including soil (36),
93 fermented food (21), aquatic environments (37, 38), as well as host-associated
94 microbiota (7, 39). Despite the ubiquity of unidirectional cross-feeding interactions in
95 nature, the rules that govern their establishment remain poorly understood (40-43). In
96 particular, it is unclear how the relationship between the metabolite donor and the
97 auxotrophic recipient affects the likelihood that a cross-feeding interaction is
98 successfully established. Two possibilities are conceivable.

99 First, kin selection theory predicts that organisms should preferentially direct
100 benefits to close relatives rather than to more distantly related individuals (44). In this
101 way, individuals indirectly favor the spread of their own genes. Even if the interaction
102 at this stage is merely created by chance and has not evolved for this purpose, certain
103 mechanisms might be in place that could maximize the chance that cells preferentially
104 interact with conspecifics. Such mechanisms could be based, for example, on
105 recognition alleles, as they play a role in other social interactions such as quorum
106 sensing (45), biofilm formation (46), and swarming (47). Moreover, the exchange of
107 metabolites could be mediated by contact-dependent interactions such as the
108 formation of intercellular nanotubes (48, 49), which might require a close phylogenetic
109 relatedness between donor and recipient for an efficient transport to operate. In the
110 following, we refer to this possibility as the *similarity hypothesis*.

111 Second, unidirectional cross-feeding interactions might favor more distantly related
112 donor-recipient pairs over interactions among close relatives. Two closely related
113 bacterial cells are more likely to share ecological preferences such as habitat or
114 resources utilized than two phylogenetically different bacterial taxa (41). Moreover, two
115 genealogically related cells have greater similarities in their metabolic network than
116 two distantly related cells (33, 50, 51). As a consequence, the biosynthetic cost to
117 produce a given metabolite, and thus its nutritional value, is more likely to be different
118 in heterospecific pairs than among members of the same species (52, 53). If these
119 differences translate into enhanced growth of the auxotrophic recipient, a positive
120 correlation between the growth of the auxotroph and the phylogenetic and/ or
121 metabolic distance to the donor cell would be observed. In the following, we refer to
122 this alternative possibility as the *dissimilarity hypothesis*.

123 Here we aim at distinguishing between these two hypotheses to better understand
124 the rules that govern the establishment of this ecologically important interaction. To
125 achieve this goal, we used unidirectional cross-feeding interactions as a model.
126 Synthetically assembling pairs consisting of an auxotrophic recipient and a prototrophic
127 amino acid donor of the same or a different species ensured that both interaction

128 partners do not share a common evolutionary history. In this way, all results will
129 represent the situation of a naïve encounter between both interaction partners and only
130 mirror effects resulting from the phylogenetic relatedness and metabolic dissimilarity
131 between partners. Using this ecological approach, we systematically determined
132 whether and how the phylogenetic or metabolic distance between auxotrophic
133 recipients and prototrophic amino acid donors affects cross-feeding in pairwise
134 bacterial consortia.

135 Our results show that in the vast majority of cases, unidirectional cross-feeding
136 interactions successfully established between a prototrophic donor and an auxotrophic
137 recipient. Strikingly, recipients' growth was positively associated with both, the
138 phylogenetic and metabolic distance between donor and recipient. This pattern could
139 partly be explained by the difference in the amino acid profiles produced by donors.
140 Finally, an *in-silico* analysis of co-growth of species from a gut microbial community
141 revealed that recipient genotypes benefitted more when interacting with metabolically
142 dissimilar partners, thus corroborating the empirical results. Our work identifies the
143 metabolic dissimilarity between donor and recipient genotypes as a key parameter for
144 the establishment of unidirectional cross-feeding interactions.

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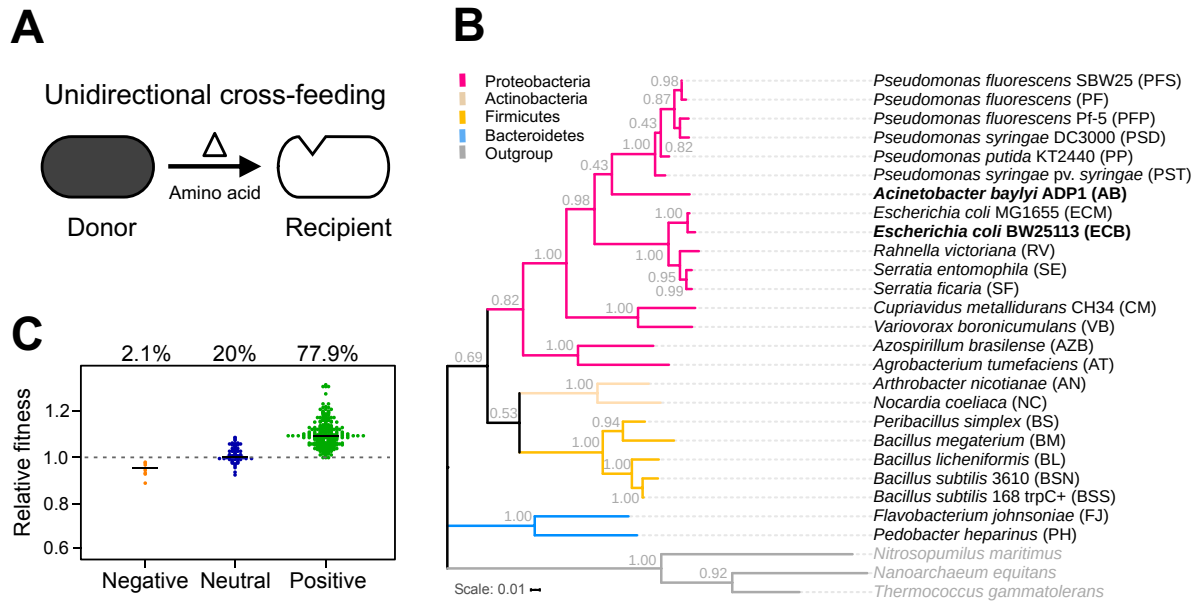
146 **Results**

147

148 **Auxotrophic recipients commonly benefit from the presence of prototrophic** 149 **donor cells**

150 To determine the probability, with which unidirectional cross-feeding interactions
151 emerge between an auxotrophic recipient and a prototrophic donor genotype, a series
152 of pairwise coculture experiments were performed (Fig. 1A). For this, 25 strains that
153 belonged to 21 different bacterial species were used as potential amino acid donors
154 (Figs. 1B and S1A). Donor strains were selected to cover both the existing diversity of
155 bacterial taxa and the phylogenetic neighborhood of the focal auxotrophs as good as
156 possible. These potential amino acid donors were individually cocultured together with
157 one auxotrophic recipient of *Escherichia coli* or *Acinetobacter baylyi*, which were both
158 auxotrophic for either histidine ($\Delta hisD$) or tryptophan ($\Delta trpB$) (Fig. S1B).

159 To test if the selected donor strains can support the growth of auxotrophic recipients,
160 the abovementioned strains were systematically cocultured in all possible pairwise
161 combinations (initial ratio: 1:1). Subsequently, the net growth of the recipient strains in
162 coculture was quantified over 24 h, and compared to the growth, the same strain
163 achieved in monoculture over the same period in the absence of amino acid. In this
164 experiment, the donor's presence affected the recipient's growth either positively,
165 negatively, or in a neutral way. Only 2% of the tested cases showed a growth reduction,
166 and in 20% of the interactions, auxotrophs did not respond at all to the presence of a
167 donor cell (Fig. 1C). In contrast, in the vast majority of cocultures tested (i.e., 78%),
168 auxotrophic cells grew significantly better in the presence of donor cells than
169 monocultures of auxotrophs, suggesting that unidirectional cross-feeding interactions
170 can readily establish (Fig. 1C).



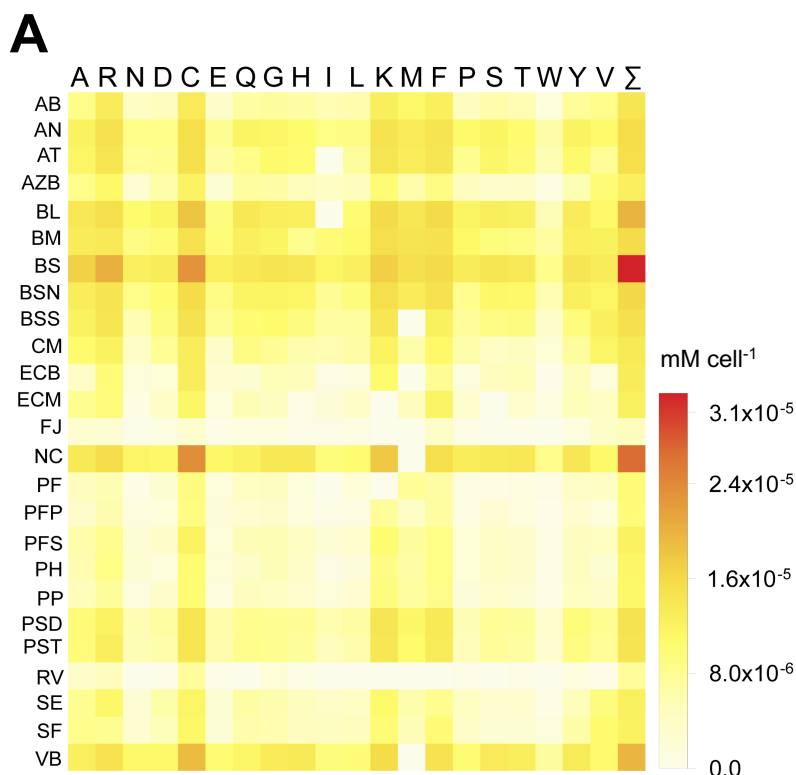
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172 **Fig. 1. Unidirectional cross-feeding between prototrophic donor cells and amino acid**
 173 **auxotrophic recipients is common. (A)** Overview over the experimental system used.
 174 Metabolically autonomous donor genotypes (dark cell) were cocultivated together with an
 175 auxotrophic recipient that was unable to produce either histidine or tryptophan (white cell).
 176 Growth of auxotrophs signifies the successful establishment of a unidirectional cross-feeding
 177 interaction, in which the focal amino acid (Δ) is exchanged between both cells. **(B)**
 178 Phylogenetic tree of bacterial species (donors and recipients) used in this study. Different
 179 colors indicate different phyla. The tree was constructed based on the 16S rRNA gene.
 180 Recipient strains used in this study are highlighted in bold. Branch node numbers represent
 181 bootstrap support values. **(C)** Growth of auxotrophic recipients in pairwise coculture with
 182 different donor genotypes. *Escherichia coli* and *Acinetobacter baylyi*, each either auxotrophic
 183 for histidine ($\Delta hisD$) or tryptophan ($\Delta trpB$), were used as amino acid recipients. The relative
 184 fitness of receivers when grown in coculture with one of X donors is plotted relative to their
 185 growth in monoculture in the absence of the focal amino acid (dashed line). CFU was
 186 calculated 24 h post-inoculation. Interactions in cocultures were classified as negative (n = 8),
 187 neutral (n = 76), and positive (n = 296), based on the statistical difference between the growth
 188 of auxotrophs in monoculture and coculture (FDR-corrected paired t-test: $P \leq 0.05$, n = 4).

189

190 Recipient growth depends on amino acid production of donor genotypes

191 The main factor causing the growth of auxotrophs in the coculture experiments
 192 was likely the amount and identity of metabolites that donor cells released into the
 193 extracellular environment (i.e., the exo-metabolome) (26). To test if amino acid
 194 production of donors could explain the observed recipient growth, the supernatant of
 195 monocultures of all 25 donor strains was collected during exponential growth.
 196 Subjecting the cell-free supernatant of these cultures to LC/MS/MS analysis revealed
 197 that all tested genotypes secreted amino acids in varying amounts (Figs. 2A and S2).
 198



B

Supernatant experiment

Total amino acids			
Recipient	n	ρ	P-value
AB-his	89	0.29	4.3x10⁻³
AB-trp	90	0.17	0.12
ECB-his	92	0.35	8.3x10⁻⁴
ECB-trp	93	0.33	1.2x10⁻³
Focal amino acid			
Recipient	n	ρ	P-value
AB-his	89	0.23	0.029
AB-trp	90	0.09	0.37
ECB-his	92	0.47	0.2x10⁻⁵
ECB-trp	93	0.43	0.13x10⁻⁴

199

200 **Fig. 2. Total amino acids production of different donors can predict unidirectional cross-**
 201 **feeding. (A)** Heatmap of amino acids released by different donor strains. Amount of amino
 202 acid (mM per cell) produced by 25 donor strains (for abbreviations see Fig. 1B) is shown (Y-
 203 axis). Cell-free supernatants of exponentially growing cultures were analyzed via LC/MS/MS.
 204 Colors indicate different amino acid concentrations (legend). Σ = total amino acid produced by
 205 the donor. **(B)** Overview over the statistical relationships between the total amount of amino
 206 acids (upper part) or the focal amino acid (lower part) in supernatants of donor cultures and
 207 the growth of the corresponding auxotrophic recipients. Results of spearman rank correlations
 208 (ρ) are shown.

209

210 In this experiment, donors are not expected to specifically produce the amino acid that
 211 the cocultured auxotroph requires for growth. Moreover, bacteria usually use generic

212 transporters that transport similar types of amino acids across the membrane (54-56).
213 Thus, auxotrophic recipients may benefit not only from the one amino acid they require
214 for growth, but also from utilizing the other amino acids that are produced by the donor.
215 To quantitatively determine whether the released amino acids could explain the growth
216 of recipient cells, the conditioned cell-free supernatant was supplied to monocultures
217 of auxotrophic cells and the resulting growth over 24 h was quantified. In addition, the
218 chemical composition of the supernatants used was determined via LC/MS/MS. This
219 experiment revealed that donor supernatants enhanced the growth of auxotrophic
220 recipients. Interestingly, recipient growth correlated positively with the total amount of
221 amino acids that were present in the supernatant (Table 2B). In addition, growth of
222 auxotrophic recipients was positively associated with the concentration of the amino
223 acid auxotrophs required for growth as well as with the total amount of amino acids
224 produced by donor cells (Table 2B). Together, these results show that auxotrophic
225 recipients not only use the amino acids they cannot produce on their own, but also take
226 advantage of the other amino acids that are produced by donor cells.

227

228 **Recipient growth correlates positively with amino acid profile dissimilarity**

229 To distinguish between the two main hypotheses, we asked whether the difference
230 in the amino acid profile produced by a closely and distantly related donor strain could
231 explain the growth auxotrophs achieved in the coculture experiment. To test this, we
232 calculated the Euclidean distance between the amino acid profiles of all 25 donor
233 strains. Assessing the statistical relationship between the normalized growth of
234 auxotrophs in coculture with the Euclidean distance in the amino acid profiles of closely
235 and distantly related donor genotypes revealed a significant positive relationship
236 between both parameters in all cases (Fig. 3A and Table 1). In other words, auxotrophs
237 grew better in coculture with a donor, which contained an amino acid mixture that was
238 different from the one a conspecific cell would have produced. Thus, these results
239 provide evidence that supports the dissimilarity hypothesis.

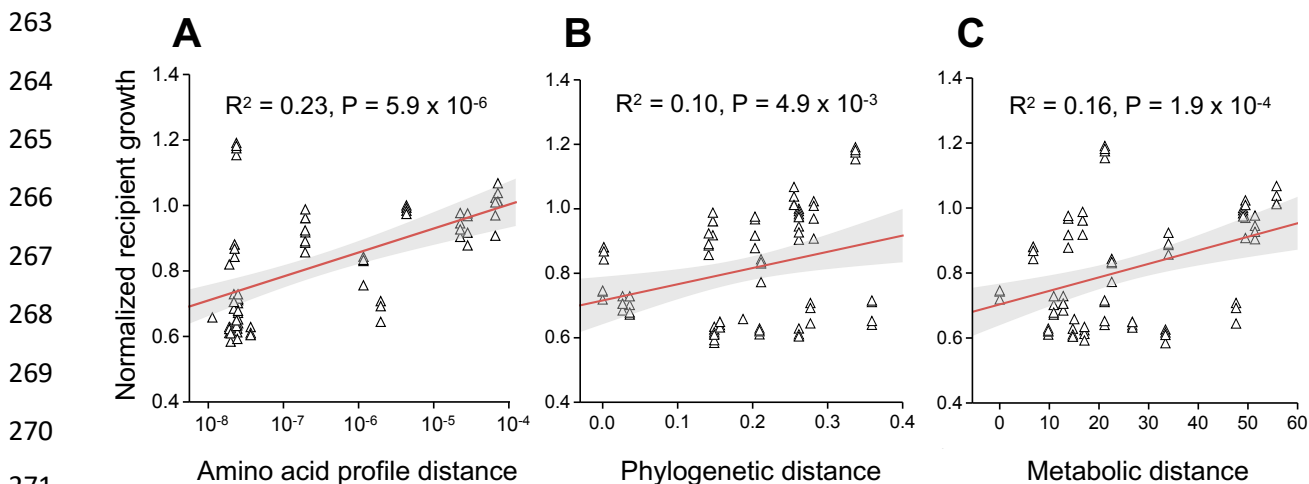
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241 **Growth of recipients scales positively with the phylogenetic and metabolic 242 distance to donor cells**

243 Next, we asked whether two phylogenetically close genotypes are more likely to
244 engage in a unidirectional cross-feeding interaction than two more distantly related
245 genotypes. To test this, we re-analyzed the results of the coculture experiment by
246 focusing on the phylogenetic relatedness between donor and recipient genotypes. In
247 this context, only those cocultures were considered, in which auxotrophs showed
248 detectable growth. These analyses revealed a positive association between the growth
249 of recipients and the phylogenetic distance to donor cells (Fig. 3B and Table 1).

250 However, given that previous analyses suggested that differences in the amino acid
251 profiles could predict auxotrophic recipients' growth (Fig. 3A and Table 1), we
252 hypothesized that the phylogenetic distance might only be a proxy for the difference in
253 the strains' metabolic networks. To verify this hypothesis, we compared genome-scale
254 metabolic networks of all donor and recipient genotypes. Using this data, a metabolic
255 similarity matrix between donor and recipient strains was calculated by identifying

256 commonalities and differences in both partners' biosynthetic pathways. Correlating the
 257 resulting data with the growth of auxotrophic recipients in coculture revealed again a
 258 positive association between the metabolic distance and recipient growth (Fig. 3C and
 259 Table 1). Together, these results provide additional support for the interpretation that
 260 cross-feeding interactions are more likely to establish between two more dissimilar
 261 genotypes.



272 **Fig. 3. Cross-feeding increases with an increasing dissimilarity to donor cells.** Shown is
 273 the net growth of the *E. coli* recipient auxotrophic for histidine ($\Delta hisD$, \triangle) as a function of (A)
 274 the amino acid profile distance, (B) the phylogenetic distance, and (C) the genome-based
 275 metabolic distance between donor and recipient. Red lines are fitted linear regressions and
 276 grey area indicates the 95% confidence interval. Sample size is 80 in all cases. Growth of
 277 recipient is displayed as a logarithm of the difference in number of CFUs between 0 h and 24
 278 h and was normalized per number of donor cells.

279
 280 **Table 1. Amino acid profile distance (AAD), phylogenetic distance (PD), and metabolic**
 281 **distance (MD), is positively associated with recipient growth.** Results of statistical
 282 regressions are shown.

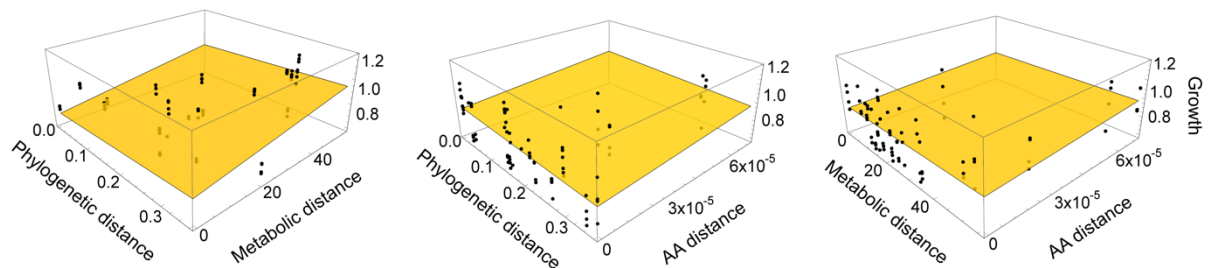
Recipient	n	AAD		PD		MD	
		R ²	P-value	R ²	P-value	R ²	P-value
AB-his	87	0.16	8.0x10⁻⁵	0.45	1.61x10⁻¹²	0.26	3.82x10⁻⁷
AB-trp	100	0.07	5.5x10⁻³	0.11	7.92x10⁻⁴	0.19	5.59x10⁻⁶
ECB-his	80	0.23	5.9x10⁻⁶	0.10	4.95x10⁻³	0.16	1.96x10⁻⁴
ECB-trp	91	0.26	2.1x10⁻⁷	0.12	6.40x10⁻⁴	0.17	3.80x10⁻⁵

283
 284
 285 **All three distance measures alone can explain recipient growth**
 286 Having observed a significant positive correlation of recipient growth with each of
 287 the three-distance metrics analyzed (i.e., amino acid profile distance (AAD),
 288 phylogenetic distance (PD), and metabolic distance (MD), Table 1), we asked whether
 289 these factors alone were sufficient to predict the growth of auxotrophic recipients. This
 290 question was addressed by replotting the data of the performed coculture experiments
 291 in three-dimensional graphs that display the growth of a given auxotroph depending

292 on two of the three measures quantified. Fitting a 2D plane into the resulting graphs
293 indicated that increasing each of the three measures also increased recipients' growth
294 (Fig. 4 and S3). Thus, these graphs suggested that the three explanatory variables are
295 likely correlating with each other. To subject this conjecture to a formal statistical test,
296 we repeated the regression analyses to examine whether MD or AAD were significantly
297 associated with the growth of auxotrophs in coculture, when the first predictor variable
298 PD was already included (Table S1). In all cases, growth of *E. coli* recipients remained
299 positively associated with metabolic distance as well as the distance of the amino acid
300 production profile (Table S1). However, this pattern no longer holds for *A. baylyi*
301 auxotrophs ($\Delta hisD$ and $\Delta trpB$) (Table S1).

302 Furthermore, we hypothesize that the amount of amino acid produced by the donors
303 should affect recipient growth. Genotypically related donors are more likely to produce
304 the same spectrum of amino acids as compared to distantly related individuals. In order
305 to consider this fact, we performed additional analyses that controlled for the role of
306 total amino acid production or focal amino acid production (his or trp). In almost all
307 cases, growth of recipients remained significantly positively associated with the three
308 distance measures (Table S2 and S3). Together, the set of analyses performed
309 demonstrates that the three different measures analyzed (i.e., AAD, PD, and MD, and)
310 can individually (in the case of *E. coli*) or in combination (both species) explain the
311 cross-feeding between prototrophic donors and auxotrophic recipients, thus,
312 corroborating the dissimilarity hypothesis.

313



314

315 **Fig. 4. Multiple distance measures interactively explain recipient growth.** The plane
316 depicts the linear regression between the growth of the histidine auxotrophic *E. coli* recipient
317 ($\Delta hisD$) and the phylogenetic distance, the metabolic distance, and the amino acid profile
318 distance between donor and recipient. Data points above the plane are shown in black. See
319 also Fig. S3 for other comparisons.

320

321 ***In-silico* models predict that metabolic dissimilarity between species enhances** 322 **cross-feeding**

323 To verify whether the patterns we observed in laboratory-based coculture
324 experiments also apply to natural microbial communities, we used *in-silico* modeling
325 to simulate the co-growth of different bacterial species that co-occur in the human
326 gastrointestinal tract. Specifically, we considered all of 334,153 pairwise combinations
327 of 818 bacteria commonly found in this environment. The *in-silico* simulations indicated
328 that the relationship between metabolic distance and metabolic exchange (i.e., total
329 metabolic fluxes between species) follows a saturation curve (Fig. 5). In fact, fitting a
330 logistic function resulted in a higher goodness of fit ($P < 0.001$, $R^2 = 0.04$) than fitting

331 a linear function ($R^2 = 0.023$). This finding suggests that also bacteria residing within
332 the human gut are more likely to engage in cross-feeding interactions with
333 metabolically more dissimilar species. Taken together, the set of computational
334 analyses performed here is in line with the experimental data shown above: both
335 datasets reveal that metabolic cross-feeding interactions are more likely to establish
336 between two metabolically more dissimilar partners.

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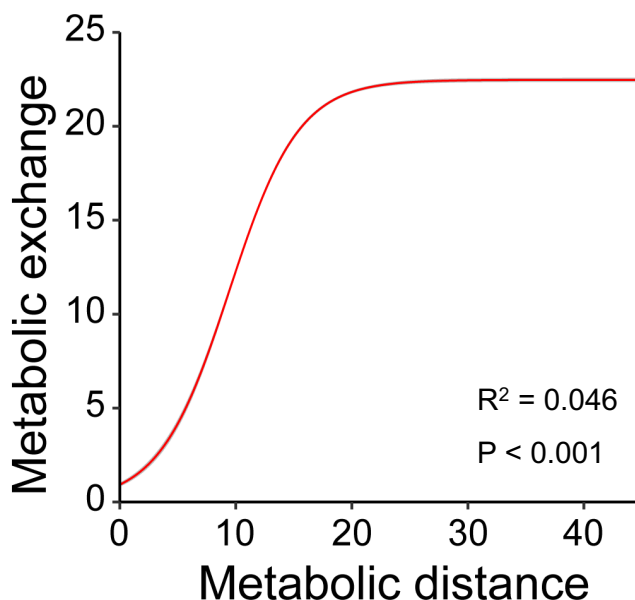
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349 **Fig. 5. Metabolic exchange increases with an increasing metabolic distance between**
350 **interaction partners in gut microbial communities.** Shown is the result of an *in-silico* flux-
351 balance-analysis of paired models analyzing 334,153 combinations of 818 bacterial species
352 residing in the human gut. Results of a logistic model (red line) and the 95% confidence interval
353 (grey area) are displayed.

354

355 Discussion

356 Metabolic cross-feeding interactions among different microbial species are
357 ubiquitous and play key roles in determining the structure and function of microbial
358 communities (16, 57, 58). However, the rules that govern their establishment remain
359 poorly understood. Here we identify the metabolic dissimilarity between donor and
360 recipient genotype as a major determinant for the establishment of obligate,
361 unidirectional cross-feeding interactions between two bacterial strains. In systematic
362 coculture experiments between a prototrophic amino acid donor and an auxotrophic
363 amino acid recipient, we show that growth of auxotrophic recipients in coculture was
364 positively associated with (i) the compositional difference in the amino acid mixtures
365 various donor produced (Fig. 3A), (ii) their phylogenetic distance (Fig. 3B), as well as
366 (iii) the difference in their metabolic networks (i.e. their metabolic distance) (Fig. 3C).
367 Furthermore, *in-silico* simulations of the co-growth of species from a gut microbial
368 community corroborated that the propensity of cross-feeding interactions to establish
369 increased when both interacting partners were metabolically more dissimilar (Fig 5).

370 In our study, we manipulated the relatedness between donor and recipient
371 genotypes. A high phylogenetic relatedness between two genotypes (donor and
372 recipient) in coculture means that they can perform similar metabolic reactions and are
373 more likely to be characterized by overlapping growth requirements (33, 43). As a
374 consequence, both the nutritional value of a given molecule and the biosynthetic cost
375 to produce it is alike (52, 53, 59). In contrast, two phylogenetically distant strains likely
376 differ in their metabolic capabilities and requirements. Thus, two more closely related
377 strains are likely to compete for environmentally available nutrients and provide an
378 increased potential for a difference in the cost-to-benefit-ratio than two distant relatives
379 (43, 60). This statistical relationship can explain why in our coculture experiments, both
380 the phylogenetic and metabolic distance was positively associated with the growth of
381 cocultured auxotrophs. Thus, our results support the dissimilarity hypothesis to explain
382 the establishment of unidirectional cross-feeding interactions. Our findings are in line
383 with previous studies that analyzed the effect of the phylogenetic relatedness and
384 metabolic dissimilarity on antagonistic interactions between two different genotypes.
385 The authors of these studies found that bacteria mainly inhibit the growth of
386 metabolically more similar and related species (41, 61). Even though the focal
387 biological process differs drastically between our (metabolic cross-feeding) and these
388 other studies (antagonistic interactions), the main finding is conceptually equivalent:
389 genotypes are more likely to compete against close relatives, yet support the growth
390 of more dissimilar strains – either by enhancing their growth (Fig. 3) or inhibiting them
391 less (41, 61).

392 In our experiments, we took advantage of synthetically assembled pairwise
393 interactions between different bacterial genotypes to assess how the similarity
394 between interacting partners affects the cross-feeding of metabolites. Even though this
395 approach is limited by the number of pairwise comparisons that can be analyzed in
396 one experiment, the obtained results provide a very clear answer to the focal question.
397 First, the selected donor strains covered a broad range of taxonomic diversity in
398 bacteria (Fig. 1B). Thus, the spectrum of ecological interactions analyzed here likely
399 reflects the range of interactions a given bacterial genotype would typically experience
400 in a natural microbial community. Second, by deliberately choosing strains that lack a
401 previous coevolutionary history, any result observed can be clearly attributed to the
402 focal, experimentally-controlled parameter (e.g., phylogenetic or metabolic distance).
403 In this way, confounding effects like an evolved preference for a certain genotype can
404 be ruled out. Finally, we analyzed bacterial consortia in a well-mixed, spatially
405 unstructured environment, in which the exchanged metabolites are transferred
406 between cells via diffusion through the extracellular environment. Such a set-up
407 minimizes factors that would be amplified in a spatially structured environment, such
408 as a local competition for nutrients or the release of metabolic waste products that
409 inhibit the growth of other cells in the local vicinity. Thus, the experimental approach
410 chosen circumvents the challenges of manipulating and detecting metabolite
411 exchange in natural environments and instead capitalizes on analyzing experimentally
412 arranged and carefully controlled coculture experiments.

413 The guiding principle discovered in this study is most likely relevant for ecological
414 interactions outside the realm of microbial communities. Mutualistic interactions, in
415 which two partners reciprocally exchange essential metabolites or services, usually
416 involve two or more completely unrelated species (60, 62-64). In contrast, cooperative
417 interactions among closely related individuals usually rely on the uni- or bidirectional
418 exchange of the same commodity or service (60). Thus, the fact that two more
419 dissimilar individuals have an increased potential to engage in a synergistic interaction
420 than two more similar individuals may be a universal rule that guides the establishment
421 of mutualistic interactions in general (43).

422 Our results highlight the utility of using synthetic, laboratory-based model systems
423 to understand fundamental principles of microbial ecology. In this study, we
424 demonstrated that the establishment of interactions in complex natural communities is
425 likely determined by simple rules of assembly. These insights not only enrich our
426 understanding of complex microbial communities, but also help us to engineer them
427 for biotechnological or medical applications.

428

429 **Material and methods**

430

431 *Bacterial strains and their construction*

432 Twenty-five bacterial wild type strains were used as potential amino acid donors
433 (Supplemental Table S4). *Escherichia coli* BW25113 and *Acinetobacter baylyi* ADP1
434 were used as parental strains, from which mutants that are auxotrophic for histidine
435 ($\Delta hisD$) or tryptophan ($\Delta trpB$) were generated. The gene to be deleted in order to
436 create the corresponding auxotrophy was identified using the KEGG (65) and the
437 EcoCyc (66) database. For *E. coli*, deletion alleles were transferred from existing single
438 gene deletion mutants (i.e. the Keio collection, (67)) into *E. coli* BW25113 using phage
439 P1-mediated transduction (68). In-frame knockout mutants were achieved by the
440 replacement of target genes with a kanamycin resistance cassette. In the case of *A.*
441 *baylyi*, deletion mutants were constructed as described previously (48). Briefly, linear
442 constructs of the kanamycin resistance cassette with 5'-overhangs homologous to the
443 insertion site were amplified by PCR, where pKD4 was used as a template (see
444 Supplemental Table S5 for primer details). Upstream and downstream regions
445 homologous to *hisD* and *trpB* were amplified using primers with a 5'-extension that was
446 complementary to the primers used to amplify the kanamycin resistance cassette. The
447 three amplified products (upstream, downstream, and kanamycin) were combined by
448 PCR, resulting in overhanging flanks with a kanamycin cassette. This PCR product
449 was introduced into the *A. baylyi* WT strain. For this, the natural competence of *A.*
450 *baylyi* was harnessed. Transformation was done by diluting 20 μ l of a 16 h-grown
451 culture in 1 ml lysogeny broth (LB). This diluted culture was mixed with 50 μ l of the
452 above PCR mix and further incubated at 30 °C with shaking at 200 rpm for 3 h. Lastly,
453 1 ml volume was pelleted, washed once with LB broth, plated on LB agar plates
454 containing kanamycin (50 μ g ml⁻¹), and incubated at 30 °C for colonies to grow.

455 Conditional lethality of constructed auxotrophic mutations in MMAB medium was
456 verified by inoculating 10^5 colony-forming units (CFU) ml⁻¹ of these strains into 1 ml
457 MMAB medium with or without the focal amino acid (100 μ M). After 24 h, their optical
458 density (OD) was determined spectrophotometrically at 600 nm using FilterMax F5
459 multi-mode microplate reader (Molecular Devices) and the mutation was considered
460 conditionally essential when growth did not exceed the OD_{600nm} of uninoculated
461 minimal medium (67, 69).

462

463 *Culture conditions and general procedures*

464 A modified minimal media for *Azospirillum brasilense* (MMAB, (70)) was used for all
465 experiments containing K₂HPO₄ (3 g L⁻¹), NaH₂PO₄ (1 g L⁻¹), KCl (0.15 g L⁻¹), NH₄Cl
466 (1 g L⁻¹), MgSO₄ · 7H₂O (0.3 g L⁻¹), CaCl₂ · 2H₂O (0.01 g L⁻¹), FeSO₄ · 7H₂O (0.0025
467 g L⁻¹), Na₂MoO₄ · 2H₂O (0.05 g L⁻¹), and 5 g L⁻¹ D-glucose as a carbon source. 10 ml
468 of trace salt solution was added per liter of MMAB media from the 1L stock. Trace salt
469 stock solution consisted of filter sterilized 84 mg L⁻¹ of ZnSO₄ · 7H₂O, 765 μ l from 0.1
470 M stock of CuCl₂ · 2H₂O, 8.1 μ l from 1 M stock of MnCl₂, 210 μ l from 0.2 M stock of
471 CoCl₂ · 6H₂O, 1.6 ml from 0.1 M stock of H₃BO₃, 1 ml from 15 gL⁻¹ stock of NiCl₂.

472 All strains were precultured in replicates by picking single colonies from LB agar
473 plates, transferring them into 1 ml of liquid MMAB in 96-deep well plate (Eppendorf,
474 Germany), and incubating these cultures for 20 h. In all experiments, auxotrophs were
475 precultured at 30 °C in MMAB, which was supplemented with 100 μ M of the required
476 amino acid. The next day, precultures were diluted to an optical density of 0.1 at 600
477 nm as determined by FilterMax F5 multi-mode microplate readers (Molecular Devices).

478

479 *Coculture experiment*

480 Approximately 50 μ l of preculture were inoculated into 1 ml MMAB, leading to a
481 starting density 0.005 OD. In case of cocultures, donor and recipient were mixed in a
482 1:1 ratio by co-inoculating 25 μ l of each diluted preculture without amino acid
483 supplementation. Monocultures of both donors and recipient (with and without the focal
484 amino acid) were inoculated using 50 μ l of preculture. Cultures were incubated at a
485 temperature of 30 °C and shaken at 220 rpm. Cell numbers were determined at 0 h
486 and 24 h by serial dilution and plating. Donor strains were plated on MMAB agar plates,
487 whereas recipients (auxotrophs) were differentiated on LB agar containing kanamycin
488 (50 μ g ml⁻¹) to select for recipient strains. For key resources, see (Supplemental Table
489 S6).

490

491 *Relative fitness measurement*

492 To quantify the effect of amino acid cross-feeding on the fitness of the recipient, the
493 number of colony-forming units (CFU) per ml was calculated for monoculture and
494 coculture conditions at 0 h and 24 h. Each donor was individually paired with one of
495 the recipients as well as grown in monoculture. Every combination was replicated four
496 times. The relative fitness of each recipient was determined by dividing the growth of
497 each genotype achieved in coculture by the value of its respective monoculture. Since
498 different donor genotypes show inherent differences in growth, the growth of recipients
499 in coculture was normalized to reduce to minimize potential effects of this variation.

500 For this, growth of recipients in monoculture was first subtracted from its growth in
501 coculture and then divided by the growth the respective donor genotype achieved in
502 coculture.

503

504 *Amino acid supernatant experiment*

505 To determine whether cross-feeding was mediated via compounds that have been
506 released into the extracellular environment, the cell-free supernatants of donor
507 genotypes were harvested and provided to receiver strains. To collect the supernatant,
508 donors were grown in 2.5 ml MMAB in 48-deep well plates (Axygen, USA) and
509 cultivated at 30 °C under shaking conditions (220 rpm). Supernatants were isolated in
510 the mid-exponential growth phase and centrifuged for 10 min at 4,000 rpm. Then,
511 supernatants were filter-sterilized (0.22 µm membrane filter, Pall Acroprep, USA) and
512 stored at -20 °C. Meanwhile, receivers were grown in 1 ml MMAB in 96-well plates for
513 24 h. After adjusting the receiver OD_{600nm} to 0.1, 5 µl of the receiver culture was added
514 to the replenished donor supernatant (total culturing volume: 200 µl, i.e. 160 µl donor
515 supernatant + 40 µl MMAB) in 384-well plates (Greiner bio-one, Austria) (total: 50 µl
516 culture). Four replicates of each comparison were grown for 24 h at 30 °C in a FilterMax
517 F5 multi-mode microplate reader (Molecular Devices). MMAB without supernatant and
518 monocultures of receiver strains were used as control. Growth was determined by
519 measuring the optical density at 600 nm every 30 minutes, with 12 minutes of orbital
520 shaking between measurements. OD_{600nm} was measured and analyzed to calculate
521 the maximum optical density (OD_{max}) achieved by the receiver strain using the Softmax
522 Pro 6 software (Table S7). For each donor supernatant-receiver pair, OD_{max} achieved
523 by receivers with supernatant was subtracted from the values achieved by cultures
524 grown without supernatant and normalized with the OD_{600nm}, the respective donor
525 strain had achieved at the time of supernatant extraction.

526

527 *Amino acid quantification by LC/MS/MS*

528 All 20 proteinogenic amino acids in the culture supernatant were analyzed. 100 µl
529 of extracted supernatant was derivatized using the dansyl chloride method (71, 72).
530 Norleucine was added as an internal standard to the sample and a calibration curve
531 was generated by analyzing all 20 amino acids at different concentrations. All samples
532 were directly analyzed via LC/MS/MS. Chromatography was performed on a Shimadzu
533 HPLC system. Separation was achieved on an Accucore RP-MS 150 x 2.1, 2.6 µm
534 column (Thermo Scientific, Germany). Formic acid 0.1% in 100% water and 80%
535 acetonitrile were employed as mobile phases A and B, respectively. The mobile phase
536 flow rate was 0.4 ml min⁻¹ and the injection volume was 1 µl. Liquid chromatography
537 was coupled to a triple-quadrupole mass spectrometer (ABSciex Q-trap 5500). Other
538 parameters were: curtain gas: 40 psi, collision gas: high, ion spray voltage (IS): 2.5
539 keV, temperature: 550 °C, ion source gas: 1: 60 psi, ion source gas 2: 70 psi. Multiple
540 reaction monitoring was used to determine the identity of the focal analyte. Analyst and
541 Multiquant software (AB Sciex) were used to extract and analyze the data.

542

543 *Amino acid profile-based distance calculation using supernatant data*

544 The similarity between closely related and distantly related donor amino acid profiles
545 was measured by calculating the Euclidean distance. If the amino acid production of a
546 closely related donor is given by $CR = (cr_1, cr_2, \dots, cr_{20})$, and the amino acid production
547 of the distant related donor is given by $DR = (dr_1, dr_2, \dots, dr_{20})$, the Euclidean distance
548 between recipient and donor is:

549

$$550 \quad ED(CR, DR) = \sqrt{(cr_1 - dr_1)^2 + (cr_2 - dr_2)^2 + \dots + (cr_{20} - dr_{20})^2}$$

551 Index numbers (1-20) refer to individual amino acids.

552

553 *Phylogenetic tree construction and distance calculation*

554 To cover a broad taxonomic diversity of donor strains, we chose 25 well-
555 characterized species, belonging to four different phyla. The 16S rRNA gene
556 sequences of 20 strains were retrieved from the NCBI GenBank and 5 strains from
557 16S rRNA sequencing (see supplementary method). The phylogenetic tree of this
558 marker gene was generated using the maximum likelihood method in MEGA X
559 software (73). 16S rRNA gene locus sequences of all strains were converted to amino
560 acid sequences and aligned with MUSCLE. Maximum-likelihood (ML) trees were
561 constructed using the Kimura 2-parameter model, where rates and patterns among
562 mutated sites were kept at uniform rates, yielding the best fit. Bootstrapping was
563 carried out with 1,000 replicates. The phylogenetic tree was edited using the iTOL
564 online tool (Table S7) (74). Pairwise phylogenetic distances between donor and
565 receiver strains were extracted from a phylogenetic distance-based matrix. The
566 resulting values quantify the evolutionary distance that separates the organisms.

567

568 *Reconstruction of metabolic networks*

569 Genome-scale metabolic networks for all organisms (Table S4) were reconstructed
570 based on their genomic sequences using the GapSeq software (version v0.9,
571 <https://github.com/jotech/gapseq>) (75). In brief, the reconstruction process is divided
572 in two main steps. First, reactions and pathway predictions, and, second, gap-filling of
573 the network to facilitate *in-silico* biomass production using flux balance analysis. For
574 the reaction and pathway prediction step, all pathways from MetaCyc database (76)
575 that are annotated for the taxonomic range of bacteria, were considered. Of each
576 reaction within pathways, the protein sequences of the corresponding enzymes were
577 retrieved from the SwissProt database (77) and aligned against the organism's
578 genome sequence by the TBLASTN algorithm (78). An enzyme, and thus the
579 corresponding reaction, was considered to be present in the organism's metabolic
580 network, if the alignment's bitscore was ≥ 200 and the query coverage $\geq 75\%$.
581 Moreover, reactions were considered to be existing, if more than 75% of the remaining
582 reactions within the pathway were predicted to be present by the BLAST-searches or
583 if more than 66% of the key enzymes, which are defined for each pathway by MetaCyc,
584 were predicted to be part of the network by the blast searches. As reaction database
585 for model construction, we used the ModelSEED database for metabolic modeling
586 (79).

587 The second step (i.e., the gap filling algorithm of gapseq) solves several optimization
588 problems by utilizing a minimum number of reactions from the ModelSEED database
589 and adding them to the network, in order to facilitate growth in a given growth medium.
590 Here, the chemical composition of the M9 medium (which is qualitatively identical to
591 MMAB) with glucose as sole carbon source was assumed.

592

593 *Calculating the genome-based metabolic distance of organisms*

594 To estimate the pairwise metabolic distance between donor and recipient
595 genotypes, the structure of their metabolic network was compared. For this, a flux
596 balance analysis was performed on each individual metabolic network model.
597 Subsequently, total flux was minimized to predict the flux distribution in M9-glucose
598 medium (80). Pairwise distances of flux distributions between organisms were
599 calculated as the Euclidean distance between the predicted flux vectors. Only
600 reactions with a non-zero flux in at least one of the two organisms were included in the
601 distance approximations.

602

603 *In-silico simulation of bacterial co-growth*

604 To further investigate the relationship between the metabolic distance between
605 organisms and the likelihood for them to enter into a cross-feeding interaction, we
606 extended our analysis to a larger number of bacterial organisms using *in-silico* co-
607 growth simulations. For this, we reconstructed 818 bacterial metabolic network models
608 as described above. The selected 818 organisms are the same as from the AGORA-
609 collection, which represent common members of the human gut microbiota (81). For
610 co-growth simulations, the models were merged in a pairwise manner as described
611 previously (82). The predicted metabolic flux distributions of bacterial co-growth
612 simulations were used to estimate the *metabolic exchange* between organisms. The
613 metabolic exchange between two organisms was calculated as the sum of absolute
614 predicted exchange rates (exchange fluxes) of metabolites. A logistic curve function of
615 the form $y(x) = a / (1 + e^{-b(x-c)})$ was fitted to the data using R (83).

616

617 *Statistical data analysis*

618 Normal distribution of data was evaluated by means of the Kolmogorov-Smirnov test
619 and data was considered to be normally distributed when $P > 0.05$. Homogeneity of
620 variance was determined using Levene's test and variances were considered
621 homogenous if $P > 0.05$. Differences in the recipient growth in coculture versus
622 monocultures were assessed with paired sample t-tests. P-values were corrected for
623 multiple testing by applying the false discovery rate (FDR) procedure of Benjamini *et*
624 *al.* (84, 85). Linear regressions were used to assess the growth support of recipients
625 in cocultures as a function of different variables (i.e. amino acid profile distance,
626 phylogenetic distances, metabolic distance). Spearman's rank correlation was used to
627 assess the relationship between amino acid production and growth of recipient as
628 maximum density when cultured with donor supernatants. The relationship between
629 each proxy tested and recipient growth was depicted as a 2D plane and analyzed by

630 fitting a linear regression. Regression analyses was also used to disentangle the effect
631 of more than one interacting predictor variable. In these cases, the phylogenetic signal
632 or amino acid produced was controlled for the respective other predictor variable (e.g.
633 metabolic distance or amino acid production profile distance) used to predict growth of
634 recipient (Table S7).

635

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650

651 **Author contributions**

652 SG and CKo conceived the project. SG, SS, and CKo designed the research. SG
653 performed all experiments. SG and LO analyzed the data. SG, LO, and CKo
654 interpreted the data. SW calculated the genome-scale metabolic distance of tested
655 strains and performed all *in-silico* analyses. SW and CKa carried out the gut
656 microbiome *in-silico* data collection. GY isolated and phenotypically characterized the
657 five environmentally-derived donor strains. SG and CKo wrote the manuscript, all
658 authors revised the manuscript. CKo provided resources and acquired funding.

659

660 **Competing interests**

661 The authors declare no competing interests.

662

663 **References**

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